Abnormal Aortic Valve Development in Mice Lacking Endothelial Nitric Oxide Synthase

Tony C. Lee, BSc; Yidan D. Zhao, MD, PhD; David W. Courtman, PhD; Duncan J. Stewart, MD

Background—Endothelium-derived nitric oxide (NO) is produced by an oxidative reaction catalyzed by endothelial NO synthase (eNOS). NO plays a crucial role in controlling cell growth and apoptosis, as well as having well-characterized vasodilator and antithrombotic actions. More recently, endothelium-derived NO was shown to be involved in postdevelopmental vascular remodeling and angiogenesis, as well as in the formation of limb vasculature during embryogenesis. Therefore, we investigated the role of endothelium-derived NO during cardiovascular development using mice deficient in eNOS.

Methods and Results—We examined the hearts of 12 mature eNOS-deficient and 26 mature wild-type mice. Five of the mature eNOS-deficient mice had a bicuspid aortic valve; none of the 26 wild-type animals exhibited identifiable valvular or cardiac abnormalities. Immunohistochemical analysis revealed prominent eNOS expression localized to the endothelium lining the valve cusps of the aorta in mature wild-type mice; expression was localized to the myocardium and endothelial cell monolayer lining the valve leaflets in the developing embryo.

Conclusions—These results show a strong association between eNOS deficiency and the presence of a bicuspid aortic valve; they provide the first molecular insight into one of the most common types of congenital cardiac abnormality. (Circulation. 2000;101:2345-2348.)

Key Words: nitric oxide • endothelium • mice, knockout • heart defects, congenital • valves

Nitric oxide (NO) is a free radical gas that is synthesized from L-arginine in a complex oxidative reaction catalyzed by 3 distinct isoforms of NO synthase: endothelial (eNOS), inducible (iNOS), and neuronal (nNOS). The neuronal isoform is highly expressed in neuronal cells and skeletal muscle, whereas the endothelial isoform is predominantly expressed in endothelial cells and produces small amounts of NO in response to intimal shear stress or agonists such as bradykinin. The inducible isoform is expressed in many cell types, including macrophages and smooth muscle cells, primarily in response to inflammatory cytokines. Once expressed, this isoform can produce large amounts of NO in a continuous manner. Endothelium-derived NO is crucial in the regulation of cell growth and apoptosis; it also has a well-characterized role as a vasodilator and antithrombotic agent. More recently, NO derived from the endothelium was shown to be involved in postdevelopmental vascular remodeling and angiogenesis, as well as in the formation of limb vasculature during embryogenesis.

The valve leaflets of the heart originate from mesenchymal outgrowths known as cardiac cushions. Cushion formation is localized to the atrioventricular canal and ventricular outflow tract regions of the primary heart tube. These formations arise from regional thickenings of the cardiac jelly, the extracellular matrix that resides between the myocardium and endocardium of the primitive heart tube. This event involves the transformation of a subset of endothelial cells of the endocardium into mesenchyme. The molecular mechanisms and the possible role of the endothelium in this developmental process remain largely unexplored. We report here that mice lacking functional eNOS demonstrate a high incidence of bicuspid aortic valves; this provides the first evidence for the involvement of endothelium-derived NO in cardiac valve morphogenesis.

Methods

Mice

Wild-type (n=29) and eNOS knockout (n=27) mice of the C57BL/6J genetic background were obtained from Jackson Laboratories (Bar Harbor, ME). Male mice weighing 25 to 30 g and embryos at day 13.5 of gestation were used for analysis. The use and care of mice were in accordance with the guidelines of the Canadian Council of Animal Care.

Histology and Immunohistochemistry

Serial sections of the hearts and aortas from mature knockout (n=12) and wild-type (n=26) mice were collected after perfusion fixation with saline and 10% buffered formalin. Embryos from knockout (n=8) and wild-type (n=8) mice were isolated and fixed in 10% buffered formalin. Immunohistochemistry was performed using a monoclonal antibody specific for eNOS (1:1000 dilution, Transduction Laboratories) and visualized using the avidin-biotin immuno-
peroxidase complex and diaminobenzidine. For a negative control, the primary antibody was substituted with nonimmune mouse serum.

**Vascular Casting**

A methylmethacrylate casting compound (Polysciences) was infused retrograde into the abdominal aorta of mature knockout (n=15) and wild-type (n=3) mice at a constant physiological pressure. The cast was allowed to set for 30 minutes, and the tissue was dissolved by immersion into a saturated solution of potassium hydroxide.

**Results**

Of the 12 adult eNOS-deficient mice examined, 5 had a bicuspid aortic valve, whereas none of the 26 wild-type mice had a bicuspid aortic valve. The proportion of bicuspid aortic valves in the eNOS-deficient group was significantly higher than in the wild-type group (42%; P=0.0015 by Fisher’s exact test). Immunohistochemical analysis revealed prominent eNOS staining of endothelial cells lining the aortic valve leaflet in the wild-type animal (C). No staining was apparent in the eNOS knockout mice (D) or the negative control in which nonimmune mouse serum was substituted for the primary antibody (E).

![Figure 1. Analysis of mature mice.](image1)

Transverse sections through the aortic root of wild-type (A) and eNOS-deficient (B) mice that were stained with hematoxylin and eosin show normal tricuspid aortic valve formation in wild-type mice and a bicuspid aortic valve in eNOS-deficient mice (42%; P=0.0015 by Fisher’s exact test). Immunohistochemical analysis revealed prominent eNOS staining of endothelial cells lining the aortic valve leaflet in the wild-type animal (C). No staining was apparent in the eNOS knockout mice (D) or the negative control in which nonimmune mouse serum was substituted for the primary antibody (E).

