Hydrogen Peroxide Activates the Gas6-Axl Pathway in Vascular Smooth Muscle Cells

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Running Title: Axl activation by H$_2$O$_2$ in VSMC
Abstract

Axl, a receptor tyrosine kinase, is involved in cell survival, proliferation, and migration. We have shown that Axl expression increases in the neointima of balloon-injured rat carotids. Since oxidative stress is known to play a major role in remodeling of injured vessels, we hypothesized that \( \text{H}_2\text{O}_2 \) might activate Axl by promoting autophosphorylation. \( \text{H}_2\text{O}_2 \) rapidly stimulated Axl tyrosine phosphorylation in rat vascular smooth muscle cells (VSMC) within 1 min that was maximal at 5 min (6-fold). The response to \( \text{H}_2\text{O}_2 \) was concentration-dependent with \( \text{EC}_{50} \) of \(~500 \mu\text{M} \). Axl phosphorylation was partly dependent on production of its endogenous ligand, Gas6, since Axl-Fc, a fragment of Axl extracellular domain that neutralizes Gas6, inhibited \( \text{H}_2\text{O}_2 \)-induced Axl phosphorylation by 50%. Axl phosphorylation by \( \text{H}_2\text{O}_2 \) was also attenuated by warfarin which inhibits Gas6 activity by preventing post-translational modification. In intact vessels Axl was phosphorylated by \( \text{H}_2\text{O}_2 \), and Axl phosphorylation inhibited by warfarin-treatment in balloon-injured carotids. Akt, a downstream target of Axl was phosphorylated by \( \text{H}_2\text{O}_2 \) in Axl\(^{+/+}\) mouse aorta, but significantly inhibited in Axl\(^{-/-}\) aorta. Intimal proliferation was decreased significantly in a cuff injury model in Axl\(^{+/+}\) mice compared to Axl\(^{-/-}\) mice. In summary, Axl is an important signaling mediator for oxidative stress in cultured VSMC and intact vessels, and may represent an important therapeutic target for vascular remodeling and response to injury.
Introduction

Axl, also known as Ark, UFO, and Tyro7, is a receptor tyrosine kinase (RTK) expressed in several types of cells including VSMC and mesangial cells (1). It is known that Gas6 (Growth Arrest Gene 6), a homolog of Protein S, is a ligand for Axl (2). Gas6 has been shown to induce Axl tyrosine-phosphorylation and promote cell survival, migration, and growth (3);(4);(5). Importantly, Axl is a member of a large RTK family that includes Sky (also named Rse, Dtk, and Tyro3) and Mer. These RTKs have important roles in inflammatory cell function (both T cells and monocytes) (6). Our laboratory has studied Axl in cardiovascular disease for many years since we identified it as the RTK most highly regulated following balloon-injury of the rat carotid (4). Recently we have established that Gas6-Axl-Akt pathway is one of the anti-apoptotic mechanisms for VSMC that may be important in the response to vascular injury (4).

Axl may be activated not only by Gas6 but also by H₂O₂ since other RTKs (e.g., EGFR, PDGFR, and FGFR) are tyrosine-phosphorylated by H₂O₂ (7-11). Thus under conditions of oxidative stress, such as commonly occur with hypertension and diabetes, Axl is likely to be activated. Most relevant are data that reactive oxygen species including H₂O₂ are increased in diabetic glomeruli and injured vessels. A role for the Gas6-Axl pathway in nephropathy was suggested by a recent report that showed diabetic Axl pathway was less severe when Gas6 expression was decreased (Gas6-knockout mice) or Gas6 activity inhibited (treatment with warfarin that blocks γ-carboxylation) (12). Based on these data we hypothesized that Gas6-Axl activation may be important in the vascular response to injury mediated by reactive oxygen species.
Materials and Methods

**Materials**— Antibodies to phospho-Akt (Ser473), Akt, and phospho-p38 were purchased from Cell Signaling Technologies (Beverly, MA). Antibodies to Axl (goat polyclonal), p38 (rabbit polyclonal), and phosphotyrosine (mouse monoclonal) were from Santa Cruz (Santa Cruz, CA). Axl-Fc was from R&D Systems (Minneapolis, MN). Rabbit polyclonal antibody for Axl (13) and Gas6 were kindly provided by Dr Brian Varnum in Amgen (Thousand Oaks, CA). All other reagents and chemicals were from Sigma (St. Louis, MO) unless specifically indicated.

