Acidosis Inhibits Endothelial Cell Apoptosis and Function and Induces Basic Fibroblast Growth Factor and Vascular Endothelial Growth Factor Expression

Daniela D’Arcangelo, Francesco Facchiano, Laura Maria Barlucchi, Guido Melillo, Barbara Illi, Lucia Testolin, Carlo Gaetano, Maurizio C. Capogrossi

Abstract—Endothelial cells are exposed to an acidic environment in a variety of pathological and physiological conditions. However, the effect of acidosis on endothelial cell function is still largely unknown, and it was evaluated in the present study. Bovine aortic endothelial cells (BAECs) were grown in bicarbonate buffer equilibrated either with 20% CO₂ (pH 7.0, acidosis) or 5% CO₂ (pH 7.4, control). Acidosis inhibited BAEC proliferation in 10% FCS, whereas by day 7 in serum-free medium, cell number was 3-fold higher in acidic cells than in control cells. Serum deprivation enhanced BAEC apoptosis, and apoptotic cell death was markedly inhibited by acidosis. Additionally, acidosis inhibited FCS-stimulated migration in a modified Boyden chamber assay and FCS-stimulated differentiation into capillary-like structures on reconstituted basement membrane proteins. Conditioned media from BAECs cultured for 48 hours either at pH 7.0 or pH 7.4 enhanced BAEC proliferation and migration at pH 7.4, and both effects were more marked with conditioned medium from BAECs grown in acidic than in control conditions. Acidosis enhanced vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) mRNA expression as well as bFGF secretion, and a blocking bFGF antibody inhibited enhanced BAEC migration in response to conditioned medium from acidotic cells. These results show that acidosis protects endothelial cells from apoptosis and inhibits their proangiogenic behavior despite enhanced VEGF and bFGF mRNA expression and bFGF secretion. (Circ Res. 2000;86:312-318.)

Key Words: acidosis ▪ apoptosis ▪ endothelium ▪ ischemia ▪ growth factors

Endothelial cells are exposed to an acidic pH in a variety of pathological conditions, including ischemia, diabetic ketoacidosis, wound healing, respiratory failure, and uremia and in response to some drugs and poisons.1 Furthermore, acidification also occurs in physiological conditions; a decrease in endothelial cell pH is observed in response to hydrodynamic shear stress,2 and intracellular acidosis is part of the physiological response to physical exercise.3 It has been demonstrated that acidification enhances endothelial cell Ca²⁺4 and induces intercellular adhesion molecule-1 expression on the endothelial cell surface.3 However, because acidification also occurs in ischemia and because this condition may induce neovascularization,6-7 it is of interest to determine how acidification modulates endothelial cell angiogenic properties. Prior studies have shown that hypoxia, which leads to intracellular acidification,8 enhances vascular endothelial growth factor (VEGF) expression via a hypoxia inducible factor 1 (HIF-1)–mediated mechanism9 and increases mRNA stability.10 The effect of hypoxia on basic fibroblast growth factor (bFGF) is still controversial.11,12 Thus, the effect of acidification per se on endothelial cell function as well as on VEGF and bFGF expression remains to be determined. These issues were addressed in the present study, and extracellular and intracellular acidification was achieved by changing from a 5% to a 20% CO₂-HCO₃⁻ buffer.4 It was found that by lowering buffer pH from 7.4 to 7.0, ie, within a range found in many clinical conditions, endothelial cell proliferation, migration, and differentiation into capillary-like structures were inhibited, whereas endothelial cells were protected from apoptosis. These effects were associated with enhanced VEGF and bFGF mRNA expression without any effect on mRNA stability or the induction of HIF-1.

Materials and Methods

Cell Preparation and Culture

Primary cultures of bovine aortic endothelial cells (BAECs) were prepared and cultured as described.13 Culture purity was consistently ≥98%. BAECs (1×10⁵ cells per 60-mm dish) were grown in serum-free or 10% FCS medium at either pH 7.4 or pH 7.0.

Acidification Protocol

Culture dishes or Boyden apparatuses were placed in airtight modular incubator chambers (Forma Scientific) infused for 20
minutes with either 5% CO2/95% air or 20% CO2/80% air to achieve pH 7.40±0.02 and pH 7.0±0.05, respectively. Chambers were placed in an incubator at 37°C for the duration of the experiment.

