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Axl receptor activation mediates laminar shear stress anti-apoptotic effects in human endothelial cells

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Abstract

Objective: Laminar Shear Stress (SS) induces cytosolic acidification and protects endothelial cells (ECs) from apoptosis. Our prior studies showed that acidification protects ECs from serum deprivation-induced apoptosis by a mechanism directly involving Axl-receptor activation. Aim of the present study was to determine whether the anti-apoptotic action of SS involves acidification-dependent Axl activation.
Methods and results: Axl mRNA and protein levels were significantly higher (5 and 8 fold, respectively) in ECs exposed to SS (12 dyne/cm^2), compared to static culture (ST). This effect was dependent on the presence of bicarbonate ion and blocked by the anion exchangers inhibitors, DIDS and SITS. Moreover, DIDS markedly inhibited the anti-apoptotic action of SS. Notably, after 5 min of SS exposure, Axl-receptor was tyrosine-phosphorylated. The over-expression in human ECs of an Axl-receptor soluble form completely reverted the anti-apoptotic SS effect. Since laminar SS exerts its effects through the activation of integrin-dependent pathways, we examined whether Axl might be associated with the $\alpha v\beta 3$ integrin complex known to be activated by SS. Co-immunoprecipitation experiments indicate that 5 min of ECs exposure to SS induced Axl-receptor/$\beta 3$-integrin complex formation, suggesting their functional association.
Conclusions: These results indicate that Axl receptor activation modulates laminar SS anti-apoptotic effects possibly through its association with specific integrin-complexes.
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Keywords: Acidification; Apoptosis; Axl tyrosine kinase receptor; Endothelial cells; Integrins; Mechanotransduction; Shear stress

1. Introduction

Vascular endothelial cells (ECs), placed at the interface between the blood and the vessel wall, are constantly subjected to the mechanical forces produced by blood flow. Laminar Shear Stress (SS) is the frictional force of flowing blood acting tangentially on the ECs which stimulates several signaling cascades including: potassium channel activation, elevation of inositol trisphosphate and diacylglycerol, increase in intracellular calcium levels, G-protein activation, MAPK phosphorylation, and transcription factors activation [1–3]. SS-dependent early signaling events are followed by changes in the chromatin structure [4], gene expression [5], and alignment of actin filaments and microtubules within the flow direction, resulting in changes in cell shape and directional migration [6–8], growth arrest [9] and apoptosis inhibition [10]. ECs growth arrest induced by SS correlates with $p21^{Cip1}$ up-regulation without affecting $p27^{Kit1}$ protein levels [11]. Notably, $p21^{Cip1}$ mediates SS-dependent anti-apoptotic function and significantly reduces ECs *in vivo* apoptosis in ischemic hind-limbs [12]. Although the nature of the SS-sensing mechanism is not completely clear, several studies demonstrated that vascular endothelial

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growth factor receptor-2 (VEGFR-2), integrins [13,14], vascular endothelial cadherin [15,16], focal adhesion kinase (FAK) [17], stretch-activated anion channels [18], K^+ ion channel [3], paxillin [19] and platelet endothelial cell adhesion molecule-1 [20] are important components of the SS mechano-transduction machinery. Cellular metabolism may also influence adaptive responses to mechanical stresses. Specifically, laminar SS is known to induce mild cytosolic acidification which seems to play an important role in ECs transduction of mechano-signals [21,22]. In this regard, our prior studies indicated that acidification has an important role in protecting ECs from serum deprivation-induced apoptosis through activation of Axl tyrosine kinase receptor [23]. It is noteworthy that many studies showed the importance of Gas6/Axl pathway both in physiology and pathology of vascular cells [24–26]. However, its role in the endothelial adaptive response to laminar SS was not completely elucidated.

Therefore, the aim of this study has been to characterize the potential role of Axl tyrosine kinase receptor in the anti-apoptotic effect of laminar SS on ECs, providing evidences of its presence in molecular complexes potentially involved in the mechano-transduction.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVEC; Cambrex) were maintained in complete medium (EGM-2; Cambrex) containing endothelial cell basal medium (EBM-2; Cambrex) supplemented with endothelial cell Bullet Kit as previously described [23]. Cells were used for the experiments between passage 3 and 5. Primary cultures of bovine aortic endothelial cells (BAEC) were prepared and cultured as previously described [27]. BAEC were used for the experiments between passage 2 and 6.

2.2. Apoptosis assessment

Apoptosis was measured by photometric enzyme-immunoassay (Cell Death Detection ELISA, Boehringer Mannheim), to quantify cytoplasmic histone-associated DNA fragments (mono- and oligo-nucleosomes) as previously described [27].

2.3. Laminar shear stress studies

HUVEC (1.5×10^6) were seeded in complete medium onto 150 mm tissue culture dishes and maintained at 37 °C in humidified air containing 5% CO_2 for 24 h. Then complete growth medium was replaced with serum-free EBM-2, after 26 h cells were exposed to laminar SS (12 dyne/cm^2) for 5, 15 min or for 1, 6, 18 and 22 h in a cone-and-plate apparatus [28] maintained at 37 °C in humidified air with 5% CO_2. Controls were kept under static (ST) cultures under similar conditions. In some experiments, before exposure to laminar SS, anion exchangers were inhibited by a 1 h pretreatment with 100 μM 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene (DIDS), or with 4-acetamido-4′-isothiocyanatostilbene-2,′-disulfonic acid (SITS, Sigma) [22]. In other experiments, before exposure to 5 min SS cells were treated for 1 h with 2 μg/ml soluble human recombinant Axl-Fc chimera (soluble hrAxl-Fc chimera, R&D Systems) or with 1 μM Warfarin (Sigma) or with 50 μg/ml RGDS (Arg-Gly-Asp-Ser, Bachem); (50 μg/ml, i.e. a concentration not affecting endothelial cell adhesion).

