Inactivation of the Lysyl Oxidase Gene Lox Leads to Aortic Aneurysms, Cardiovascular Dysfunction, and Perinatal Death in Mice

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Background—The lysyl oxidases are extracellular copper enzymes that initiate the crosslinking of collagens and elastin. Five human isoenzymes having been characterized so far. The crosslinks formed provide the tensile strength and elastic properties for various extracellular matrices, including vascular walls. We studied the role of the first described isoenzyme Lox by inactivating its gene in mice.

Methods and Results—Murine Lox gene was disrupted by routine methods. Lox−/− mice died at the end of gestation or as neonates, necropsy of the live-born pups revealing large aortic aneurysms. In light microscopy, hazy and unruffled elastic lamellae in the Lox−/− aortas were observed, and electron microscopy of the aortic walls of the Lox−/− fetuses showed highly fragmented elastic fibers and discontinuity in the smooth muscle cell layers in Lox−/− fetuses. The wall of the aorta in the Lox−/− fetuses was significantly thicker, and the diameter of the aortic lumen was significantly smaller than that in the Lox+/+ aortas. In Lox−/− fetuses, Doppler ultrasonography revealed increased impedance in the umbilical artery, descending aorta, and intracranial artery blood velocity waveforms, decreased mean velocities across cardiac inflow and outflow regions, and increased pulsatility in ductus venosus blood velocity waveforms.

Conclusions—Lox has an essential role in the development and function of the cardiovascular system. Inactivation of the Lox gene causes structural alterations in the arterial walls, leading to abnormalities in the cardiovascular functions. Alterations in LOX activity may also play a critical role in certain human cardiovascular diseases. (Circulation. 2002;106:2503-2509.)

Key Words: aneurysm ■ aorta ■ crosslink ■ lysyl oxidase

Lysyl oxidases are extracellular copper enzymes that initiate the formation of the lysine and hydroxylysine-derived crosslinks in collagens and lysine-derived crosslinks in elastin. These crosslinks are essential for the mechanical stability of the fibers and other supramolecular assemblies formed by these proteins and the elasticity of elastin. Because collagens and elastin are important components of the extracellular matrix, abnormalities in their modification can be expected to affect many tissues, as seen in lathyrism, a connective tissue disorder caused by the administration of β-aminopropionitrile, an irreversible inhibitor of lysyl oxidases. The manifestations of lathyrism include kyphoscoliosis, bone deformities, weakening of tendons and ligament attachments, dislocation of joints, weakening of skin and cartilage, hernias, and dissecting or saccular aneurysms of the aorta.

Five human lysyl oxidase isoenzymes, referred to as lysyl oxidase, LOX, and lysyl oxidase-like proteins, LOXL, LOXL2, LOXL3, and LOXL4, have been characterized so far, but little is known about their specific functions. We inactivated the mouse Lox gene and report here that the isoenzyme Lox has an essential role in the development and functioning of the cardiovascular system.

Methods

Generation of a Mouse Line With an Inactivated Lox Gene

A 10.2-kb genomic clone containing the 5′ end of the murine Lox gene was isolated from the 129SV library using human LOX cDNA as a probe. The loxP sequence was inserted into a NeoR cDNA a positive selection cassette flanked by the loxP sites was inserted 90 bp downstream of the first exon into the BamHI site. The linearized targeting construct was electroporated into R1 embryonic stem (ES) cells. Genomic DNA from the G418-resistant clones was digested with SpeI and hybridized with the BstI/Spel external probe. Cells from correctly targeted ES clones were then electroporated with the pIC-Cre plasmid (a gift from Dr Werner Müller, University of Cologne, Germany) and grown in the presence...
of Gancyclovir. DNA from clones that had survived selection was digested with Nsil and hybridized with the B1-E1/E1 probe. Cells in which the selection cassette and the first exon of the Lox gene had been deleted were injected into C57BL/6 blastocysts, which were then implanted into pseudopregnant females. The resulting chimeras were bred with C57BL/6 mice to produce heterozygous mutants that were identified by genotyping tail DNA.

Total RNA was extracted from whole E18.5 fetuses using TriPure Isolation Reagent (Roche Molecular Biochemicals), and Northern blots were prepared by routine methods. All animal care and experimental procedures were approved by the Animal Research Committee of University of Oulu, Finland.

