Axl, A Receptor Tyrosine Kinase, Mediates Flow-Induced Vascular Remodeling

Vyacheslav A. Korshunov, Amy M. Mohan, Mary A. Georger, Bradford C. Berk

Abstract—Intima–media thickening (IMT) in response to hemodynamic stress is a physiological process that requires coordinated signaling among endothelial, inflammatory, and vascular smooth muscle cells (VSMC). Axl, a receptor tyrosine kinase, whose ligand is Gas6, is highly induced in VSMC after carotid injury. Because Axl regulates cell migration, phagocytosis and apoptosis, we hypothesized that Axl would play a role in IMT. Vascular remodeling in mice deficient in Axl (Axl−/−) and wild-type littermates (Axl+/+) was induced by ligation of the left carotid artery (LCA) branches maintaining flow via the left occipital artery. Both genotypes had similar baseline hemodynamic parameters and carotid artery structure. Partial ligation altered blood flow equally in both genotypes: increased by 60% in the right carotid artery (RCA) and decreased by 80% in the LCA. There were no significant differences in RCA remodeling between genotypes. However, in the LCA Axl−/− developed significantly smaller intima + media compared with Axl+/+ (31±4 versus 42±6×10−6 μm², respectively). Quantitative immunohistochemistry of Axl−/− LCA showed increased apoptosis compared with Axl+/+ (5-fold). As expected, p-Akt was decreased in Axl−/−, whereas there was no difference in Gas6 expression. Cell composition also changed significantly, with increases in CD45+ cells and decreases in VSMC, macrophages, and neutrophils in Axl−/− compared with Axl+/+. These data demonstrate an important role for Axl in flow-dependent remodeling by regulating vascular apoptosis and vascular inflammation. (Circ Res. 2006;98:1446-1452.)

Key Words: Axl ■ flow ■ carotid artery ■ remodeling ■ mouse ■ apoptosis ■ inflammation

The receptor tyrosine kinase Axl (also known as Ufo and Tyro7) belongs to a family of tyrosine receptors that includes Tyro3 (Sky) and Mer (Tyro12). A common ligand for Axl family is Gas6 (Growth arrest-specific protein 6). Important cellular functions of Gas6/Axl include cell adhesion, migration, phagocytosis, and inhibition of apoptosis. Gas6 and Axl family receptors are highly regulated in a tissue and disease specific manner. A recent genetic study found a significant association of a Gas6 mutation with stroke in humans. Both Gas6 and Axl-family receptor knockout mice show abnormalities in platelet function, further supporting a role for Axl family in the mammalian immune system. Recent data suggest that the Axl family is a central regulator of macrophage activation and phagocytosis.

We developed a reproducible mouse model of flow-dependent vascular remodeling that resembles human intima–media thickening (IMT). In response to decreased blood flow IMT occurs, which involves critical interactions among cells of the vessel wall. Based on the significant roles described for Axl in EC, inflammatory cells, platelets, and VSMC, we hypothesized that Axl plays an important role in vascular remodeling associated with changes in flow.

Materials and Methods

Animals

Male and female Axl wild-type (Axl+/+) and Axl knockout (Axl−/−) mice (8 weeks old) were kindly provided by Dr Stephen Goff (Columbia University), bred in house and used in accordance with the guidelines of the National Institutes of Health and American Heart Association for the care and use of laboratory animals (approved by the University of Rochester Animal Care Committee). To genotype animals, DNA was isolated from tails at weaning and PCR performed.

Original received December 22, 2005; resubmission received March 22, 2006; accepted April 11, 2006.

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/01.RES.0000223322.16149.9a

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TABLE 1. Physiological Parameters in Axl+/+ and Axl−/− Mice

<table>
<thead>
<tr>
<th></th>
<th>Axl+/+ Males (n=7)</th>
<th>Axl+/+ Females (n=9)</th>
<th>Axl−/− Males (n=10)</th>
<th>Axl−/− Females (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>21.3±0.7*</td>
<td>17.4±0.5</td>
<td>20.4±0.5*</td>
<td>17.2±0.7</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>129±2</td>
<td>126±3</td>
<td>127±3</td>
<td>125±2</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>625±7</td>
<td>637±9</td>
<td>641±9</td>
<td>655±10</td>
</tr>
</tbody>
</table>

*P<0.05 compared with females (Student t test).