2.4. RNA extraction

Total cellular RNA was isolated from HUVEC monolayers maintained in serum-free medium either in ST or in SS for 6, 18 and 22 h using TriZOL Reagent (Invitrogen). RNA was then size-fractionated by electrophoresis to assess its integrity and stored at −80 °C.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was converted to cDNA by reverse transcription using the Superscript Preamplification System Strand for first strand cDNA Synthesis (Invitrogen), according to manufacturer's instructions. PCR was performed using the Ampli Taq DNA Polymerase (Perkin Elmer Roche Molecular System Inc.). The primers' sequences used for amplification of PCR conditions were as previously described [23].

2.6. Construction of Axl adenovirus vector

A recombinant adenovirus expressing the Axl cDNA (AdCMV.Axl) was recently generated in our laboratory by homologous recombination in bacteria by using a standard procedure [29]. Briefly, the Axl full length cDNA was inserted into the pTrack-CMV vector (gift of Dr. B. Volgelstein), co-transformed in bacteria with the adenovirus backbone plasmid pAdEasy-1 followed by selection onto kanamycin-containing plates. The linearized recombinant plasmid was then transfected into the adenovirus packaging 293 cell line, and viral lysate were obtained 7–10 days after transfection. In order to generate higher titer viral stocks, 293 cells were infected with repeated rounds at 1–5 multiplicity of infection (M.O.I.) and viral DNA obtained following CsCl banding followed by exhaustive dialysis to remove CsCl traces.

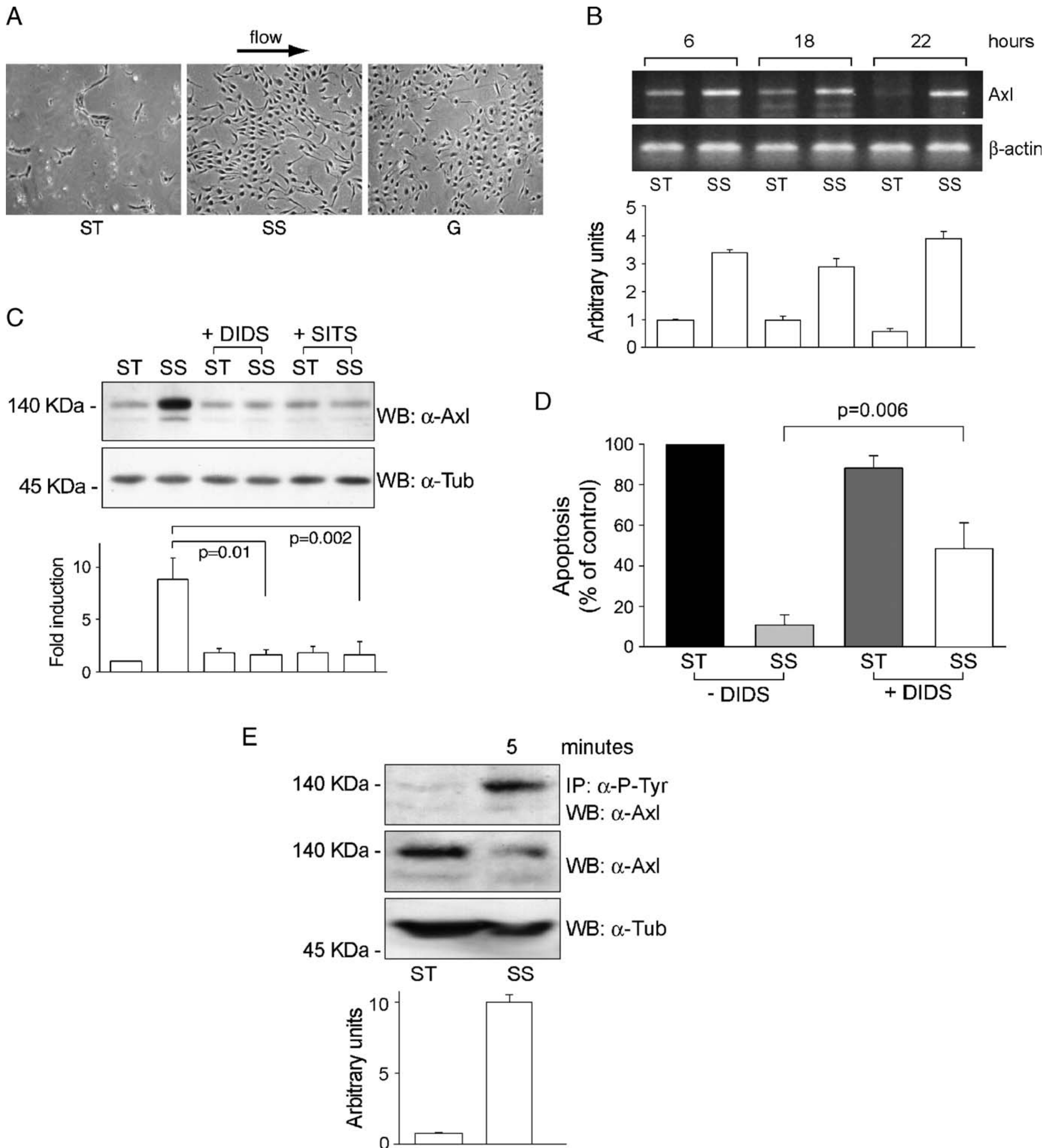
2.7. Lentivirus production

The MISSIOM shRNA clones constructed within the lentivirus plasmid vector pLKO.1-Puro were purchased from Sigma. The vectors were grown and purified according to manufacturer's instructions.