**Cell Culture**—VSMC were isolated from the thoracic aorta of 200-250 g male Sprague-Dawley rats and maintained in DMEM containing 5.5 mM glucose, supplemented with 10% fetal bovine serum (Gibco BRL/ Life technologies, Rockville, MD) as previously described (14).

**Immunoblot Analysis**—Western blot analyses were performed as described previously (15). VSMC were lysed in Triton-based lysis buffer (1% Triton X100, 20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 0.1% Protease inhibitor cocktail (Sigma)), scraped off the dish and centrifuged at 10,000 x g for 10 min, and the supernatant was collected as a total cell lysate. The equal amounts of cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond™ EDL, Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were incubated with appropriate primary antibodies and membrane-bound antibodies were visualized by HRP-conjugated secondary antibodies and ECL system (Amersham Pharmacia Biotech). To detect Axl or phospho-Axl in the membranes prepared from vessel lysates, SuperSignal West Femto (Pierce, Milwaukee, WI) was used for a chemiluminescent reagent according to the manufacturer's protocols. For lysate preparation from
aortae or carotids, vessels were ground with a pellet pestle (Kimble Kontes, Vineland, NJ) in 1.5 ml tube cooled with liquid nitrogen and suspended in lysis buffer. After a centrifugation (10,000 x g, 10 min), the supernatant was collected as a total cell lysate.

**Immunoprecipitation**—Lysates containing equal amounts of protein were incubated with anti-Axl antibody (goat polyclonal) overnight at 4 °C. After incubation with protein G agarose (Gibco BRL/ Life technologies, Rockville, MD) for 2 h, precipitates were washed with lysis buffer and then resuspended in SDS-PAGE sample buffer. After being denatured at 100 °C for 5 min, samples were separated by SDS-PAGE.

**Animal experiments:** Axl−/− mice were kindly provided by Dr Stephen Goff (16). Mice homozygous for null-mutation of Axl were viable and fertile, and displayed no anatomical abnormality as described before (16). Femoral artery cuff injury was performed exactly as previously described (17). Balloon injury of the rat left carotid was performed exactly as described using male Sprague-Dawley rats (300 to 400 g, Charles River Laboratories, Wilmington, MS) (4). Warfarin was administered to rats in drinking water (0.25 mg/L, warfarin sodium, Sigma) beginning 2 days prior to carotid injury. The dosage was determined based on a previous report (12). At the end of the experiment, the injured (cuff or balloon) and uninjured contralateral vessels were removed and snap-frozen in liquid nitrogen. Animals whose vessels were used for histological analysis were perfusion-fixed at 130 mm Hg with 10% formalin. All procedures were carried out in a specific pathogen-free animal care facility at the University of Rochester and were approved by the University Committee on Animal Resource at University of Rochester.
**Histological Analysis**—Paraffin-embedded carotid and femoral arteries (5 μm sections) were stained with hematoxylin-eosin and photographed with a digital camera equipped with a microscopy. The intima / media (I/M) ratio was measured using Image J software.

**Statistics**—Cell culture results are representative of 2 or 3 different experiments. Data with error bars were expressed as mean ± SEM. The comparison between uninjured and injured arteries was performed using 2-way ANOVA. The comparison of phospho-Axl between vehicle and warfarin treatment was performed using Student’s-t test.
Results