Experimental Protocol
Blood flow in the left common carotid artery (LCA) was reduced by partial ligation of the left external and internal carotid arterial branches. For sham operation, the branches of the LCA were exposed but not ligated.23 The animals were allowed to recover and housed individually under SPF conditions with 12-hour/12-hour light/dark cycle for 3, 7, and 14 days after the surgery. Systolic blood pressure (BP) and heart rate (HR) were measured in nonanesthetized mice by tail-cuff plethysmography (Visitech Systems). Blood flow was measured on both carotids using an ultrasonic transit-time volume flowmeter (Transonic Systems Inc). Shear stress was calculated as described.25

Morphometry and Immunohistochemistry
Animals were perfusion fixed with 10% paraformaldehyde as described.23 The carotids were harvested, embedded in paraffin, and stained with hematoxylin and eosin (DAKO) and analyzed using computerized morphometry (ImagePro Plus). The vessel compartment volumes were calculated as described.23 Selected samples (~1 mm proximal from carotid bifurcation) from both genotypes were evaluated using Gas6 (1:100; Santa Cruz Biotechnology), Ki-67 (1:500; DAKO), and α1-actin antibody (1:1000; DAKO) with hematoxylin and eosin stain. The vessel compartment volumes were calculated as described.23 Sections were exposed but not ligated.23 The animals were allowed to recover and housed under SPF conditions with 12-hour/12-hour light/dark cycle for 3, 7, and 14 days after the surgery. Systolic blood pressure (BP) and heart rate (HR) were measured in nonanesthetized mice by tail-cuff plethysmography (Visitech Systems). Blood flow was measured on both carotids using an ultrasonic transit-time volume flowmeter (Transonic Systems Inc). Shear stress was calculated as described.25

Statistical Analysis
Results are reported as mean±SEM. Statistical tests were done with StatView for Macintosh, version 5.1.2. Comparison for 2 groups was performed using Student t test. Differences among 3 or more groups were analyzed by means of a repeated-measures 1-way ANOVA, followed by a Fisher’s post hoc test. The level of P<0.05 was regarded as significant.

Results
Physiological and Hemodynamic Characteristics of Axl Mouse
Axl+/− mice have similar physiological parameters compared with age- and sex-matched Axl+/+ mice (Table 1). Males were heavier (~3 g) than females in both genotypes (Table 1). Ligated animals gained less weight (~3%) compared with sham 2 weeks after ligation. There were no significant differences in systolic BP or HR at baseline or following ligation (data not shown).

Blood flow in carotids was similar in both genotypes (Table 2). In response to partial ligation, flow significantly decreased (~80%) in the left carotid artery (LCA) and increased (~60%) in the right carotid artery (RCA) 2 weeks after ligation (Table 2). There were no significant hemodynamic differences between Axl+/+ and Axl+/− mice after ligation (Table 2). Shams exhibited equal shear stress in Axl+/+ (28 dyne/cm²) and Axl+/− (27 dyne/cm²). Changes in shear stress were similar in Axl+/+ and Axl+/− mice 2 weeks after ligation: decreased in the LCA of Axl+/+ and Axl+/− (to 8 and 10 dyne/cm², respectively) and slightly increased in the RCA (30 and 30 dyne/cm², respectively). These results show that compensatory enlargement of RCA lumen to maintain shear stress constant was equivalent in both genotypes.

Vascular Remodeling After Flow Alteration in Axl Mice
In Axl+/+ mice, the major differences in vascular remodeling between ligated and sham animals occurred in the low flow LCA where an intima formed (white box, Figure 1A). The media thickened (bracket) compared with the sham LCA (inset, Figure 1A). The LCA adventitia (gray box in Figure 1A) also increased in Axl+/+ mice. There was no IMT formation in the high flow RCA of Axl+/+ mice (Figure 1B). Strikingly, remodeling was significantly attenuated in all vessel layers of the LCA of Axl+/− compared with Axl+/+
mice (Figure 1C versus 1A). Specifically, thickness of the intima, media, and adventitia were obviously decreased (Figure 1A versus 1C). There was no intima formation in Axl<sup>+/−</sup>/H11002<sup>RCA</sup> (Figure 1D).