Lentiviral supernatants were produced by using a standard procedure [30] by calcium phosphate transient co-transfection of a three-plasmid expression system and the pLKO.1-Puro containing MISSION shRNA for Axl or the MISSION non-target control vector in 293 T packaging human embryonic kidney cell line. The calcium-phosphate DNA precipitate was removed after 14–16 h by replacing the medium. Viral supernatant was collected 48 h after transfection, filtered through 0.22-μm pore nitrocellulose filters, concentrated by ultracentrifugation at 50,000 × *g* for 140 min at RT and stored at −80 °C.

2.8. Transient transfections and viral infections

Either pCMVAxl, pCMVslAxl or pcDNA$_3$ (Invitrogen) control vector were co-transfected with pEGFP-N1



(Clontech) reporter vector (3:1 ratio). Co-transfection with 2 independent vectors results in the internalization of both plasmids by the same cell [31]. HUVEC or BAEC (1×10^6 cells/100 mm Petri dish) were transfected for 6 h with FuGENE 6 Transfection Reagent (Roche) according to the manufacturer's instructions and recovered in complete fresh medium for additional 24 h. Subsequently, ECs (1.5×10^6 cells) were plated onto 150 mm Petri dish and cultured in complete medium (EGM-2) for 24 h. Then medium was replaced with serum-free medium (EBM-2) and cells were cultured for 26 h in ST then exposed to laminar SS for 22 h or maintained in ST culture. HUVEC (1×10^6 cells/100 mm Petri dish) were infected for 2 h with AdCMV.Axl or with AdCMV.null (100 M.O.I.) or with lentivirus supernatants containing 6 μg/ml of polybrene to improve the infection efficiency. ECs were then recovered in complete fresh medium for additional 24 h. Subsequently, HUVEC (1.5×10^6 cells) were plated onto 150 mm Petri dish and cultured in complete medium (EGM-2) for 24 h. Medium was replaced with serum-free medium (EBM-2) and cells were cultured for 26 h in ST then exposed to laminar SS for 22 h or maintained in ST culture.

2.9. Immunoprecipitation and western blot analysis of Axl and Gas6

HUVEC were cultured in serum free-medium EBM-2 for 26 h in ST conditions then were exposed to laminar SS for 22 h and lysed with a lysis buffer containing 20 mM Tris/HCl (pH 7.5), 135 mM NaCl, 1% NP-40, 10% glycerol, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 50 mM NaF, 1 mM sodium orthovanadate, 10 μg/ml Leupeptin, 10 μg/ml Aprotinin and 1 mM PMSF (Sigma). Equal amount of total proteins (100 μg/lane) were electrophoresed in 8% SDS-PAGE under reducing conditions and electroblotted to a nitrocellulose membrane Optitran BA-S 83 (Schleicher & Schuell). Axl was detected by incubating the membrane with 1 μg/ml of affinity-purified goat polyclonal antibody against human Axl (αAxl, Santa Cruz Biotechnology Inc.). The complexes were evidenced by incubation with a secondary peroxidase-conjugated antibody and ECL™ detection system (Amersham Pharmacia Biotech), according to manufacturer's instructions. Loading of equal amount of total proteins and normalization were assessed by densitometric measures of tubulin bands. The Axl bands quantification was carried out on the major band (140 kDa). In some experiments, before exposure to laminar SS, the anion exchangers were inhibited by a 1 h pretreatment with DIDS or SITS (100 μM, Sigma). Tyrosine-phosphorylated Axl was immunoprecipitated from HUVEC cultured in serum-free medium for 24 h in ST then exposed to laminar SS for 5 min, by using the monoclonal anti-phosphotyrosine IgG clone4G10 (Upstate Biotechnology) followed by the goat polyclonal antibody against human Axl (αAxl, Santa Cruz Biotechnology Inc.). Immunoprecipitation of Axl in the integrin complex was performed with lysates from BAEC transfected either with $pcDNA_3$ or with pCMVAxl (see below) by using the goat polyclonal antibody against human integrin β3 subunit (Santa Cruz Biotechnology Inc.) followed by the goat polyclonal antibody against human Axl (αAxl, Santa Cruz Biotechnology Inc.).

Gas6 was detected into CM collected from HUVEC maintained in serum free-medium EBM-2 for 26 h then maintained for 5 to 15 min, or 1, 18, and 22 h either in ST or in laminar SS. CM was concentrated 50 fold through a 10 kDa cutoff at +4 °C using Centriprep 10 filters (Amicon). Equal amount of total proteins (100 μg) were electrophoresed in 8% SDS-PAGE and electroblotted as described above. Gas6 was detected by incubating the nitrocellulose with 1 μg/ml of affinity-purified goat polyclonal antibody against human Gas6 (αGas6, Santa Cruz Biotechnology Inc.) followed by secondary antibody hybridization and ECL™ detection.

