Extracellular acidosis elevates carbonic anhydrase IX in human glioblastoma cells via transcriptional modulation that does not depend on hypoxia

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Abstract. Most solid tumors display extracellular acidosis, which only partially overlaps with hypoxia and induces distinct adaptive changes leading to aggressive phenotype. Although acidosis is mainly attributable to excessive production of lactic acid, it also involves carbonic anhydrase (CA) IX-mediated conversion of CO_2 to an extracellular proton and a bicarbonate ion transported to cytoplasm. CA IX is predominantly expressed in tumors with poor prognosis and its transcription and activity are induced by hypoxia. Here we investigated whether low extracellular pH in absence of hypoxia can influence CA IX expression in cell lines derived from glioblastoma, a tumor type particularly linked with acidosis. Our data show that extracellular acidosis increased the level of CA IX protein, mRNA and the activity of minimal *CA9* promoter that contains binding sites for HIF-1 and SP-1 transcription factors. Mutation within each of these two biding sites reduced the promoter activity, but did not eliminate the increase by acidosis. Transfection of HIF-1α cDNA produced additive inducing effect with acidosis. Normoxic acidosis was accompanied by HIF-1α protein accumulation and transiently increased phosphorylation of ERK1/2. Expression of a dominant-negative mutant of ERK2 reduced the *CA9* promoter activity in both standard and acidic conditions. Similar result was obtained by inhibitors of MAPK and PI3K pathways, whose combination completely suppressed CA IX expression and abolished induction by acidosis. Altogether, our results suggest that acidosis increases the CA IX expression via a hypoxia-independent mechanism that operates through modulation of the basic *CA9* transcriptional machinery.

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Abbreviations: CA IX, carbonic anhydrase protein; *CA9*, carbonic anhydrase gene; ERK, extracellular signal-regulated kinase; HIF, hypoxia inducible factor; MAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol-3-kinase, VEGF, vascular endothelial growth factor

Key words: tumor microenvironment, acidosis, carbonic anhydrase IX, hypoxia, promoter, transcriptional regulation

Introduction

Extracellular acidosis and hypoxia frequently occur in microenvironment of solid tumors due to poor blood perfusion and/or oncogenic metabolism (1). These physiological stresses induce ample changes in genome, transcriptome and proteome of tumor cells, leading to acquisition of malignant tumor phenotype linked with invasion, metastasis and treatment resistance (2-4). Although there is a strong correlation between mean profiles of pH and partial oxygen pressure (pO_2), they show discordant local relationships (5). Thus, acidosis and hypoxia may either act separately or cooperate to alter behavior of tumor cells.

Acidosis stimulates production of growth factors, angiogenic factors and proteases, impairs immune functions, stimulates invasion and motility, modulates responses to drugs and provides selective growth advantage to tumor cells (2). From the physiological point of view, acidosis may result either from inherently oncogenic metabolism with high rate of glycolysis in the presence of oxygen or from anaerobic metabolism induced by hypoxia, in which oxidative phosphorylation is replaced by glycolysis to satisfy energy demands of tumor cells (3,6). Both situations lead to excessive production of lactate and CO_2, which cannot be efficiently eliminated from extracellular fluid because of defective vasculature and elevated interstitial pressure in solid tumors (7). Acidic microenvironment then causes transient intracellular acidification, which is incompatible with the cell growth and survival. However, tumor cells actively regulate their cytoplasmic pH to maintain neutral or slightly alkaline values favorable for various intracellular processes, via increased levels and/or activities of pumps, ion transporters and exchangers that extrude protons (1).

In addition to anaerobic glycolysis, hypoxia induces multiple responses including stimulation of angiogenesis and erythropoiesis, reduction of cell proliferation and adhesion, and modulation of cell survival to select for more aggressive tumor cells (4). These effects are mediated by a concerted action of numerous proteins including glucose and lactate transporters (GLUT-1, MCT-4), glycolytic enzymes (PGK, LDH), vascular endothelial growth factor (VEGF) and its receptors (VEGFR), erythropoietin (EPO-1) etc. All of these molecules are transcriptional targets of hypoxia inducible factor (HIF), which binds to hypoxia responsive elements (HRE consensus 5'-RCGTG-3') present in the regulatory regions of their genes. HIF is a heterodimer consisting of a constitutive ß subunit and an oxygen-regulated α subunit, whose stability is determined by hydroxylation of two prolyl residues in oxygen-dependent degradation domain and transcriptional activity depends on hydroxylation of asparaginyl residue in C-terminal transactivation domain. In the presence of oxygen, HIF-α is modified by hydroxylases and targeted by pVHL tumor suppressor protein for proteolytic degradation. Under hypoxia, HIF-α remains non-hydroxylated, escapes recognition by pVHL, accumulates, translocates to nucleus and binds to HIF-ß to form active HIF, which induces transcription of target genes (8).

In contrast to hypoxia, no master regulator of responses to low extracellular pH has been identified to date. There are only few published studies investigating transcription factors mediating the effects of acidosis. They suggest some role for AP1 and SP1, whose DNA binding capacity is pH-dependent, and show that low pH may induce nucleolar sequestration of pVHL and accumulation of HIF-α, thus supporting an involvement of HIF transcription factor (9-11).

Although extracellular acidosis in tumors is mainly attributable to increased accumulation of lactic acid, it also involves hydration of pericellular CO_2 by carbonic anhydrase IX (CA IX), a highly active, tumor-associated transmembrane carbonic anhydrase isoform, which works well even in the microenvironment rich in lactic acid (12-14). CA IX possesses an extracellular catalytic domain, whose performance is induced by hypoxia (15). Hydration of CO_2 by CA IX generates an extracellular proton, which contributes to low pH outside of cell, and bicarbonate ion, which is delivered to cytoplasm through the bicarbonate exchangers directly interacting with CA IX and utilized to neutralize intracellular pH [15; Morgan *et al*, FASEB J 19 (Suppl.): abs. 637, 2005].

