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

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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. Because oxidative stress is known to play a major role in remodeling of injured vessels, we hypothesized that H2O2 might activate Axl by promoting autophosphorylation. H2O2 rapidly stimulated Axl tyrosine phosphorylation in rat vascular smooth muscle cells within 1 min that was maximal at 5 min (6-fold). The response to H2O2 was concentration-dependent with EC50 of ~500 μM. Axl phosphorylation was partly dependent on production of its endogenous ligand, growth arrest gene 6 (Gas6), because Axl-Fc, a fragment of Axl extracellular domain that neutralizes Gas6, inhibited H2O2-induced Axl phosphorylation by 50%. Axl phosphorylation by H2O2 was also attenuated by warfarin, which inhibits Gas6 activity by preventing post-translational modification. In intact vessels Axl was phosphorylated by H2O2, and Axl phosphorylation was inhibited by warfarin treatment in balloon-injured carotids. Axl, a downstream target of Axl, was phosphorylated by H2O2 in Axl+/− mouse aorta but significantly inhibited in Axl−/− aorta. Intimal proliferation was decreased significantly in a cuff injury model in Axl−/− mice compared with Axl+/+ mice. In summary, Axl is an important signaling mediator for oxidative stress in cultured vascular smooth muscle cells and intact vessels and may represent an important therapeutic target for vascular remodeling and response to injury.

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times. Cell lysates were immunoprecipitated with goat anti-Axl antibody and blotted with antiphosphotyrosine antibody. The membrane was stripped and reprobed with rabbit anti-Axl antibody. B, quantified densitometry data expressed as fold increases relative to control.

FIG. 1. Axl tyrosine phosphorylation is induced by H2O2 in a time-dependent manner. A, VSMC were incubated with serum-free DMEM for 24 h and then treated with H2O2 (600 μM) for the indicated times. Cell lysates were immunoprecipitated with goat anti-Axl antibody and blotted with antiphosphotyrosine antibody. The membrane was stripped and reprobed with rabbit anti-Axl antibody. B, quantified densitometry data expressed as fold increases relative to control.

FIG. 2. Axl tyrosine phosphorylation is induced by H2O2 in a dose-dependent manner. A, VSMC were incubated with serum-free DMEM for 24 h and then treated with the indicated concentrations of H2O2 for 5 min. Cell lysates were immunoprecipitated with goat anti-Axl antibody and blotted with antiphosphotyrosine antibody. The membrane was stripped and reprobed with rabbit anti-Axl antibody. B, quantified densitometry data expressed as fold increases relative to control.

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RESULTS

To initiate our studies of Axl, we measured the activation in response to H2O2. As shown in Fig. 1, 600 μM H2O2 rapidly stimulated Axl tyrosine phosphorylation (onset within 1 min) with a 6-fold increase at 5 min. Immunoprecipitation of Axl yielded three immunoreactive bands (140, 120, and 104 kDa) as reported previously (18). These differences are because of varied N-glycosylation, because N-glycosidase treatment reduced molecular mass to 104 kDa. The major protein phosphorylated was the 140-kDa protein, which was used for all quantification. No obvious differences in regulation of the three Axl immunoreactive proteins were observed. The response to H2O2 was concentration-dependent with EC50 of ~500 μM (Fig. 2). The magnitude of Axl phosphorylation by 500 μM H2O2 was ~2-fold greater than phosphorylation by 50 nM Gas6, an endogenous ligand (not shown). The mechanism by which H2O2 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) (8, 11). To characterize the relative roles of these pathways in H2O2-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 H2O2-mediated tyrosine phosphorylation at 5 min by ~50%, suggesting an important role for extracellular Gas6. To confirm the role of Gas6 we also investigated 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 H2O2-dependent Axl tyrosine phosphorylation in a concentration-dependent manner (IC50 ~100 nM). In summary, these data indicate that endogenous Gas6 is partially required for H2O2-mediated Axl tyrosine phosphorylation.