2.10. In vitro interaction of soluble Axl with full length Axl receptor

The *in vitro* interaction of soluble Axl with the full length Axl receptor was carried out with lysates from HUVEC infected either with AdCMV.Axl or with AdCMV.null. Equal amount of total proteins (100 μg/lane) were electrophoresed in 7.5% SDS-PAGE under reducing conditions and electroblotted to a nitrocellulose membrane Optitran BA-S83 (Schleicher & Schuell) as described above. For the interaction experiment, the membrane was incubated 3 h at RT with 4 μg/ml of soluble hrAxl-Fc chimera (R&D Systems) followed by incubation with an anti-human IgG HRP-conjugated antibody (Amersham Pharmacia Biotech)

Fig. 1. Effect of SS on Axl, Gas6 mRNA and protein expression and Axl phosphorylation. Panel A: Phase-contrast images of HUVEC cultured for 48 h in serum-free medium in ST or exposed to 22 h SS or maintained in complete medium (growing cells, G). Arrow indicates the flow direction. (200× magnification). Panel B: Semiquantitative RT-PCR is shown. After 6, 18 and 22 h exposure to SS, Axl mRNA level was significantly higher than in static culture (ST) and at 22 h was 5 fold higher. Densitometric values were normalized for β-actin levels and are reported in the histogram. Panel C: Western blot analysis confirmed that, after 22 h exposure to SS, Axl protein expression in HUVEC was significantly higher than in ST (8 fold; $p<0.0004$). When cells were pre-incubated for 1 h with 100 μM DIDS or 100 μM SITS and maintained during all length of the experiment, the Axl levels were the same both in ST and in SS. Panel D: Effect of anion exchanger inhibitor on the protective action of SS. HUVEC were cultured in serum-free medium for 26 h, and maintained in ST or exposed to laminar SS for additional 22 h before apoptosis quantification with ELISA. In HUVEC grown in serum-free medium, SS decreased the DNA fragmentation by 89.2±4.7% ($p<0.0004$). Apoptosis was 10.7±4.7% of control in SS−DIDS; 88.9±4.7% of control in ST+DIDS; 48.7.3±12.0% in SS+DIDS. DIDS reverted the protective effect of laminar SS ($p<0.006$). Results represent the average of 3 experiments in duplicate. Panel E: Immunoprecipitation of tyrosine-phosphorylated-Axl from HUVEC cultured in serum-free medium in ST or exposed to laminar SS shows that Axl was strongly phosphorylated after 5 min exposure to SS. Equal amounts of proteins (100 μg/lane) were loaded and densitometric values were normalized for tubulin bands (reported in the histogram). Figure shows a representative example of 3 independent experiments.

for the complex detection. Signals were visualized by standard ECL™ detection system.

2.11. Precipitation of Axl in an RGDS complex

This method was based on the Dynabead-streptavidin biomagnetic separation of small biotinylated molecules. Briefly, 1 nmol of amino-terminal-biotinylated-RGDS (Sigma-Genosys) was coupled to 1 mg of Dynabeads M-270 Streptavidin (Dynal Biotech ASA) by mixing these components in a racking system at RT for 1 h, according to manufacturer's instructions. The Dynabeads M-270 Streptavidin-biotinylated-RGDS complex was washed twice with lysis buffer using Magnetic Particle Concentrator (Promega). Then the complex was added to 3 mg of cell lysates from HUVEC cultured in EGM-2 medium or in EBM-2 medium both in ST or in SS conditions and incubated in a rotating system at +4 °C for 18 h. For non-specific complex precipitation, a Dynabeads M-270 Streptavidin without biotinylated-RGDS was used. Then, the complex was washed three times with lysis buffer. Western blot analysis and immunodetection of Axl and integrin β3 subunit was performed as described above.

2.12. Statistical analysis

Continuous variables were analyzed by Student's *t*-test two tails uncoupled. Data are expressed as mean ± SD. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Laminar SS sustains Axl mRNA, protein expression and induces Axl-phosphorylation

Laminar SS induces alignment of actin filaments and microtubules in the flow direction, redistribution of focal adhesion complexes, leading to changes of cell shape and directional migration [6,19]. Fig. 1A shows representative images of HUVEC cultured for 48 h in serum-free medium in ST or exposed to 22 h SS or maintained in complete medium (growing cells, G). Arrow indicates the flow direction. To investigate the effect of laminar SS on Axl regulation, HUVEC were cultured in serum-free medium either in ST or exposed to laminar SS for 6, 18, 22 h. Total RNA was purified and semi-quantitative RT-PCR was performed. In ST condition, Axl mRNA was time dependently down-regulated while it remained at a constant level in cells exposed to SS; the maximum difference of 5 fold occurred at the 22 h time-point (Fig. 1B).

The effect of SS on Axl protein level was evaluated by Western blot analysis. HUVEC were cultured in serum-free medium and exposed to laminar SS as described above and cell lysates were prepared at the 22 h time-point. Consistently with RT-PCR experiments, in these experimental conditions, the levels of Axl protein were 8 fold higher in cells exposed to flow compared to those kept in ST (Fig. 1C). In order to investigate the role of SS-induced acidification [22] in the modulation of Axl protein level, two different inhibitors of Cl^-/HCO_3^-, DIDS and SITS were used. Interestingly, under both conditions in which anion exchangers were irreversibly inhibited, Axl protein level was not sustained by SS when compared to ST (Fig. 1C), confirming that a pH-dependent mechanism is involved in the regulation of Axl expression as previously reported [23]. The effect of anion exchangers inhibitors on Axl expression prompted us to evaluate the role of SS-dependent acidification on the anti-apoptotic effect of laminar flow. SS is known to induce mild cytosolic acidification which seems to play an important role in ECs transduction of mechano-signals [21,22]. Our previous studies showed that acidification has an important role protecting ECs from serum deprivation-induced apoptosis through activation of Axl tyrosine kinase receptor [23]. In this study, to examine the role of SS-induced intracellular acidification in the modulation of ECs apoptosis, HUVEC were maintained in serum-free medium for 26 h, pretreated with DIDS for 1 h, then exposed to laminar SS or maintained in ST culture for 22 h. The inhibition of the anion exchangers partially but significantly reverted the protective effect of SS. In fact, in cells treated with DIDS, apoptosis increased 4.5 fold in the presence of SS while no significant difference was observed in ST (Fig. 1D).