Expression of CA IX is limited to only few normal tissues mainly including epithelia of gastrointestinal tract (16). On the other hand, CA IX is closely associated with a broad range of tumors that either suffer from hypoxia or contain inactive pVHL (17-19). This expression pattern is principally determined by a strong HIF-1-mediated transcriptional activation of *CA9* gene, which contains HRE element localized on a negative DNA strand immediately upstream of transcription start site at -10/-3 position (20). The HRE element cooperates with an adjacent SP1 binding site (at -32/-38 position) within a minimal *CA9* promoter region in response to both hypoxia and high cell density (21,22).

Despite dramatic induction by hypoxia, intratumoral distribution of CA IX protein only partially overlaps with distribution of low pO_2 measured by microelectrodes and with distribution of other markers of hypoxia, such as pimonidazole, HIF-1α, GLUT-1 and VEGF. This difference can be explained by the position of HRE element near the transcription start, which allows for especially tight transcriptional regulation of the *CA9* gene, as well as by a high post-translational stability of CA IX protein, which reflects both actual and expired hypoxia (23). In addition, it may be caused by microenvironmental factors other than hypoxia, particularly by extracellular acidosis.

In order to distinguish a contribution of low extracellular pH to expression of CA IX, we investigated the effect of normoxic acidosis on the level of CA IX protein and on the *CA9* promoter activity in 8-MG-BA and 42-MG-BA human cell lines derived from glioblastoma multiforme, which is a highly malignant tumor particularly linked with acidosis (24). We demonstrated that cultivation of these cells in acidic medium (pH 6.4) resulted in elevation of CA IX protein, mRNA and *CA9* promoter activity. We also showed that this effect was independent of hypoxia, but could be achieved only when the signaling to basic *CA9* transcriptional machinery through MAPK and/or PI3K pathways was at least partially preserved. Our data suggest that acidosis does not work *per se*, but modulates ongoing transcriptional responses of *CA9* gene to oncogenic and hypoxic stimuli.

Materials and methods

Cell culture. 8-MG-BA and 42-MG-BA human glioblastoma cell lines (kindly provided by Dr Ivana Macikova and Dr Anna Perzelova, School of Medicine, Comenius University, Bratislava, Slovakia) were grown in MEM supplemented with 10% FCS under standard conditions as described (24). The cells were cultivated in sparse monolayer throughout the study to prevent the density-induced expression of CA IX. To simulate acidosis, MEM medium was supplemented with 5% FCS and adjusted to initial pH value of 5.8 with 1 M HCl immediately before the experiment. Then the medium was added to cells, where it spontaneously achieved pH 6.6 within one hour of cultivation and declined to pH 6.4 by the end of 24-h incubation period. No adjustment was done for control conditions, where the initial medium pH 8.2 equilibrated to pH 7.4 during the cell incubation. To mimic hypoxia, the cells were treated with an iron chelator deferoxamine mesylate (DFO, Sigma, St. Louis, MO) at 100 μM final concentration. Effect of acidosis on cell proliferation was determined by cell counting and [^{3}H]-thymidine incorporation. Extent of cell death was assessed by FACS analysis performed with the Coulter Epics Altra flow cytometer using Annexin-V-FITC staining as described elsewhere (25).

Inhibitors, antibodies and plasmids. The MAPKK inhibitor PD98059 (Sigma) and the PI3K inhibitor LY294002 (Calbiochem, Cambridge, MA) were dissolved in dimethyl sulfoxide (DMSO) at 10 mM and stored in aliquots at -20˚C. Prior to use, the inhibitors were diluted in culture medium to working concentrations of 20 μM PD98059 and 5 μM LY294002. Cultures were pre-incubated with the inhibitors 1 h before the acidic treatment and/or addition of DFO.

The primary antibodies were as follows: M75 anti-human CA IX mouse monoclonal antibody was described earlier (26), anti-phospho ERK1/2 mouse monoclonal antibody

(Santa Cruz Biotechnology, Inc.), anti-ERK1/2 polyclonal rabbit serum (Sigma), anti-ß-actin mouse monoclonal antibody (Sigma) and anti-HIF-1α mouse monoclonal antibody (Chemicon Int.). Secondary anti-mouse antibodies conjugated with horseradish peroxidase were purchased from Sevapharma (Prague, Czech Republic) and anti-rabbit antibodies conjugated with peroxidase were obtained from Sigma.

Promoter constructs were generated by an insertion of PCR-amplified -50/+37 and -174/+37 *CA9* genomic fragments upstream of the firefly luciferase gene in pGL3-Basic luciferase reporter vector (Promega). HRE and SP1 binding sites in -174/+37 construct were mutated by PCR-based *in vitro* mutagenesis. The resulting mutations were verified by sequencing. pRL-TK renilla vector (Promega) served for the control of the transfection efficiency. HIF-1α cDNA in pcDNA1/Neo/HIF-1α expression plasmid (27) was kindly provided by Professor Patrick Maxwell (Renal Section, Imperial College, London, UK). Dominant-negative mutants of ERK1 (pcDNA-ERK1) and ERK2 (pcDNA-ERK2) mutated in their ATP binding sites were kindly provided by Dr M.H. Cobb from Southwestern Medical Center, Dallas, TX (28).

Transient transfection and luciferase assay. The cells were plated into 30-mm Petri dishes to reach approximately 60% monolayer density on the next day. Transfection was performed with 1 μg of promoter-containing luciferase construct and 100 ng of pRL-TK plasmid DNA using a GenePorterII reagent (Gene Therapy Systems, San Diego, CA) according to the manufacturer's recommendation. One day later, the transfected cells were trypsinized and plated in triplicates into 24-well plates. The cells were allowed to attach for 20 h. After pre-treatment with inhibitors for 1 h, the medium was removed and replaced with acidic or standard medium containing 5% FCS and appropriate concentration of inhibitor (where relevant). The cells were incubated for additional 24 h and then the reporter gene expression was assessed using the Dual-Luciferase Reporter Assay System (Promega). The luciferase activity was normalized against the renilla expression. To analyze the effect of HIF-1α overexpression and ERK1/2 dominant-negative mutants, the promoter constructs (0.75 μg) were co-transfected with 0.75 μg of corresponding cDNAs in expression vectors as described elsewhere (29).

