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Am J Physiol Heart Circ Physiol 284:1460-1467, 2003. First published Dec 12, 2002;
doi:10.1152/ajpheart.00700.2002

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

P. Bianchi, O. Kunduzova, E. Masini, C. Cambon, D. Bani, L. Raimondi, M.-H. Seguelas, S. Nistri, W. Colucci, N. Leducq and A. Parini
Circulation, November 22, 2005; 112 (21): 3297-3305.
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Age-dependent increase in hydrogen peroxide production by cardiac monoamine oxidase A in rats

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Submitted 4 October 2002; accepted in final form 5 December 2002

Maurel, Agnès, Carole Hernandez, Oksana Kunduzova, Guy Bompard, Claudie Cambon, Angelo Parini, and Bernard Francés. Age-dependent increase in hydrogen peroxide production by cardiac monoamine oxidase A in rats. *Am J Physiol Heart Circ Physiol* 284: H1460–H1467, 2003; 10.1152/ajpheart.00700.2002.—Oxidative stress is one of the factors involved in age-related impairment of cardiac function. In the present study, we investigated the role of the catecholamine-degrading enzyme monoamine oxidase (MAO) in H₂O₂ production in the hearts of young, adult, and old rats. MAO-dependent H₂O₂ production, measured by a chemiluminescence-based assay, increased with age, reaching the maximum in 24-mo-old rats (7.5-fold increase vs. 1-mo-old rats). The following observations indicate that the age-dependent increase in H₂O₂ generation was fully related to the MAO-A isoform: 1) at all the ages tested, chemiluminescence production was inhibited by the MAO-A inhibitor clorgyline but not by the MAO-B inhibitor RO-19 6327; 2) enzyme assay, Western blot, and semiquantitative RT-PCR analysis showed an age-dependent increase in cardiac MAO-A activity, immunodetection, and mRNA expression, respectively; and 3) the MAO-B isoform was undetectable by enzyme assay and Western blot analysis. These results suggest that MAO-A could be a major source of H₂O₂ in the aging heart.

monoamine oxidases; oxidative stress

REACTIVE OXYGEN SPECIES (ROS) occupy a prominent position in aging processes (45, 57). These substances may participate in cell aging by different mechanisms, including protein and lipid oxidation and DNA damage (33, 46, 57). ROS can be generated by multiple sources, such as xanthine oxidase (21), NADPH oxidase (5), and the mitochondrial respiratory chain (19, 26, 27). In normal situations, the physiological intracellular redox state is maintained by the equilibrium between ROS production by the different sources and inactivation by antioxidant systems (51). The alteration of such an equilibrium leads to an increase in intracellular ROS concentration and to the subsequent oxidative stress. In aging, the increase in intracellular ROS and the oxidative stress-dependent decline of cell functions have been related, in part, to impairment of the mitochondrial respiratory chain (2, 26–28). The mitochon-

drial enzymes monoamine oxidase (MAO) A and B, which play a major role in the oxidative deamination of biogenic [catecholamines and serotonin (5-hydroxytryptamine, 5-HT)] and exogenous amines (i.e., tyramine) (56), are a source of hydrogen peroxide (H₂O₂). Convergent evidences suggest their involvement in aging processes (9, 47). Indeed, the increase in MAO activity has been associated to the detrimental structural and functional processes of aging in some brain regions (18, 29, 40–42, 47).

Recently, we have shown that H₂O₂ generated by MAOs during substrate degradation is involved in cell proliferation (52, 53) and apoptosis (P. Bianchi, M. H. Séguélas, A. Parini, and C. Cambon, personal communications), two events associated with aging (31, 39, 54, 55).

In the aging heart, the increase in ROS production has been considered as one of the factors involved in myocyte apoptosis and reactive hypertrophy, two processes contributing to the development of cardiac failure (3, 4, 34–36, 43). Moreover, the impairment of cardiac metabolic and functional tolerance toward oxidative stress and a decrease in some cardiac scavenger enzymes have been implicated in the process of cardiac aging (1). As reported for other tissues, the age-dependent oxidative stress in the heart is related, in part, to mitochondrial dysfunction (38, 43). Indeed, these organelles have increasing capacities to produce H₂O₂ and generate lipid peroxidation in the aging heart (32). MAOs are highly expressed in the rat heart (10, 43). Enzyme assay using specific MAO-A and MAO-B substrates showed a predominant expression of the MAO-A isoenzyme in the rat heart. However, these studies supplied intriguing results. Indeed, the degradation of the MAO-B substrate β -phenylethylamine (β -PEA) was prevented by the MAO-A inhibitor RO-41 1049 and poorly affected by the MAO-B inhibitor RO-19 6327 (49). At present, it is still unknown whether this unusual pattern of β -PEA degradation is related to the expression of atypical forms of MAOs or to particular conformational states of the enzymes.