Histological Analysis
Tissue samples were fixed overnight in 10% buffered formalin and embedded in paraffin. Sections were stained with H&E or Masson’s trichrome, the H&E-stained sections being examined under UV light. Processing and sectioning of Lox+/− and Lox−− samples were performed parallel and were inspected without knowledge of the genotype.

For evaluation of the cardiac chamber sizes, thickness of the ventricular and aortic walls, and diameter of the aortic lumen, digital images were captured from paraffin sections at the atrioventricular valve level. Measurements were carried out using an objective micrometer as a reference.

Transmission Electron Microscopy
Biopsies from the descending aorta of E18.5 fetuses were fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer, postfixed in 1% osmium tetroxide, dehydrated in acetone, and embedded in Epon LX112. Thin sections were cut with a Reichert Ultracut ultramicrotome and examined in a Phillips CM100 transmission electron microscope.

Ultrasoundographic Examination
E18.5 fetuses were examined using an Acuson Sequoia 512 Doppler ultrasonograph with a 13-MHz linear probe.29 The dam was anesthetized, and the fetuses were located in each uterine horn. The fetal heart was identified by color Doppler, and the sample volume of the pulsed Doppler was placed over it to cover the entire heart. The high-pass filter was set at its minimum. Different views of the heart were examined to minimize the angle between the Doppler beam and the inflow and outflow regions to obtain their maximal velocities. The descending aorta, intracranial arteries, umbilical artery, and ductus venosus were located on the sagittal view of fetus with the help of color Doppler, and blood velocity waveforms were obtained by the pulsed Doppler method. Immediately after examination, the dam was killed, the abdomen was opened, and fetuses were identified according to their location in ultrasonography. The detection rate of the fetuses by ultrasonography was 96.5% (n=85 of 88). The ultrasonographic examinations were videotaped and analyzed later using the cardiovascular measurement package included with the ultrasound equipment. Time-velocity integrals (TVIs) were measured from the inflow and outflow blood velocity waveforms by planimetry of the area underneath the Doppler spectrum. The fetal heart rate (FHR) was obtained, and the inflow and outflow mean velocities, which are directly proportional to volume blood flow, were calculated (Vmax=FHR×TVI). The presence of valve regurgitation was documented.30 Pulsatility index values [PI=(peak systolic velocity−end-diastolic velocity)/time-averaged maximum velocity over the cardiac cycle] were obtained from the descending aorta, intracranial arteries, and umbilical artery blood velocity waveforms, and pulsatility index values for veins [PIV=(peak systolic velocity−velocity during atrial contraction)/time-averaged maximum velocity over the cardiac cycle] were calculated from the ductus venosus blood velocity waveforms. Three consecutive cardiac cycles were analyzed for every measurement, and their means were used for additional analysis. Intraobserver variability was analyzed in 15 fetuses from 3 litters by performing a second examination ~30 minutes later under the same anesthesia. The mean intraobserver variability of the inflow and outflow TVI measurements ranged from 6.0% to 6.5% (95% CI, 4.0 to 8.4), the corresponding variabilities in the PI and PIV calculations being 3.6% and 6.7% (95% CI, 2.3 to 8.8), this repeatability being similar to that reported in human studies.11

Statistical Analysis
Comparisons between 3 groups were made by ANOVA, the Scheffe F-test being used for additional analysis. Comparisons between 2 groups were made by Student’s t test.

Results
Generation of Mice With an Inactivated Lox Gene
ES cells carrying the inactivated Lox gene were generated by a two-step targeting method comprising homologous recombination followed by cre recombination (Figures 1A through 1D). The targeted ES cells were used to generate chimeras, from which 1 male transmitted the mutation to his offspring when mated with wild-type females. Deletion of the first exon led to complete inactivation of the Lox gene, as seen in the Northern blot (Figure 1E) and confirmed using probes from several regions of the gene. Heterozygous mice, which were indistinguishable from wild type, were crossbred, and the offspring were genotyped at 2 weeks of age. From 111 pups analyzed, 32.4% were Lox+/− and 67.6% were Lox−−, but no Lox−− pups were present.