RT PCR. Total RNA was extracted from the cells using NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). Precipitated RNA was dissolved in DEPC-treated water and reverse transcribed with the M-MuLV reverse transcriptase (Finnzymes OY, Finland) using random hexanucleotides as primers. PCR reactions were performed using the primers specified below. Semiquantitative PCR was carried out with EXT DNA polymerase (Finnzymes). Following an initial denaturation at 94˚C for 3 min, the amplification program was set as follows: denaturation at 94˚C for 30 sec, annealing at 60˚C for 30 sec, and extension at 72˚C during 40 sec for a total of 30 cycles, and finally 7 min at 72˚C. The amplification products were analyzed on a 2% agarose gel. Quantitative PCR was performed with DyNAmo™ HS kit SYBR® Green qPCR kit (Finnzymes) on Techne Quantica (Duxford, Cambridge, UK) at similar amplification conditions. The primers were as follows (S, sense; A, antisense): *CA9* S: 5'-CCGAGCGACGCAGCCTTTGA-3' and *CA9* A: 5'-GGC TCCAGTCTCGGCTACCT-3' (252 bp product), ß-actin S: 5'-CCAACCGCGAGAAGATGACC-3' and ß-actin A: 5'-GATCTTCATGAGGTAGTCAGT-3' (236 bp product).

Immunobloting. The cells were plated at different densities (10000-30000 cells/cm^2) depending on the purpose of the experiment and incubated for 24 h. Then the cultures were pre-treated with inhibitors for 1 h and exposed to acidosis for 24 h. Parallel control dishes were pre-treated and maintained in the standard conditions for the same time period.

For the detection of CA IX, the cells were rinsed and extracted with the cold extraction buffer composed of 0.3% SDS, 20 mM Tris pH 8.8, Complete Mini protease inhibitors (Roche Applied Science) and 20 μl of the solution containing 0.5 M Tris pH 7.0, 50 mM $MgCl_2$, 0.032 mM DNase I and 0.036 mM RNase (MP Biomedicals, Inc.). The extracts were centrifuged (15 min at 13000 rpm) and total protein concentrations were determined by BCA assay (Pierce, Rockford, IL). Samples of 40 μg total proteins were separated by the electrophoresis using 10% SDS-PAGE and blotted onto the PVDF membrane (Hybond-P, Amersham). Before immunodetection, the membrane was treated by the blocking buffer containing 5% non-fat milk in PBS with 0.2% Nonidet P-40 for 1 h and incubated for 1 h with M75 MAb diluted 1:2 in the blocking buffer. Then the membrane was washed, incubated for 1 h with the anti-mouse secondary antibody, washed again and developed with the ECL detection system.

To analyze the accumulation of HIF-1α protein, the cells were scraped into extraction buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF, supplemented with Complete Mini protease inhibitors), sonicated on ice for 30 sec and centrifuged. Samples containing 100 μg of total proteins were electrophoresed on 8% SDS-PAGE, transferred onto PVDF membrane and detected with anti-HIF-1α antibody diluted 1:500 in blocking buffer using the same procedure as described for CA IX.

Activation of ERK1/2 was detected in cell extracts obtained with RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, containing the protease inhibitor cocktail). Samples of 40 μg total proteins were run on 8% SDS-PAGE gel and blotted. Two parallel membranes were treated either with antibodies to phospho-ERK1/2 at 1:500 dilution or with antibodies to total ERK1/2 at 1:40 000. After incubation with the secondary antibodies, the membranes were processed and the signals were visualized as above.

Results

Effect of extracellular acidosis on cell growth and survival. Glioblastoma multiforme is the most malignant type of brain tumor, which is characterized by hypoxia and acidosis. Recent immunohistochemical analyses of human tissue sections revealed that this tumor type readily expresses carbonic anhydrase IX and that high CA IX level is significantly and independently associated with poor prognosis of brain tumor patients (30). Due to these facts, we have chosen two human glioblastoma cell lines as a suitable model for our present investigation. Both 8-MG-BA and 42-MG-BA cell lines possess

