Acidification Prevents Endothelial Cell Apoptosis by Axl Activation

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Abstract—Prior studies have shown that acidification due to hypercarbia protects endothelial cells from serum deprivation–induced apoptosis. However, the mechanism(s) responsible for the antia apoptotic effect of acidification is still unclear. cDNA array screening was performed on human umbilical vein endothelial cells cultured in a bicarbonate medium equilibrated either with 5% CO₂ (pH 7.4) or with 20% CO₂ (pH 7.0). Tyrosine kinase receptor Axl expression was 3.3-fold higher after 6 hours at pH 7.0 compared with pH 7.4; this modulation was confirmed by reverse transcriptase–polymerase chain reaction (3.0±0.9-fold, P<0.03; n=3), Northern blot (3.6±1.0-fold, P<0.0003; n=3), and Western blot (10±1.8-fold, P<0.004; n=3). In a time-course study, both Northern and Western blot analyses showed that the most marked difference in Axl expression between pH 7.4 and pH 7.0 occurred after 24 to 48 hours. Furthermore, Axl phosphorylation was enhanced at pH 7.0. Axl ligand, the survival factor growth arrest–specific gene 6 product (Gas6), was released into the conditioned medium, and by Western blot analysis, similar amounts of protein were found at pH 7.0 and 7.4. Full-length Axl cDNA overexpression reduced serum deprivation–induced apoptosis by 64.4±11.9% in human umbilical vein endothelial cells cultured at pH 7.4 compared with mock-transfected cells (P<0.0004). Furthermore, overexpression of either soluble Axl or antisense Gas6 mRNA partially reverted the protective effect of acidification, increasing ≈2.5-fold the number of apoptotic cells at pH 7.0 (control 19.3±2.7%, soluble Axl 48.9±9.7%, P<0.001; antisense Gas6 49.3±14.3%, P<0.03). In conclusion, Gas6/Axl signaling may play an important role in endothelial cell survival during acidification. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2002;91:e4-e12.)

Key Words: acidosis ■ apoptosis ■ endothelium ■ ischemia ■ Gas6/Axl
Materials and Methods

Cell Culture
HUVECs (Clonetics) and human pulmonary artery endothelial cells (HPAECs, Clonetics) were maintained in complete medium containing endothelial cell basal medium (EBM-2, Clonetics) supplemented with an endothelial cell Bullet Kit (Clonetics) containing 2% FCS, human endothelial growth factor (EGF)-2, human fibroblast growth factor-2, human vascular endothelial growth factor, R3-insultralike growth factor-1, ascorbic acid, hydrocortisone, heparin, gentamycin, and amphotericin B. Cells were grown at 37°C in a humidified atmosphere with 5% CO2/95% air. The culture medium was changed every 2 or 3 days, and when cells were subconfluent, monolayers were harvested by 1 to 2 minutes of exposure to 0.1% Trypsin-EDTA (Life Technologies Inc). Cells were used for the experiments between passages 3 and 5. Primary cultures of bovine aortic endothelial cells (BAECs) were prepared as previously described.1 BAECs were grown in DMEM (Hyclone Inc) with 10% FCS (Hyclone Inc) and maintained at 37°C in a humidified atmosphere with 5% CO2/95% air. BAECs were used for the experiments between passages 2 and 6.

Acidification Protocol
Complete growth medium was replaced with serum-free EBM-2. Culture dishes were placed in airtight modular incubator chambers (Forma Scientific Inc) flushed for 20 minutes with 5% CO2/95% air, 20% CO2/80% air, and 25% CO2/75% air to achieve a pH of 7.4±0.02, 7.0±0.05, and 6.6±0.02, respectively. Thereafter, chambers were sealed, made airtight, and placed in an incubator at 37°C for the duration of the experiment.1

