Long-Term Serotonin Administration Induces Heart Valve Disease in Rats

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Background—The purpose of this study was to investigate whether rats dosed with serotonin develop changes similar to those seen in human carcinoid heart disease.

Methods and Results—Ten Sprague-Dawley rats were given serotonin injections subcutaneously once daily for 3 months; controls were given saline. A long-lasting hyperserotonemia with a >10-fold increase in both platelet-poor plasma and dialysate from the femoral muscles appeared. The animals developed clinical signs such as flushing and loose stools. After 3 months, 6 of 10 rats given serotonin had pathological echocardiographs. Two animals had a combination of aortic and pulmonary valve insufficiency, 1 had isolated aortic valve insufficiency, and 3 had isolated pulmonary valve insufficiency. Histopathological examination revealed shortened and thickened aortic cusps and carcinoidlike plaques characterized by a collection of myofibroblasts within an extracellular matrix of collagen ground substance. Immunostaining for Ki-67 demonstrated an increased number of proliferating subendocardial cells. In the control group, no pathological changes were seen. With the use of reverse-transcription polymerase chain reaction, normal rat aortic cusps were shown to express mRNA for serotonin receptors 5-HT1A, 5-HT2A, and 5-HT2B and the serotonin transporter 5-HTT.

Conclusions—For the first time, long-term serotonin administration was performed in rats. Morphological and echocardiographic changes similar to those seen in human carcinoid heart disease developed. This study demonstrates that serotonin most likely is involved in the pathogenesis of carcinoid heart disease. (Circulation. 2005;111:1517-1522.)

Key Words: carcinoid heart disease ● receptors ● serotonin ● valves

Serotonin [5-hydroxytryptamine (5-HT)] is a well-known neurotransmitter. Outside the central nervous system, serotonin is produced mainly by the enterochromaffin cells of the gut and participates in the regulation of intestinal motility, fluid secretion, and regional blood flow. After release, serotonin is rapidly taken up by an active transport mechanism into a number of cell types, with platelets serving as the major reservoir.

Midgut carcinoids are derived from the enterochromaffin cells of the gut. The primary tumor is often small and asymptomatic because its hormonally active secretagogues (serotonin, neuropeptide K, bradykinin, and substance P) are broken down by first-pass metabolism in the liver. When metastasizing to the liver, hormonally active tumor products are secreted into the liver vein, giving rise to the carcinoid syndrome with vasomotor symptoms, bronchospasm, diarrhea, and in 50% of the patients, valvular heart disease. In a study at the Mayo Clinic, preoperative examination of 75 patients with carcinoid heart disease revealed tricuspid regurgitation in all 75, tricuspid stenosis in 15, pulmonary valve regurgitation in 58, pulmonary stenosis in 50, mitral regurgitation in 33, and aortic regurgitation in 24 patients.

Histologically carcinoid plaques are seen in areas subjected to the greatest concentrations of tumor products. Carcinoid plaques contain subendocardial deposits of myofibroblasts, fibroblasts, and smooth muscle cells in a myxoid matrix. The origin of this unique valvular derangement remains unknown, but among patients with the carcinoid syndrome, those with heart disease have higher levels of tachykinin and serotonin in serum and 5-hydroxyindoleacetic acid (5-HIAA) in urine. Serotonin is broken down by monoaminooxidase in the liver and lungs, which may explain the predominance of right-sided carcinoid heart lesions when tumor products are secreted into the liver vein.

Studies on cell cultures have shown that serotonin has mitogenic effects on fibroblasts, smooth muscle cells, and...
osteoblasts, mesangial cells, and endothelial cells. Serotonin mediates its actions by interaction with multiple serotonin receptor subtypes. Serotonin and serotonin receptors play a crucial role in normal heart embryogenesis. Ablation of the 5-HT2B receptors in mice leads to abnormal cardiac development with hypoplastic ventricles. The presence of serotonin receptors in human cardiac valves suggests that valvular interstitial cells have the potential to respond to serotonin. It has recently been shown that serotonin induces upregulation of transforming growth factor-β in aortic valve interstitial cells via 5-HT1 receptors and that transforming growth factor-β stimulates glycosaminoglycan production in sheep aortic valve interstitial cells.