Lox−− Mice Develop to Full Term but Are Not Viable
To study the time of death of the Lox−− mice, pregnant Lox−− females were killed at different times after breeding with Lox−− males, and the fetuses were genotyped. At the end of gestation, E18.5, a normal Mendelian ratio of all 3 genotypes was obtained (26.7% Lox−−, 45% Lox+/−, 28.3% Lox−−, n=127), indicating loss of Lox+/− pups immediately after birth. Pregnant females were then observed during the assumed day of delivery, and in this way, 4 Lox−− pups were obtained alive and 3 were found dead in a total number of 27 pups in 5 litters. The gross appearance of the newborn Lox−− pups that were alive was normal, but they had cyanotic skin and poor condition. They were breathing but sluggish and did not suck milk (Figure 2A) and would presumably have been cannibalized if left with the dams. Necropsy revealed large aneurysms in the aortas of 3 of the 4 live-born pups (Figures 2B through 2D). In addition, a narrowed lumen was observed in the abdominal aorta of all 4 pups (Figures 2C and 2D). The 3 Lox−− pups that had died immediately after birth had a diaphragmatic hernia, and 2 also had a large hemorrhage in the upper chest region suggesting aortic rupture, but rupture sites could not be localized because of the tissue degeneration in the cadavers.

Structural Alterations in the Aortic Wall but Not in the Heart of Lox−− Fetuses
Because preliminary analyses indicated defects in blood vessels, aortas from fetuses at different stages were taken for detailed analysis. Alterations in the structure of the aortic wall were seen at the earliest time point analyzed, E14.5, but were most obvious at E18.5. Light microscopy revealed hazy and unruffled elastic lamellae in all Lox−− samples, whereas the
lamellae in the wild-type aorta were well defined and ruffled (Figures 3A and 3B). Furthermore, the aortic wall in the Lox$^{-/}$/H11002 fetuses was thicker ($P<0.005$), and the diameter of the aortic lumen was smaller ($P<0.001$) than that in the Lox$^{+/+}$/H11002 (Figures 2C and 2D, Figures 3A and 3B, Table 1). In contrast to the aorta, no major abnormalities were found in the superior vena cava. Aortic aneurysms were observed in 4 and diaphragmatic hernias in 2 of 30 analyzed E18.5 Lox$^{-/-}$ fetuses; however, the incidence of aneurysms is probably higher, because some of the samples were not sectioned through the whole aorta length.

In electron microscopy of the Lox$^{-/-}$ aorta, the smooth muscle cell layer appeared discontinuous, and elastic laminae were fragmented. These findings differ distinctly from the uniform elastic lamina and smooth muscle cell layers seen in the media of the wild-type E18.5 aorta (Figures 4A through 4C). In the remnants of elastic fibers, amorphous elastin was seen, but in fragments (Figure 4C). Most of the endothelial

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**Figure 1.** Targeted inactivation of the Lox gene. A, Top, 5' end of the Lox gene. Exons are depicted as black boxes and numbered. In the targeting construct, the HSV-tk-neo' selection cassette, flanked by loxP sequences (arrowheads), was inserted into the first intron and an additional loxP sequence in front of exon 1. B, Targeting events were identified using the B1/SpeI probe in Southern blots, where 7.7-kb wild-type and 4.9-kb targeted SpeI fragments are seen. C, ES cells with the targeted allele were treated with cre recombinase and analyzed in Southern blots with the B1/E1-E1 probe that hybridizes to the 5.8-kb wild-type and 4.5-kb Cre-deleted NsiI fragments. D, Southern blot with genomic DNA from E18.5 fetuses digested with NsiI and hybridized with the B1/E1-E1 probe. E, Northern analysis of RNA from E18.5 fetuses using a 613-bp polymerase chain reaction fragment from exon 1 of the Lox gene as a probe. The 4.5- and 6.0-kb mRNAs were observed in the Lox$^{-/-}$ and Lox$^{+/+}$ samples. An $\alpha$-actin probe was used to verify the quality and quantity of the RNA.
Ventricular wall thickness, mm

Abdominal aorta in the same animal. Bars, C and D, 100 μm.