Endothelial Cell Proliferation Assay and Apoptosis Assessment
HUVECs seeded at a density of 1×104 cells/60-mm dish were grown in serum-free EBM-2 at either pH 7.4 or pH 7.0. The medium was replaced daily, and cells were harvested and counted with a hemocytometer. For the evaluation of apoptosis, 1×105 cells were plated on glass coverslips in 60-mm Petri dishes and maintained in serum-free medium at pH 7.4 or pH 7.0. After 1, 2, and 3 days, cells were fixed with 4% paraformaldehyde, and apoptotic nuclei were counted after terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL, Roche), and nuclear staining was accomplished with the DNA-binding fluorochrome Hoechst 33258 (1 μg/mL, Sigma Chemical Co).10 Cells were examined with an Axiosplan 2 microscope (Zeiss). The number of apoptotic nuclei was determined by counting 10 different fields (magnification ×400) per sample. At least 200 nuclei per sample were counted. In some experiments, HUVECs were plated on glass coverslips as described above, and then the cells were treated with BAEC conditioned medium (CM) alone or supplemented with 5 μg/mL of either human soluble Axl/Fc Chimera (R&D Systems) or the irrelevant receptor GFRα4/Fc (Sigma). In these experiments, BAEC CM was prepared by incubating cells in serum-free EBM-2 for 96 hours at pH 7.0. Thereafter, the medium was collected and concentrated 100-fold through a 10-kDa cutoff at 4°C by using Centricon prep filters (Amicon). The effect of BAEC CM on HUVECs was examined by diluting the concentrated CM (1:50) with EBM-2. After 24 hours, cells were fixed, and apoptotic nuclei were counted as described above.

cDNA Array Screening
Differential gene expression in HUVECs cultured for 6 hours in complete medium at pH 7.4 or pH 7.0 was evaluated with a human cDNA expression array (Clontech Laboratories, Inc) containing 588 genes, including 3 negative control cDNAs and 9 housekeeping genes. Polya’ DNA was isolated from the cells at the indicated conditions by using the FastTrack 2.0 Kit (Invitrogen). 32P-labeled cDNA probes were generated from each polya’ DNA sample and hybridized to the atlas array according to the manufacturer’s instructions. Equivalent amounts of polya’ DNA were used to synthesize cDNA probes, and equivalent counts per minute were added to hybridizations. After high-stringency washes, the expression profile was quantified by exposing the membranes to Kodak BioMax MS x-ray film with a BioMax MS intensifying screen at −70°C for 24 hours, and a densitometric analysis was performed.

RNA Extraction
Total cellular RNA was isolated from HUVEC monolayers maintained in serum-free medium at either pH 7.4 or pH 7.0 for 48 hours with the use of TriZOL reagent (Life Technologies Inc). RNA was then size-fractionated by electrophoresis to assess its integrity and stored at −80°C.

Reverse Transcriptase–Polymerase Chain Reaction
Total RNA was converted to cDNA by reverse transcription with the use of the Superscript Preamplification System Strand (Life Technologies Inc) for first-strand cDNA synthesis according to the manufacturer’s instructions. Polymerase chain reaction (PCR) was performed by using Ampli Taq DNA Polymerase (Perkin-Elmer Roche Molecular Systems Inc). PCR was performed in a Perkin-Elmer thermal cycler for 25 cycles. The primers’ sequences used for amplification of a 507-bp product of Axl by PCR were as follows: upper 5’-AACTCTCAACTCTGCCTTCT-3’, lower 5’-TCGAGACCTCGGAACACAGC-3’. The primers’ sequences used for amplification of a 798-bp product of Gas6 by PCR were as follows: upper 5’-GACCTGCAAGACATAGACG-3’, lower 5’-CTGGATGGTTGTTCTTCTC-3’. The primers’ sequences used for amplification of a 208-bp product of Rse by PCR were as follows: upper 5’-CTCGAGTGTTGAGGAGAGTCCGG-3’, lower 5’-GCCACACTGCGTGGGAGATCCTCGG-3’. The primers’ sequences used for amplification of a 154-bp product of the housekeeping β-actin gene by PCR were as follows: upper 5’-TCTACACATT-GGCAATGAG-3’, lower 5’-GTGTTGGGCTACAGTT-3’. PCR conditions used for Axl, Gas6, and β-actin amplification were as follows: denaturation at 94°C for 1 minute, annealing at 58°C (Axl, Gas6, and Rse) and 52°C (β-actin) for 1 minute, and extension at 72°C for 1 minute. To obtain a semiquantitative measurement and for PCR normalization, β-actin was amplified from the same amount of cDNA template used for the amplification of other genes (for 10, 15, 20, 25, and 30 cycles) to determine whether the amplification was in the linear phase.

