Hypoxia activates the capacity of tumor-associated carbonic anhydrase IX to acidify extracellular pH

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Abstract Acidic extracellular pH (pHe) is a typical attribute of a tumor microenviroment, which has an impact on cancer development and treatment outcome. It was believed to result from an accumulation of lactic acid excessively produced by glycolysis. However, metabolic profiles of glycolysis-impaired tumors have revealed that CO_2 is a significant source of acidity, thereby indicating a contribution of carbonic anhydrase (CA). The tumor-associated CA IX isoform is the best candidate, because its extracellular enzyme domain is highly active, expression is induced by hypoxia and correlates with poor prognosis. This study provides the first evidence for the role of CA IX in the control of pHe. We show that CA IX can acidify the pH of the culture medium in hypoxia but not in normoxia. This acidification can be perturbed by deletion of the enzyme active site and inhibited by CA IX-selective sulfonamides, which bind only to hypoxic cells containing CA IX. Our findings suggest that hypoxia regulates both expression and activity of CA IX in order to enhance the extracellular acidification, which may have important implications for tumor progression.

Keywords: Tumor microenvironment; Acidosis; Carbonic anhydrase IX; Hypoxia; Sulfonamide

1. Introduction

Acidic extracellular pH (pHe) has been associated with tumor progression via multiple effects including upregulation of angiogenic factors and proteases, increased invasion, and impaired immune functions [1–5]. In addition, it can influence the uptake of anticancer drugs and modulate the response of tumor cells to conventional therapy [1,6]. Acidification of the tumor microenvironment was generally attributed to the accumulation of lactic acid excessively produced by glycolysis and poorly removed by inadequate tumor vasculature. A high rate of glycolysis is especially important for the hypoxic cells that largely depend on anaerobic metabolism for the energy generation. However, experiments with glycolysis-deficient cells indicate that production of lactic acid is not the only mechanism leading to tumor acidity. The deficient cells produce only diminished amounts of lactic acid, but form acidic tumors in vivo [7,8]. A comparison of the metabolic profiles of the glycolysis-impaired and parental cells revealed that CO_2, in addition to lactic acid, is a significant source of acidity in tumors [9]. These data strongly imply possible contribution of carbonic anhydrase (CA), a zinc metalloenzyme catalyzing the reversible conversion of CO_2 to bicarbonate and a proton. Mammalian CA exists in at least 15 isoforms differing in molecular properties, subcellular localization, tissue distribution, and kinetic properties. The active CA isoenzymes are fundamental for various physiological processes that involve ion exchange and pH balance. They can be efficiently inhibited by sulfonamides, which are clinically exploited for the treatment of disorders associated with abnormal CA activity [10].

The CA IX isoform appears to be the best candidate for a role in acidification of the tumor microenvironment. First, CA IX is an integral plasma membrane protein with an extracellularly exposed enzyme active site [11,12]. Second, CA IX has a very high catalytic activity with the highest proton transfer rate among the known CAs [13]. Third, CA IX is present in few normal tissues, but its ectopic expression is strongly associated with many frequently occurring tumors [14]. Finally, CA IX level dramatically increases in response to hypoxia via a direct transcriptional activation of *CA9* gene by hypoxia inducible factor-1 (HIF-1) [15] and its expression in tumors is a sign of poor prognosis [16]. Taken together, CA IX has all necessary predispositions needed to act in the control of tumor pH.

To obtain support for this concept, we focused on the capacity of CA IX to acidify extracellular pH (pHe) in culture. Using MDCK epithelial cells that constitutively express a human CA IX protein, we proved that CA IX is able to decrease the pHe of the culture medium in response to low oxygen, suggesting that hypoxia stimulates its enzymatic activity.

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Abbreviations: AE, anion exchanger; CA, carbonic anhydrase; ΔCA, deletion mutant of CA IX lacking the catalytic domain; CAI, carbonic anhydrase inhibitor; ΔPG, deletion mutant of CA IX lacking the proteoglycan-like domain; HIF, hypoxia inducible factor; MAb, monoclonal antibody; pHe, extracellular pH