**Apoptosis Assessment**

Fluorescence-activated cell sorter (FACS) analysis was carried out with cells stained with propidium iodide (50 μg/mL) by use of a FACScan (Becton Dickinson) and CellQuest software as reported.14 Apoptosis was also analyzed by Cell Death Detection ELISA (Boehringer-Mannheim), terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) assay,15 and Hoechst 33258 dye nuclear staining.16

**Preparation of BAEC Conditioned Medium**

BAECs in complete medium (1×10⁶ cells per 100-mm dish) were grown for 3 days, then medium was replaced with serum-free DMEM, and dishes were placed in airtight chambers. After 48 hours, conditioned medium (CM) was collected.

**Migration Assay**

Migration was assessed in modified Boyden chambers.17 Medium without (control) or with 10% FCS was used as a chemoattractant, and cells were incubated for 5 hours at 37°C in a 5% CO₂ or 20% CO₂ atmosphere. In other experiments, either BAEC CM or serum-free medium was used with or without an anti-bFGF neutralizing antibody (100 ng/mL, R&D) or with the denatured antibody (10 minutes, 95°C).

**Differentiation on Matrigel**

BAEC differentiation on Matrigel (Collaborative Research) was performed as described.18 Capillary-like structures were quantified by counting internodal points formed in 6 fields per dish.

**RT-PCR Experiments**

Total cellular RNA was isolated from BAECs grown in serum-free medium either at pH 7.4 or pH 7.0 for 48 hours by use of TRIzol reagent (GIBCO Life Technologies). RNA was converted to cDNA by reverse transcription (RT) with the Superscript Preamplification System (Life Technologies). Polymerase chain reaction (PCR) was performed for 10, 15, 20, 25, and 30 cycles. Sequences of the primers were as follows: for VEGF (250-bp product), upper 5'-TCTATGATGTTATCTCAG-3', lower 5'-TGCTTAGAAAGCTCAT-3'; for bFGF (220-bp product), upper 5'-TCAAAGTTTACACTCTCAAGCAG-3', lower 5'-ATACGCTGCAGTTGTTTC-3'. PCR conditions for VEGF and bFGF amplification were as follows: denaturation at 94°C for 1 minute; annealing at 52°C and 56°C, respectively, for 1 minute; and extension at 72°C for 1 minute. Actinomycin D chase studies were performed as described10 to determine mRNA stability on BAECs maintained at either pH 7.4 or pH 7.0 for 48 hours, followed by additional 1 to 8 hours with actinomycin D (5 μg/mL).

**Detection and Quantification of bFGF into BAEC CM**

CM was concentrated 30-fold through a 10-kDa cutoff (Centriprep 10), and then proteins (40 μg) were subjected to SDS-PAGE under reducing conditions and electroblotted. bFGF was detected with 0.2 μg/mL antibody (100 ng/mL, R&D) or with the denatured antibody (10 minutes, 95°C). Double-strand oligonucleotide containing an HIF-1 consensus binding site (40 ng) was labeled with Klenow Enzyme (Boehringer) for 30 minutes at 37°C with the use of 25 μCi [α-32P]dATP. Nuclear extracts14 were incubated on ice with probe alone (HIF-1), with double-strand competitor oligonucleotides (HIF-1 or mutated HIF-1, 100-fold nuclear excess), with an affinity-purified anti–arylic hydrocarbon receptor translocator (ARNT) antibody (αARNT, 1 μg) or with a nonspecific antibody, anti–arylic hydrocarbon receptor complex (AHRC) antibody (αAHRC).

**Statistical Analysis**

Continuous variables were analyzed by the Student t test and 1-way ANOVA, along with post hoc Student-Newman-Keuls tests when appropriate. Data are expressed as mean±SE. A value of P<0.05 was considered statistically significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

**Results**

**Effect of Acidosis on BAEC Proliferation and Apoptosis**

The effect of acidosis on BAEC proliferation was examined both in the absence and in the presence of 10% FCS. Figure 1A shows strong inhibition of serum-stimulated BAEC proliferation under acidic conditions compared with cells grown at normal pH. Figure 1B shows the BAEC proliferation curve under serum-free conditions. After day 2, cells grown at normal pH exhibited a progressive decrease in number, and this effect was prevented at acidic pH. In fact, the cell number at day 7 was 3-fold higher for cells grown under acidic conditions versus cells grown at normal pH. It is noteworthy that at pH 7.4, the cell number at day 7 was lower than the cell number at time 0, suggesting that acidosis inhibited endothelial cell death occurring under starvation. By FACS analysis, in serum-free medium there was an increase in apoptotic cells, and acidosis decreased the sub-G₁ population by ~50% (Figure 2A). Furthermore, the level of cytoplasmic histone-associated DNA fragments in BAECs grown at...
pH 7.0 was significantly lower than that at pH 7.4, and this effect of acidosis was observed both in 10% FCS and in serum-free medium (Figure 2B). Additional experiments were performed to evaluate the amount of apoptotic cells by TUNEL assay and Hoechst 33258 staining. Acidosis at days 3, 5, and 7 of the growth curve strongly reduced apoptotic cell number both in serum-free medium and in 10% FCS (Figure 2C).