The areas of individual components of the vessel wall in sham and ligated Axl mice (Figure I in the online data supplement available at http://circres.ahajournals.org) were similar to those previously described in C57Bl/6J mice. Quantitative morphometric analyses of the vessel component volumes showed no differences in sham-operated Axl<sup>+/−</sup> and Axl<sup>+/−</sup> vessels (filled and open bars, Figure 2; supplemental Figure II). There were no significant differences in any RCA measurements comparing ligated to sham or Axl<sup>+/−</sup> and Axl<sup>+/−</sup>, although RCA lumens exhibited a trend (P<0.10) to increase compared with shams in both genotypes (Figure 2A).

In contrast to the RCA, there were many differences in LCA morphometry. In response to decreased flow in the LCA, Axl<sup>+/−</sup> exhibited trends (P<0.09) toward increases in media and intima formation (supplemental Figure IIA and IIB), whereas intima+media and adventitia were significantly increased compared with shams (compare thin hatched with filled bars, Figure 2B and 2C, respectively). In contrast, vessel wall components in Axl<sup>+/−</sup> LCAs were not statistically different from shams 2 weeks after ligation (compare thick hatched with open bars, Figure 2B and 2C, respectively). However, Axl<sup>+/−</sup> LCAs showed a significant decrease in media compared with Axl<sup>+/+</sup> (compare thick hatched with thin hatched bars, supplemental Figure IIB), with a ≈50% reduction in intima (supplemental Figure IIA) that resulted in significantly attenuated intima+media remodeling in Axl<sup>+/−</sup> mice (Figure 2B). In addition, the adventitia response was also significantly decreased in Axl<sup>+/−</sup> compared with Axl<sup>+/+</sup> vessels (Figure 2C). Finally, the LCA lumen and vessel sizes (EEL) were similar between genotypes. The LCA lumen was significantly decreased compared with the RCA lumen after ligation (hatched bars, Figure 2A). Analysis of the vessel size measured by EEL did not show differences between sham and ligated mice in either genotype (supplemental Figure IIC). However, there was a trend (P=0.10) to increase RCA EEL compared with LCA EEL in both genotypes (supplemental Figure IIC). The relationship between intima+media size and vessel size (EEL) was similar in both genotypes (slope=0.7) and comparable to previous data for C57Bl/6J. Thus, Axl deficiency resulted in a significant decrease (~30%) in intima–media and adventitia thickening induced by low blood flow in the carotid.

**Cell Proliferation and Apoptosis in Ligated Carotids From Axl Mice**

To gain further insight into mechanisms by which Axl alters IMT, we evaluated cell apoptosis and proliferation in the LCA (Figure 3). There was no cell proliferation (measured by

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**Figure 1.** Photomicrographs of carotid cross-sections from Axl mice 2 weeks after ligation. LCA (Axl<sup>+/−</sup> [A]; Axl<sup>+/−</sup> [C]) and RCA (Axl<sup>+/−</sup> [B]; Axl<sup>+/−</sup> [D]), insets are sham-operated mice. Bracket shows area between internal and external elastic lamina; open boxes, intima formation; gray boxes, adventitia. Magnification is ×10 Bar=100 μm.

**Figure 2.** Vessel component volumes. Lumen (A), intima+media (B), and adventitia (C). Black bars indicate Axl<sup>+/−</sup> shams; thin hatched bars, Axl<sup>+/−</sup> ligated; white bars, Axl<sup>+/−</sup> shams; thick hatched bars, Axl<sup>+/−</sup> ligated. Values are mean±SEM. *P<0.05 compared with sham, †P<0.05 compare with Axl<sup>+/−</sup>, ‡P<0.05 compared with LCA (ANOVA).
Ki-67) in the LCA from sham-operated animals of both genotypes (insets, Figure 3A and 3B), as previously reported. In shams of both genotypes, a small number of apoptotic cells (ApopTag) were observed in the media (area within brackets in insets, Figure 3C and 3D). Quantitative analysis of the media of shams did not show any statistically significant differences in either apoptosis or proliferation between Axl+/+ and Axl−/− (data not shown).