Additional experiments were performed to investigate SS effect on Axl tyrosine-phosphorylation. HUVEC were cultured for 24 h in serum-free medium before 5 min exposure to laminar SS or ST; under these conditions, Axl was found strongly phosphorylated in the presence of laminar SS (Fig. 1E).

3.2. Role of GAS6

To determine whether laminar SS modulated the production of the Axl ligand Gas6, conditioned medium (CM) was collected at 5, 15 min, 1, 18, 22 h and analyzed for Gas6 expression. Notably, in our experimental conditions, Gas6 protein levels were not modulated by laminar SS at all time points tested, even at very short time (5 min) of SS treatment, indicating a specific effect of SS on Axl receptor protein levels (Fig. 2A). To further investigate the role of Gas6, we studied the effect of warfarin, which prevents γ-carboxylation of Gas6 by decreasing its interaction with Axl. As shown in Fig. 2B, warfarin did not affect Axl phosphorylation in HUVEC exposed to laminar SS (5 min) compared to cells maintained in ST culture. These results suggest that the involvement of GAS6 is dispensable at least at an early time-point when SS induced Axl-tyrosine phosphorylation.

3.3. Soluble Axl inhibits Axl-phosphorylation and reverts SS anti-apoptotic effect

To assess the potential role of Axl pathway in the SS-dependent protection from apoptosis, HUVEC were

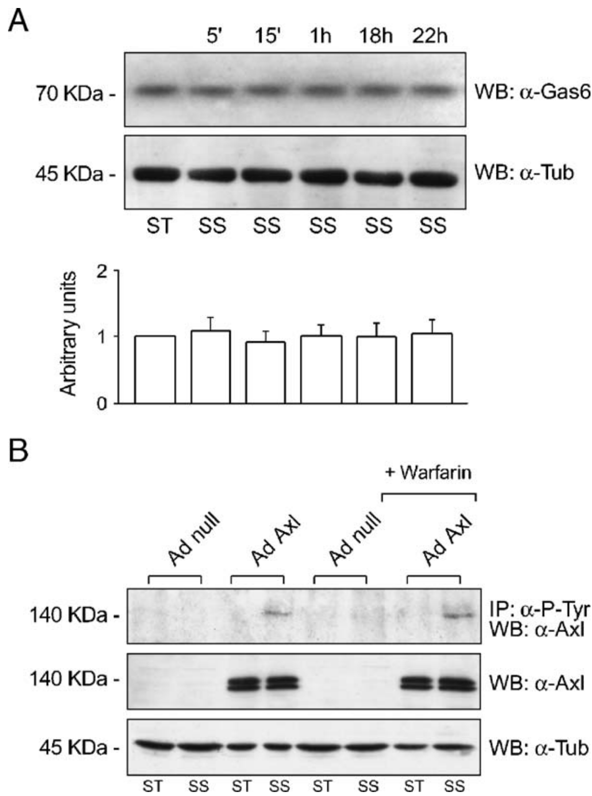
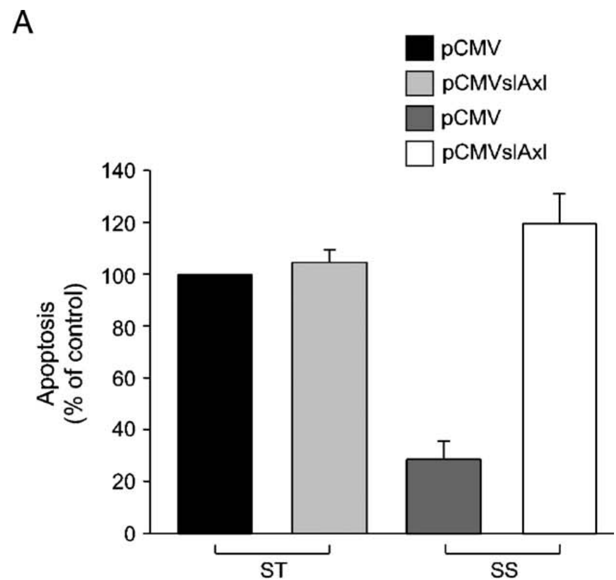
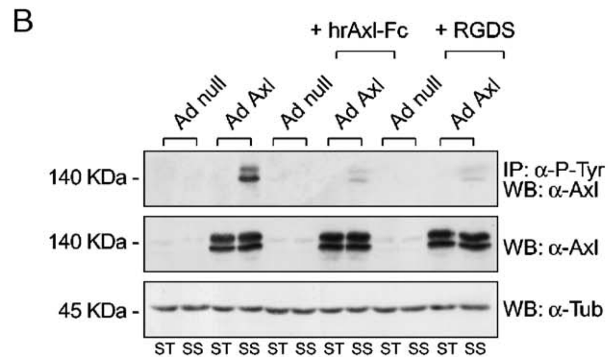


Fig. 2. Role of Gas6 in SS-mediated Axl activation. Panel A: Western blot analysis of Gas6 released into CM by HUVEC cultured in serum-free medium in ST or exposed to SS for 5, 15 min, 1, 18 and 22 h shows no significant difference. Figure shows a representative example of 3 independent experiments. Panel B: Warfarin does not affect Axl tyrosine phosphorylation induced by SS. Immunoprecipitation of tyrosine-phosphorylated-Axl in HUVEC over-expressing Axl, pretreated with 1 μM Warfarin and exposed to laminar SS (5 min) or maintained in ST culture, shows no significant differences. Figure shows a representative example of 3 independent experiments.