In the present study, we have subcutaneously injected rats with serotonin once daily for 3 months. The aim of the study was to investigate whether long-term serotonin overload in rats leads to pathological changes similar to those seen in human carcinoid heart disease.

Methods

Animals

The Animal Welfare Committee at Trondheim University Hospital approved this study. Forty-eight Sprague-Dawley female rats (200 g) were housed solely in wire-top cages with aspen woodchip bedding from B&K Universal Ltd. Room temperature was 24±1°C with a relative humidity of 40% to 50% and a 12-hour light/dark cycle. The Rat and Mouse Diet of B&K and tap water were provided ad libitum.

Before procedures, the animals were anesthetized with 2 mL/kg body weight of a combination of fluanison (2.5 mg/mL), fentanyl (0.05 mg/mL), and midazolam (1.25 mg/mL). During echocardiography, animals were anesthetized with ketamine hydrochloride (40 mg/kg) and xylazine (8 mg/kg) intraperitoneally. Serotonin (5-Hydroxytryptamine Creatinin Sulfate Complex) purchased from Sigma-Aldrich was dissolved in physiological saline (25 mg/mL) before subcutaneous injection.

Twelve rats were given daily serotonin injections subcutaneously (50 mg/kg for the first 3 days and 20 mg/kg thereafter); 10 controls were given saline. The serotonin was dissolved in saline just before it was injected. To avoid trauma and ulcers of the neck skin, the animals were immobilized in a specially built cage during the injections, which were given strictly subcutaneously with a 30-gauge BD syringe. The last injections were given 1 week before the rats were euthanized. At euthanization, the animals were weighed and decapitated, and blood was collected.

Because of peripheral vasodilatation after the serotonin injections, it was not possible to collect blood by peripheral vein puncture. We therefore performed an additional experiment on 33 animals to determine the serotonin levels in platelet-poor plasma (PPP), whole blood, and dialysate from skeletal muscle.

Echocardiography

The animals were anesthetized and examined with echocardiography 4 days before they were sacrificed. A GE Vingmed Ultrasound System Five scanner with an 8-MHz phased-array probe was used. Two-dimensional color-flow Doppler in the parasternal long-axis and apical views was used to visualize valvular regurgitation, which was confirmed by continuous-wave Doppler recordings. Treatment group identity was unknown during echocardiography.

Histopathology and Immunohistochemistry

The hearts were weighed and fixed in formalin 4% for 24 hours. After fixation, they were cut in the frontal plane, through the aortic arch, and down to the apex; then, they were embedded in paraffin, cut, and stained with hematoxylin-erytrosin and van Gieson. Immunohistochemical staining for proliferating cells was performed with the monoclonal antibody Ki-67, MIB-3 (code M7248, Dako), as described.

Blood Sampling and Microdialysis

To describe the pharmacokinetics of the serotonin administration protocol, we divided 18 animals into 3 different groups, 6 in each group, and then treated them with daily serotonin injections (50 mg/kg SC) for 1, 2, and 5 days respectively. In addition, 3 controls were given saline. In each group, blood was collected from 3 animals (via the inferior vena cava) 24 hours after their last injection. The remaining 3 animals in each group were given an additional serotonin injection, followed by blood sampling 2 hours later.

To obtain PPP and whole blood, we followed a protocol earlier described. We also performed microdialysis to determine the free fraction of serotonin. We assumed that the interstitial serotonin level would reflect the serotonin level in blood. Nine rats were divided into 3 groups and treated with daily serotonin injections for 1, 2, and 5 days before the microdialysis; 3 control animals were given saline. Three hours before an additional and final serotonin injection, a flexible microdialysis probe (MAB 7.80.10; 10-mm membrane length, 0.6-mm outer diameter, 15-kDa cutoff; AgnTho’s AB) was implanted in the femoral muscles. The microdialysis probe was connected to a microinfusion pump (CMA 107, CMA Microdialysis AB) and perfused with perfusion fluid (T1, CMA Microdialysis, Na+ [147 mmol/L], K+ [4 mmol/L], Ca2+ [1.23 mmol/L], Cl− [156 mmol/ L]) at a flow rate of 2 μL/min. After a 60-minute rest, samples were collected at 30-minute intervals for 2 hours and at hourly intervals thereafter for another 4 hours. The samples were protected from light during the whole procedure and immediately frozen at −80°C until further analysis. In vitro recovery of serotonin was 31%.