Northern Blot Analysis
Total RNA was isolated from HUVECs cultured at pH 7.4 or 7.0 at different time points (1, 6, 12, 24, and 48 hours) by using TriZOL Reagent (Life Technologies Inc). RNAs (15 μg) were electrophoresed on a 1% formaldehyde-agarose gel, capillary-blotted on Hybond-N membrane (Amersham Pharmacia Biotech), prehybridized in QuikHyb (Stratagene) at 42°C for 4 hours, and hybridized overnight at 42°C in prehybridization buffer containing 1×104 cpm/mL of randomly 32P-labeled PCR probe corresponding to 507 bp of Axl, to 798 bp of Gas6, and to full-length GAPDH. Membranes were first washed with 2× SSC and 0.1% SDS at room temperature for 15 minutes and then with 0.5× SSC and 0.1% SDS at 50°C for 15 minutes. Filters were exposed on Kodak Biomax MS Films, and densitometric analysis was performed.

Immunoprecipitation and Western Blot Analysis of Axl and Gas6
HUVECs, HPAECs, and BAECs were cultured in 0% FCS at pH 7.4 or 7.0 and lysed at different time points (1, 6, 12, and 24 hours) with a lysis buffer containing 50 mmol/L HEPES (pH 7.5), 100 mmol/L NaCl, 1% Triton, 2.5 mmol/L β-glycerophosphate, 10% glycerol, 5 mmol/L MgCl2, 1 mmol/L EGTA, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride. Equal amounts of total proteins (100 μg) were electrophoresed in 5% SDS-PAGE under reducing conditions and electroblotted to a nitrocellulose membrane (Hybond-C, Amersham Pharmacia Biotechnology). Loading an equal amount of total proteins was assessed by Coomassie staining of
separate gels. Axl was detected by incubating the membrane with 1 μg/mL affinity-purified goat polyclonal antibody against human Axl (αAxl, Santa Cruz Biotechnology Inc). The complexes were evidence by incubation with a secondary peroxidase-conjugated antibody and by the ECL detection system (Amersham Pharmacia Biotech) according to manufacturer’s instructions. Tyrosine-phosphorylated Axl was immunoprecipitated both from HUVECs cultured in 0% FCS at pH 7.4 or pH 7.0 for 48 hours and from HUVECs transfected either with pcDNA, or with pCMVAxl (where the prefix p indicates plasmid). Tyrosine-phosphorylated Axl was immunoprecipitated from the cell lysates with the monoclonal anti-phosphotyrosine IgG clone 4G10 (Upstate Biotechnology), followed by the goat polyclonal antibody against αAxl (Santa Cruz Biotechnology Inc).

Gas6 was detected into CM collected from HUVECs maintained in serum-free medium EBM-2 for 48 hours at either pH 7.4 or pH 7.0 or from cells transiently transfected with pCMVslAxl (where slAxl indicates soluble Axl) or with pCMVasGas6 (where asGas6 indicates antisense Gas6) as described below. Gas6 was detected into CM of BAECs maintained in serum-free medium (DMEM) for 96 hours at either pH 7.4 or pH 7.0. CM was concentrated 50-fold through a 10-kDa cutoff at 4°C by using Centriprep 10 filters (Amicon). Equal amounts 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 affinity-purified goat polyclonal antibody against human Gas6 (αGas6, Santa Cruz Biotechnology Inc), followed by ECL (Amersham Pharmacia Biotech).

Cloning

PCR was performed by using Platinum Pfx DNA Polymerase (Life Technologies Inc). The primers’ sequences used for amplification of a 2684-bp product of full-length human Axl were as follows: upper 5′–CCAGCAACTTCTGAGGAAGT-3′, lower 5′–GGCACCATT- CCTCCTGCCCCTG-3′. The primers’ sequences used for amplification of a 1211-bp product of slAxl were as follows: upper 5′–CCAGCAACTTCTGAGGAAGT-3′, lower 5′–TCGCAAGTCCAGGGGTACCTC-3′. The primers’ sequences used for amplification of a 2060-bp product of full-length human Gas6 were as follows: upper 5′–GCTCTGAGCGGCTCCCCGG-3′, lower 5′–GGCTGGCGGCGGGCTCCACGG-3′.