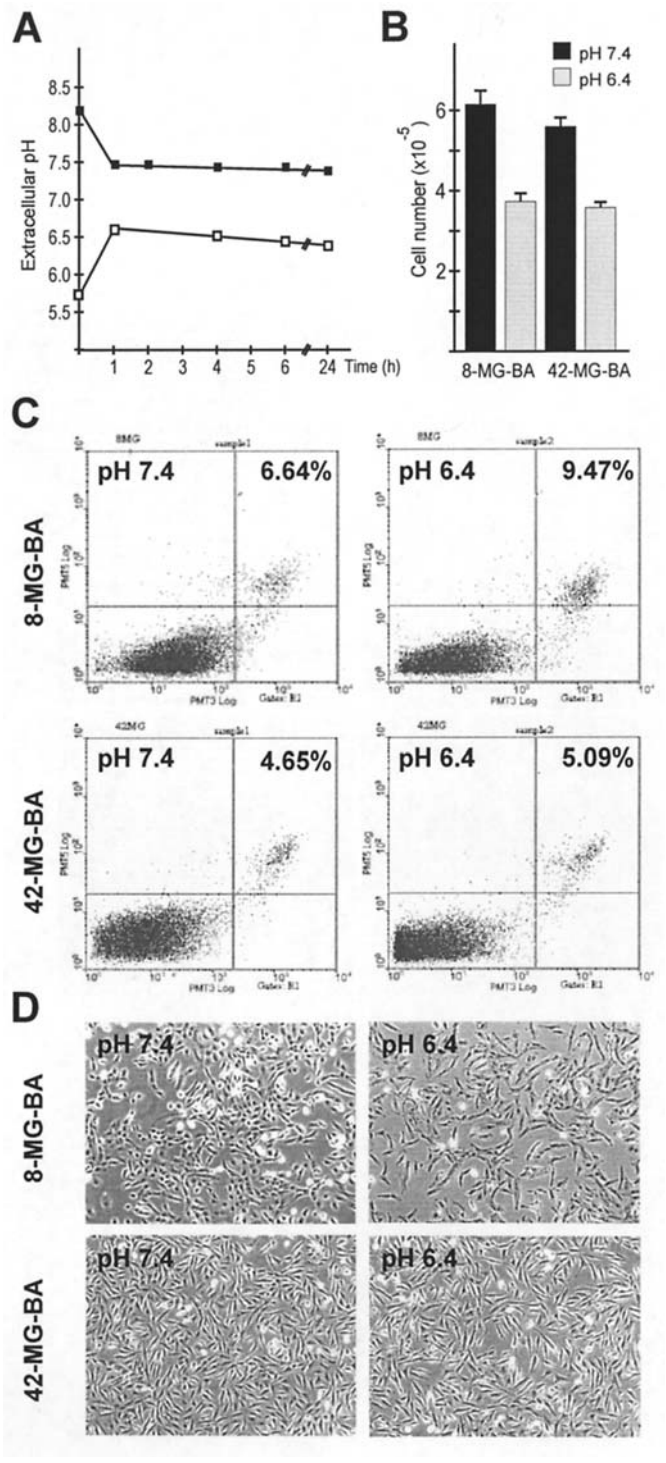

Figure 1. Proliferation and survival of 8-MG-BA and 42-MG-BA glioblastoma cells exposed to extracellular acidosis. (A) Time-dependent spontaneous equilibration of extracellular pH in acidic and standard culture medium throughout the experimental treatment of cells. The medium pH was measured in triplicates at indicated time intervals. (B) Cell numbers in the cultures plated at the same initial density (10 000 cells/cm^2) 24 h before the experiment and then exposed in parallel to acidic and standard medium for additional 24 h. The cells were counted in triplicate samples and data are illustrated on the histogram showing the mean values and standard deviations. (C) Dot plots obtained from FACS analysis of Annexin-V-FITC stained cells after their exposure to extracellular acidosis versus standard conditions. Living cells remain unstained (bottom left quadrant), while both the apoptotic (bottom right quadrant) and necrotic (top right quadrant) cells emit green fluorescence. The number in the top right quadrant represents the percentage of dead cells. (D) Phase contrast microphotographs of cell monolayers plated in parallel at the same density and then incubated for 24 h in acidic and standard media. Original magnification x20.

typical morphological features of glioblastoma multiforme and express corresponding protein markers (24,31).

In the initial experiments, we examined the effect of acidic culture medium adjusted to pH 6.4 on the growth properties and survival of cells in comparison with the cells maintained in medium with pH (pHe) of 7.4 (Fig. 1A). In accordance with the published data obtained with other glioblastoma cell lines (32), proliferation of both 8-MG-BA and 42-MG-BA cells was decreased following their exposure to low extracellular pH for 24 h as determined by cell counting (Fig. 1B). This was confirmed by [^{3}H]-thymidine incorporation, which revealed that the proliferation of acidic cultures was reduced to about 20% of the controls (data not shown). Nevertheless, Annexin-V staining has detected only a negligible increase in the fraction of dead cells (Fig. 1C). As a consequence, the cell cultures subjected to acidosis remained viable, but reached lower density than the control cultures incubated in the standard cultivation medium (Fig. 1D).

Acidosis increases CA IX protein levels in the absence of hypoxia. In spite of the reduced cell proliferation, lowered extracellular pH led to significantly elevated expression of CA IX protein. The CA IX level was higher under more acidic conditions (pH 6.4) than in the moderate acidosis (pH 6.8) and was proportional to initial cell density at plating, with higher expression of CA IX in more dense cells (Fig. 2). Positive relationship to cell density indicated possible involvement of pericellular hypoxia, which is typical for crowded cell cultures and which could augment the effect of acidosis on CA IX expression (21). Therefore, both acidic and control cultures were subjected to continuous stirring in order to ensure thorough cell oxygenation throughout the experiment. Expectedly, stirring led to overall reduction of CA IX levels irrespective of extracellular pH (Fig. 2B). However, the cells maintained in acidic medium (pH 6.4) still revealed considerably higher expression of CA IX protein than the control cells (pH 7.4), suggesting that acidosis induces CA IX independently of pericellular hypoxia and that both factors have additive effects.

CA9 gene responds to acidosis via minimal promoter. As demonstrated on Fig. 3A by RT PCR analysis using mRNAs from glioblastoma cells incubated for 24 h in medium with pH adjusted to 7.4 and 6.4, extracellular acidosis stimulated the transcription of *CA9* gene. Both 8-MG-BA and 42-MG-BA cell lines showed remarkably increased levels of the *CA9* mRNA when maintained in acidic medium. This fact prompted us to look at the activation of the *CA9* promoter. We first compared the effect of extracellular acidosis on activities of two constructs, in which *CA9* promoter regions were cloned upstream of the reporter luciferase gene. The longer construct consisted of the *CA9* genomic region corresponding to -174/+37 nucleotides with respect to transcription initiation site. This promoter fragment contained HRE element just upstream of the transcription start and five regions (PR1-PR5) protected in DNAse I footprinting (33), see the scheme on Fig. 3. PR1 and PR2 regions bind SP1/3 and AP1 transcription factors important for the basic activation of *CA9* transcription (34). HRE plays a crucial role in response of *CA9* to hypoxia and density via HIF-1 transcription complex cooperating with