Two major differences were observed in ligated LCA. First, there was a dramatic increase in ApopTag staining in the intima of Axl−/− compared with Axl+/+ mice (area to right of brackets, white arrows, compare Figure 3C versus 3D). In the intima the number of positive cells was significantly elevated in Axl−/− compared with Axl+/+ mice when measured per area (≈5-fold, Figure 3E) and per total cells (≈3-fold; Figure 3F). Second, both Axl−/− and Axl+/+ mice showed a significant increase in proliferation in the intima (white arrows, Figure 3A and 3B) when normalized to area (Figure 3E) or total cell number (Figure 3F). However, the 2-fold increase in proliferation was significantly lower than the increase in apoptosis (5-fold) in LCA Axl−/− (Figure 3E and 3F). Taken together, these data indicate that in response to flow reduction in the LCA, Axl−/− vessels exhibited a dramatic increase in apoptosis compared with Axl+/+ vessels.

**Gas6/Axl/p-Akt Pathway in Intima–Media in Axl Mice**

To characterize the signal transduction pathways that might explain differences in Axl−/− and Axl+/+ carotid remodeling, we studied expression of Gas6 and phosphorylation of Akt (p-Akt), a downstream target of Gas6/Axl (Figure 4). We observed low levels of Gas6 and p-Akt in the media of LCA from shams (Figure 4A and 4B and 4E and 4F, respectively). Gas6 expression was significantly upregulated after flow reduction in intimal cells and in extracellular matrix to a similar extent in both genotypes (Figure 4C and 4D). However, there was a dramatic decrease in p-Akt positive staining in the vessel wall of Axl−/− compared with Axl+/+ (Figure 4H versus 4G). Thus, we confirmed that the Gas6/Axl/p-Akt pathway is activated during flow-induced IMT in Axl+/+ and decreased in Axl−/−.

**Immunohistochemistry of Intima–Media in Axl Mice**

To gain further insight into mechanisms responsible for different remodeling in Axl−/− and Axl+/+ mice, we characterized vascular matrix and cell composition (Figure 5 and supplemental Figures III and IV). There were no differences in staining for elastin and collagen of the LCA between genotypes 2 weeks after ligation (supplemental Figure IIIb versus IIIc). However, there was a notable decrease in LCA cell nuclei (≈1.5-fold) in Axl−/− compared with Axl+/+ after ligation (brown staining, supplemental Figure IIIId versus IIId).

To characterize VSMC and myofibroblasts we analyzed smooth muscle α1-actin expression. We did not observe any differences in α1-actin staining between sham-operated Axl+/+ and Axl−/− mice (compare insets in Figure 5A versus 5B), whereas there was obvious decreased intensity of α1-
actin staining in the intima and media of Axl−/− compared with Axl+/+ (Figure 5B versus 5A).

We next focused on inflammatory cells because of the known role for Axl in white blood cell function.22 We found that there was a significant decrease in mononuclear inflammatory cells adherent to the internal elastic lamina in Axl−/− compared with Axl+/+ 3 days after ligation (white arrows, supplemental Figure IVB versusIVA). In addition, a significant decrease in media cell number was evident in Axl−/− compared with Axl+/+ 3 days after ligation (supplemental Figure IVB versusIVA). Despite prior evidence that Gas6/Axl modulates platelet function,13,14 we did not see any platelet adhesion in shams or ligated carotids 2 weeks after ligation (Figure 1 and supplemental Figure IV).

As we previously reported in C57Bl/6J mice,23 inflammatory cells were not detected in LCA from sham-operated mice of both genotypes (insets, Figure 5C through 5H). In LCAs from Axl+/+ mice, evidence for inflammation was clearly present 2 weeks after ligation. First, Axl+/+ mice showed intimal macrophages assayed by the specific F4/80 antibody (Figure 5C). Second, a large number of neutrophils were present in the intima of Axl+/+ mice assayed by the specific 7/4 antibody that does not recognize tissue macrophages (compare Figure 5E with 5C). Third, a significant number of CD45-positive cells consistent with hematopoietic lineage were present in carotids from Axl+/+ after ligation. Although the number of CD45-positive cells was less than F4/80 positive cells, several cells were positive for both antigens (compare Figure 5G with 5C).

The profile of inflammatory cell markers in Axl−/− LCA after ligation differed significantly from Axl+/+ LCA. First, no macrophages were present in the intima of Axl−/− mice (Figure 5D). Second, very few neutrophils were seen in the intima of Axl−/−, although substantial numbers were present in the adventitia (Figure 5F). Third, the greatest difference between genotypes was a dramatic increase in mononuclear cells positive for the CD45− in the intima of Axl−/− vessels (compare Figure 5F versus 5E).