Although an increase in cardiac MAO activity with age has been reported (10, 30), the involvement of

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MAOs in H₂O₂ production in the aging heart and the relative role of each isoenzyme have not been investigated.

In the present report, we studied H₂O₂ production by MAOs in the hearts of young (1 mo), adult (3 and 6 mo), and old (24 mo) rats. Our results show that aging induces a strong increase in H₂O₂ production by MAOs. By combining chemiluminescence (CL) assay, enzyme activity, Western blot, and semiquantitative RT-PCR analysis, we demonstrated that the age-dependent raise in H₂O₂ production by MAOs is fully related to the increase in the expression of the MAO-A form. This isoenzyme maintains the ability to oxidize the MAO-B substrate β -PEA and displays molecular properties typical of the classical MAO-A form.

MATERIALS AND METHODS

Materials. 5-[¹⁴C]HT (specific activity = 52.3 Ci/mol) and β -[¹⁴C]PEA (specific activity = 52 Ci/mol) were obtained from DuPont NEN (Life Science Products; Boston, MA). Tyramine, pargyline, clorgyline, RO-41 1049, and lazabemide (RO-19 6327) were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit β -ATPase antibody was a generous gift from Dr. Lunardi (Biochemical Laboratory; Grenoble, France).

Crude total membrane fraction preparation. Hearts of male Sprague-Dawley rats (Harlan; Gannat, France) at the ages of 1, 3, 6, and 24 mo were washed in ice-cold phosphate buffer (50 mM, pH 7.4) supplemented with protease inhibitors (0.1 mM PMSF, 10 μ g/ml bacitracin, and 2 μ g/ml soybean trypsin inhibitor), hereafter referred to as phosphate buffer, and ground in a Turrax homogenizer (IKA Labortechnik; Staufen, Germany). After 10 additional passes in a Dounce homogenizer, the homogenate was centrifuged for 10 min at 1,800 g and 4°C. The resulting pellet was discarded, whereas the supernatant was homogenized for a second time in a Dounce homogenizer and centrifuged for 20 min at 16,000 g and 4°C. Finally, the supernatant was eliminated, and the pellet was resuspended in ice-cold phosphate buffer to obtain a concentration of 1 mg/ml protein and stored at -80°C.

MAO activity. Crude total membrane fractions (20–40 μ g) were incubated at 37°C for 20 min in a final volume of 50 μ l phosphate buffer with increasing concentrations of 5-[¹⁴C]HT (0–500 μ M) or β -[¹⁴C]PEA (0–10 μ M) to measure MAO-A and MAO-B activities, respectively. The irreversible MAO inhibitor pargyline (10 μ M) was used to defined nonspecific MAO-A or MAO-B activity. After 20 min, the reaction was ended by adding 100 μ l of 4 N HCl at 4°C. The deaminated products were the extracted by adding 1 ml toluene-ethyl acetate (vol/vol) and vigorous mixing (92% efficiency). Finally, 750 μ l of the organic phase were counted in a liquid scintillation spectrophotometer with 97% of performance (Packard 1900 TR). MAO activity was expressed as picomoles of oxidized substrate during 1-min incubation per milligram of protein.

MAO steady-state kinetic parameters were evaluated using a nonlinear least-square curve-fitting procedure (Graph-Pad Prism; San Diego, CA).

H₂O₂ production assay. As previously reported, the luminol-amplified CL assay is a sensitive procedure to measure the specific contributions of MAO-A and MAO-B to H₂O₂ production (37).

H₂O₂ production was measured by CL assay on heart homogenates (100 μ g protein) in the presence of luminol (10 μ M) and horseradish peroxidase (0.1 U/ml) by using a ther-

mostatically (37°C) controlled luminometer (Bio-Orbit 1251; Turku, Finland). The generation of CL triggered with tyramine (20 μ M), a common MAO substrate, was continuously monitored during 85 min, and the area under the curve (total CL emission) was analyzed by the Bio-Orbit MultiUse program. Inhibition of luminescence by selective MAO-A inhibitor (10 μ M clorgyline) or MAO-B inhibitor (10 μ M RO-19 6327) was measured to control the specificity of H₂O₂ production.