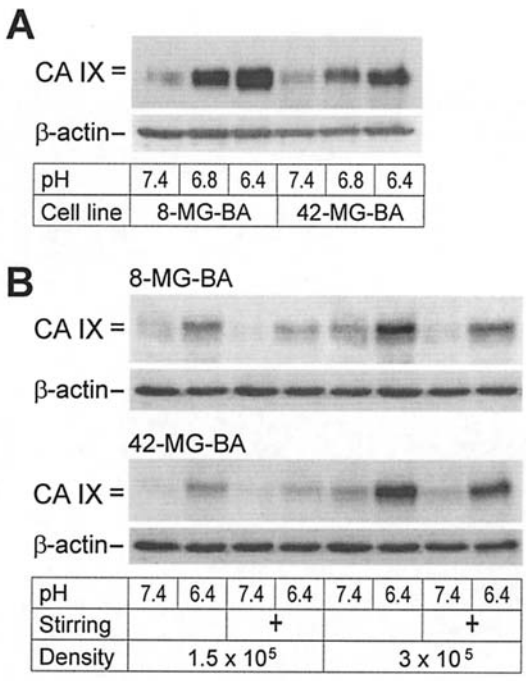

Figure 2. CA IX protein expression in the cells exposed to extracellular acidosis. (A) 8-MG-BA and 42-MG-BA cells were plated in parallel dishes 24 h before the experiment and incubated for additional 24 h in freshly replenished media with different pH values. (B) The cells were seeded at two different initial densities. One day after plating, the cultivation medium was replaced with the acidic and standard medium, respectively, for another 24 h. First parallel of each sample was incubated under static conditions whereas the second parallel was subjected to stirring at 60 rpm/min. In both (A) and (B) experiments, the cells were extracted and CA IX protein expression was analyzed by immunoblotting using M75 MAb. The blots were reprobed with ß-actin-specific antibody for the control of loading.

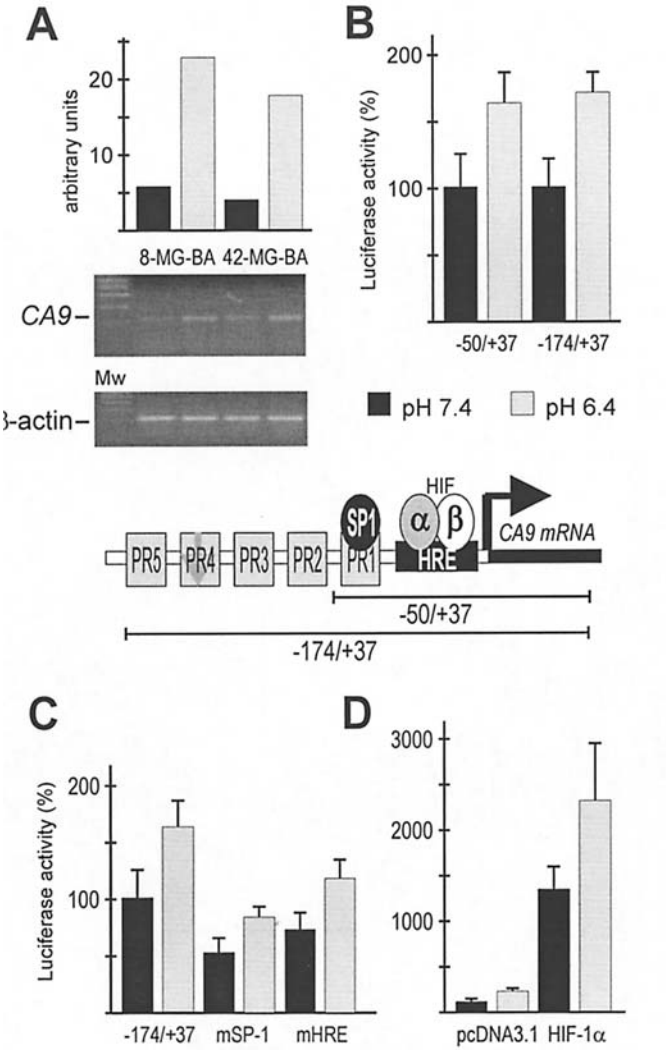

Figure 3. Effect of extracellular acidosis on *CA9* gene transcription and promoter activity. (A) Above histogram illustrates the data obtained with the quantitative RT PCR analysis of mRNAs isolated from 8-MG-BA and 42-MG-BA cells exposed to 24 h of extracellular acidosis versus control cells incubated in the standard medium. The data represent the ratio between the values obtained for *CA9* and *ß-actin*. Below is the representative result of semi-quantitative PCR. Level of *CA9* amplification product was compared to the level of ß-actin. (B) Transcriptional activity of the *CA9* promoter fragments, whose extent and *cis*-element composition is illustrated on the scheme below. 8-MG-BA cells were co-transfected with the promoter-luciferase constructs and renilla plasmid, re-plated to triplicates and subjected to acidosis. Promoter activity was measured 48 h after the transfection and calculated as a ratio between the luciferase and renilla-related values. Results are expressed as the percentage of activity obtained in standard conditions. Bars represent the mean values including standard deviations. (C) Transcriptional activity of the *CA9* promoter mutated in SP-1 binding site and HRE, respectively. Transfection, measurement and calculation of results were performed as in the previous experiment. (D) Effect of HIF-1α overexpression on the *CA9* promoter activity under standard and acidic conditions. 8-MG-BA cells were triple transfected with the promoter-luciferase construct, renilla plasmid and either cDNA encoding HIF-1α or empty pcDNA3.1 plasmid as a negative control. Treatment of cells, assessment of the *CA9* promoter activity and illustration of results were as described above.

SP1 (21). The shorter construct contained minimal *CA9* promoter encompassing nucleotides from -50 to +37 and corresponding to the PR1-HRE module. Each promoter-luciferase construct was co-transfected with the renilla coding plasmid to 8-MG-BA glioblastoma cells, which were then maintained for 24 h in acidic medium. Interestingly, both promoter regions exhibited approximately the same increase of the activity in the cells exposed to extracellular acidosis (Fig. 3B). This finding strongly suggested that the -50/+37 promoter region containing PR1-HRE module is both responsible and sufficient for the observed inducing effect of low extracellular pH.