Discussion

The major conclusion of this study is that Axl, a receptor tyrosine kinase, plays an important role in vascular remodeling induced by changes in blood flow. We found that genetic deletion of Axl significantly attenuated IMT of the mouse carotid, by increasing cell apoptosis and by altering vascular inflammation. The first major finding was that Axl−/− mice demonstrated a critical role for apoptosis in IMT, as predicted by the known effect of Axl to prevent VSMC apoptosis.20 A second important finding was that the Gas6/Axl pathway regulated the function of several cell types in the vessel wall (Figure 5) and appeared to act in an autocrine/paracrine fashion (based on the increase in Gas6 expression). Third, remodeled carotids from Axl−/− mice had a significantly altered inflammatory response compared with Axl+/+ mice. Specific alterations included relatively more monocytes than VSMC, decreased macrophages and neutrophils in the intima, and increased neutrophils in the adventitia. There was a significant delay in the inflammatory response in Axl−/− during vascular remodeling (supplemental Figure IV), which could be attributable to apoptosis and/or a failure to complete phagocytosis caused by impaired macrophage function.22 These data provide novel insights into the pathophysiological importance of Axl in vascular remodeling.

The most significant conclusion of this study is that apoptosis plays a key role in IMT. Previously, we and others23-26 reported that proliferation is a driving force for remodeling in response to low flow. Indeed, there was increased proliferation in both Axl+/+ and Axl−/− LCAs, as anticipated (Figure 3). However, the rate of apoptosis was dramatically increased in Axl−/− (5 times greater than proliferation), providing an obvious explanation for the decreased IMT. In addition to changes in the intima and media, a significant decrease in adventitia in Axl−/− vessels may also contribute to decreased IMT.25 The importance of coordination between proliferation and apoptosis was previously shown in flow-dependent remodeling in the rabbits.27 There are also 2 publications in transgenic mice showing that flow-induced remodeling is regulated by apoptosis.28,29 First, the Fasl-deficient mouse that lacks the proapoptotic Fas
ligand exhibited increased intima formation after flow cessation.28 Second, the neurotrophin receptor P75NTR-deficient mouse exhibited increased intima associated with decreased apoptosis.29 Our data suggest that inhibiting Axl would be a useful strategy to treat vascular diseases by promoting macrophage apoptosis, as proposed recently.30 The finding that the intima in Axl−/− mice lacked intimal macrophages suggests a significant role for Axl in resolving vascular inflammation, which is a key feature of IMT (Figure 5). This concept is supported by the findings that Mer (a member of the Axl/UFO tyrosine-receptor family) is required for receptor-mediated phagocytosis of apoptotic cells by macrophages. In particular, Mer knockout macrophages exhibited a significant delay in phagocytosis and clearance of apoptotic thymocytes.6 Despite the fact that we were unable to detect apoptotic cells engulfed by intimal macrophages in LCA in Axl+/− mice, a macrophage marker (F4/80) that recognizes tissue macrophages also recognizes cells that have phagocytosed latex or Leishmania major in vitro.31 Because the ratio of cells positive for ApopTag versus F4/80 was impressively different between Axl−/− and Axl+/− mice after ligation, we can speculate that phagocytosis is altered during IMT. It is likely that phagocytic defects in Axl−/− vessels increased monocyte recruitment to the intima because of loss of feedback inhibition (Figure 5H). Specifically, phagocytotic clearance of apoptotic cells inhibited monocyte recruitment to atherosclerotic lesions via TGF-β signaling in the apoE−/− mouse.32 However, the biggest difference between genotypes in our study was a dramatic decrease in macrophages and neutrophils in the intima of Axl−/− mice, suggesting that lack of Axl may also prevent differentiation of peripheral mononuclear cells in the vessel wall. Under physiological conditions, Axl is expressed only by peripheral monocytes and macrophages, and not by lymphocytes or granulocytes.33 Several studies suggest that Axl plays a role in differentiation of early myeloid progenitors,33 thymic stromal cells,34 and mast cells.35 In addition, a triple knockout of all Axl-family receptors exhibited lymphoproliferative abnormalities attributable to increased expression of proinflammatory cytokines by activated macrophages.7 Similarly, our data showed that decreased survival and/or differentiation of intimal macrophages and neutrophils resulted in a greater neutrophil recruitment to the LCA adventitia of Axl−/− mice (Figure 5F). Future studies to compare cytokine expression in remodeling vessels in Axl mice will be useful to define specific roles for monocyte- and neutrophil-derived proteins.

