Serotonin produces monoamine oxidase-dependent oxidative stress in human heart valves

Ricardo A. Peña-Silva,1,3 Jordan D. Miller,2 Yi Chu,2 and Donald D. Heistad1,2,4

Departments of 1Pharmacology and 2Internal Medicine, University of Iowa Carver College of Medicine, Iowa City School of Medicine, Iowa City; 3Veterans Affairs Medical Center, Iowa City, Iowa; and 4Universidad de los Andes, Bogotá, Colombia

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Peña-Silva RA, Miller JD, Chu Y, Heistad DD. Serotonin produces monoamine oxidase-dependent oxidative stress in human heart valves. Am J Physiol Heart Circ Physiol 297: H1354–H1360, 2009.—Heart valve disease and pulmonary hypertension, in patients with carcinoid tumors and people who used the fenfluramine-phenetermine combination for weight control, have been associated with high levels of serotonin in blood. The mechanism by which serotonin induces valvular changes is not well understood. We recently reported that increased oxidative stress is associated with valvular changes in aortic valve stenosis in humans and mice. In this study, we tested the hypothesis that serotonin induces oxidative stress in human heart valves, and examined mechanisms by which serotonin may increase reactive oxygen species. Superoxide (O2⁻) was measured in heart valves from explanted human hearts that were not used for transplantation. O2⁻ levels (lucigenin-enhanced chemoluminescence) were increased in homogenates of cardiac valves and blood vessels after incubation with serotonin. A nonspecific inhibitor of flavin-oxidases (diphenyliodonium), or inhibitors of monoamine oxidase [MAO (tranylcypromine and clorgyline)], prevented the serotonin-induced increase in O2⁻. Dopamine, another MAO substrate that is increased in patients with carcinoid syndrome, also increased O2⁻ levels in heart valves, and this effect was attenuated by clorgyline. Apocynin [an inhibitor of NAD(P)H oxidase] did not prevent increases in O2⁻ during serotonin treatment. Addition of serotonin to recombinant human MAO-A generated O2⁻, and this effect was prevented by an MAO inhibitor. In conclusion, we have identified a novel mechanism whereby MAO-A can contribute to increased oxidative stress in human heart valves and pulmonary artery exposed to serotonin and dopamine.

serotonin; reactive oxygen species; carcinoid syndrome; superoxide; valvulopathy

CARCINOIND VALVE DISEASE is present in ~50–60% of patients with the carcinoid syndrome (2, 39, 54). Several circulating hormones including serotonin (5-hydroxytryptamine) and dopamine are released by carcinoid tumors (2, 18, 23, 44). Serotonin is released by carcinoid tumors, and its metabolism is associated with development and progression of myxomatous changes in heart valves and pulmonary hypertension (12, 38, 47). Similar findings were described in patients taking fenfluramine-phenetermine for weight control (10, 16, 48, 52), pergolide for Parkinson’s disease (60, 61), and other drugs such as ergot derivatives (48). Interestingly, fenfluramine also increases circulating serotonin levels (49). The combination fenfluramine-phenetermine was removed from the market in 1997, and pergolide was removed in 2007, after Food and Drug Administration advisories about increased risk of valvular disease. However, mechanisms whereby serotonin elicits myxomatous valvular diseases have remained poorly understood.

Several studies in animals suggest a role for serotonin in carcinoid heart disease and drug-induced valve disease. Tryptophan hydroxylase I, the limiting enzyme in serotonin synthesis, is increased in canine myxomatous valve disease (14). In rats exposed to long-term administration of serotonin, increased cell proliferation and thickening of the heart valves, that resembles the changes reported in patients with carcinoid heart disease, are observed (15, 20). In addition, mice with the serotonin transporter gene knocked out also manifest valvular dysfunction, hyperplasia, and fibrosis of the valve leaflets (34). In several cell types, including valvular interstitial cells (21, 43) and vascular smooth muscle cells (SMC) (9, 28–30), serotonin increases cell proliferation. There is evidence for activation and nuclear translocation of mitogen-activated protein kinases, transforming growth factor-β1, and other proliferative pathways by serotonin (28–30).