To initiate our studies of Axl we measured the activation in response to H$_2$O$_2$. As shown in Fig. 1, 600 µM H$_2$O$_2$ rapidly stimulated Axl tyrosine phosphorylation (onset within 1 min) with a 6-fold increase at 5 min. Immunoprecipitation of Axl yielded 3 immunoreactive bands (140, 120 and 104 kD) as reported previously(18). These differences are due to varied N-glycosylation as N-glycanase treatment reduced molecular mass to 104 kD). The major protein phosphorylated was the 140 kD protein which was used for all quantification. No obvious differences in regulation of the three Axl immunoreactive proteins were observed. The response to H$_2$O$_2$ was concentration dependent with EC$_{50}$ of approximately 500 µM (Fig. 2). The magnitude of Axl phosphorylation by 500 µM H$_2$O$_2$ was approximately 2-fold greater than phosphorylation by 50 nM Gas6, an endogenous ligand (not shown). The mechanism by which H$_2$O$_2$ stimulates Axl phosphorylation is unknown. Previously, RTK activation has been shown to involve both intracellular (e.g. via c-Src activation and protein tyrosine phosphatase inhibition) and extracellular pathways (via generation of HB-EGF) (11);(8). To characterize the relative roles of these pathways in H$_2$O$_2$-mediated Axl activation we studied the effect of Axl-Fc, a fragment of the extracellular domain of Axl that binds Gas6 and inhibits Gas6-Axl signaling (19). Inhibition of Axl phosphorylation in the presence of Axl-Fc implicates Gas6 as the activating ligand. As shown in Fig. 3, 2 µg/ml Axl-Fc inhibited H$_2$O$_2$-mediated tyrosine phosphorylation at 5 min by ~50% suggesting an important role for extracellular Gas6. To confirm the role of Gas6 we also investigate the effect of warfarin which inhibits γ-glutamyl carboxyltransferase and prevents γ-carboxylation of Gas6, thereby decreasing its interaction with Axl (4). As shown in Fig. 4, warfarin inhibited H$_2$O$_2$-dependent Axl tyrosine phosphorylation in
a concentration dependent manner (IC$_{50}$ ~100 nM). In summary these data indicate that endogenous Gas6 is partially required for H$_2$O$_2$-mediated Axl tyrosine phosphorylation.

To prove the physiological relevance of H$_2$O$_2$-mediated Axl activation, we characterized tyrosine phosphorylation of intact rat aortae. Aortae were cultured ex vivo, and stimulated with 1 mM H$_2$O$_2$. Axl phosphorylation peaked at 5 min similar to cultured VSMC (Fig. 5). We and others (3,4) have shown that Axl mediates anti-apoptotic effects in part via activation of Akt. To evaluate the importance of Axl in H$_2$O$_2$-mediated Akt activation we used mouse aortae that lack the Axl receptor (Axl$^{-/-}$). As shown in Fig. 6A, H$_2$O$_2$-mediated activation of Akt (measured by phosphoserine 473-Akt) was dramatically inhibited in Axl$^{-/-}$ aortae. Interestingly, H$_2$O$_2$ activation of p38 measured by phospho-p38 was not inhibited (Fig. 6B).

To clarify further the physiological importance of Axl activation in VSMC in vivo, we studied phosphorylation of Axl in injured vessels as well as the effects of warfarin on Axl phosphorylation and the neointimal response to injury. We chose the balloon-injured rat carotid as a standard model to assess the VSMC response to injury. To evaluate the effect of warfarin, rats were given 0.25 µg/ml warfarin in the drinking water for 2 days prior and 7 days after injury. Previously, we found that Axl expression reached a plateau at 7 days (4). Lysates of injured and uninjured vessels were collected and Axl expression, as well as Axl phosphorylation, were studied by Western blot. As we have already shown (4), Axl expression in injured vessels was significantly increased both in vehicle- and warfarin-treated animals compared to uninjured vessels (1.41 ± 0.18 fold in vehicle group and 1.26 ± 0.12 fold in warfarin group, p < 0.05 vs uninjured). Furthermore, Axl phosphorylation was increased in injured vessels compared with uninjured vessels as shown in Fig. 7A. Warfarin-treatment significantly inhibited the increase in
Axl phosphorylation normalized to Axl expression (Fig. 7B), demonstrating that the inhibition of Gas6 activity by warfarin attenuated Axl activation in injured carotids.

Axl activation may increase VSMC migration, proliferation, and survival (4,5,20) potential mechanisms involved in neointima formation. To study the effect of warfarin on neointima formation, animals were treated for 15 days after balloon injury. The animals were perfused, vessels harvested, and morphometry performed. The intima/media ratio of injured vessels was determined as a measure of neointima formation. The ratios were 0.953 ± 0.093 and 0.921 ± 0.137 in control and warfarin-treated groups respectively (N = 11). Thus, there was no statistically significant effect of warfarin to inhibit neointima formation (p > 0.05).