The major limitation of the present study is the absence of dramatic differences in carotid EEL volumes following ligation, despite significant differences in IMT (compare supplemental Figure IIC versus Figure 2C). This phenomenon is probably attributable to the genetic background (C57Bl/6J) of Axl mice. This mouse strain did not respond with significant neointima formation to complete ligation of the carotid artery.36 Previously we reported that C57Bl/6J inbred mouse strain exhibited small changes in carotid EEL 2 weeks after partial ligation.23,24 Despite this disadvantage of the background mouse strain, we did find significant differences in luminal and EEL volumes between LCA and RCA (Figure 2A and supplemental Figure IIC). Other limitations are the possibility of compensatory mechanism(s) occurring in Axl−/− mice after ligation, increased monocyte recruitment to the intima (Figure 5H), and an increased proliferation rate compared with Axl+/− (Figure 3E and 3F). Finally, future studies will be needed to clarify the specific mechanism(s) of Gas6/Axl pathway activation during IMT. We previously found that angiostatin II or thrombin can upregulate Axl in VSMC.19 In addition, the Gas6/Axl pathway can be upregulated by ROS in blood vessels.21 Increasing evidence suggests that the Gas6/Axl/Akt pathway is important for vascular homeostasis and represents a therapeutic opportunity because of its key role in function of several cell types.14,37,38 For example, inhibiting Gas6 transglutamination by warfarin showed beneficial effects in nephropathy models.7,39,40 Recently, Axl polymorphisms were associated with salt-induced hypertension38 and osteoarthritis.41 An important role for the Gas6/Axl/Akt pathway in thrombosis has been shown because both Gas6 and Axl-family receptor knockout mice exhibited abnormalities in platelet function,13,14 and a Gas6 mutation is associated with thrombotic stroke in humans.12 However, it is likely that Gas6-dependent signaling is important only during pathological processes because no correlation between plasma Gas6 and platelet aggregation in healthy people was found.42 Our current data on vascular remodeling and previous reports19,21...
support a key role for the Gas6/Axl pathway in vascular remodeling and vascular inflammation.

Acknowledgments

V.A.K. received an American Heart Association Scientist Development Grant (0432067N). This study was also supported in part by NIH grant HL-64836 (to B.C.B.). We thank Drs Jun-ichi Abe and Klaus Ley for thoughtful discussions of the manuscript.

References


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Circ Res. 2006;98:1446-1452; originally published online April 20, 2006;
doi: 10.1161/01.RES.0000223322.16149.9a
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Figure I. The carotid component areas are plotted versus the length of the carotid artery from Axl mice. A, LCA lumen; B, RCA lumen; C, LCA intima+media; D, RCA media; E, LCA adventitia; F, RCA adventitia. Open squares are Axl+/+ shams, black squares are Axl+/+ ligated, open triangles are Axl-/- shams, black triangles are Axl-/- ligated. LCA, left carotid artery. RCA, right carotid artery. Values are mean±SEM.

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Figure II. Vessel component volumes. Intima (A), media (B) and external elastic lamina (C). Black bars are Axl\(^{+/+}\) shams; thin hatched bars are Axl\(^{+/+}\) ligated; white bars are Axl\(^{-/-}\) shams; thick hatched bars are Axl\(^{-/-}\) ligated. LCA, left carotid artery. RCA, right carotid artery. Values are mean±SEM. *, p<0.05 compare with Sham; †, p<0.05 compare with Axl\(^{+/+}\) (ANOVA).

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Figure III. Representative microphotographs of Van Gieson staining of LCA from Axl mice. Elastic fibers are black; nuclei, brown; collagen, red; other tissue structure yellow. Magnification bar is 20 µm. Magnification is 60x.
**Figure IV.** Representative microphotographs of Hematoxylin and Eosin staining of LCA from Axl mice. 3 days (A, Axl\(^{+/+}\); B, Axl\(^{-/-}\)) and 7 days after ligation (C, Axl\(^{+/+}\); D, Axl\(^{-/-}\)). Insets are shams. Bracket shows area between internal and external elastic lamina. White arrows show inflammatory cells. Magnification is 60x. Bar is 20 \(\mu m\).