Transcriptional activation of the *CA9* gene is principally mediated via two cis-regulatory elements, namely the HIF-binding HRE site and the SP1-binding site within PR1. In order to reveal their possible participation in response to acidosis, we analyzed the *CA9* promoter activity using the -174/+37 promoter constructs with mutations introduced separately into each of these two elements. Luciferase activities measured in 8-MG-BA cells transfected with the promoter construct containing the 5'-TCCTCCC-3' to 5'-TCCT**AA**C-3' mutation in SP1 site were significantly lower in both acidic and control conditions, but the acidic induction remained preserved to similar extent as observed with the wild-type promoter (Fig. 3C). The other promoter construct with the 5'-TGC ACGTA-3' to 5'-TGC**TTT**TA-3' mutation in HRE behaved analogously as it exhibited generally lowered activity, but the ratio between the luciferase values normalized against renilla at extracellular pH 6.4 versus 7.4 was approximately equal to the ratio obtained with the wild-type promoter (Fig. 3C). These data indicated that both HRE and SP1 sites primarily determine the basic level of the *CA9* promoter activation, on top of which then acidosis can apply its modifying effect.

The above assumption was indirectly supported by the next experiment. The -174/+37 *CA9* promoter construct was co-transfected to 8-MG-BA cells together with the cDNA coding for HIF-1α subunit of HIF-1 transcription factor and then its activity was analyzed in relationship to extracellular pH. As could be expected, overexpression of HIF-1α led to highly elevated activity of the *CA9* promoter in the standard conditions (medium pH 7.4). This elevation was further improved by extracellular acidosis (pH 6.4) so that the ratio between the acidic and control luciferase values was roughly the same in the absence and presence of HIF-1α cDNA (Fig. 3D).

Acidosis induces accumulation of HIF-1α and transient phosphorylation of ERK1/2. HIF-1 plays a crucial role in activation of *CA9* transcription by hypoxia and high cell density. However, it has been recently demonstrated that acidosis can cause accumulation of an α subunit of HIF and thereby influence the expression of the HIF-regulated genes (11). Because this phenomenon is dependent on the cell type and on the extent of extracellular acidosis, we wanted to know whether it occurs in the glioblastoma cells at an extracellular pH 6.4 and whether HIF-1α accumulation might be involved in acidic induction of CA IX observed in this study. Therefore, we performed immunoblot analysis to visualize the level of HIF-1α in lysates of 8-MG-BA cells maintained in acidic medium in comparison to control cells grown in the standard conditions. We also followed the levels of CA IX protein in the same samples. Moreover, we included a sample of the control cells treated by a hypoxia-mimicking chemical deferoxamine mesylate (DFO). As shown on Fig. 4A, acidosis induced slow but clearly visible accumulation of HIF-1α over the 24-h incubation period. The resulting level of HIF-1α was similar to the level of HIF-1α obtained by DFO treatment for 4 h. The acidosis-induced elevation of HIF-1α was accompanied by considerably increased level of CA IX protein. However, despite the similar levels of HIF-1α in 24-h acidosis and in 4-h hypoxia, the cells exposed to acidosis for 24 h contained higher amount of CA IX protein suggesting contribution of additional factor(s)/signal(s).

One of the possible players is the MAPK pathway that increases the transactivation capacity of HIF-1α without influencing its level (28,35). Indeed, in our experimental conditions acidosis induced transient activation of MAPK pathway via phosphorylation of ERK1/2, which temporally preceded the accumulation of CA IX (Fig. 4B). This result indicated that MAPK pathway could be involved in acidosis-induced signaling leading to augmented expression of CA IX, but its precise role requires further study.

Simultaneous inhibition of MAPK and PI3K pathways completely blocks CA IX expression and eliminates the effect of acidosis. MAPK pathway has been already implicated in molecular response of tumor cells to acidosis elsewhere (9). In order to better understand potential involvement of MAPK in acidosis-induced expression of *CA9* gene, we co-transfected the -174/+37 promoter construct with the cDNAs coding for the dominant-negative (DN) mutants of ERK1 and ERK2. These DN mutants lack the kinase activity and prevent the signal transduction via the MAPK pathway (28). The transfected 8-MG-BA cells overexpressing DN-ERK2 but not DN-ERK1 displayed reduced *CA9* promoter activity when