PCR conditions used for Axl and Gas6 amplification were as follows: denaturation at 94°C for 15 seconds, annealing at 58°C for 30 seconds, and extension at 68°C for 2 minutes. PCR was performed in a Perkin-Elmer thermal cycler for 25 cycles. PCR products of full-length slAxl and asGas6 were cloned into a pcMV expression vector with the use of the pcDNA3.1/V5-His Topo TA cloning kit (Invitrogen) according to the manufacturer’s instructions (Figure 1).

Transient Transfections

Either pCMVAxl, pCMVslAxl, pCMVasGas6, or pcDNA, (Invitrogen) control vector was cotransfected with pEGFP-N1(Clontech) reporter vector (3:1 ratio). Cotransfection with two independent vectors results in the internalization of both plasmids by the same cell.11 HUVECs (1×10⁶ cells/100-mm Petri dish) were transfected for 6 hours with FuGENE 6 Transfection Reagent (Roche) according to the manufacturer’s instructions, and they were recovered in complete fresh medium for an additional 24 hours. Subsequently, 1×10⁵ cells were plated on glass coverslips in 60-mm Petri dishes and maintained in serum-free medium at either pH 7.4 or pH 7.0. After 48 hours, cells were fixed with 4% paraformaldehyde, Hoechst 33258 dye nuclear staining was performed, and both normal and highly condensed and fragmented apoptotic nuclei were counted. The results were normalized by transfection efficiency (15% to 20%) determined by the number of green fluorescent protein (GFP)-positive cells. Therefore, in these experiments, the numbers of all Hoechst 33258–positive nuclei and of those Hoechst 33258–positive nuclei that exhibited evidence of apoptosis were counted. Furthermore, the number of GFP-positive cells (ie, the cells transfected with the cDNA of interest) was counted. Thereafter, the percentage of apoptotic nuclei was determined, and this value was normalized by the number of GFP-positive cells. By this approach, it was possible to determine the effect on apoptosis of different cDNAs transfected into cells and to express the final results as percentage of apoptotic nuclei in transfected cells. This analysis was applied to the results depicted in Figures 5, 6, and 7.

Statistical Analysis

Continuous variables were analyzed by the Student t test. Data are expressed as mean±SD. A value of P≤0.05 was considered statistically significant.

Results

Effect of Acidification on HUVEC Apoptosis

The effect of acidification on serum deprivation–induced apoptosis was examined in HUVECs. Cells maintained in serum-free medium in normal (pH 7.4) or acidic (pH 7.0 or 6.6) conditions were kept in culture for 1, 2, and 3 days before counting or Hoechst nuclear staining. Acidification strongly protected HUVECs from apoptosis, and this effect was statistically significant at days 2 (P<0.0002) and 3 (P<0.0001); at these time points, acidification inhibited the progressive decrease in cell number by 80% and 99%, respectively (Figure 2A), and decreased the number of apoptotic cells evaluated with Hoechst staining by 84% and 88.5%, respectively (Figure 2B). It is noteworthy that the effect of pH 6.6 on viable cell numbers was comparable to that of pH 7.0. Under these experimental conditions, similar results were obtained by TUNEL (not shown). These results

Figure 1. Constructs. PCR products of full-length Axl (2684 bp) and the extracellular domain (ECD) of Axl (1211 bp) were cloned in a frame and a sense orientation into a pcMV expression vector, whereas full-length Gas6 (2060 bp) was cloned in an antisense orientation. These constructs were used for overexpression studies. TM indicates transmembrane domain; TK, tyrosine kinase domain.
are in agreement with prior studies from our laboratory, performed under similar conditions, in which endothelial cell apoptosis was evaluated with different morphological and biochemical techniques.1

Effect of Acidification on Axl mRNA Expression
In these experiments, the expression of 588 different genes was evaluated with a cDNA array. HUVECs were exposed to pH 7.0 for 6 hours, and in this condition, it was found that the expression of the tyrosine kinase receptor Axl was 3.3-fold higher than the expression at pH 7.4 (Figure 3, inset). In this preliminary screening, it was found that some other genes involved in proliferation control and apoptosis regulation appeared to be modulated by a decrease in pH (not shown). However, because of their relevance as antiapoptotic signaling molecules in the cardiovascular system,12–15 it was decided to examine in greater detail the Axl/Gas6 pathway rather than to focus on other genes.