transiently transfected with soluble Axl expression plasmid (pCMVslAxl) [23] (Fig. 3A) and maintained in serum-free medium for 48 h in order to induce apoptosis. In this condition, soluble Axl completely reverted the protective effect of SS. In fact, the amount of apoptosis in cells exposed to SS and transfected with pCMVslAxl was similar to controls kept in ST. These results indicate that Axl pathway plays an important role in the ability of laminar SS to protect ECs from programmed cell death. To investigate the





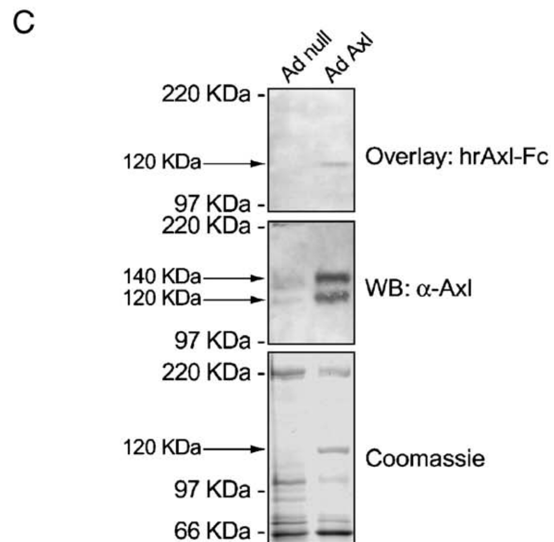


Fig. 3. Effect of soluble Axl over-expression on the protective action of SS. Panel A: HUVEC were co-transfected with expression vector for soluble Axl and pEGFP-N1 reporter vector. Cells were cultured for 24 h after transfection in complete medium; thereafter they were cultured in serum-free medium for 26 h, and cultured in ST or exposed to laminar SS for additional 22 h before apoptosis quantification with ELISA. In HUVEC grown in serum-free medium, SS decreased the DNA fragmentation by 71.2±5.8% ($p<0.0001$). Apoptosis was 104.8±4.8% of control in ST pCMVslAxl; 28.8±5.8% of control in SS pCMV; 120.3±12.8% in pCMV slAxl. Soluble Axl completely reverted the protective effect of laminar SS ($p<0.00003$). Results represent the average of 3 experiments in duplicate. Panel B: Immunoprecipitation of tyrosine-phosphorylated-Axl shows that in HUVEC over-expressing Axl, pretreated with 2 μg/ml soluble hrAxl-Fc Chimera or with 50 μg/ml RGDS and exposed to laminar SS (5 min) or maintained in ST culture, levels of SS-induced tyrosine Axl phosphorylation were strongly reduced (70% and 90% respectively). Panel C: *In vitro* binding studies show that soluble hrAxl-Fc Chimera can specifically bind a 120 kDa band protein recognized, in Western blot, by the anti-Axl receptor antibody, in HUVEC over-expressing Axl (AdAxl). Equal amounts of proteins (100 μg/lane) were loaded as confirmed in the Coomassie stained panel.

mechanism by which soluble Axl reverts SS anti-apoptotic effect, HUVEC over-expressing Axl were cultured for 24 h in serum-free medium, pretreated for 1 h with the soluble hrAxl-Fc chimera and exposed to laminar SS or ST (5 min). In these conditions, Axl tyrosine-phosphorylation was markedly reduced (70%) (Fig. 3B), indicating that Axl soluble receptor was able to inhibit the SS-dependent Axl activation. To investigate whether a direct interaction of soluble Axl with the membrane anchored Axl receptor could be involved in this process, an *in vitro* binding study was performed (Fig. 3C). The results of these experiments showed that soluble hrAxl-Fc chimera specifically bind to the full length Axl receptor, since it interacted with a protein migrating exactly as the same MW (120 kDa) recognized by the anti-Axl antibody. This confirms its capacity of homophilic interaction [32,33].

3.4. Axl silencing markedly reduced the SS-dependent anti-apoptotic effect

In order to functionally investigate the role of Axl receptor in SS-dependent anti-apoptotic effect, a specific RNA-interference approach was used. HUVEC were infected with the lentiviral particles carrying the Axl-specific short hairpin RNA sequence to interfere with endogenous Axl expression, or with the control virus. Under these conditions, the level of Axl protein was reduced about 70% (Fig. 4A). In order to induce apoptosis, infected cells were starved for 26 h before SS treatment for 22 h. Remarkably, the reduction of Axl expression, as documented by Western blot analysis, significantly inhibited the anti-apoptotic effect of SS (Fig. 4B) when compared to controls.