TABLE 1. Aortic and Cardiac Measurements in E18.5 Lox+/+ and Lox−/− Fetuses

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<th>Lox+/+</th>
<th>Lox−/−</th>
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<tr>
<td></td>
<td>Mean±SD</td>
<td>n</td>
</tr>
<tr>
<td>Descending aorta, μm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter of the lumen</td>
<td>285±41</td>
<td>13</td>
</tr>
<tr>
<td>Thickness of the wall</td>
<td>20±5</td>
<td>13</td>
</tr>
<tr>
<td>Ventricular size, mm</td>
<td></td>
<td></td>
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<tr>
<td>Right ventricular circumference</td>
<td>2.89±0.45</td>
<td>13</td>
</tr>
<tr>
<td>Left ventricular circumference</td>
<td>3.35±0.42</td>
<td>13</td>
</tr>
<tr>
<td>Ventricular wall thickness, μm</td>
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<td></td>
</tr>
<tr>
<td>Right ventricle</td>
<td>167±56</td>
<td>13</td>
</tr>
<tr>
<td>Interventricular septum</td>
<td>339±102</td>
<td>13</td>
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<tr>
<td>Left ventricle</td>
<td>236±71</td>
<td>13</td>
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*P<0.005; †P<0.001.

Figure 2. Neonatal Lox−/− mice are of poor viability. A, Newborn Lox+/+ pup has normal morphology but is cyanotic. B, Saccular aneurysm (arrows) in the thoracic aorta, observed by stereomicroscopy in necropsy of a Lox−/− neonatal pup. The heart and lungs were removed to reveal the aorta, still against the body wall. C and D, Histological analysis of the thoracic aorta in a newborn wild-type (C) and Lox−/− (D) mouse. Masson’s trichrome staining. Aortic lumen is indicated with an asterisk. D, An aneurysmal dilatation and thickened wall of the thoracic aorta is seen in the Lox−/− sample. Inset, the narrowed lumen of the abdominal aorta in the same animal. Bars, C and D, 100 μm.

were seen between the Lox−/− and Lox+/+ fetuses by electron microscopy (not shown).

Fetal hearts were first inspected for gross morphology and size and then by histology. Cardiac structure was indistinguishable, and the chamber sizes and wall thicknesses were similar among E18.5 Lox−/− and Lox+/+ fetuses (Table 1).

Ultrasonographic Signs of Cardiovascular Dysfunction

Cardiovascular functions of the fetuses were examined in utero by Doppler ultrasonography of anesthetized Lox+/+ females at day 18.5 after mating with Lox−/− males (Figure 5A). All 3 genotypes could thus be examined under the same conditions. No difference in FHR was found between the 3 groups, but the PIs for the descending aorta, umbilical artery, and intracranial arteries were significantly higher, and the outflow and inflow mean velocities were significantly lower in the Lox−/− fetuses (Table 2). The ductus venosus PIVs were significantly higher in the Lox−/− fetuses (Table 2). In addition, fetuses with the highest PIs in the descending aorta had semilunar valve regurgitation (Figures 5A through 5D).

Discussion

All 5 lysyl oxidase isoenzymes are likely to use both elastin and collagens as their substrates, as has been shown so far for 3 of them. Therefore, the neonatal lethality associated with the lack of only 1 isoenzyme, Lox, was unexpected, especially because mice totally lacking elastin survive for up to 4 days, and mice totally lacking collagen III can survive for up to 6 months, although most die at birth. Our study shows that Lox cannot be compensated for by other isoenzymes in terms of its contribution to the development and maintenance of the structure and mechanical properties of the aortic wall. Besides the abnormalities in the aortic wall, diaphragmatic hernias were detected in a few Lox−/− fetuses, whereas no other major abnormalities were observed.

A deficiency in Lox activity is likely to affect several aortic wall components, mainly elastin and collagen types I and III. The early development of the aorta and the translation and...
secretion of aortic wall components are likely to occur normally in Lox−/− embryos, as demonstrated by the normal number of elastic lamellae and smooth muscle cell layers. In elastin-null mice, smooth muscle cells proliferate and thereby stabilize the arterial structure but finally obliterate the arterial lumen.14 No increase in smooth muscle cell proliferation was observed in the Lox−/− aortas, probably because normal amounts of elastin were produced, at least during the early stages. Rupture of a large blood vessel is the major cause of death in mice lacking collagen III.15 The arrangement of elastic fibers and smooth muscle cells is normal in the aortas of these mice, but collagen fibrils are reduced or absent in the media, and the fibrils in the adventitia are of variable diameter.15 No analogous changes were seen in the Lox−/− aortas, where the collagen fibrils were of normal appearance in electron microscopy.