To evaluate the role of Axl in VSMC function further we studied the vessel response to injury in the Axl<sup>−/−</sup> mouse. Neointima formation was induced by placement of a polylethylene cuff around the left femoral artery (LFA, right femoral artery (RFA) was control). After 28 days of cuff injury, vessels were harvested for analysis. The uninjured RFA showed no intima formation in either Axl<sup>−/−</sup> or Axl<sup>+/+</sup> (not shown). The injured LFA in Axl<sup>+/+</sup> exhibited substantial intima formation (Fig. 8A, 8D). In contrast, intima formation was markedly less (~80% inhibition) in the injured LFA of Axl<sup>−/−</sup> mice (Fig. 8B, 8D). There were no significant differences in lumen, media or adventitia area in Axl<sup>−/−</sup> compared to Axl<sup>+/+</sup> (Fig. 8C, 8E, 8F). These data show that neointima formation induced by vascular injury is significantly reduced in the Axl<sup>−/−</sup> mice.

**Discussion**

The major findings of the present study are that Axl is a redox sensitive receptor tyrosine kinase activated by H<sub>2</sub>O<sub>2</sub> in VSMC (both in culture and intact aorta) as well as by injury in intact carotid and femoral arteries. Specifically, we found that Axl was tyrosine-phosphorylated by
H₂O₂ via a mechanism that was partially dependent on endogenous Gas6, as shown by inhibition with Axl-Fc or warfarin. There was a significant increase in Axl phosphorylation in the injured artery. Importantly, mice deficient in Axl expression exhibited markedly smaller neointima formation in response to injury. These data demonstrate the essential role for Axl in the vessel response to injury, and represent the first report of Axl activation by a pathophysiological process in the vasculature.

It has been previously shown that several RTKs including EGFR (7,8,10), PDGFR (11), and FGFR (9) are activated by H₂O₂ at concentrations ranging from 20 μM to 5 mM, although the precise mechanisms of receptor activation are unknown. Several investigators have shown that proteolytic shedding of an endogenous ligand, HB-EGF, is involved in EGFR tyrosine-phosphorylation upon stimulation with H₂O₂ or angiotensin II (8,21,22). Therefore, we assessed the role of endogeneous Gas6 in Axl phosphorylation by inhibiting Gas6. Axl phosphorylation was partially attenuated by both Axl-Fc and warfarin (Fig. 3 and 4), suggesting that endogenous Gas6 was involved in H₂O₂-induced Axl activation. Interestingly, a Gas6 splice variant is proteolytically cleaved releasing a peptide that has full capability to activate Axl receptor phosphorylation (23,24). Because Gas6 can localize on the cell surface through its γ-carboxyglutamic acid domain (25), it is possible that active fragments might be proteolytically released (similar to HB-EGF) upon stimulation with H₂O₂. An alternative mechanism of activation is inhibition of tyrosine phosphatases which has been proposed to be responsible for PDGFR activation.(26). Although the tyrosine phosphatase modulating Axl activation has not been identified, it has been reported that a protein containing a putative tyrosine-phosphatase domain specifically associates with Axl (27). Thus we speculate that Axl receptor activation by H₂O₂ may be regulated both by intrinsic tyrosine-kinase activity (stimulated by receptor
dimerization in part due to Gas6 binding) and by tyrosine-phosphatase activity (inhibited by \( \text{H}_2\text{O}_2 \)) (28).

In the present study we showed Axl phosphorylation ex vivo in intact vessels using a relatively high dose of \( \text{H}_2\text{O}_2 \) (1 mM, Fig. 5). We believe that the high concentration of \( \text{H}_2\text{O}_2 \) was necessary because of the very low expression levels of Axl and Gas6 in the normal aorta (4). In contrast, Axl expression was increased in injured carotids (Fig. 7A) in which Gas6 as well as \( \text{H}_2\text{O}_2 \) production would also be augmented (29). It is likely that a large amount of \( \text{H}_2\text{O}_2 \) would be required to activate Axl in normal vessels, whereas only a small increase in oxidative stress may be enough under pathologic conditions in which Axl and Gas6 are already increased.