Immunoblots. Crude total membrane fractions (30 μ g) were solubilized in 4 \times Laemmli sample buffer (187 mM Tris·HCl, 2% SDS, 20% glycerol, 0.7 M β -mercaptoethanol, and 0.025% bromophenol blue; pH 6.8) and loaded per well onto 8% SDS-PAGE gels (migration buffer: 124 mM Tris·HCl, 0.5% SDS, and 0.96 glycine; pH 8.3). Electrophoretically resolved proteins were transferred to polyvinylidene difluoride membranes (NEN Life Science Products) by semidry electroblotting (Trans-blot SD, Bio-Rad Laboratories; Richmond, VA). Blots were blocked overnight at 4°C with 5% milk in wash buffer [PBS (pH 7.4)-0.1% Tween 20], washed, and incubated for 1 h at room temperature with rabbit anti-MAO-A/MAO-B polyclonal antiserum. The polyclonal antiserum was obtained from immunized rabbits with the peptide TNGGQERKQFVGGSGGQ, corresponding to amino acids 210–225 in MAO-A and 202–217 in MAO-B. The specificity of the antibody was previously determined by peptide competition in heterologous expression systems and various tissues (37). After being washed, blots were incubated with peroxidase-labeled anti-rabbit IgG for 30 min. Bound antibodies were detected using enhanced CL (ECL kit, Amersham Pharmacia Biotech; Buckinghamshire, UK) and exposure to Hyperfilm MR (Amersham Pharmacia Biotech). Immunoblot analysis with the rabbit anti- β -ATPase polyclonal antiserum was used as an internal mitochondrial standard to quantify proteins.

Semiquantitative RT-PCR. Total RNA were extracted from frozen powdered tissues (100–200 mg) using the acid guanidium thiocyanate-phenol-chloroform method as described by Chomczynski and Sacchi (13).

First-strand cDNA was synthesized from 1 μ g total RNA by RT for 60 min at 42°C in a final volume of 20 μ l RT buffer with 100 units SuperScript II, 0.25 μ g oligo(dT)_{12–18}, 0.5 mM dNTPs, 5 mM DTT, and 32 units RNase inhibitor (Invitrogen; Paisley, UK).

Five microliters of denaturated (94°C for 2.5 min) first-strand cDNA were then used to amplify MAO-A, MAO-B, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) fragments by PCR. Fifty microliters of the reaction mix containing PCR buffer with 1.5 mM MgCl₂, 0.2 mM dNTPs, 60 nM primer couples, 2 units *Taq* polymerase (Invitrogen), and the RT product were amplified with a DNA thermal cycler (TRIO, Thermoblock Biometra; Göttingen, Germany). To evaluate PCR products comparatively, we amplified the MAO and GAPDH products for 30 and 20 cycles at the same time, respectively. A cycle was composed of a denaturation step at 95°C for 1 min, a primer annealing step at 56°C for 1 min, and an extension step at 72°C for 2 min. The final extension step was prolonged to 10 min. The absence of contaminants was routinely checked by RT-PCR assays of negative control samples in which the SuperScript II was omitted.

Primers used. Primers for MAO-A were defined by bases 1,537–1,556 [5'-GTGGCTCTTCTCTGCTTTGT-3' (forward)] and 2,037–2,016 [5'-AGTGCCAAGGGTAGTGTGTATCA-3' (reverse)]. Primers for MAO-B were defined by bases 1,415–1,434 [5'-TCCCAGCAAGACCCATTACC-3' (forward)] and 2,252–2,229 [5'-TGACAAAGACAAGACTCCCATTCTC-3' (reverse)] (23). Primers for GAPDH were defined by bases

510–529 [5'-AATGCATCCTGCACCACCAA-3' (forward)] and 980–960 [5'-GTCATTGAGAGCAATGCCAGC-3' (reverse)] (20). The expected sizes of the amplification products were 500 and 837 bp for MAO-A and MAO-B, respectively, and 470 bp for GAPDH.

Protein determination. Protein concentration was performed by using a modified Lowry procedure (DC protein assay, Bio-Rad Laboratories) using γ -globulin as a standard.

Statistical analysis. Values are expressed as means \pm SE. The statistical significance of differences among the experimental groups was evaluated by one-way ANOVA, followed by a Newman-Keuls post test (GraphPad Prism).

RESULTS

H₂O₂ production in aging hearts. To determine the involvement of MAOs in H₂O₂ production in the aging heart, we used a luminol-based CL assay. We have previously shown that the use of the MAO-A/MAO-B substrate tyramine in this technique allows evaluation of H₂O₂ production by each MAO isoenzyme (37). As shown in Fig. 1A, experiments performed in 3-mo-old rats showed that incubation of heart homogenates with tyramine (20 μ M) led to a time-dependent increase in CL that was inhibited by the MAO-A inhibitor clorgyline (10 μ M) but not by the MAO-B inhibitor RO-19 6327 (10 μ M). Tyramine-dependent CL generation in-

creased with age, reaching the maximum in 24-mo-old rats (7.5-fold increase compared with 1-mo-old rats). At all the ages tested, CL production was inhibited by clorgyline and unaffected by RO-19 6327 (Fig. 1B). These results show an increase in H₂O₂ production by MAOs and suggest the predominant role of the MAO-A isoenzyme in this effect.

Characterization of MAOs in aging hearts. Previous studies showed an unusual pattern of substrate selectivity of rat cardiac MAOs, suggesting the potential expression of atypical MAOs in the heart (49). To identify the MAOs responsible for H₂O₂ production during heart development and aging, we characterized rat cardiac MAOs by combining enzyme assays, Western blot, and semiquantitative RT-PCR analysis.