High-Performance Liquid Chromatography

For serotonin measurements, we developed a sensitive high-performance liquid chromatography (HPLC) analysis method. Serotonin levels were determined in platelet-poor plasma (PPP), whole blood, and dialysate from skeletal muscle.

Primer Sets Used in RT-PCR Analysis of Gene Expression in Rat Aortic Cusps

<table>
<thead>
<tr>
<th>Primers</th>
<th>mRNA Position</th>
<th>Sequence</th>
<th>Product Length, Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT1A</td>
<td>1032–1050</td>
<td>5′-GAAGACTCTGGGCAATCATC-3′</td>
<td>169 bp, 54°C</td>
</tr>
<tr>
<td>5-HT2A</td>
<td>1200–1181</td>
<td>5′-ATAAATACCGGAGCA-3′</td>
<td>190 bp, 52°C</td>
</tr>
<tr>
<td>5-HT2B</td>
<td>1875–1894</td>
<td>5′-CTTCGAGAAGAAGGAACCTTTG-3′</td>
<td>420 bp, 58°C</td>
</tr>
<tr>
<td>5-HT2C</td>
<td>2294–2275</td>
<td>5′-CCCTGTACTCGGACTGATG-3′</td>
<td>190 bp, 52°C</td>
</tr>
<tr>
<td>5-HTT</td>
<td>1101–1020</td>
<td>5′-GAAGAGGCTGGGAGTCTG-3′</td>
<td>190 bp, 52°C</td>
</tr>
<tr>
<td>5-HTC</td>
<td>1209–1190</td>
<td>5′-TCGAGAAGAAGGAGAAAG-3′</td>
<td>498 bp, 60°C</td>
</tr>
<tr>
<td>5-HTT</td>
<td>1135–1153</td>
<td>5′-CACATAGCCATCAAACAAAC-3′</td>
<td>158 bp, 52°C</td>
</tr>
</tbody>
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tonin and internal standard were extracted from the samples with a basic liquid-liquid extraction. The organic phase was collected and the solvent was evaporated; the samples were then reconstituted in methanol. Samples were analyzed by reverse-phase chromatography on an Agilent 1100 LCMS system consisting of a quaternary pump with online degasser, an automatic liquid sampler, a thermostatted column compartment, and a single quadropol mass selective detector using selected ion monitoring. The limit of detection for the HPLC method was found to be 23.5 nmol/L; the limit of quantification was 38.9 nmol/L. The standard deviations for the HPLC at different serotonin concentrations were as follows: 47±5.5, 470±54.2, and 4700±201.1 nmol/L.

Reverse-Transcriptase Polymerase Chain Reaction
Total RNA was isolated from the aortic cusps of 5 control rats with the RNeasy Fibrous Tissue Mini Kit (Qiagen). Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed according to standard procedures with the OneStep RT-PCR kit (Qiagen) with the primers as listed in the Table and 40 cycles of amplification.

Data Analysis
Values are expressed as group mean±SEM. Differences between the 2 groups were analyzed by use of a 2-sided Student t test. For all evaluations, values of P<0.05 were considered statistically significant.

Results
Serotonin Measurements and Clinical Signs
The serotonin level in whole blood increased from 6986±577 nmol/L in controls to 21 236±745 nmol/L in the serotonin-treated animals. A steady-state level was reached after the second injection, reflecting the fact that the platelets were saturated with serotonin. The PPP serotonin level in untreated controls was 51.8±3.0 nmol/L. In the serotonin-treated animals, the PPP serotonin (in blood samples collected 24 hours after the fifth serotonin injection) was 794.8±194.1 nmol/L. The peak PPP serotonin levels (10 940±739.2 nmol/L) were seen when the animals had been treated with serotonin for 5 days, 2 hours after an additional injection (Figure 1A).