Reactive oxygen species (ROS), especially superoxide (O2⁻), appear to participate in serotonin-induced mitogenesis (19, 26, 28–30). Antioxidants can prevent the mitogenic effects of serotonin (19, 26, 28–30). Nicotine adenine dinucleotide phosphate oxidase [NAD(P)H oxidase] may be a significant source of O2⁻ after stimulation of SMC with serotonin, because inhibitors of NAD(P)H oxidase such as diphenyliodonium (DPI) inhibit proliferative signals in response to serotonin in pulmonary artery SMC and rat mesangial cells (19, 29, 30). Furthermore, recent evidence suggests that ROS may contribute to development of structural changes in stenotic aortic valves in humans and mice (36, 37). However, it is not known if serotonin increases oxidative stress in heart valves. Thus it is possible that increased oxidative stress in heart valves exposed to high concentrations of serotonin might increase proliferation of valve interstitial cells and contribute to the development of myxomatous valve disease.

In this study, we tested the hypothesis that serotonin increases ROS in human heart valves and explored mechanisms for production of ROS by serotonin in heart valves. We found that high concentrations of serotonin increase O2⁻ in heart valves and blood vessels. Because amines, including serotonin and dopamine, are metabolized by monoamine oxidase (MAO) (4, 42, 55), we incubated the valves with an MAO inhibitor to prolong and augment the effects of serotonin. Surprisingly, the MAO inhibitor greatly reduced the serotonin-induced increase in O2⁻ in cardiac valves and blood vessels. We also found that MAO-dependent metabolism of dopamine increased O2⁻ lev-
els. We used pharmacological interventions and recombinant human protein to demonstrate that MAO-A-mediated degradation of serotonin can be a critical contributor to O$_{2}^{{-}}$ production in heart.

**METHODS**

Normal human cardiac valves (pulmonary, tricuspid, and mitral) and proximal segments of pulmonary artery and aorta were obtained from donor hearts that were not suitable for transplantation. Hearts were obtained through the Iowa Donor Network and the National Disease Research Interchange (Philadelphia, PA) <12 h after organ harvesting and maintained in cold University of Wisconsin solution as described previously (36). Because clinical information was not obtained from the donor patients (except for age and sex), the University of Iowa Institutional Review Board indicated that informed consent was not required from each patient. Tissue was homogenized in a cocktail of protease inhibitors in PBS and stored at −80°C until analysis.

**O$_{2}^{{-}}$ measurement.** Homogenized heart valve and vascular tissue were used to examine the levels of O$_{2}^{{-}}$ using lucigenin-enhanced chemoluminescence. Tissue homogenates have been used in the past to study the activity of MAO in several animal tissues, including brain, heart, and liver (1, 5, 17, 22). Homogenates from pulmonary artery were used as a positive control because it is known that serotonin increases O$_{2}^{{-}}$ in this tissue (28–30). Protein was quantified in each sample. Tissue homogenate containing 250 µg of protein was placed in a cuvette containing 5 µM lucigenin in PBS to obtain a total volume of 500 µl. Samples were then incubated in the presence of serotonin at 37°C in a mixture of 95% O$_{2}$ and 5% CO$_{2}$ for 4 h. Oxygen concentration was calibrated with an oxygen analyzer (Beckman OM11). These conditions have been used for examination of MAO in several animal tissues, including brain, heart, and liver (1, 5, 17, 22). Oxygen concentration was calibrated with an oxygen analyzer (Beckman OM11). These conditions have been used for examination of MAO in several animal tissues, including brain, heart, and liver (1, 5, 17, 22). Oxygen concentration was calibrated with an oxygen analyzer (Beckman OM11). These conditions have been used for examination of MAO in several animal tissues, including brain, heart, and liver (1, 5, 17, 22).

Enzyme inhibitors were added to the samples 30 min before the addition of serotonin and maintained during the incubation period. The following drugs were used: 10 µM DPI (a nonspecific flavin-containing enzyme inhibitor), 10 µM N$^{6}$-nitro-l-arginine methyl ester [L-NAME, an inhibitor of all three isoforms of the nitric oxide synthase (NOS)], 10 µM allopurinol (a xanthine oxidase inhibitor), 100 µM apocynin [an NAD(P)H oxidase inhibitor], 10 µM indomethacin (a cyclooxygenase inhibitor), 200 U/ml catalase (36), 1–10 µM tranylcypromine (an MAO-A/B inhibitor), and 1 µM clorgyline (an MAO-A inhibitor) (56). All reagents were obtained from Sigma.