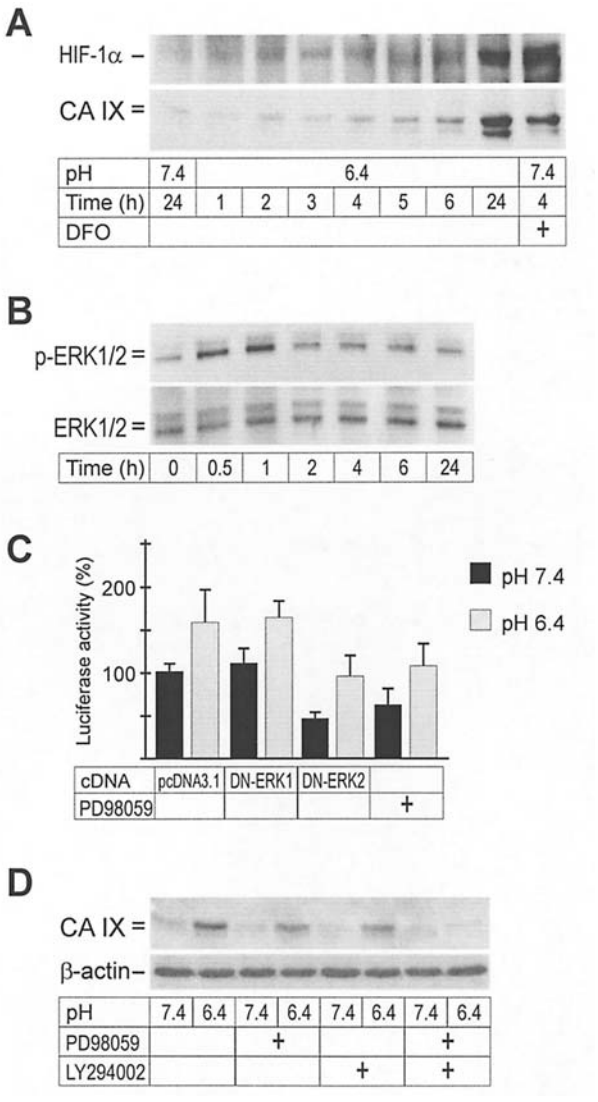

Figure 4. Involvement of signal transduction in the acidosis-induced CA IX expression. (A) Immunoblot analysis of HIF-1α accumulation in 8-MG-BA cells exposed for 24 h to normoxic acidosis. The cells were seeded in multiple parallels, exposed to acidosis and analyzed at indicated time intervals. Expression of HIF-1α and CA IX was detected by immunoblotting on the same membrane (divided to two parts based on the molecular weights of the analyzed proteins) using specific monoclonal antibodies. The cells incubated in the standard medium for 24 h served as a negative control for the effect of acidosis. The cells incubated in the standard medium and treated for 4 h with 100 μM DFO served as a positive control for the effect of hypoxia. (B) Immunoblot analysis of ERK1/2 activation during the exposure to extracellular acidosis. The cells were incubated as described above and parallel samples of extracts were analyzed using phospho-ERK1/2-specific and total-ERK-1/2-specific antibodies. (C) Transcriptional activity of the *CA9* promoter following overexpression of dominant-negative mutants of ERK (DN-ERK) and treatment with MAPKK inhibitor PD98059. Transfections, cell incubations, measurements and calculation of results were as in Fig. 2. Treatment of transfected cells with PD98059 was initiated 1 h before and continued throughout the exposure to acidic and standard medium, respectively. (D) Expression of CA IX following inhibition of MAPK pathway (20 μM PD98059), PI3K pathway (5 μM LY294002), or both together. The cells were plated, pre-treated with inhibitors for 1 h, exposed to acidosis and inhibitors for additional 24 h and analyzed by immunoblotting with M75 MAb. The blot was reprobed with ß-actin-specific antibody for the control of loading.

compared to control cells (Fig. 4C). Nevertheless, this reduction occurred both in the standard and acidic medium so that the remaining activity was still higher at lower extracellular pH. Similar effect was achieved using PD98059, a chemical inhibitor of MEK1, which is the MAPK kinase acting directly upstream of ERK1/2 (Fig. 4C). The decrease in overall expression without elimination of acidic induction was clearly visible following MAPK pathway inhibition by PD98059 also at the level of CA IX protein (Fig. 4D). As we have shown previously, MAPK signaling works concurrently with PI3 kinase to activate CA IX expression under both high density and hypoxia (29). Thus, we also examined the effect of PI3K pathway inhibitor LY294002 with the same result showing overall decrease in expression, but preserved acidic induction (Fig. 4D). However, the induction by acidosis was finally lost upon simultaneous inhibition of MAPK and PI3K pathways leading to complete block of CA IX expression (Fig. 4D). These data indicate that the effect of acidosis requires ongoing signal transduction that activates basal transcription of *CA9*.

Discussion

In this study, we investigated the effect of extracellular acidosis on expression of carbonic anhydrase IX. This was important from several reasons: a) acidosis is a hallmark of most solid tumors with direct impact on their phenotype and response to anticancer treatment; b) acidosis partly overlaps and cooperates with hypoxia in tumor progression; c) it induces adaptive responses of tumor cells to maintain neutral or slightly alkaline intracellular pH via activation of ion transport mechanisms that increase proton extrusion; c) carbonic anhydrase IX contributes to extracellular acidosis under hypoxia; d) CA IX is involved in ion transport machinery as a component of bicarbonate transport metabolon; e) CA IX is expressed in many solid tumors, but not in the corresponding normal tissues and its expression is strongly induced by hypoxia; f) intratumoral distribution of CA IX extends beyond the hypoxic regions detected by other markers of hypoxia. All these facts evoked a question, whether the expression of CA IX is induced by acidosis in absence of hypoxia. Positive answer to this question could nominate CA IX among the candidate mediators of adaptive responses of tumor cells to low extracellular pH and support its role in tumor progression.

Herein we showed that this is true for the glioblastoma multiforme-derived tumor cell lines 8-MG-BA and 42-MG-BA, which exhibited significantly increased CA IX levels with decreasing extracellular pH under normoxic conditions. This increase was clearly observed even though the acidic cultures achieved considerably lower density than the control cells and their basic, density-regulated expression was thus reduced. Similar effect was demonstrated by Willam *et al* (36) in HeLa cervical carcinoma cells and Hep3B hepatoma cells at pH 7.0 in both normocapnic-low bicarbonate and hypercapnic acidosis, whereas Vordermark *et al* (37) failed to achieve CA IX induction in the normoxic HT 1080 fibrosarcoma cells and FaDu pharyngeal carcinoma cells at extracellular pH 6.7. Also Kaluz *et al* (21) could not see any increase in CA IX level in highly dense HeLa cells in the normoxic acidosis at pH 6.6. These inconsistent data obtained with different cell lines in different experimental conditions indicate that extracellular acidosis may have cell-specific consequences. Nevertheless, the available evidence obviously supports the general idea that acidosis can induce the expression of CA IX in tumor cells independently of hypoxia.

The above conclusion is not surprising, since acidosis influences also some other hypoxia-regulated genes, including VEGF (38). However, acidosis and hypoxia work separately to stimulate expression of VEGF and have no additive effect when applied simultaneously (9). In the case of CA IX, both pericellular hypoxia in dense culture and overexpression of HIF-1α from the transfected cDNA resulted in increased expression level, which was further augmented by acidosis. This finding was compatible with the earlier observations that *CA9* mRNA levels induced by low oxygen or chemical hypoxia were further elevated by acidosis in HeLa and Hep3B cells (36). It is well possible that CA IX, which efficiently catalyzes conversion of CO_2 to bicarbonate and proton, and which is believed to deliver bicarbonate to transmembrane transporters in order to buffer cytoplasmic pH, is increasingly needed for the improved protection of tumor cells exposed to combined hypoxic and acidic stress. Irrespective of the functional involvement, the additive inducing effect of hypoxia and acidosis provides further explanation for the intratumoral distribution of CA IX, which incompletely overlaps with distribution of VEGF and other hypoxic markers and which has been so far mainly attributed to high posttranslational stability of CA IX protein (23,39).