3.5. Axl tyrosine kinase receptor complexes with integrins in cells exposed to SS

Integrins play a key role in SS signaling and cell survival [34–36]. The evidence that Axl was rapidly phosphorylated upon cell exposure to SS prompted us to investigate whether SS stimulates Axl association to integrin complexes which are activated by laminar SS. Cell lysates were obtained from HUVEC cultured for 24 h in serum-free medium before exposure to SS or ST. Lysates from cells grown in complete medium (G) were used as an additional control. To isolate integrins and integrin-complexes, each cell lysate was precipitated with RGDS-biotinylated peptide conjugated to paramagnetic streptavidin beads and analyzed by western blot. Fig. 5A shows that Axl receptor forms complexes and co-precipitates with RGDS peptide. Co-immunoprecipitation studies with anti-β3 integrin specific antibodies were also performed. BAEC were transiently transfected with Axl full length expression vector (pCMVAxl) [23] or empty vector (pCMV) as control. Cells were cultured in serum-free medium and kept in ST or exposed to SS or maintained in complete medium as described above. Immunoprecipitation/Western blot analyses revealed the presence of Axl in protein

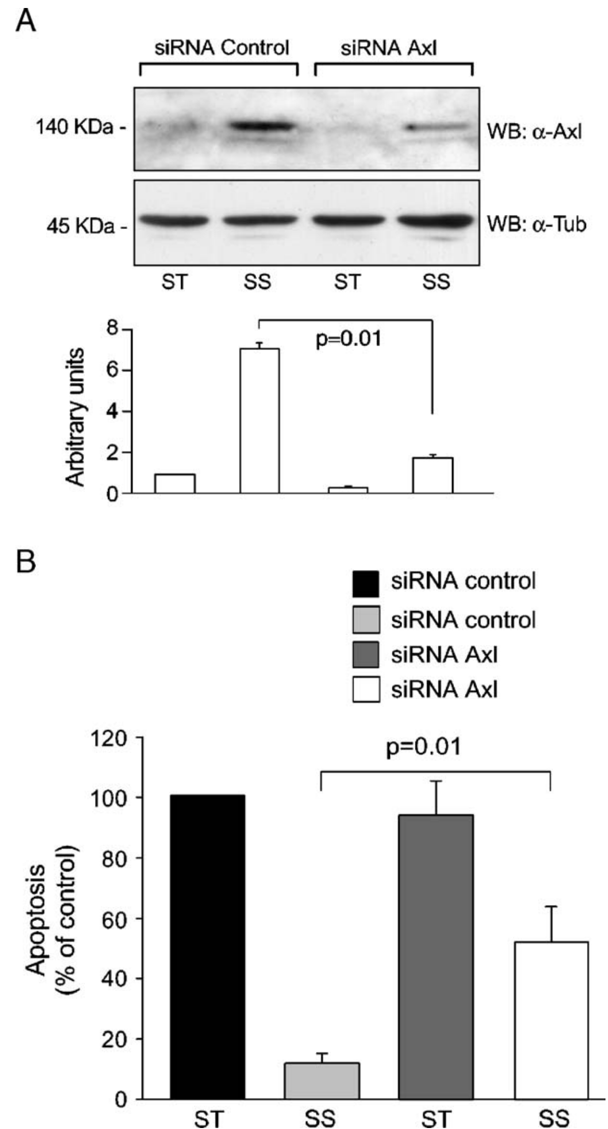


Fig. 4. Effect of siRNA Axl on the SS-dependent anti-apoptotic effect. Panel A: Western blot analysis shows that after 22 h exposure to SS, Axl protein expression in HUVEC was significantly higher than in ST (as shown above). When cells were infected with lentiviral particles carrying the sequence to specifically block Axl expression, Axl protein levels were reduced by 70%. Figure shows a representative example of 3 independent experiments. Panel B: HUVEC were infected with lentiviral particles carrying Axl siRNA sequences or non-target sequences as control. Cells were cultured for 72 h after transfection in complete medium; thereafter they were cultured in serum-free medium for 26 h, and cultured in ST or exposed to laminar SS for additional 22 h before apoptosis quantification with ELISA. In HUVEC grown in serum-free medium, SS decreased the DNA fragmentation by 87.6±2.9% (p < 0.0002). Apoptosis was 12.3±2.9% of control in SS siRNA control; 94.27±10.6% of control in ST siRNA Axl; 51.6±11.3% in SS siRNA Axl. The inhibition of Axl expression markedly reduced the anti-apoptotic effect of SS (p = 0.01) when compared to control (non-target infected cells). Results represent the average of 3 experiments in duplicate.

complexes recognized by the anti-β3 antibody (Fig. 5B) only in cells exposed to laminar SS for 5 min or maintained in complete medium, while it was not detected in lysates obtained from cells cultured in ST conditions. These results

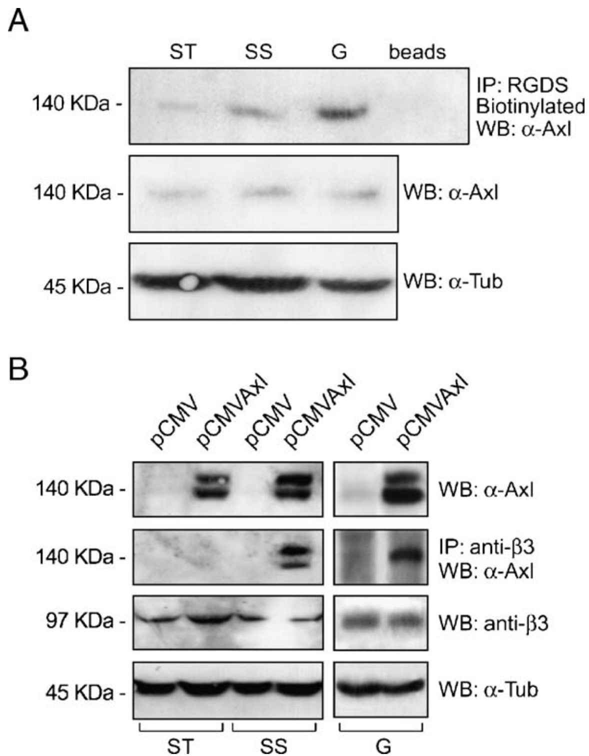


Fig. 5. Axl tyrosine kinase receptor is present in an integrin complex. Panel A: HUVEC were cultured for 24 h in serum-free medium then exposed for 5 min to laminar SS or maintained in ST cultures. Cells were also cultured in complete medium (G). Cell lysates were prepared and used for the precipitation with RGDS-biotinylated peptide conjugated to paramagnetic streptavidin beads. Western blot analysis shows that Axl co-precipitates with RGDS peptide. Panel B: BAEC were transiently transfected with the expression vector for Axl full length or with the empty vector. Cells were cultured in serum-free medium and kept in ST or exposed to SS (5 min) or maintained in complete medium (G) and cells lysates were prepared. Western blot analysis shows that Axl was immunoprecipitated by the anti-β3 antibody in SS-exposed cells only, or maintained in complete medium, while it did not co-precipitated in ST conditions.