These findings suggest that the lower cell number observed in medium with 10% FCS at pH 7.0 versus pH 7.4, shown in the proliferation assay depicted in Figure 1A, was due to diminished proliferation rate rather than to increased cell death. This conclusion is also supported by the lower number of detached cells in the supernatant of BAECs grown at pH 7.0 than at pH 7.4 in 10% FCS and also in serum-free medium (not shown).

Taken together, the results of Figures 1 and 2 indicate that acidosis slows down BAEC proliferation and protects these cells from apoptosis.

Effect of Acidosis on BAEC Migration

The results depicted in Figure 3 show that 10% FCS was a powerful chemoattractant for BAECs and that acidosis markedly inhibited BAEC migration in response to FCS. A decrease of basal migration was also observed in acidotic conditions in the absence of a chemoattractant in the lower chamber of the Boyden apparatus.

Effect of Acidosis on BAEC Differentiation

The effect of acidosis on BAEC differentiation on Matrigel20 was examined in 0% and in 10% FCS. BAECs developed a...
diffuse network of capillary-like structures in 10% FCS at pH 7.4. (Figure 4B), and this effect was strongly inhibited at pH 7.0 (Figure 4D). In 0% FCS, BAECs failed to differentiate regardless of pH. The average results from the above experiments are reported in Figure 4E.

Effect of BAEC CM on BAEC Proliferation and Migration

BAECs were grown for 48 hours in serum-free conditions either at pH 7.0 or at pH 7.4. Thereafter, CM was collected and tested on BAEC proliferation assays carried out for 48 hours at pH 7.4. CM from BAECs grown at pH 7.0 enhanced BAEC proliferation, and cell number after 48 hours was 183 ± 8% of control versus 121 ± 6% of control for CM from BAECs grown at pH 7.4 (Figure 5). In addition, CM from BAECs grown at both pH levels stimulated BAEC migration, but with different potency; CM from BAECs grown at pH 7.0 induced the migration of 102 ± 4 cells per field, and CM from BAECs grown at pH 7.4 induced the migration of 61 ± 2 cells per field. These results show that BAEC CM enhances BAEC proliferation as well as migration and that these effects are more marked when CM is obtained from cells grown under acidic conditions.

Characterization of BAEC CM

The mitogenic activity of CM produced under acidic conditions was evaluated in a 48-hour proliferation assay; it was abolished by treatment at 100°C for 5 minutes and reduced by 33 ± 6% after 15 minutes at 65°C (not shown). Furthermore, the mitogenic effect of CM produced under acidic conditions was not modified by ultrafiltration with a 10-kDa cutoff, indicating that a proteic factor(s) responsible for the mitogenic activity had a molecular size >10 kDa (not shown).
Effect of Acidosis on VEGF and bFGF

Semiquantitative PCR amplification of reverse-transcribed mRNAs derived from BAECs grown at pH 7.4 or pH 7.0 showed that VEGF and bFGF mRNA levels in acidotic conditions were 2-fold higher than those at pH 7.4 (Figures 6A and 6B). This effect was not due to increased mRNA stability, as assessed by actinomycin D chase studies (Figure 6C). Furthermore, the amount of bFGF in CM from BAECs cultured at pH 7.0 for 48 hours was 2.8-fold higher than the amount of bFGF in CM from BAECs cultured at pH 7.4 (Figure 6D). This increase was confirmed by Western blot analysis (Figure 6E). The presence and the activity of bFGF in BAEC CM were confirmed in migration assays. Figure 7 shows that the addition of an anti-bFGF antibody to the CM inhibited the chemotactic activity of CM from BAECs grown at pH 7.4 and pH 7.0 by 34% and 41%, respectively (P < 0.005). The results are in agreement with the data in Figure 6D showing enhanced bFGF secretion at pH 7.0. Because bovine VEGF antibodies are not commercially available, it was not possible to detect VEGF in BAEC CM, and VEGF-mediated modulation of BAEC migration could not be determined.