Enzyme assays were performed using 5-[¹⁴C]HT and β -[¹⁴C]PEA as selective substrates for MAO-A and MAO-B, respectively. As shown in Fig. 2, the equilibrium parameters of 5-[¹⁴C]HT oxidation showed an age-dependent increase in MAO-A activity. The rate of 5-[¹⁴C]HT oxidative deamination was 32-fold higher in 24-mo-old rats than in 1-mo-old rats. We also observed a weak but significant increase in the *K_m* of the 5-[¹⁴C]HT degradation rate, suggesting a possible age-related conformational change of heart MAO-A.

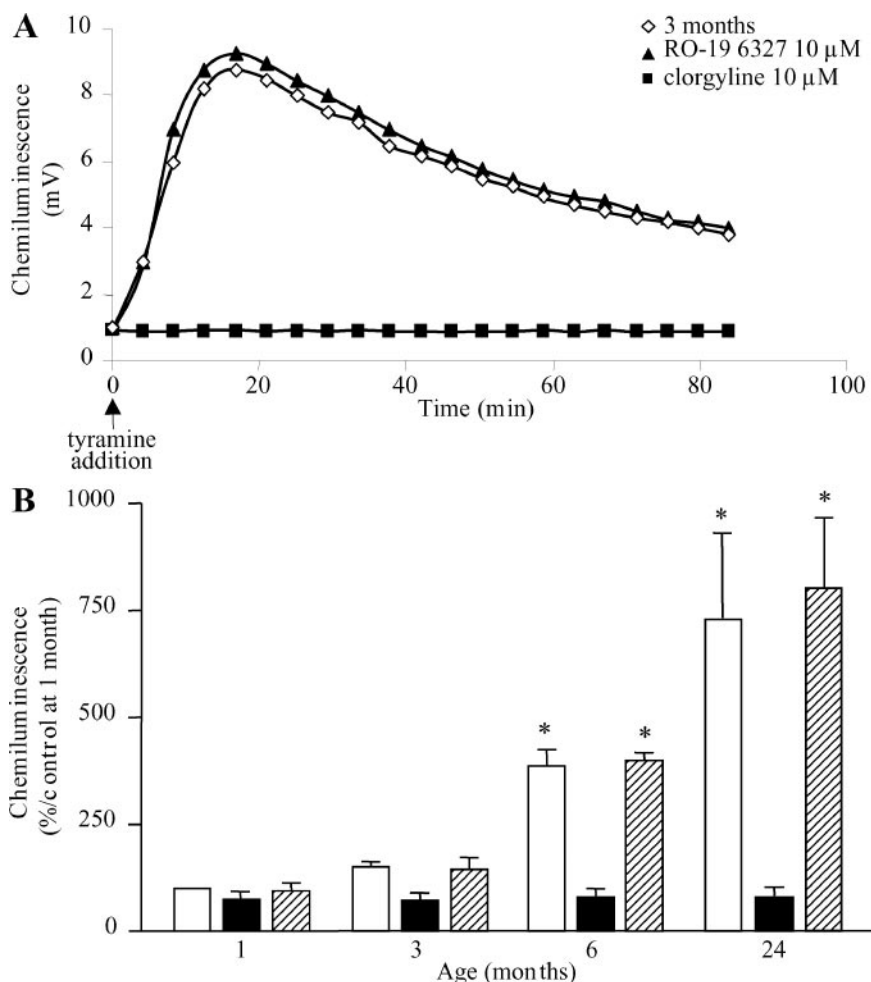


Fig. 1. Monoamine oxidase (MAO)-dependent H₂O₂ production in heart homogenates. **A:** generation of chemiluminescence monitored for 85 min after 20 μ M tyramine addition to 3-mo-old rat heart homogenates, as described in MATERIALS AND METHODS. Heart homogenates were pre-incubated 20 min with 10 μ M clorgyline or 10 μ M RO-19 6327 before tyramine addition. The plot is representative of four separate experiments, and results are expressed in millivolts. **B:** H₂O₂ production measured in 1-, 3-, 6-, and 24-mo-old rat heart homogenates (open bars) with 10 μ M clorgyline (solid bars) or 10 μ M RO-19 6327 (hatched bars). The histograms represent the means \pm SE of four separated experiments and are expressed as a percentage of control CL at 1 mo. **P* < 0.05 vs. 1 mo.