indicate that Axl can associate with β3 integrin-bearing complex both in proliferating cells and in cells exposed to laminar SS cultured in serum-free medium. These results suggest a potential functional association between β3 integrin complexes and Axl in laminar SS stimulated ECs, indicating that activated Axl may interact with β3-integrin. Further, this association was confirmed by an additional set of experiments, reported in Fig. 3B, in which 1 h pretreatment with RGDS prevented by >90% SS-dependent Axl tyrosine-phosphorylation.

4. Discussion

The present study shows that in the presence of laminar SS the levels of Axl receptor mRNA and protein remain elevated compared to ST controls and an early Axl tyrosine phosphorylation is induced. The inhibition of the anion exchangers reverted the effect of SS on Axl expression and abolished the protective effect of SS on ECs apoptosis. In this context, the Axl ligand, Gas6, was not modulated by laminar SS and the early Axl tyrosine-phosphorylation was independent on Gas6 activity. Remarkably, abrogating Axl function completely reverted the protective effect of SS in ECs sensitized to apoptosis by serum deprivation and inhibited the Axl tyrosine phosphorylation, as well as, the reduction of Axl protein levels strongly reduced the protective effect of SS in ECs apoptosis. Biochemical analyses revealed that Axl receptor associates with β3 integrin in a SS-dependent fashion, forming an Axl-integrin complex, which may be important for laminar SS sensing and apoptosis protection. The Gas6/Axl pathway is an important mediator of cell growth and apoptosis inhibition in several cell types [37,38] including vascular smooth muscle cells (VSMCs) [24] and ECs [23,25,39,40], suggesting an important role for this signaling pathway in vascular remodeling and function. We demonstrate here a key role for Axl in SS-mediated ECs survival. In this context, the role of the Axl ligand, Gas6, which is not modulated by flow, appears dispensable as demonstrated by the warfarin treatment of ECs which did not prevent SS-dependent Axl activation (Fig. 2). This observation differs from our prior finding about the importance of Axl activation by its ligand Gas6 in cells cultured in the presence of an extracellular acidic pH [23]. In that condition, in fact, both Axl and Gas6 levels were significantly increased. In the present manuscript, we report that in human ECs exposed to laminar flow, which only slightly decreases the intracellular pH [22], the SS-dependent activation of integrins seems to be more important to provide those signals leading to Axl phosphorylation and function. Consistently, the soluble Axl reverted the protective effect of SS and inhibited the Axl tyrosine phosphorylation (Fig. 3A–B), at least in part, by an homophilic protein–protein interaction with the membrane anchored Axl receptor, rather than a simple decoy effect (Fig. 3C) [41,42]. Notably similar ligand-independent mechanisms of action were reported for other soluble receptors [43].

Axl receptor exerts its anti-apoptotic activity by c-Src-, PI3K-activation and Akt phosphorylation and induces a rapid and transient increase in nuclear NF-kB binding activity [40,44,45]. Since similar anti-apoptotic pathways are regulated by SS via integrin activation [34,46], we investigated whether Axl receptor may be associated to integrins in ECs activated by SS. Surprisingly, we found that Axl receptor associates with integrins on ECs in a SS-dependent manner, as indicated by co-immunoprecipitation experiments (Fig. 5). Laminar SS stimulates many pathways regulated by integrin binding to ECM, triggering activation of MAP kinase and changes in Rho activity [14]. Further, other pathways characteristic of integrin signaling, like FAK and c-Src activation [17,47] are triggered by SS. Notably, integrin antagonists potently interfere with SS signaling [48,49]. Moreover, blocking integrins with an RGD peptide abolishes the anti-apoptotic effect of SS [34]. Other studies showed the SS triggered association of integrins with Shc, and this interaction was ECM-specific [35]. The role of acidification in ECs exposed to SS is largely unclear. This

work provides the evidence that intracellular acidification is important for the SS-dependent anti-apoptotic function. This effect is associated with the apparent stabilization of Axl protein and mRNA levels occurring under SS. Further experiments are required to elucidate the role of ion channels in the regulation of Axl expression and function in the presence of laminar SS and its potential implication in pathological conditions associated to flow alterations. Our experiments suggest for the presence of a functional link among pH regulation, Axl expression and ECs survival.

The present study, showing for the first time a SS-dependent modulation of Axl signal and Axl interaction with integrin complexes, indicates that Axl may be a very important player in vascular homeostasis, likely by participating to the formation of an integrin-based pro-survival specialized mechano-receptor complex.

In summary, the results of the present study show that activation of Axl signaling plays a key role in the anti-apoptotic effect of SS on ECs and represent an important evidence supporting the role of the interaction of TK receptors with adhesion molecules (such as integrins) in the ECs homeostasis, opening novel interesting ways, likely involving Axl axis, to explain the SS-mediated events in vascular remodeling.

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