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-HT2B 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 development1 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 vasoconstriction8 and reactive oxygen species (ROS)—depent 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 (H2O2) 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 H2O2 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

Received December 8, 2004; revision received August 23, 2005; accepted August 24, 2005.

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.).

Guest Editor for this article was Roberto Bolli, MD.

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

© 2005 American Heart Association, Inc.

Circulation is available at http://www.circulationaha.org DOI: 10.1161/CIRCULATIONAHA.104.528133
regulation of cardiac functions is still not clearly defined. Some studies indicated that cardiac MAO-A regulates noradrenaline concentrations. Our demonstration that MAO-A is also an important source of H2O2 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. Briefly, hearts were perfused retrogradely with Ca2+-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 g/mL BSA (Sigma-Aldrich) to separate ventricular myocytes from nonmyocytes as described by Ellingsen et al. 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. 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 g/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/L) and fluorescein-12-dUTP for 1 hour at 37°C. After rinsing in 1 PBS, the slides were immersed in propidium iodide solution (1 g/L) for 15 minutes.

### TABLE 1. Area at Risk and Infarct Size

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

LV indicates left ventricular; WT, weight; P, paragyline; C, clorgyline; and SB, SB 206553. Data are mean±SEM.

*P<0.001 vs I/R.

### TABLE 2. Scoring Method of Myocyte and Endothelial Injury

<table>
<thead>
<tr>
<th>Injury</th>
<th>Score</th>
<th>Degree of Injury</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myocyte</td>
<td>0</td>
<td>Normal myocyte</td>
<td>Mild intracellular edema</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Slight</td>
<td>Mild mitochondrial swelling</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Moderate</td>
<td>Mild intracellular edema; Contracture of myofibrils</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Severe</td>
<td>Severe mitochondrial swelling; Presence of intramitochondrial dense granules</td>
</tr>
<tr>
<td>Endothelial</td>
<td>0</td>
<td>Normal endothelium</td>
<td>Nuclear degeneration (apoptosis or karyolysis)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Slight</td>
<td>Mild to moderate endothelial swelling</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Moderate</td>
<td>Marked endothelial swelling; Disarrangement of myofibrils</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Severe</td>
<td>Severe mitochondrial swelling; Plasma membrane rupture</td>
</tr>
</tbody>
</table>

|        |       | Neutrophil adhesion and extravasation |
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 assessed 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 was 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

H₂O₂ production was determined by the oxidation of 2′,7′-dichlorodihydro-fluorescein (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-12 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 000g for 15 minutes. Then, 5% metaphosphoric acid was added to the supernatant. After centrifugation at 10 000g 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 (86/600/ 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 (Pentothal, 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) 30 minutes before ischemia and subjected to 30 minutes of ischemia followed by 60 minutes of reperfusion; group 5 (n=6), rats pretreated for 3 days with the 5-HT₂BR antagonist SB 206553 before addition of 5-HT (10 μmol/L) for 5 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).
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 $\times 3000$ to $\times 20000$).

Evaluation of Myeloperoxidase Activity
Myeloperoxidase (MPO) activity, a marker for neutrophil accumulation, was evaluated according to the method of Bradley et al.\textsuperscript{21} Frozen samples of left ventricular tissue (100 mg) were homogenized in 5 mmol/L potassium phosphate buffer and then centrifuged at 30 000g 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 000g 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$_2$O$_2$. 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.\textsuperscript{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.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{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.}
\end{figure}
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 rat cardiomyocyte mortality (35% versus control; \( P < 0.001 \)) and caspase-3 activity (2.5-fold versus control; \( P < 0.01 \)) with a maximal effect at 10 \( \mu \text{mol/L} \) 5-HT for 16 hours in the absence or presence of 1 \( \mu \text{mol/L} \) pargyline, 1 \( \mu \text{mol/L} \) pyruvate, or 50 \( \mu \text{mol/L} \) defereroxamine. B. Effect of 5-HT on H2O2 production (DCF fluorescence) in the presence or absence of pargyline (1 \( \mu \text{mol/L} \)). Data are mean ± SEM from 3 independent experiments. ***\( P < 0.001 \) vs control; ###\( P < 0.001 \) vs pargyline treatment.