To confirm the effect of acidification on Axl obtained with the cDNA expression array, semiquantitative reverse transcriptase (RT)-PCR (Figure 3A) and Northern blot analysis (Figure 3B) for Axl and its ligand, Gas6, were performed. HUVECs were cultured in 0% FCS at pH 7.4 or 7.0, and total RNA was purified after 1, 6, 12, 24, and 48 hours. It was found that over the 48-hour time course of these experiments, Axl mRNA was downregulated at pH 7.4, whereas it was not modulated significantly at pH 7.0; the peak difference occurred between 24 and 48 hours, and at these time points, Axl mRNA was 3.0- to 3.6-fold higher at pH 7.0 versus pH 7.4 (Figure 3). Furthermore, Northern blot analysis showed that acidification enhanced Gas6 2-fold (Figure 3).

Effect of Acidification on Axl and Gas6 Protein Expression
The modulation of Axl protein by acidification was evaluated by Western blot analysis. HUVECs were cultured in serum-free medium as described above, and the cellular lysates were prepared at 1, 6, 12, and 24 hours. In agreement with the results on mRNA expression, Axl protein exhibited a time-dependent decrease at pH 7.4, whereas this decrease was much less pronounced at pH 7.0 (Figure 4A), and comparable results were obtained at pH 6.6 (Figure 4B). In the experimental conditions of the present study, the same results were obtained with the use of HPAECs; in fact, at 24 hours, the amount of Axl protein was 5-fold higher at pH 7.0 than at pH 7.4 (Figure 4C). In other experiments, Axl tyrosine phosphorylation was investigated by immunoprecipitation at both pH 7.4 and pH 7.0; under these conditions, Axl detected at pH 7.0 was partially phosphorylated (Figure 4D). Additional experiments were performed with HUVECs and with BAECs to determine whether, under our experimental conditions, endothelial cells release Gas6 into the CM and, eventually, whether Gas6 levels in CM differ between pH 7.4 and pH 7.0. Western blot analysis indicated that Gas6 was released into CM by both endothelial cell types and that Gas6 protein levels were similar at pH 7.0 and pH 7.4 (Figure 4E).
Effect of Full-Length Axl Overexpression on HUVEC Apoptosis

To test the involvement of Axl in the protective action of acidification on endothelial cells, HUVECs were transiently transfected with an expression plasmid containing the full-length cDNA of human Axl (pCMVAxl) (Figure 5). After 48 hours, a strong increase of Axl expression and phosphorylation was present (inset), and a marked reduction of apoptosis (64.4%/1100611.9%) was observed in cells cultured at pH 7.4 compared with mock-transfected cells. In contrast, Axl overexpression had no effect on HUVECs cultured at pH 7.0; this result was likely due to the fact that cells cultured at low pH were already protected by sustained Axl expression (see Figures 3A, 3B, 4A, and 4C).

Effect of slAxl and asGAS6 on HUVEC Apoptosis

To demonstrate the direct role of the Gas6/Axl pathway in acidification-dependent protection from apoptosis, HUVECs were transiently transfected with slAxl (pCMVslAxl) or asGas6 (pCMVasGas6) plasmids, and apoptosis was evaluated as described above. In both cases, the inhibition of the Gas6/Axl pathway partially reversed the protective effect of acidification on serum deprivation–induced apoptosis (Figure 6). In fact, in cells transfected with slAxl, the number of apoptotic cells at pH 7.0 increased 2.5-fold, whereas no
significant difference was observed at pH 7.4 (Figure 6A). Western blot analysis of CM and cell lysates of HUVECs transfected with pCMVslAxl showed that the soluble receptor of the expected molecular weight was present both in CM and in the cell lysate (Figure 6A, inset).

In other experiments, cells were transfected with asGas6, and the number of apoptotic nuclei, after 48-hour exposure to pH 7.0, was enhanced \( \approx 2.5 \)-fold versus pcDNA3-transfected cells. Similar to what had been observed with slAxl, there was no effect of asGas6 overexpression at pH 7.4 (Figure 6B).