In addition, some samples were incubated with 1 mM dopamine dissolved in PBS. Clorgyline (1 µM) was added 30 min before dopamine to inhibit MAO. Luminescence in each vial was read in a luminometer after 4 h of incubation.

To test the specificity of the MAO inhibitors, we examined effects of the inhibitors on NAD(P)H oxidase, which is another potential source of O$_{2}^{{-}}$ that has been associated with serotonin-induced oxidative stress, in pulmonary artery SMC (29, 30). For the evaluation of NAD(P)H oxidase-mediated O$_{2}^{{-}}$ production, homogenates were preincubated for 30 min in the presence of DPI, tranylcypromine, clorgyline, or vehicle (PBS). Samples were exposed to 100 µM NAD(P)H, and luminescence in each cuvette was measured.

**O$_{2}^{{-}}$ generation by recombinant MAO-A.** Recombinant human MAO-A (Sigma) was incubated with serotonin for 4 h in 5 µM lucigenin in PBS at 37°C in a mixture of gases of 95% O$_{2}$ and 5% CO$_{2}$. MAO-A was preincubated in the presence of clorgyline (10 µM) or PBS (vehicle) for 30 min before the addition of 100 µM serotonin or PBS (control). O$_{2}^{{-}}$ generation was detected by lucigenin-enhanced chemoluminescence in a luminometer.

**Electron paramagnetic resonance.** Recombinant human purified 100 µg/ml MAO-A (Sigma) was added to a solution containing 0.1 mM serotonin or vehicle (PBS), 100 mM phosphate buffer, 250 µM of the iron-chelating agent diethylenetriaminepentaacetic acid (DETPAC), 1% albumin, and 50 mM 5,5-dimethylpyrroline-1-oxide (DMPO) as a spin trap. Electron paramagnetic resonance (EPR) was performed with a Bruker EMX spectrometer. Data were collected during 30 min at room temperature and ambient air. EPR parameters were 3510.3 G center field; 80 G scan width; 9,854 GHz microwave frequency; 20 mW power; 2 × 10$^{5}$ receiver gain; modulation frequency of 100 kHz; modulation amplitude of 1.0 G; with the conversion time and time constant both being 40.96 ms with five scans for each 1,024-point spectrum.

**Quantitative real-time RT-PCR.** Heart valve and vascular tissues were homogenized in 0.5 ml of TRIzol (Invitrogen) and stored at −80°C until collection was complete. RNA extraction, quantification, and RT were performed as described previously (8). RT (1 µl) was used for TaqMan real-time PCR to obtain the cycle threshold ($C_{t}$) for MAO-A and β-actin. TaqMan Primers/probes were obtained from Applied Biosystems. Expression levels of MAO-A were determined relative to that of β-actin (which was constant among samples), using the $ΔΔC_{t}$ method as described previously (8, 36).

**Statistics.** Results are expressed as means ± SE. Statistical significance was determined by one-way ANOVA and post hoc analysis with the Tukey test using the statistical program SAS (SAS Institute, Cary, NC) and VassarStats calculator (Vassar College, Poughkeepsie, NY). A significant difference was considered as $P < 0.05$.

**RESULTS**

Incubation of homogenates of human heart valves (tricuspid and pulmonary) and pulmonary artery with 100 µM serotonin significantly increased levels of O$_{2}^{{-}}$ (Fig. 1). Serotonin also increased O$_{2}^{{-}}$ levels in the mitral valve and proximal aorta.

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**Fig. 1.** Superoxide levels are increased in human tricuspid and pulmonary valves, and pulmonary artery, after incubation with serotonin (5-HT, 10 µM and 100 µM) compared with control tissue [incubated with vehicle (PBS)]. *$P < 0.05$ vs. control; $n = 8–10$ valves and pulmonary arteries.
MAO-A was also studied with EPR. A DMPO-OH signal, produced a significant increase in O$_2^-$ in heart valves and pulmonary arteries. Apocynin [an inhibitor of NAD(P)H oxidase] did not attenuate the increase in O$_2^-$ in response to serotonin in any of the tissues (Fig. 4). Tranylcypromine and clorgyline also prevented the increase in O$_2^-$ after treatment with serotonin in mitral valve and aorta (Supplemental Fig. 1).