Elastin is present in elastic fibers as soluble monomers and as a highly crosslinked insoluble protein in the amorphous component.2 Crosslinking has been suggested to be a critical step in the nucleation of elastin assembly.2 Amorphous elastin was found in Lox−/− aortic walls, indicating that other lysyl oxidase isoenzymes are also likely to function in elastin crosslinking, but the fragmentation of elastic fibers implies that these isoenzymes did not compensate completely for the deficiency in Lox. The incomplete crosslinking may make elastin more susceptible to proteolytic degradation,16 which would lead to fragmentation of the elastic fibers, so that they are unable to bear the hemodynamic stress, which would lead in turn to dilatation of the aortic wall and probably also rupture.

The increased PIs measured in the umbilical artery, descending aorta, and intracranial arteries in Doppler ultrasonography are likely to be attributable to abnormalities in the arterial walls. Comparable increased PIs in the umbilical artery and descending aorta are observed in human pregnancies with placental insufficiency, but they are connected with

![Figure 4. Electron micrographs of Lox−/− (left) and Lox−/− aortic wall (right). A, Smooth muscle cells (SMC) in the Lox−/− aortic wall are organized between elastic fibers (arrows), whereas those in Lox−/− are disorganized, and elastic fibers are fragmented and endothelial cells (uppermost cell layer) are rounded. B, The endothelial cells (EC) in the Lox−/− aortic wall are in contact with the intact elastic lamina (arrow), whereas those in Lox−/− are poorly associated with the fragmented elastic lamina (arrow). C, In the Lox−/− aortic wall, amorphous elastin (aEL) is detected in a continuous elastic fiber, whereas in Lox−/−, the elastic fiber is fragmented, with remnants of amorphous elastin (arrows). Original magnifications: A, ×1600; B, ×4400; and C, ×8400.](image-url)
A concomitant decrease in PI values in the cerebral circulation, so that an adequate oxygen supply to the coronary and cerebral circulation is maintained. Structural alterations are likely to cause a lower relative pulse amplitude in the arteries, leading to an increased afterload of the heart. In addition, the decreased mean velocities in the inflow and outflow regions suggest a lower cardiac output than in \( Lox^{+/+} \) fetuses. Increased fetal cardiac afterload may lead to a drop in cardiac output and valve regurgitation. In our series, fetuses with the greatest increase in the afterload had semilunar valve regurgitation, most likely pulmonary valve regurgitation. It is not possible to distinguish between the right and left ventricular inflow and outflow regions in mouse fetuses because of the small cardiac size, but both the ductus arteriosus and the foramen ovale are wide open in the fetal circulation, so that the fetal cardiac ventricles function in parallel and the

\[ Lox^{+/+} \]

\[ Lox^{-/-} \]
pressure faced by the ventricles is equal. Thus, the pathophysiological effects would be similar in both ventricles. Increased PIVs in the ductus venosus implies an increase in systemic venous pressure, which, if detected in human fetuses, is interpreted as a sign of congestive heart failure.\(^\text{11}\) The ventricular sizes and wall thicknesses in the fetuses, is interpreted as a sign of congestive heart failure.\(^\text{11}\) Our results show that when the activity of the Lox isoenzyme is completely abolished, aneurysms and cardiovascular dysfunction already occur during fetal development, indicating that a certain level of Lox activity in the aortic wall is irreplaceable. Furthermore, the results underline the importance of the structural and functional properties of the arteries for normal cardiovascular development.

In conclusion, the pathophysiological sequence of events in the cardiovascular system of Lox\(^/-\) fetuses suggests that deficient crosslinking of elastin and collagens causes a decline in the resilience and tensile strength of the arterial walls, giving rise to abnormalities in cardiovascular functions and leading to aortic aneurysms. Our findings suggest that alterations in LOX activity may also play a critical role in human cardiovascular diseases, and in this sense our mouse model will be a useful tool for additional studies.

**Acknowledgments**

This work was supported by grants from the Health Sciences Council of the Academy of Finland, from the Finnish Centre of Excellence Programme 2000-2005 (44843), and from FibroGen Inc (South San Francisco).

**References**

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\textit{Circulation}. 2002;106:2503-2509
doi: 10.1161/01.CIR.0000038109.84500.1E

\textit{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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