It is noteworthy that HUVECs transiently transfected with pCMVasGas6 versus pcDNA3-transfected control cells exhibited a significant reduction of Gas6 released into CM after 48-hour incubation at pH 7.0 (Figure 6B, inset) or pH 7.4 (not shown). Similar results were obtained with BAECs (not shown).

Taken together, these results show that the Gas6/Axl pathway plays an important role in the ability of acidification to protect endothelial cells from programmed cell death.

**Effect of CM on HUVEC Apoptosis**

In these experiments, it was determined whether endothelial cell CM protected HUVECs from apoptosis and, eventually, whether the CM antiapoptotic effect was due to the presence of Gas6. CM was prepared from BAECs as described in Materials and Methods and was known to contain Gas6 (Figure 4E). All experiments were performed at pH 7.4 with HUVECs transfected either with pcDNA3 or with pCMVslAxl.

As previously shown (Figure 5), pCMVasGas6 decreased HUVEC apoptosis at pH 7.4. BAEC CM exhibited a marked antiapoptotic effect in both pcDNA3- and pCMVslAxl-transfected cells (Figure 7). Because CM can contain a variety of antiapoptotic agents, BAEC CM was supplemented with slAxl/Fc to bind Gas6 present in the CM. The protective effect of CM was abolished by slAxl/Fc in cells overexpressing Axl, whereas it was not significantly modified in mock-transfected cells. This contrasting effect could be at least partially explained by the low amount of Axl receptor present in mock-transfected cells at pH 7.4 in serum-free conditions and/or by the possible interference elicited by Axl overexpression with survival pathways depending on other growth factors/receptors. An irrelevant receptor, GFRα-3/Fc, did not modify the results obtained with BAEC CM or with EBM-2 in either pcDNA3- or pCMVslAxl-transfected cells.

**Effect of Acidification on Tyrosine Kinase Receptor Rse Expression**

In these experiments, the effect of acidification on Rse, another member of the Axl tyrosine kinase receptor family, was examined. Rse mRNA expression was determined by RT-PCR. HUVECs were cultured either in complete medium or in 0% FCS, both at pH 7.4 and at pH 7.0, for 6, 12, 24, and 48 hours. In our experimental conditions, this receptor was present in HUVECs maintained in growing conditions but was not expressed at any time point in cells kept in 0% FCS at either pH 7.4 or pH 7.0 (Figure 8).

Figure 6. Effect of slAxl or asGas6 overexpression on the protective effect of acidification. HUVECs were cotransfected with expression vector for slAxl (A) or asGas6 (B) and pEGFP-N1 reporter vector. Cells were cultured for 24 hours after transfection in complete medium; thereafter, they were cultured in serum-free medium at either pH 7.4 or pH 7.0 for an additional 48 hours before apoptosis quantification with Hoechst 33258 nuclear dye staining. Both slAxl and asGas6 partially reverted the protective effect of acidification. A. Apoptotic nuclei at pH 7.0 were 19.3±2.7% in pcDNA3-transfected cells and 48.9±9.7% in slAxl-transfected cells (P<0.001). In contrast, no significant difference was observed at pH 7.4. Results are expressed as percentage of apoptotic pcDNA3-transfected cells at pH 7.4; under these conditions, 5.3±0.6% of Hoechst 33258-stained nuclei exhibited evidence of apoptosis. Bar graphs represent the average of 3 experiments performed in duplicate. Inset, CM and cell lysate WB analysis using an anti-tag antibody (anti-V5) showed that after HUVEC transfection with pCMVslAxl, the soluble receptor of the expected molecular weight was present both in CM and in cell lysates. B. Apoptotic nuclei at pH 7.0 were 19.3±2.7% in pcDNA3-transfected cells and 49.3±14.3% in asGas6-transfected cells (P<0.003). Results are expressed as percentage of apoptotic pcDNA3-transfected cells at pH 7.4; under these conditions, 5.2±0.6% of Hoechst 33258-stained nuclei exhibited evidence of apoptosis. Bar graphs represent the average of 3 experiments in duplicate. Inset, WB analysis showed that Gas6 released into CM was \( \approx 50% \) lower in pCMVgasGas6-transfected cells vs pcDNA3-transfected cells. Inset shows a representative example of 3 independent experiments.
Figure 7. Effect of slAxl on CM-induced protection from apoptosis. HUVECs were cotransfected either with full-length pCMV/Axl or with pcDNA3 control vector and pEGFP-N1 reporter vector. Cells were cultured for 24 hours after transfection; thereafter, the culture medium was replaced with fresh EBM-2 supplemented with concentrated BAEC CM (final concentration 2%×; see Materials and Methods) and kept at pH 7.4 for additional 48 hours before apoptosis quantification. BAEC CM exerted a significant protection from apoptosis both in mock-transfected cells (59%, P<0.0002) and in Axl-overexpressing cells (51%, P<0.0001). The protective effect of CM was abolished by the slAxl/Fc in cells overexpressing Axl and was not significantly modified in pcDNA3-transfected cells. slAxl/Fc added to EBM-2 abolished the antiapoptotic effect of Axl overexpression. An irrelevant receptor, GFRα3/Fc, did not modify the results obtained with BAEC CM or with EBM-2, in either pcDNA3- or pCMV/Axl-transfected cells. Results are expressed as percentage of apoptotic pcDNA3-transfected cells in EBM-2; under these conditions, 9.8±1.2% of Hoechst 33258–stained nuclei exhibited evidence of apoptosis. Bar graphs represent the average of 3 experiments in duplicate.