To prove the physiological relevance of H2O2-mediated Axl activation, we characterized tyrosine phosphorylation of intact rat aortas. Aortas were cultured ex vivo and stimulated with 1 mM H2O2. Axl phosphorylation peaked at 5 min, similar to cultured VSMC (Fig. 5). We and others (3, 4) have shown that Axl mediates antiapoptotic effects, in part, via activation of Akt. To evaluate the importance of Axl in H2O2-mediated Akt activation we used mouse aortas that lack the Axl receptor (Axl−/−). As shown in Fig. 6A, H2O2-mediated activation of Akt (measured by phosphoserine 473-Akt) was dramatically inhibited in Axl−/− aortas. Interestingly, H2O2 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

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 (300–400 g; Charles River Laboratories, Wilmington, MS) (4). Warfarin was administered to rats in drinking water (0.25 mg/die, 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 injured (cuff or balloon) and uninjured contralateral vessels were re-
injured and uninjured vessels were collected, and Axl expression, as well as Axl phosphorylation, were studied by Western blot. The membrane was stripped and reprobed with rabbit anti-Axl antibody.

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 further the role of Axl in VSMC function, we studied the vessel response to injury in the Axl−/− mouse. Neointima formation was induced by placement of a polyethylene cuff around the left femoral artery (LFA) (right femoral...
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Fig. 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 ×20. Inset magnification is ×60. 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 × 10$^{-3}$ μm$^2$) compared with Axl$^{+/+}$ (18.9 ± 1.5 × 10$^{-3}$ μm$^2$). Also note that LFA external elastic lamina failed to increase in Axl$^{-/-}$ (45.9 ± 1.9 × 10$^{-3}$ μm$^2$) compared with Axl$^{+/+}$ (57.3 ± 5.7 × 10$^{-3}$ μm$^2$), whereas RFA were 40 ± 5 × 10$^{-2}$ μm$^2$. Values are mean ± S.E. *p < 0.05 compared with Axl$^{+/+}$ LFA.

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$^{-/-}$ or Axl$^{+/+}$ (not shown). The injured LFA in Axl$^{+/+}$ exhibited substantial intima formation (Fig. 8, A and D). In contrast, intima formation was markedly less (∼80% inhibition) in the injured LFA of Axl$^{-/-}$ mice (Fig. 8, B and D). There were no significant differences in lumen, media, or adventitia area in Axl$^{-/-}$ compared with Axl$^{+/+}$ (Fig. 8, C, E, and F). These data show that neointima formation induced by vascular injury is significantly reduced in the Axl$^{-/-}$ mice.

DISCUSSION

The major findings of the present study are that Axl is a redox-sensitive receptor tyrosine kinase activated by H$_2$O$_2$ 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 H$_2$O$_2$ 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 pathophysiologic process in the vasculature.

It has previously been shown that several RTKs, including EGF receptor (7, 8, 10), platelet-derived growth factor receptor (11), and fibroblast growth factor receptor (9), are activated by H$_2$O$_2$ at concentrations ranging from 20 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 EGF receptor tyrosine phosphorylation upon stimulation with H$_2$O$_2$ or angiotensin II (8, 21, 22). Therefore, we assessed the role of endogenous Gas6 in Axl phosphorylation by inhibiting Gas6. Axl phosphorylation was partially attenuated by both Axl-Fc and warfarin (Figs. 3 and 4), suggesting that endogenous Gas6 is involved in H$_2$O$_2$-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-carboxylglutamic acid domain (25), it is possible that active fragments might be proteolytically released (similar to HB-EGF) upon stimulation with H$_2$O$_2$. Inhibition of tyrosine phosphatases, which has been proposed to be responsible for platelet-derived growth factor receptor activation, is an alternative mechanism of 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 H$_2$O$_2$ 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 H$_2$O$_2$) (28).

In the present study we showed Axl phosphorylation ex vivo in intact vessels using a relatively high dose of H$_2$O$_2$ (1 mM, Fig. 5). We believe that the high concentration of H$_2$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 H$_2$O$_2$ production would also be augmented (29). It is likely that a large amount of H$_2$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 with 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 although 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, fibroblast growth factor, and platelet-derived growth factor 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
and nephropathy in which increases in Axl expression and the potential role of Gas6-Axl in hypertension, atherosclerosis, Axl-specific tyrosine kinase inhibitor has yet to be identified, of increased Axl expression and oxidative stress. Although an they suggest a mechanism for Axl activation under conditions cultured VSMC and intact vessels are of importance because little effect. Finally, transactivation of Axl by H$_2$O$_2$ will not be affected by warfarin to the extent that transactivation is neointima formation it is logical that Axl inhibition may have 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 H$_2$O$_2$ 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 H$_2$O$_2$ production are evident makes this pathway an attractive therapeutic target (4, 12, 19, 34, 35).

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