Effect of Acidosis on HIF-1

HIF-1, a heterodimer formed by HIF-1α and ARNT, is a transcription factor involved in hypoxia-induced VEGF gene-

Figure 6. Effect of acidosis on VEGF and bFGF. A, Semiquantitative RT-PCR for VEGF and bFGF is shown. Representative example shows that acidosis enhanced both VEGF and bFGF expression. B, Average results of densitometric analysis confirm that acidosis enhanced VEGF and bFGF mRNA expression (n = 7). Results were normalized for β-actin level. C, Actinomycin chase analysis is shown. BAECs were cultured at pH 7.4 or at pH 7.0 for 48 hours. Cells were treated with actinomycin D for an additional 0, 1, 2, 4, 6, or 8 hours, then total mRNA was isolated, and VEGF and bFGF mRNAs were determined by RT-PCR. Acidosis did not modulate VEGF (n = 4) and bFGF (n = 4) mRNA stability. D, Acidosis induced a significant increase of bFGF secreted into the medium compared with results at pH 7.4 (P < 0.001). Conditioned media were obtained after 48 hours incubation at different pH levels (n = 3). E, Western blot analysis confirms the significant increase of bFGF secreted into the medium versus pH 7.4. Equal amount of proteins (40 μg/lane) from concentrated CM were used. Recombinant human (hr) bFGF and unconditioned DMEM were used as controls.

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Figure 7. Effect of BAEC CM on BAEC migration. Serum-free CM from BAECs grown for 48 hours either at pH 7.4 or pH 7.0 was used as chemoattractant in migration assays at pH 7.4. Compared with unconditioned medium, CM enhanced migration; furthermore, CM from BAECs grown at pH 7.0 was a more powerful chemoattractant than CM from cells grown at pH 7.4 (n = 6, P < 0.0001). An anti-bFGF neutralizing antibody (Ab-bFGF) added to the CM reduced BAEC migration both at pH 7.0 and at pH 7.4, and inhibition was more marked with CM from BAECs grown at pH 7.0 than at pH 7.4 (n = 3, P < 0.005). A heat-denatured bFGF antibody did not affect migration. Results represent the average of 3 to 6 experiments in duplicate.
Acidosis Modulates Endothelial Cell Function

D’Arcangelo et al

The results of the present study show that hypercarbic acidosis inhibits endothelial cell proliferation, migration, and differentiation into capillary-like structures and protects cells from apoptosis. Furthermore, acidosis enhances VEGF and bFGF mRNA expression as well as bFGF secretion. The increase in bFGF and VEGF mRNA is not due to increased mRNA stability, and VEGF gene expression is not modulated via an HIF-1–dependent mechanism. The effect of acidosis to inhibit endothelial cell function despite enhanced expression of VEGF and bFGF may be explained by different mechanisms, including diminished affinity of the growth factors for their cell-associated receptors, diminished receptor number, or inhibition of the intracellular signals triggered by the agonist-receptor interaction.21–24 Alternatively, it is possible that this may represent a time-dependent phenomenon and that >7 days (Figure 1) may be required to observe an enhancement in BAEC proliferation.

The protective effect of acidosis on endothelial cell apoptosis is a new finding and is in agreement with prior results that have shown that extracellular acidosis protects primary neurons as well as p53+/− and p53−/− mouse embryo fibroblasts from serum deprivation–induced apoptotic death.25 In the present study, the mechanism by which acidosis protects endothelial cells from apoptosis was not elucidated. However, because acidification per se increases endothelial cell Ca2+4, the protective effect of acidosis was unrelated to inhibition of the Ca2+ overload, whereas enhanced production of VEGF and bFGF may have played a role in the inhibition of apoptotic cell death.26,27

Acidosis-induced increase of VEGF and bFGF mRNA is of interest because an increase in VEGF mRNA has also been described in hypoxic cells and was related to the activation of HIF-1 and enhanced VEGF mRNA stability.19 HIF-1 is a transcription factor that is considered to be a key regulator of the cell hypoxia response pathway because it activates the transcription of a variety of genes that help cells survive in hypoxic conditions. Because hypoxia leads to intracellular acidosis,2 it was hypothesized that acidosis may also modulate HIF-1 activity. However, the results of the experiment depicted in Figure 8 show no activation of HIF-1 in BAECs at pH 7.0. Furthermore, we found no effect of acidosis on VEGF mRNA stability. Thus, the enhanced VEGF and bFGF mRNA expression reported in the present study is compatible with increased gene transcription and may have been due to activation of unidentified pH-sensitive transcription factor(s).