Neointima formation in response to cuff injury was significantly reduced in Axl\(^{-/-}\) mice compared to Axl\(^{+/+}\) mice. These data demonstrate an essential role for Axl in the vessel response to injury. Axl expression likely contributes to neointima formation by two non-exclusive mechanisms. First, Axl expression is required for VSMC migration, proliferation and protection from apoptosis. All three processes have been shown to augment neointima formation in response to vascular injury. Second, Axl expression may contribute to stem cell-dependent neointima formation. Axl is highly expressed in hematopoietic stem cells and these cells have been shown to participate in vascular injury, repair and atherosclerosis lesion formation (30).

Interestingly, we could not detect a significant decrease in neointima formation by warfarin-treatment, although the same dose of warfarin clearly reduced Axl phosphorylation in injured carotids (Fig. 7B). There are several explanations why warfarin had no effect on neointima formation in the present study while other investigators showed a clear benefit in nephropathy models (12,19). First, warfarin may have both beneficial and harmful effects on the injured vessel. It has been shown that warfarin inhibits platelet adhesion to de-endothelialized vessels, a
process that may protect VSMC from oxidative stress (31). Second, inhibition of Axl by warfarin is likely to be partial as shown in Fig 4, and insufficient to inhibit VSMC proliferation and migration in vivo. Third, other pathways including EGF, FGF and PDGF that stimulate neointima formation (32);(33) are not blocked by warfarin. Fourth, the dominant effect of Gas6-Axl signaling may be to prevent apoptosis (rather than promote proliferation) as suggested by the difference in Akt activation in Axl-/- mouse aorta (Fig. 6) and previous studies(3). If apoptosis is not as important as proliferation in neointima formation it is logical that Axl inhibition may have little effect. Finally, transactivation of Axl by H₂O₂ will not be affected by warfarin to the extent that transactivation is independent of Gas6 (20). To address these questions we are studying the effect of Axl deficiency in mouse vascular injury models.

The present observations that Axl is activated by H₂O₂ in cultured VSMC and intact vessels are of importance because they suggest a mechanism for Axl activation under conditions of increased Axl expression and oxidative stress. Although an Axl-specific tyrosine kinase inhibitor has yet to be identified, the potential role of Gas6-Axl in hypertension, atherosclerosis and nephropathy in which increases in Axl expression and H₂O₂ production are evident makes this pathway an attractive therapeutic target (4,12,19,34,35).

Acknowledgements

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References

Figure Legends

Figure 1. Axl tyrosine-phosphorylation is induced by H$_2$O$_2$ in a time-dependent manner.
A. VSMC were incubated with serum-free DMEM for 24 h, then treated with H$_2$O$_2$ (600 µM) for the indicated times. Cell lysates were immunoprecipitated with goat anti-Axl antibody and blotted with anti-phosphotyrosine antibody. The membrane was stripped and reprobed with rabbit anti-Axl antibody. B. Quantified densitometry data expressed as fold increases relative to control.

Figure 2. Axl tyrosine-phosphorylation is induced by H$_2$O$_2$ in a dose-dependent manner.
A. VSMC were incubated with serum-free DMEM for 24 h, then treated with the indicated concentrations of H$_2$O$_2$ for 5 min. Cell lysates were immunoprecipitated with goat anti-Axl antibody and blotted with anti-phosphotyrosine antibody. The membrane was stripped and reprobed with rabbit anti-Axl antibody. B. Quantified densitometry data expressed as fold increases relative to control.

Figure 3. Axl-Fc partially inhibits Axl-phosphorylation induced by H$_2$O$_2$.
VSMC were incubated with serum-free DMEM containing 0.1 % bovine serum albumin with or without Axl-Fc (2 µg/ml) for 24 h, then treated with H$_2$O$_2$ (600 µM) for the indicated times. Cell lysates were immunoprecipitated with goat anti-Axl antibody and blotted with anti-phosphotyrosine antibody. The membrane was stripped and reprobed with rabbit anti-Axl antibody.