Discussion

The present study shows that acidification modulates Axl expression in endothelial cells. Gas6 produced under these conditions activates Axl signaling and, via this mechanism, plays a key role in the protective effect of acidification on serum deprivation–induced endothelial cell apoptosis. To our knowledge, this is the first report showing, in endothelial cells, the functional autocrine/paracrine role of the Gas6/Axl pathway in a clinically relevant condition, ie, acidification.

Gas6 is a secreted protein that was initially was identified in growth-arrested NIH 3T3 cells14,16 and was subsequently shown to have a high degree of homology with protein S, a negative regulator of the coagulation cascade.17,18 It has been shown that Gas6 plays a role in cell survival,19 proliferation,20 growth arrest,17 and adhesion.21 Gas6 binds, with a different affinity, three different receptors with tyrosine kinase activity: Axl (also named Ufo, Ark, and Tyro7), Rse (also named Sky Brt, Tif, Dtk, Etk, and Tyro 3), and Mer (also named Eyk, Nyc, and Tyro 12). A mouse knockout for any single receptor or any combination of two receptors was viable and did not show any gross anatomic defect. In contrast, mice lacking all three receptors produced no mature sperm and also exhibited increased apoptosis in many tissues, including the vascular wall.26 Recent studies have called attention to the potential role of the Gas6/Axl pathway in the response of vascular smooth muscle cells (SMCs) to injury. It has been shown that Gas6 is present in SMC CM and that it can potentiate SMC growth,27 prevent growth arrest–induced SMC death,28 and act as an SMC chemoattractant.29 Finally, in the rat carotid artery model of balloon injury, an enhanced expression of both Axl and Gas6 2 weeks after injury has been shown. The role of the Gas6/Axl pathway in endothelial cell physiology and pathophysiology is less well defined than that in SMCs. For several years, it has been known that Gas6 is expressed in endothelial cells,17 but its function was unknown. Recently, it has been shown that exogenously added Gas6 inhibits granulocyte adhesion to activated endothelial cells.30 Previously published work on primary endothelial cells has demonstrated that Axl is also expressed by HUVECs and that exogenously added Gas6 protects these cells from serum deprivation– and tumor necrosis factor–α–induced cell death.31 Similarly, it has also been found that treatment with Gas6 protects HPAECs from serum deprivation–induced apoptosis via Axl signaling.14 In the present study, little Axl expression was observed after 24 to 48 hours of serum-free medium culture at normal pH. This result differs from that reported by O`Donnell et al13 and Healy et al14 and is likely due to the different cell density at which the experiments were performed. In fact, in the present study, the experiments were performed at low cell density (3500 cells/cm2), whereas significantly higher cell density conditions were used in the other studies.13,14 This issue was addressed in additional experiments with HUVECs kept in serum-free conditions for 24 and 48 hours; it was observed that the Axl protein level was significantly higher in cells grown to confluence versus sparse cultures (not shown).