The results of the present study suggest that acidification may inhibit new blood vessel formation and must be reconciled with the results of other studies that have shown that ischemia in vivo triggers an angiogenic response:6,7 A possible explanation for this apparent discrepancy is related to the enhancement of bFGF and VEGF expression by acidosis. The induction of these growth factors may increase their storage in the extracellular matrix and set the stage for enhanced angiogenesis on return to normal pH. Because most patients with coronary artery28,29 and peripheral vascular disease have normal resting blood flow and develop ischemia in response to exercise, it is possible that acidosis during transient ischemia may enhance growth factor secretion and protect enhanced expression. In hypoxic conditions, it is possible to detect nuclear HIF-1 bound to its 32P-labeled consensus binding site (ACGTG) by electrophoretic mobility shift assay, with use of a specific antibody. The experiments reported in the present study investigated the effect of acidosis on HIF-1. Figure 8 shows that HIF-1 is not induced in acidotic cells, because there was no supershift with an anti-ARNT affinity-purified antibody (αARNT), or with a nonspecific antibody (αAHRC). HIF-1 (ACGTG) and mutated HIF-1 (mHIF-1, AAAAG) were oligonucleotide competitors. B, Nuclear extracts from BAECs grown for 48 hours at pH 7.4 with 100 μmol/L CoCl2 (left) or for 24 hours (chemical hypoxia) at pH 7.4 with 100 μmol/L CoCl2 (left). In chemohypoxic conditions, HIF-1 expression was induced in BAECs. Acidosis did not induce HIF-1 expression, because no supershift was detected in the presence of αARNT. Supershifted complex is indicated by the arrow. Experimental conditions were otherwise similar to those used in panel A. This experiment was performed 3 times with similar results.

Discussion

The protective effect of acidosis on endothelial cell apoptosis is a new finding and is in agreement with prior results that have shown that extracellular acidosis protects primary neurons as well as p53+/− and p53−/− mouse embryo fibroblasts from serum deprivation–induced apoptotic death.25 In the present study, the mechanism by which acidosis protects endothelial cells from apoptosis was not elucidated. However, because acidification per se increases endothelial cell Ca2+, the protective effect of acidosis was unrelated to inhibition of the Ca2+ overload, whereas enhanced production of VEGF and bFGF may have played a role in the inhibition of apoptotic cell death.26,27

Acidosis-induced increase of VEGF and bFGF mRNA is of interest because an increase in VEGF mRNA has also been described in hypoxic cells and was related to the activation of HIF-1 and enhanced VEGF mRNA stability.19 HIF-1 is a transcription factor that is considered to be a key regulator of the cell hypoxia response pathway because it activates the transcription of a variety of genes that help cells survive in hypoxic conditions. Because hypoxia leads to intracellular acidosis,2 it was hypothesized that acidosis may also modulate HIF-1 activity. However, the results of the experiment depicted in Figure 8 show no activation of HIF-1 in BAECs at pH 7.0. Furthermore, we found no effect of acidosis on VEGF mRNA stability. Thus, the enhanced VEGF and bFGF mRNA expression reported in the present study is compatible with increased gene transcription and may have been due to activation of unidentified pH-sensitive transcription factor(s).

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Figure 8. Effect of acidosis on HIF-1. A, Nuclear extracts from BAECs grown for 48 hours at pH 7.4 were incubated with probe alone, with different double-stranded oligonucleotide competitors, with an anti-ARNT affinity-purified antibody (αARNT), or with a nonspecific antibody (αAHRC). HIF-1 (ACGTG) and mutated HIF-1 (mHIF-1, AAAAG) were oligonucleotide competitors. B, Nuclear extracts from BAECs grown for 48 hours at pH 7.0 (right) or for 24 hours (chemical hypoxia) at pH 7.4 with 100 μmol/L CoCl2 (left). In chemohypoxic conditions, HIF-1 expression was induced in BAECs. Acidosis did not induce HIF-1 expression, because no supershift was detected in the presence of αARNT. Supershifted complex is indicated by the arrow. Experimental conditions were otherwise similar to those used in panel A. This experiment was performed 3 times with similar results.
cells from apoptosis. On restoration of blood flow and return to normal pH, the growth factors stored in the extracellular matrix would be available to trigger an angiogenic response and, by this mechanism, would account for the ability of ischemia to induce neovascularization.

In summary, acidification inhibits those endothelial functions that are required for neovascularization to occur and protects endothelial cells from apoptosis. Additional studies will be necessary to characterize the molecular mechanisms by which pH modulates endothelial cell function and gene expression as well as the pathophysiological relevance of these findings.

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