![Figure 2. Aortic casts.](image2)

Representative casts of the aorta and its major branches from wild-type (left) and knockout (right) mice showed no evidence of aortic coarctation. The levels at which the aortic lumen diameters were measured are depicted on the left.
C57BL/6J animals exhibited identifiable valvular or cardiac abnormalities (Figures 1A and 1B). Immunohistochemical analysis revealed prominent eNOS expression localized to the endothelium lining the valve cusps of the aorta in adult mice, but no staining was seen in either the mature eNOS knockout mice or negative controls (Figures 1C through 1E). Measurements from vascular casts at the 5 levels depicted in Figure 2 showed no differences in aortic lumen diameter between mutant and wild-type mice (Table). Furthermore, representative casts of the aorta and its major branches in mature mice showed no evidence of aortic coarctation (Figure 2). In the small number of mutant and wild-type embryos examined, there was no evidence of the presence of severe cardiac abnormalities (Figures 3A and 3B). In the developing embryo at day 13.5 of gestation, immunohistochemical examination revealed prominent eNOS staining localized to the myocardium and the endothelial cell monolayer lining the valve leaflets (Figure 3C). Staining for eNOS was not observed in the negative control (Figure 3D).

**Discussion**

A bicuspid aortic valve is one of the most common forms of human birth defect in which familial clustering has been described; it occurs in ≥1% of newborn infants. Although bicuspid aortic valves can be functionally normal, this congenital abnormality accounts for a substantial disease burden because of a propensity toward complications, including aortic stenosis and bacterial endocarditis, which often lead to valve failure. A functionally normal bicuspid valve may also develop progressive incompetence; therefore, it is an important cause of anatomically isolated aortic regurgitation in adults. The use of targeted gene disruption has provided valuable insight into the molecular basis of cardiovascular development. A number of these genetic mutations have resulted in severe cardiac abnormalities that have led to embryonic death. In the present study, we showed that mice lacking functional eNOS are predisposed to developing a bicuspid aortic valve, a condition that has never before been reported in mice. Insights previously gained from eNOS-deficient mice support a role for endothelium-derived NO as a major mediator of vascular remodeling and angiogenesis. Furthermore, the expression of eNOS is increased during early cardiomyogenesis, and NOS inhibitors prevent the maturation of terminally differentiated cardiomyocytes in an embryonic

### Table: Aortic Lumen Diameters Taken From Vascular Casts

<table>
<thead>
<tr>
<th>Level 1</th>
<th>Level 2</th>
<th>Level 3</th>
<th>Level 4</th>
<th>Level 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.72±0.07</td>
<td>0.77±0.06</td>
<td>0.78±0.11</td>
<td>0.71±0.17</td>
</tr>
<tr>
<td>eNOS knockout</td>
<td>0.72±0.08</td>
<td>0.75±0.09</td>
<td>0.72±0.14</td>
<td>0.73±0.18</td>
</tr>
<tr>
<td>P</td>
<td>0.88</td>
<td>0.70</td>
<td>0.28</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Measurements were taken from the vascular casts made at each of the 5 levels depicted in Figure 2. No differences were observed in aortic lumen diameters between mutant (n=3) and wild-type (n=15) mice (Student’s t test). Values are expressed in millimeters as the mean±SD.

**Figure 3.** Analysis of mouse embryos. Transverse sections through the aortic valve of wild-type (A) and eNOS-deficient (B) mouse embryos at day 13.5 of gestation were stained with hematoxylin and eosin. Immunohistochemical analysis revealed intense eNOS staining in the myocardium and endothelial cells lining the valve leaflets in wild-type mice (C); no staining was apparent from the negative control in which nonimmune mouse serum was substituted for the primary antibody (D).
stem cell system, which suggests a possible role for NO in cardiac development. Mice deficient in eNOS also possess limb reduction defects, possibly due to vascular insufficiency. These limb abnormalities were seen in \( \approx 10\% \) of the null animals, and they resemble the limb reduction defects seen in patients with Holt-Oram Syndrome. Recently, a novel heart-hand syndrome was described; it consisted of a patent duc tus arteriosus, a bicuspid aortic valve, hand abnormalities, and pseudocoarctation of the aorta. The presence of a bicuspid aortic valve, together with abnormal limb development in the eNOS knockout mouse, suggests that altered NO activity may contribute either directly or indirectly to the heart-hand abnormalities seen in some patients.

The mechanism by which an eNOS deficiency contributes to the formation of a bicuspid aortic valve remains to be determined. It is possible that the valvular endothelium is responsible for transducing luminal events, such as shear stress, and generating signals that fine-tune the developmental program of the primitive ventricular outflow tract to ensure the formation of a normal, low-profile tricuspid structure. Given that NO has been implicated in vascular remodeling in response to changes in luminal flow conditions, it is also reasonable to speculate that a lack of eNOS may also interfere with the remodeling of the aortic isthmus during the transition from the fetal to the adult pattern of circulation. Interestingly, a bicuspid aortic valve is found in \( \approx 50\% \) of patients with coarctation of the aorta. In the present study, there was no evidence of aortic coarctation in the 15 eNOS-deficient mice examined. However, a bicuspid aortic valve is a far more common clinical condition than aortic coarctation and, therefore, a much larger number of animals would be needed to exclude the possibility of a low incidence of coexisting aortic coarctation in these animals.

The present results show a strong association between eNOS deficiency and the presence of a bicuspid aortic valve, and they provide the first molecular insight into the development of this congenital cardiac abnormality. Further experiments are required to elucidate the precise mechanisms by which endothelium-derived NO modulates valve morphogenesis in the developing embryo.

Acknowledgments

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References

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