Involvement of MAOs in the Postreperfusion Cardiac Damage

To determine the relevance of H2O2 production by MAOs in vivo, we investigated the effects of MAO inhibition in cardiac damage after IR. 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, the amine transporter inhibitor imipramine, the iron chelator defereroxamine, or the antioxidant pyruvate). In contrast, the increase in 5-HT-dependent cardiomyocyte apoptosis was unaffected by cell pretreatment with the 5-HT2B receptor antagonist SB 206553. Taken together, these data show that 5-HT induces cardiomyocyte apoptosis by a mechanism involving H2O2 generation by MAO-A.
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 postsischemic semithin sections demonstrated attenuated myofibrillar 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 as 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 ventricles 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 H2O2 production. In addition, we demonstrate that H2O2 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(2) receptors, 5-HT induces cardiac hypertrophy27 and prevents cardiomyocytes from apoptosis.3 The 5-HT(2) receptor–mediated antiapoptotic effect of 5-HT involves inhibition
by guest on April 17, 2017 http://circ.ahajournals.org/ Downloaded from

acid metabolism, and nonphagocyte NADPH oxidase, have been
ROS, including mitochondrial electron-transport chain, arachidonic
5-HT in cardiac cells requires ROS production. Various sources of
progression of ventricular dysfunction.
conceivable that, in vivo, these dual concentration-dependent effects
5-HT may have opposite effects on cardiomyocyte survival. It is
depending on the concentrations and the mechanisms of action,
those previously reported by Nebigil and coworkers suggest that,
in vivo, these dual concentration-dependent effects
of 5-HT (5 \text{ mol/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.

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 \text{ mmol/L}) higher than those observed for
the antiapoptotic activity (1 \text{ mmol/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_3B receptor antagonist SB 206553 and
was completely prevented by the amine transporter inhibitor imip-
ramine. The effect of 5-HT involves the activation of the mitochon-
drial 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 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. Concerning serotonin, we have identified the
mitochondrial enzyme MAO-A as the intracellular source of ROS
involved in cardiomyocyte apoptosis. During the oxidative deami-
nation 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 mem-
brane 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. According to these results, we showed that MAO-dependent ROS
generation induced an increase in the Bax/Bcl-2 ratio and mitochon-
drial cytochrome c release, leading to cardiomyocyte apoptosis. These
findings point out the critical role of ROS produced by MAO-A
during serotonin degradation in triggering the proapoptotic mito-
chondrial cascade.

Oxidative stress and mitochondrial dysfunction are considered
key mediators of cardiomyocyte apoptosis associated with post-I/R
cardiac damage. In addition, large amounts of serotonin are
released by activated platelets during vascular injury and I/R. It is
noteworthy that the 5-HT concentration inducing cardiomyocyte
apoptosis in vitro (10 \text{ mmol/L}) is compatible with those potentially
observed in vivo. Indeed, it has been shown that, in cats and mice, platelets can release up to 27-\text{mmol/L concentrations of 5-HT. In
addition, Fu and Longhurst have recently shown that large amounts
of 5-HT (5 \text{ mmol/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_3B 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 in-
hibitors 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 postre-
perfusion 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, includ-
ing myofibril hypercontraction, interstitial edema, and mitochon-
drial 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 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, respec-
tively. Data are expressed as mean \pm SEM from 8 separate
experiments. **P<0.01 and ***P<0.001 vs sham-operated

group; #P<0.05 vs I/R 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. 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.

Acknowledgments
This work was supported by the Institut National de la Santé et de la Recherche Médicale France and by a grant from the University of Florence (Italy). Drs Bianchi and Kunduzova were supported by a postdoctoral fellowship from the Fondation pour la Recherche Médicale. We would like to thank XinXin Guo and Kara Clemente for their technical support.

References


31. Takano S, Hoshino Y, Li L, Matsuoka I, Ono T, Kimura J. Dual roles 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 H2O2 production. In addition, we demonstrate that H2O2 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 cardiac myocyte 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.}

**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 H2O2. 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 H2O2 production. In addition, we demonstrate that H2O2 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.
Oxidative Stress by Monoamine Oxidase Mediates Receptor-Independent Cardiomyocyte Apoptosis by Serotonin and Postischemic Myocardial Injury
Pascale Bianchi, Oxana Kunduzova, Emanuela Masini, Claudie Cambon, Daniele Bani, Laura Raimondi, Marie-Helene Seguelas, Silvia Nistri, Wilson Colucci, Nathalie Leducq and Angelo Parini

Circulation. 2005;112:3297-3305; originally published online November 14, 2005;
doi: 10.1161/CIRCULATIONAHA.104.528133
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/112/21/3297

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/