In dialysate collected from the femoral muscles, in saline and in serotonin-treated rats, 24 hours after the injections, serotonin was below the detection limit (23.5 nmol/L) of the HPLC method. The serotonin levels in femoral muscle reached a peak 2 hours after the final injection (302.0±11.0 nmol/L), when the animals had been treated with serotonin for 5 days, 277.1±5.9 nmol/L after 3 days (data not shown), and 180.2±47.5 nmol/L after 1 day (Figure 1B).

The serotonin injections induced clinical signs, including flushing, loose stools, drowsiness, and tachypnea. The flushing and drowsiness persisted 4 to 5 hours after the injections. On the third day, 2 animals died a few minutes after injection, and the rest of the treated animals were in a poor condition. The serotonin dose was therefore reduced as described in Methods. At sacrifice, serotonin-treated animals tended to have a lower body weight (306±5.2 g) compared with the control group (322±5.2 g; P=0.08), whereas their hearts were significantly heavier (1.312±0.036 g) than those of controls (1.203±0.023 g; P<0.02; Figure 2).

Echocardiography
The echocardiographic examinations did not show any pathological changes in the control group. Of 10 rats treated with serotonin, 6 had pathological echocardiographs. Two animals had a combination of aortic and pulmonary insufficiency, 1 animal had isolated aortic insufficiency, and 3 animals had isolated pulmonary insufficiency (Figure 3). No mitral valve

![Figure 1](http://circ.ahajournals.org/)

![Figure 2](http://circ.ahajournals.org/)

Figure 1. A, PPP serotonin levels in animals given daily subcutaneous serotonin injections for 1, 2, and 5 days. Blood was collected 2 hours after additional (second, third, and sixth) serotonin injection. B, Serotonin levels in dialysate from femoral muscles in animals given daily subcutaneous serotonin injections for 1 (○) and 5 (■) days. Dialysate was collected 2 hours before (serotonin not detectable) and for 6 hours after (second and sixth) additional serotonin injection (↑). Values are mean±SEM.

Figure 2. Animal (A) and heart (B) weights at end of study. Values are mean±SEM. *P=0.08, nonsignificant decrease in body weight vs controls; **P<0.02, significant increase in heart weight vs controls.
failure was detected, whereas the tricuspid valve was not properly visualized.

**Histopathology and Immunohistochemistry**

Histopathological examination of the hearts in the serotonin group revealed shortened and thickened aortic cusps, with an increased cellularity of myofibroblasts in a collagenous matrix (Figure 4A through 4C). There was a correspondence between pathological echocardiography and pathology in the histological picture. In the sections stained for immunoreactivity against Ki-67, there were no differences between the groups after 3 months. When we repeated the staining on hearts from animals treated with serotonin for only 5 days, however, we found a significant increase in Ki-67-positive cells compared with control rats (Figure 4D). The pulmonary valves were not visualized in the sections. Because of a less rigid shape and a tendency to rotate around their axis, the mitral and tricuspid valves were cut in different angles, making it difficult to compare the treated and untreated groups. However, some valves were clearly abnormal with distinct plaque formations (Figure 5). Subendocardial plaques characterized by a collection of myofibroblasts within an extracellular matrix of collagen myxoid ground substance developed in the atria and ventricles (Figure 6). In the control group, no histopathological changes were seen.

**RT-PCR**

To investigate whether rat aortic valves express mRNA for serotonin receptors and the serotonin transporter, we performed RT-PCR. The RT-PCR revealed the expression of 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{3A} serotonin receptors and the serotonin transporter 5-HTT in rat aortic cusps. The 5-HT_{2C} receptor, however, was not expressed (Figure 7).

**Discussion**

We present here, for the first time, a long-term animal model mimicking the human carcinoid syndrome, with excessive levels of circulating serotonin. The free fraction of serotonin in blood is probably the biological active substance causing the carcinoid syndrome and carcinoid heart disease. Because >99% of circulating serotonin is stored in platelets, it is crucial to avoid platelet activation during blood sampling and analyses of free circulating serotonin. In our preparation of PPP, the serotonin levels in healthy rats are in the range

![Figure 3. Continuous-wave Doppler recordings showing systolic aortic antegrade flow (AF) and diastolic aortic regurgitation (AR) in rat given serotonin.](image)