Results obtained with endothelial cells are consistent with other studies with non–endothelial cell types, which have demonstrated an antiapoptotic effect of the Gas6/Axl pathway.15,19 Our results show a key role for Gas6/Axl for the antiapoptotic effect of low pH. It is noteworthy that Axl overexpression decreased the number of apoptotic cells at pH 7.4, whereas at pH 7.0, Axl overexpression did not cause a further decrease in the number of apoptotic cells, possibly because the antiapoptotic effect of acidification was already maximal, and no further improvement was possible (Figure 5). In contrast, disabling the Gas6/Axl pathway either by overexpressing slAxl or asGas6 (Figure 6) increased the number of apoptotic cells at pH 7.0; however, the number of apoptotic cells was still ≈50% of that observed at pH 7.4. This result was expected because under our experimental conditions, not all cells are transfected. Alternatively, it is possible that other pathways, in addition to Gas6/Axl, may be responsible for the antiapoptotic effect of acidification.
The molecular mechanism(s) by which Gas6/Axl signaling protects cells from death has not been fully elucidated, but it appears to depend on phosphatidylinositol 3-OH kinase activation, leading to Akt phosphorylation and, as a consequence, to phosphorylation and inactivation of the proapoptotic protein Bad.26–31 This response is paralleled by an Akt-mediated increase in nuclear factor-κB-binding activity, an induction of the antiapoptotic gene Bcl-xL, and an increase in Bcl-xL protein.31 Furthermore, it has been previously shown that endothelial cell intracellular acidification, to levels comparable to those achieved in the present study, releases Ca2+ into the cytosol from intracellular Ca2+ stores32 and that Gas6 potentiates the mitogenic effects of Ca2+, mobilizing growth factors.27 This raises the possibility that an acidification-induced increase in cytosolic Ca2+ may amplify the antiapoptotic effect of Gas6/Axl. In addition, this increase in cytosolic Ca2+ may be linked to endothelial cell synthesis of NO,33 which is known to exercise an antiapoptotic effect on endothelial cells.34 However, a link between NO and Gas6/Axl signaling remains to be established.

We also investigated the effect of acidification on Rse, another member of the Axl tyrosine kinase receptor family. In our experimental conditions, this receptor was not expressed in cells kept in serum-free medium at pH 7.4 and 7.0 (Figure 8). Therefore, it appears unlikely that Rse may play a significant role in the antiapoptotic effect of acidification on endothelial cells.

It is noteworthy that acidification can also have harmful effects on cell viability,3 and the results of the present study refer to a relatively moderate decrease in pH and are limited to in vitro experiments. Therefore, it is possible that in vivo (under conditions in which acidification is also frequently associated with a decrease in oxygen tension and with changes both of the intracellular and extracellular concentration of some ions) the effects of a decrease in pH on endothelial cell viability may differ from those reported in the present work. Nevertheless, the results of the present study provide a working hypothesis to explain the antiapoptotic effect of acidification observed in a variety of cell types and experimental conditions.1,2,4,6,7,35–37 Endothelial cells are exposed to low pH in many conditions, including ischemia and metabolic or respiratory acidosis,38 exposure to drugs that inhibit Na+/H+ exchange in the plasma membrane,7 and laminar shear stress,39 which also has a marked antiapoptotic effect on these cells.34 Finally, because Gas6/Axl signaling may be activated by low pH in other cell types also, a potential role for this pathway may be considered in ischemic preconditioning of the heart. In fact, it has been shown that brief episodes of ischemia protect the heart from a subsequent prolonged episode of ischemia and improve cardiac function and metabolism on reperfusion.9 The mechanisms through which ischemic preconditioning protects the heart are still largely unknown, but cardiac protection can also be achieved by exposing the heart to brief episodes of acidification, without interruption of flow, before the prolonged ischemic episode.8

In summary, the results of the present study show that autocrine/paracrine activation of Gas6/Axl signaling plays a key role in the antiapoptotic effect of acidification on endothelial cells and raise the possibility that this mechanism may also be involved in other cell types in which cell death is inhibited by low pH.

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References


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