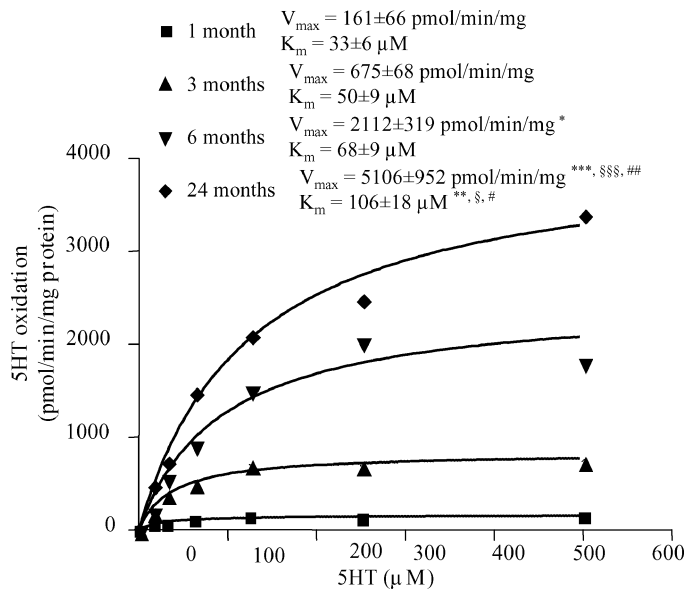


Fig. 2. MAO-A activity in the aging heart. The specific MAO-A activity was assessed by oxidation of the MAO-A substrate 5-[¹⁴C]hydroxytryptamine (5-[¹⁴C]HT; 0–500 μ M) in the absence or presence of the irreversible MAO inhibitor pargyline (10 μ M), as described in MATERIALS AND METHODS. Data show representative saturation curves of 5-[¹⁴C]HT oxidative deamination in heart homogenates at the ages of 1, 3, 6 and 24 mo. Maximal velocity (V_{max} ; in pmol·min⁻¹·mg protein⁻¹) and K_m (μ M) values are means \pm SE; $n = 4$ animals. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. 1 mo; § $P < 0.05$ and §§§ $P < 0.001$ vs. 3 mo; # $P < 0.05$ and ## $P < 0.01$ vs. 6 mo.

As observed for 5-[¹⁴C]HT oxidation, β -[¹⁴C]PEA degradation increased with age (Fig. 3A). However, β -PEA oxidation was a linear function and was inhibited by the MAO-B inhibitor RO-19 6327 with an affinity ($IC_{50} = 286 \pm 1.3 \mu$ M) lower than that reported for MAO-B (7). In contrast, at all the ages tested, β -PEA degradation was inhibited with high affinity by the MAO-A inhibitors clorgyline ($IC_{50} = 14.5 \pm 1.1 \mu$ M) and RO-41 1049 ($IC_{50} = 33 \pm 1.2 \mu$ M) (Fig. 3B). These results indicate that, at all the ages tested, MAO-B cannot be quantified using β -PEA and that the increase in β -PEA oxidation with aging is unrelated to a modification of MAO-B expression. This is further demonstrated by the lack of detection of MAO-B protein by Western blot (Fig. 3C). The selective age-dependent modification of MAO-A expression and the lack of MAO-B regulation with age were further confirmed by Western blot and semiquantitative RT-PCR analysis. Indeed, Western blots (Fig. 4) showed an immunoreactive 60-kDa band corresponding to MAO-A in crude total membrane fractions of 1-mo-old rats. The quantification of MAO-A protein, expressed as the ratio of the optical densities relative to MAO-A and mitochondrial β -ATPase bands, showed that MAO-A increased progressively with age, reaching the maximum in 24-mo-old rats (4-fold at 6 mo and 6-fold at 24 mo vs. 1 mo). In contrast, the 55-kDa immunoreactive band corresponding to MAO-B was undetectable at all the ages tested. The results of Western blot analysis were confirmed by semiquantitative RT-PCR analysis.

GADPH, as previously reported (48), was used as a reference to normalize the changes in expression of MAO-A and MAO-B genes. As shown in Fig. 5, the intensity of RT-PCR products corresponding to MAO-A significantly increased with age, being 2.9-fold and 4-fold more intense in 6- and 24-mo-old rats compared with 1-mo-old rats, respectively. Semiquantitative RT-PCR allowed the amplification of a cDNA fragment corresponding to MAO-B. The identity of this amplification product was confirmed by DNA sequencing. The intensity of this RT-PCR product was much lower than that corresponding to MAO-A and was not modified by aging.