![Figure 4. Photomicrographs showing aortic valves. A, Thickened and retracted aortic cusp (bold arrow) and deposits of collagen tissue at base of cusp (thin arrow) in a rat given serotonin (hematoxylin-erytosin). B, Aortic valve in control rat (hematoxylin-erytosin). C, Shortened, thickened, and collagen-rich cusp in serotonin-treated rat with aortic valve insufficiency (van Gieson). D, Aortic valve in animal dosed with serotonin for 5 days. Ki-67-positive nuclei (stained red) are indicated by arrows. LV indicates left ventricle.](image)
previously reported by others.27 If we correct for the recovery in the microdialysis protocol, there is still a 10-fold-higher peak serotonin level in PPP compared with the dialysates from femoral muscles. One explanation could be that free circulating serotonin in vena cava is 10 times higher than the levels in the femoral muscles. Another perhaps more plausible explanation is that there still is some serotonin leaking out of the platelets during blood sampling and subsequent handling of the blood. Nevertheless, we have clearly demonstrated that administration of serotonin as daily subcutaneous injections increases free serotonin levels in blood and musculature >10 times and that the hyperserotoninemia lasts for >6 hours.

Cell culture studies have shown that serotonin has a direct mitogenic effect on cardiac valvular subendocardial cells and that this effect is mediated by serotonin receptors.28 Carcinoid heart disease is thought to be a consequence of hyperserotoninemia, but other substances such as tachykinins and bradykinin have not been ruled out as pathogens.2,5 We found that serotonin injections induced clinical signs such as flushing, loose stools, and anorexia. After 3 months, cardiac disease with pathological echocardiograms and histopathological changes similar to those seen in the carcinoid heart disease appeared. We also found an increased number of proliferating subendocardial cells by immunohistochemical staining with Ki-67 antibodies. The increased proliferation rate was not seen after 3 months of treatment but was obvious after 5 days. The explanation for this finding could be that we did not inject the 3-month animals with serotonin the week before sacrifice. Another possible explanation is that the proliferation rate decreases over time, a phenomenon also seen in other cell types.29 In addition, we demonstrated that rat aortic cusps express mRNA for the serotonin receptors 5-HT1A, 5-HT2A, and 5-HT2B, indicating that serotonin probably exerts a direct effect on valvular and subendocardial cells in rats.

Fenfluramine, a serotonergic drug used as an appetite suppressant, was withdrawn from the market in 1997 because it induced a valvular heart disease similar to that seen in the carcinoid syndrome.30 This effect might be mediated via the 5-HT2B receptor.19,31 Medications interacting with the serotonergic system are becoming increasingly common in clinical practice. 5-HT1A receptor agonists are used to treat migraine, and 5-HT3 receptor antagonists are used for chemotherapy-induced emesis. Recently, serotonin receptor–interacting medications for treating irritable bowel syndrome also have become available. Selective serotonin reuptake inhibitors are frequently used to treat depression and anxiety syndromes. These medications are defined by their selective affinity for the serotonin transporter 5-HTT. A recent study showed that blockade of serotonin reuptake by paroxetine inhibits serotonin-induced proliferation in cultured human fetal heart cells; the authors conclude that misuse of 5-HT uptake blocker may alter heart development.17 This study demonstrates that the serotonin receptors 5-HT1A, 5-HT2A, and 5-HT2B and the serotonin transporter 5-HTT are expressed in rat aortic cusp cells.

Our future studies will focus on long-term effects of serotonergic drugs on heart and heart valves. We will also investigate whether serotonin receptor antagonists can prevent cardiac changes resulting from hyperserotoninemia. The present study describes an animal model suitable for investigating consequences of hyperserotoninemia. The study demonstrates that serotonin most likely is involved in the pathogenesis of carcinoid heart disease.

Acknowledgments

This study was supported in part by the CarciNors foundation (Oslo, Norway), Ingrid and Torleif Hoels Legacy, and Funds for Medical Research at Trondheim University Hospital. We are grateful to the following members of our staff: Kari Stordahl, Anne Kristensen, and Bjørn Munkvold for technical assistance in this project.
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Circulation. 2005;111:1517-1522; originally published online March 21, 2005;
doi: 10.1161/01.CIR.0000159356.42064.48
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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