Figure 4. Warfarin partially inhibits Axl-phosphorylation induced by H$_2$O$_2$.
VSMC were incubated with serum-free DMEM containing warfarin at the indicated concentrations for 24 h, then treated with H$_2$O$_2$ (600 µM) for 5 min. Cell lysates were
immunoprecipitated with goat anti-Axl antibody and blotted with anti-phosphotyrosine antibody. The membrane was stripped and reprobed with rabbit anti-Axl antibody.

**Figure 5. Axl tyrosine-phosphorylation by H₂O₂ in intact aortae.**

Aortae from genetically hypertensive (GH) rats (male, 300-400 g) were incubated with serum-free DMEM for 24 h, then treated with H₂O₂ (1 mM) for the indicated times. The aortae were snap-frozen in liquid nitrogen and lysates were prepared. Lysates were immunoprecipitated with goat anti-Axl antibody and blotted with anti-phosphotyrosine antibody. The membrane was stripped and reprobed with rabbit anti-Axl antibody.

**Figure 6. Akt is a downstream target of Axl activation induced by H₂O₂.**

A. Aortae from Axl⁻/⁻ mice or wild-type littermates (Axl⁺/⁺) were incubated with serum-free DMEM for 24 h, then treated with H₂O₂ (600 µM) for 5 min. The aortae were snap-frozen in liquid nitrogen and lysates were prepared. Lysates were blotted with anti-phospho-Akt or anti-phospho-p38 antibody, then stripped and reprobed with anti-Akt or anti-p38 antibody, respectively. B. Quantified densitometry data expressed as percentage of phospho-Akt or phospho-p38 in Axl⁻/⁻ aorta treated with H₂O₂. Results are representative of two experiments using aortae pooled from two animals for each measurement.

**Figure 7. Warfarin attenuates Axl phosphorylation in injured carotids.**

A. Rats were harvested on day 7 after balloon injury. Lysates from injured (I) and uninjured (U) carotids were immunoprecipitated with goat anti-Axl antibody and blotted with anti-phosphotyrosine antibody (N = 4). The membrane was stripped and reprobed with rabbit anti-Axl antibody, then with anti-goat IgG. B. Densitometry data of phospho-Axl was quantified and expressed as the ratio of I/ U normalized with Axl expression. *, p < 0.05.
Figure 8. Analysis of femoral arteries after cuff-injury in Axl+/+ and Axl−/− mice. A, Photomicrograph of LFA from Axl+/+. B, Photomicrograph of LFA from Axl−. Arrows show neointima. Magnification is 20x. Inset magnification is 60x. C, Lumen area. D, Intima area. E, Media area. F, Adventitia area. C-F, open bars are Axl+/+ (n=8), solid bars are Axl−/− (n=8). In addition, intima+media area was significantly reduced in Axl−/− mice (13.4±1.2 x10⁻³ μm²) compared with Axl+/+ (18.9±1.5 x10⁻³ μm²). Also note that LFA external elastic lamina failed to increase in Axl−/− (45.9±1.9 x10⁻³ μm²) compared with Axl+/+ (57.3±5.7 x10⁻³ μm²), while RFA were ~40±5 x10⁻³ μm². Values are mean±SEM. †, p<0.05 compared with Axl+/+ LFA.
Fig. 1
Fig. 2

Panel A: Immunoprecipitation (IP) of Axl with the indicated concentrations of hydrogen peroxide ($\text{H}_2\text{O}_2$, $\mu$M).

Panel B: Graph showing the arbitrary units of Axl as a function of $\text{H}_2\text{O}_2$, $\mu$M concentrations.
Fig. 3
Fig. 4

H$_2$O$_2$ 600 μM

IP: Axl
pY

Axl

Warfarin, nM 0 0 1 10 100 1000
Fig. 7

A

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B

![Bar chart](BarChart)
Fig. 8

A. Axl+/+, LFA

B. Axl-/-, LFA

C. Lumen

D. Intima

E. Media

F. Adventitia

Area, x10^-3 µm^2

A. Axl+/+, LFA B. Axl-/-, LFA

†