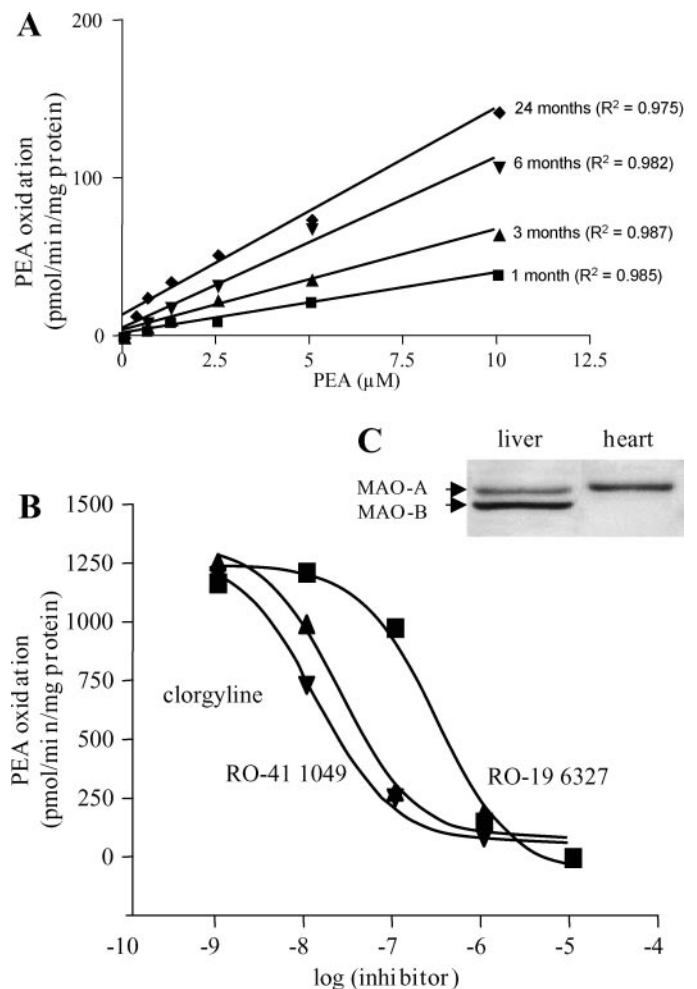


Fig. 3. MAO-B activity in the aging heart. A: MAO-B activity assessed by oxidation of β -[¹⁴C]phenylethylamine (β -[¹⁴C]PEA; 0–10 μ M) in the absence or presence of the irreversible MAO inhibitor pargyline (10 μ M), as described in MATERIALS AND METHODS. R^2 values show that β -PEA oxidative deamination was a linear function of β -PEA concentration. The plot is representative of four independent experiments. B: data showing the inhibition curves of 10 μ M β -[¹⁴C]PEA oxidative deamination by clorgyline (\blacktriangledown), RO-41 1049 (\blacktriangle), or RO-19 6327 (\blacksquare) in heart crude total membrane fractions of 3 mo old rats. The plot is representative of four independent experiments. C: heart and liver crude total membrane fractions of 3-mo-old rats immunoblotted with the rabbit polyclonal anti-MAO-A/MAO-B antiserum, as described in MATERIALS AND METHODS. Blot is representative of four independent experiments.

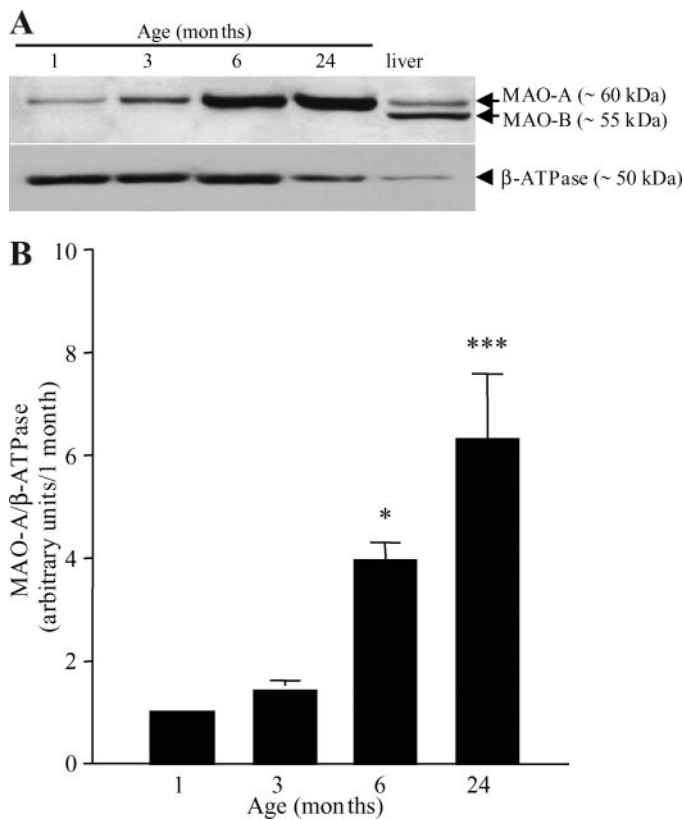


Fig. 4. MAO protein expression in aging heart. Western blots were performed on the rat heart crude total membrane fractions of 1-, 3-, 6-, and 24-mo-old rats. Thirty micrograms of crude extract were loaded onto gels for electrophoresis and processed as described in MATERIALS AND METHODS. The liver sample was used as a control to locate MAO-A and MAO-B bands at 60 and 55 kDa, respectively. The histograms represent the ratio of optical densities corresponding to cardiac MAO-A and β -ATPase bands, expressed as means \pm SE; $n = 4$ animals. * $P < 0.05$ and *** $P < 0.001$ vs. 1 mo.