In an attempt to better understand a mechanism through which acidosis operates to induce CA IX expression, we analyzed the transcriptional activation of *CA9* promoter. The *CA9* promoter is characterized by HRE element localized between the downstream transcription start site and the upstream SP1 binding site. Due to this particular position, both HIF-1 and SP1 transcription factors are critically encompassed within the basal transcriptional machinery and elimination or mutation of their binding sites significantly decreases the density- and hypoxia-induced transcription of *CA9* gene (20-22). Based on our data, the -50/+37 core promoter region containing intact HRE and SP1 sites appears sufficient to mediate the inducing effect of extracellular acidosis under normoxia. However, it remains unclear how this could be accomplished, since mutation of each of these sites leads to generally reduced *CA9* expression, but does not abolish the effect of acidosis. One possible explanation is offered by Torigoe *et al* (10) who showed that low pH significantly enhances the DNA binding activity of SP1 *in vitro* and proposed that this could be a part of an adaptive response to transient decrease of intracellular pH due to hypoxia and acidosis in tumor microenvironment. Although the experimental evidence remains to be obtained, it seems quite plausible that the basal *CA9* transcription might be induced by acidosis via tighter SP1 binding to *CA9* promoter. Alternatively or concurrently, acidosis-induced stabilization of HIF-1α subunit of HIF transcription factor could be involved as recently described by Mekhail *et al* (11). They demonstrated that normoxic acidosis can neutralize the function of pVHL by triggering its nucleolar sequestration, which enables HIFα to evade destruction in the presence of oxygen and activate its target genes. In accordance with this idea, we did find

accumulation of HIF-1α protein in 8-MG-BA glioblastoma cells exposed to acidosis and this increase was accompanied with an increase of the CA IX protein level.

In contrast to *CA9* gene, neither SP1 nor HIF, but rather AP1 transcription factor has been implicated in acidosis-stimulated expression of *VEGF* gene (9). The mechanism involved an improved binding of AP1 to *cis*-element located rather distally from the VEGF transcription start site at -937/-931 position downstream of the HRE element. However, any important role of AP1 transcription factor in acidosis-mediated effect on *CA9* promoter in glioblastoma cells can be declined, since AP1 binding site is located out of the -50/+37 region, which showed approximately equal induction as the larger -174/+37 region that includes AP1 site.

Despite the differences in the composition of *VEGF* and *CA9* promoters, particularly with respect to positions of the binding sites for HIF, SP1 and AP1 transcription factors and their utilization in transcriptional responses to microenvironmental stresses, regulation of both genes was shown to involve signaling from the MAP kinase pathway. Both Xu *et al* (9) and we showed transient ERK activation in glioblastoma cells exposed to extracellular acidosis and found reduced *VEGF*/*CA9* promoter activities following MAPK pathway inhibition. Based on these findings, Xu *et al* (9) concluded that acidic extracellular pH induces *VEGF* transcription via ERK1/2 MAPK signaling pathway. This conclusion seemed tempting also for *CA9*. However, when we analyzed the effect of MAPK pathway inhibition either by the chemical compound PD98059 or by expression of the dominant-negative mutant of ERK2, we observed reduced *CA9* expression also in the absence of acidosis, in the cells cultivated under standard conditions. This negative control, which was missing in the VEGF-study (9), revealed that the inducing effect of low extracellular pH was preserved also upon inhibition of MAPK signaling. The same deduction could be raised from the experiment, in which we inhibited PI3 kinase pathway by LY294002 inhibitor. These results are fully consistent with the previous investigations showing that MAPK and PI3 kinase pathways concomitantly mediate the signaling to HIF and SP1 and via these transcription factors influence the expression level of the *CA9* gene under both basal and hypoxia/density-induced conditions (21,29). Similarly as shown before in HeLa cells, simultaneous inhibition of both pathways in glioblastoma cells resulted in complete block of basal *CA9* expression under standard conditions. This block could not be overcome by acidosis, which failed to show an inducing effect. Altogether, the data presented here reinforce the proposal that the response of *CA9* gene to acidosis is accomplished through an adverse mechanism, which does not operate on its own. It rather appears to modulate the transcriptional machinery assembled at the minimal *CA9* promoter that involves HRE and SP1 sites and is activated by MAPK and/or PI3K signal transduction. Nonetheless, precise acidosis-induced modifications that occur at the *CA9* promoter require further detailed study.

It also remains to be determined, how acidosis is sensed to modulate the activity of MAPK and/or PI3K pathways which ultimately lead to increased *CA9* expression. Based on recent knowledge, it is quite plausible that ion transporters and other components of metabolons, which are functionally involved in adaptive responses of tumor cells to extracellular acidosis, can sense a transiently increased concentration of intracellular protons and transmit this signal via their cytoplasmic domains to signal transduction pathways, which then trigger their own expression and/or activation. This would represent a meaningful feedback mechanism facilitating the expression/activation of ion transport machinery in stressful conditions. Indeed, activity of several ion transporters is regulated by signal transduction-induced phosphorylation of their cytoplasmic domains in response to changes in pH and *vice versa*, activated ion transporters transmit this phosphorylation signal to the signal transduction pathways, including ERK (40). Interestingly, analogous signal transduction feedback has been recently described for CA IX whose intracellular tail can be phosphorylated following EGF stimulation and this phosphorylation leads to activation of PI3K-Akt pathway in renal carcinoma cells (41). Future studies will show whether this phenomenon is involved in CA IX regulation by microenvironmental stresses.

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