Addition of exogenous NADPH to homogenates of tricuspid or pulmonary valves or pulmonary artery increased O$_2^-$ (Supplemental Fig. 3). Preincubation with DPI [NAD(P)H oxidase and flavin oxidases inhibitor] attenuated the increase in O$_2^-$ and MAO inhibitors (tranylcypromine and clorgyline) did not attenuate the increase in O$_2^-$ after addition of NADPH to homogenates of cardiac valves or pulmonary artery.

Incubation of recombinant human MAO-A with serotonin produced a significant increase in O$_2^-$ levels, which was attenuated by tranylcypromine (Fig. 5). Recombinant human MAO-A was also studied with EPR. A DMPO-OH signal, suggestive of the presence of O$_2^-$ in the sample, was obtained when serotonin and MAO-A were added together (Fig. 6).

Incubation of pulmonary valve homogenates with another MAO-A substrate, dopamine, increased O$_2^-$ significantly (Fig. 7). The dopamine-induced increase in O$_2^-$ was significantly attenuated by clorgyline.

**DISCUSSION**

Elevated levels of serotonin are associated with development of myxomatous valve disease and pulmonary artery hypertension by mechanisms that are currently not well understood (2, 11, 38, 47). Because recent findings from our laboratory suggest a role for oxidative stress in the pathogenesis of aortic valve disease (36, 37), we hypothesized that oxidative stress may be found in heart valves exposed to high concentrations of serotonin. In the present study, we report two major findings. First, high concentrations of serotonin or dopamine increase O$_2^-$ radicals in human heart valves and in vascular tissue. Second, MAO-A is a novel source of O$_2^-$ in human heart valves. The data support a model in which increased O$_2^-$, derived from metabolism of amines by MAO-A, may contribute to the pathogenesis of valve disease.

About two-thirds of patients with carcinoid syndrome, especially those who tend to have the highest concentrations of serotonin in plasma (47), and high serotonin metabolism (38), develop carcinoid heart disease. Carcinoid heart disease in humans and in animal models is characterized by cellular proliferation, fibro-myxoid changes, and thickening of heart valves (2, 11, 15, 20, 34, 38, 47, 54). These changes are more frequent in the tricuspid and pulmonary valves than in the left side valves (2, 38, 39), presumably because lungs are a major site of metabolism for serotonin and other amines (24, 41), and the aortic and mitral valves therefore are exposed to lower concentrations of serotonin. In the present study, we examined human cardiac valves, and proximal segments of pulmonary artery and aorta. We focused especially on the tricuspid and pulmonary valves, and the pulmonary artery, because they are more commonly involved in carcinoid heart disease.
The data indicate that serotonin increases $O_2^-$ in heart valves and blood vessels. Data from cultured pulmonary artery SMC suggest that $O_2^-$ may participate in serotonin-induced mitogenesis in multiple pathways including: 1) activation and translocation of mitogen-activated protein kinases (28), and the phosphatidylinositol 3-kinase pathway (29); 2) activation of cell cycle proteins (53); and 3) transactivation of other mitogenic receptors such as the platelet-derived growth factor receptor (30). Antioxidants attenuate the mitogenic effects of serotonin in pulmonary artery SMC (28–30). Similarly, dexfenfluramine-induced proliferation of SMC requires ROS and is attenuated by antioxidants (27). Therefore, we speculate that serotonin or fenfluramine-induced oxidative stress may also play a critical role in proliferation of valve interstitial cells and valvular thickening in vivo.

A key finding in this study is that serotonin-mediated increases in $O_2^-$ are not primarily dependent on activation of NAD(P)H oxidase. Some studies in which DPI [an NAD(P)H oxidase inhibitor] was used concluded that NAD(P)H oxidase. Some studies in which DPI [an NAD(P)H oxidase inhibitor] was used concluded that NAD(P)H oxidase. Some studies in which DPI [an NAD(P)H oxidase inhibitor] was used concluded that NAD(P)H oxidase is the primary source of $O_2^-$ in serotonin or dexfenfluramine-induced oxidative stress in cultured pulmonary artery SMC (27, 29, 30). DPI, however, inhibits multiple flavin oxidases and is not specific for NAD(P)H oxidase (45). Apocynin [a somewhat more specific inhibitor of NAD(P)H oxidase] did not attenuate the increase in $O_2^-$ mediated by serotonin.