DISCUSSION

In the present study, we show that MAO-A is the major source of MAO-dependent H₂O₂ production in the rat heart. We also demonstrated that cardiac H₂O₂ generation by MAO-A strongly increases with age. In contrast, we found that MAO-B does not contribute to cardiac H₂O₂ generation in young, adult, and old rats.

Oxidative stress plays a pivotal role in the development of cardiac diseases (16, 43). One of the most classical examples demonstrating the impact of ROS in cardiac injury is postischemia-reperfusion syndrome. This situation occurs frequently, as seen by the large number of patients treated by coronary angioplasty and heart transplantation. In such conditions, ROS may participate in the apoptotic and necrotic cell death, leading to the irreversible loss of cardiac tissue and, in many cases, to heart failure (25).

In addition to postischemia-reperfusion syndrome, H₂O₂ generated by MAOs may also participate in cardiomyocyte dysfunctions associated with left ventricular hypertrophy and/or dilatation (11, 26, 35, 43). This modification of normal left ventricular structure and function, which is typical of different pathologies

whose frequency increase with age (i.e., hypertension, myocardial infarction, and valvulopathies), is related to ventricular hemodynamic overload and reactive cardiac remodeling. Several studies have shown that ROS play a critical role in cardiomyocyte hypertrophy and apoptosis, two processes leading to left ventricular remodeling and cardiac failure (11, 26, 35, 43). The increase in cardiac ROS in ventricular hypertrophy and dilatation has been related, in part, to the decrease in the expression of the antioxidant enzyme superoxide dismutase (1, 6, 15, 44). However, it has been suggested that a concomitant decrease in the antioxidant systems and an increase in ROS production may be necessary to induce oxidative stress and cell damage in cardiomyocytes. At present, the intracellular sources responsible for the increase in ROS production in ventricular hypertrophy and heart failure have not been clearly identified. Despite the therapeutical properties of the xanthine oxidase inhibitor allopurinol in the prevention of ventricular hypertrophy and dilatation (12, 17), the low expression of this enzyme in cardiomyocytes makes questionable its role in the promotion

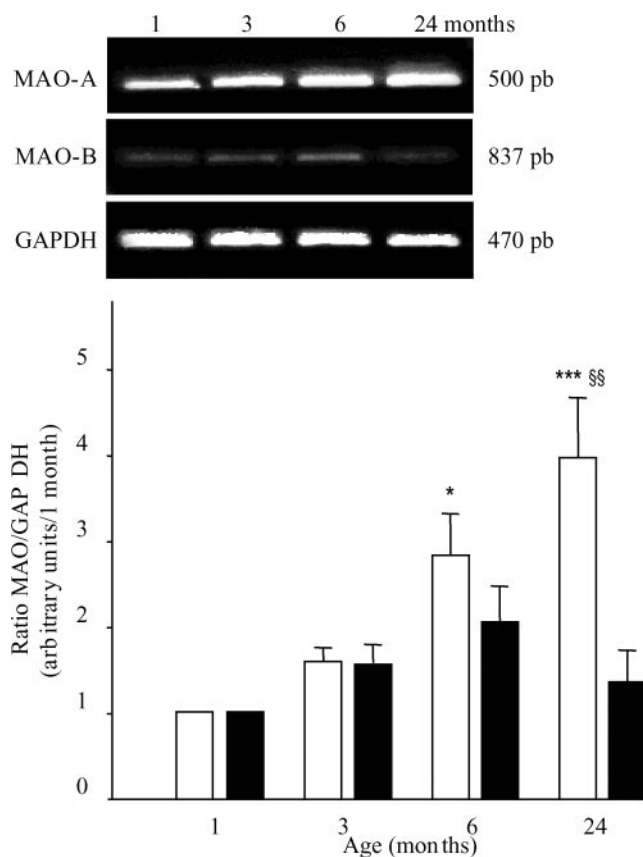


Fig. 5. MAO mRNA expression in the aging heart. Semiquantitative RT-PCR was performed, as described in MATERIALS AND METHODS, from isolated total RNA of rat hearts at the age of 1, 3, 6, and 24 mo. The relative expression of MAO mRNA was determined as the ratio of optical densities of MAO (MAO-A, open bars; MAO-B, solid bars) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) bands in the heart. The histograms represent the means \pm SE of four separate experiments. * $P < 0.05$ and *** $P < 0.001$ vs. 1 mo; §§ $P < 0.01$ vs. 3 mo.

of oxidative stress-dependent cell damage. The cardiac homologs of phagocyte NADPH oxidase subunits have been recently described in cardiac myocytes (22). In phagocytes, NADPH oxidase is responsible for a large production of O₂⁻ and H₂O₂ by the means of superoxide dismutase. Converging evidence supports the potential role of cardiac NADPH oxidase in cardiomyocyte hypertrophy and apoptosis. However, additional studies are necessary to define the functional properties of this enzyme in the heart and its involvement in physiological and pathological situations. Although both xanthine and NADPH oxidases are potentially relevant in term of ROS generation in the heart, their role in cardiac dysfunction associated with aging is far from being evident. Indeed, at present, there is no strong evidence showing an age-dependent increase in cardiac xanthine and NADPH oxidase activities.

Cardiomyocytes are extremely rich in mitochondria, and these organelles are one of the major sources of ROS. In different situations, the impairment of the mitochondrial respiratory chain leads to leakage of electrons to oxygen to form O₂⁻. In different animal models of cardiac hypertrophy or dilatation, the increase in ROS production is related to mitochondrial dysfunction (32, 50). On the other hand, a decline in mitochondrial function leading to enhanced oxidant production has been described in different aging processes, not only in the heart but also in various organs (38). The mechanisms responsible for mitochondrial decay have not been entirely identified. Recent studies performed in isolated mitochondria have suggested that H₂O₂ generated by MAOs during substrate degradation may be involved in the impairment of the mitochondrial respiratory chain (14). According to these results, we have recently shown that, in epithelial renal cells, H₂O₂ production by MAOs is responsible for cytochrome *c* release by mitochondria, leading to the activation of the proapoptotic cascade (P. Bianchi, M. H. Séguélas, A. Parini, and C. Cambon, personal communications). With the use of a rat model of renal ischemia-reperfusion, we confirmed the essential role of H₂O₂ generated by MAOs in postreperfusion tissue injury (24). Indeed, we showed that in the kidney, MAO inhibition dramatically prevented oxidative stress, activation of the proapoptotic cascade, and cell apoptosis/necrosis occurring after reperfusion. Experiments in progress in our laboratory also strongly support the role of MAOs in postischemia-reperfusion tissue injury in the heart. The demonstration that, in the heart, H₂O₂ generation by MAOs is dramatically increased in old rats proposes these enzymes as one of the major sources of ROS involved in age-related mitochondrial dysfunction and oxidative stress.

In addition to the demonstration that MAOs may play a role in cardiac dysfunctions associated with aging, our results supply new insights on the characteristics of MAOs expressed in the heart and in the mechanisms of their regulation. Enzyme assay and Western blot analysis did not reveal the expression of functional MAO-B protein in the rat heart. According to previous results (49), we showed that degradation of

the MAO-B substrate β-PEA is provided by MAO-A and that this peculiarity is maintained during heart development and aging. The unusual substrate specificity of MAO-A suggested the expression of an atypical form of the MAO-A isoenzyme in the rat heart (49). In our study, Western blot analysis of heart crude total membrane fractions with the polyclonal anti-MAO-A/MAO-B antiserum revealed a single band with an apparent molecular weight corresponding to that of "classic" MAO-A. In addition, semiquantitative RT-PCR analysis with primers covering the entire coding region of MAO-A cDNA did not reveal amplification products different from those of MAO-A (T. Guimaraes, C. Vindic, and A. Parini, personal communications). These results suggest that the unusual substrate selectivity of rat cardiac MAO-A may be related to a particular conformational state of MAO-A rather than to the expression of an additional form of the isoenzyme.

We have previously shown that, in the rat kidney, the change in MAO-A activity during development or steroid treatment was more likely related to modification of substrate accessibility rather than to a difference in protein expression (8). Our results suggest that the mechanisms of development and age-dependent regulation in the heart differ from those observed in the kidney. Indeed, Western blot analysis showed an age-dependent increase in MAO-A protein concomitant to an augmentation in the intensity of MAO-A cDNA fragments amplified and quantified by semiquantitative RT-PCR analysis. Therefore, in the heart, the increase in MAO-A activity seems to be related to a modification of the transcriptional rate of the MAO-A gene and/or to a higher stability of the corresponding mRNA. It is noteworthy that, although we have not been able to show the expression of MAO-B protein in the heart, we could detect a weak RT-PCR amplification product corresponding to MAO-B. At present, we cannot define whether these results reflect a very low expression of MAO-B protein in the heart. Nevertheless, it is clear that the intensity of the MAO-B RT-PCR products did not change with age, making unlikely the potential role of the MAO-B isoenzyme in the increase of MAO-dependent H₂O₂ generation in the heart.

In conclusion, the demonstration that MAO-A-dependent H₂O₂ production strongly increases in the senescent heart proposes this enzyme as one of the major factors involved in cardiac oxidative stress during aging. Additional experiments are needed to define the functional role of MAO-A in physiological and pathological situations. It is conceivable that the inhibition of MAOs may represent a therapeutic approach to prevent cardiac hypertrophy and dilatation, two diseases associated with aging and induced, at least in part, by oxidative stress.

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