A major finding in this study is that the increase in $O_2^-$ induced by serotonin was attenuated by inhibition of the flavin-containing enzyme MAO (46). Serotonin is metabolized avidly by MAO, which is localized in the outer mitochondrial membrane (4, 42, 55). MAO is present in two isoforms, MAO-A (which is expressed in a variety of tissues, including brain, liver, heart, kidney, and blood vessels and catalyzes the degradation of serotonin, dopamine, and norepinephrine) and MAO-B (which is expressed predominantly in the central nervous system and degrades dopamine and phenylalanine) (4, 55). In this study, we found that MAO-A was expressed in tricuspid and pulmonary valves, and that serotonin-induced oxidative stress, it was

Although the chemistry of metabolism of serotonin and other amines by MAO is not clear (51), it appears that ROS are released as a byproduct during the reaction (58, 59). In another flavin oxidase, xanthine oxidase, the reaction with xanthine produces hydrogen peroxide or $O_2^-$.

Generation of different ROS varies with electron flux through the flavin group (6, 32). We speculate that a similar mechanism may exist for MAO.

MAO-dependent oxidative stress is associated with multiple pathological processes, including proliferation of SMCs (9) and renal epithelial cells (57), cardiomyocyte hypertrophy (3), and renal ischemia-reperfusion injury (25). Oxidative stress produced by MAO-dependent degradation of amines is also increased in aged hearts in rats (31). Therefore, ROS generated from MAO-dependent degradation of serotonin may be important for the understanding of proliferation of valve interstitial cells in carcinoid heart disease and drug-induced valvulopathies.

We found that, in homogenates of cardiac valves, MAO (and not other oxidases), appears to be the primary source of $O_2^-$ after stimulation with serotonin. Because we used two different MAO inhibitors (tranylcypromine and clorglyline) to study the role of MAO in serotonin-induced oxidative stress, it was important to test the selectivity of these compounds. This is critical, because it is known that tranylcypromine may inhibit

Fig. 4. Inhibition of MAO with tranylcypromine (TC, 10 μM) significantly attenuated the increase in superoxide in tricuspid and pulmonary valves, and pulmonary artery homogenates incubated with 100 μM 5-HT. A specific inhibitor of MAO-A [1 μM clorglyline (Clorg)] also attenuated increases in superoxide. Vehicle was PBS at pH 7.4. P < 0.05 vs. control (*) and vs. 5-HT only-Veh (#); n = 4 for tricuspid valve, n = 6 for pulmonary valve, and n = 7 for pulmonary artery. Analysis used controls shown in Fig. 1.

Fig. 5. MAO-A-derived superoxide. Superoxide is generated by human recombinant purified MAO-A incubated with 1 mM 5-HT or PBS as vehicle (control). The increase in superoxide was markedly attenuated by coincubation with an inhibitor of MAO (10 μM tranylcypromine). Results were obtained from 2 independent experiments, with 2 samples of MAO in each.

MAO-INDUCED OXIDATIVE STRESS IN HEART VALVES

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Fig. 6. Superoxide generation by MAO-A. **Left:** 1 mM 5-HT dissolved in a buffer solution [1% albumin, 50 mM 5,5-dimethylpyrroline-1-oxide (DMPO), 250 mM DETAPAC] was analyzed in the spectrometer. There is no change in the baseline recording during a 30-min period. **Right:** a signal indicative of DMPO-OH adducts (product of the DMPO + superoxide reaction) appears when human purified recombinant MAO-A is added to a buffer solution containing 1 mM 5-HT. Figure shows a representative trace.

Fig. 7. Dopamine-induced increase in superoxide in homogenates of human pulmonary valve (n = 3). Dopamine (1 mM) significantly increased superoxide levels in pulmonary valve compared with control tissue (incubated with PBS). The increase in superoxide produced by dopamine was attenuated by an MAO-A inhibitor (1 μM clorgyline). P < 0.05 vs. control (*) and vs. dopamine treatment (#).
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