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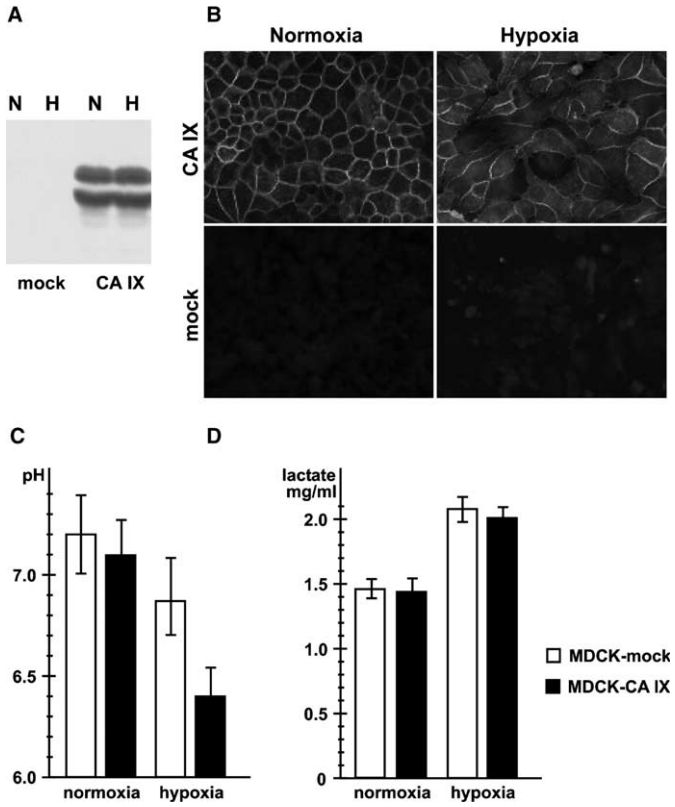

Fig. 1. CA IX-mediated acidification of the extracellular pH in hypoxia. (A) CA IX-transfected MDCK cells and mock-transfected controls were either maintained in normoxia (N, 21% O_2) or exposed to hypoxia (H, 2% O_2) for 48 h and analyzed by immunoblotting using M75 MAb. (B) Immunofluorescence analysis of the transfected MDCK cells. Values of pHe (C) and lactate concentrations (D) in the cells grown in constant medium volumes were obtained in five independent experiments with three different clones of the transfectants and three parallel dishes for each clone. Results are illustrated on the histogram showing the mean values and standard deviations.

Expression of a deletion variant lacking the catalytic domain perturbed the extracellular acidification exerted by endogenous CA IX protein produced in hypoxic HeLa cells. In both models, CA IX-selective sulfonamides reduced the medium acidification and bound only to hypoxic cells containing the wild-type CA IX. Our results suggest that CA IX can contribute to acidification of the hypoxic extracellular milieu, which may have important implications for the development of cancer.

2. Materials and methods

2.1. Cell culture

MDCK, SiHa, HeLa cells and their transfected derivatives were grown in DMEM with 10% FCS and buffered with 22.3 mM bicarbonate [17]. To maintain the standard conditions, the cells were always plated in 3 ml of culture medium at a density of $0.8–1 \times 10^6$ per 6 cm dish 24 h before the transfer to hypoxia (2% O_2 and 5% CO_2 balanced with N_2) generated in a Napco 7000 incubator. Parallel normoxic dishes were incubated in air with 5% CO_2. At the end of each experiment, pH of the culture medium was immediately measured, the medium was harvested for the determination of the lactic acid content with the standard assay kit (Sigma), the cells were counted to ensure that the resulting cultures are comparable and then processed either for immunofluorescence or extracted for immunoprecipitation and/or immunoblotting.

2.2. Sulfonamide synthesis and treatment of cells

CAI#1 sulfonamide [4-(2-aminoethyl)-benzenesulfonamide] was obtained from Sigma–Aldrich. The membrane-impermeable CAI#2 [4-(2,4,6-trimethylpyridinium-*N*-methylcarboxamido)-benzenesulfonamide perchlorate] was prepared by reaction of homosulfanilamide with 2,4,6-trimethyl pyrilium perchlorate [18]. The fluorescent CAI#3 sulfonamide was obtained from homosulfanilamide and fluorescein isothiocyanate [19]. CAIs showed the following K_I values assessed by CO_2 hydration methods using the purified CA domain of CA IX: CAI#1 36 nM, CAI#2 38 nM and CAI#3 24 nM. The sulfonamides were dissolved in PBS with 20% DMSO at 100 mM concentration and diluted in a culture medium to a required final concentration just before their addition to cells. The cells were incubated for 48 h in hypoxia and normoxia, respectively, pH of the culture medium was measured and the binding of the FITC-labeled CAI#3 to living cells, washed three times with PBS, was viewed by a Nikon E400 epifluorescence microscope.

2.3. Cloning of CA IX mutants and transfection

Cloning of CA IX deletion mutants lacking either the N-terminal PG domain or the central CA domain was performed as described [20]. MDCK and HeLa cell lines constitutively expressing CA IX protein or its mutants were obtained by cotransfection of recombinant plasmids pSG5C-CA IX, pSG5C-ΔCA and pSG5C-ΔPG with pSV2neo plasmid in a 10:1 ratio using a GenePorter II transfection kit from Gene Therapy Systems. The transfected cells were subjected to selection in 500–1000 μg/ml G418, cloned, tested for CA IX and expanded. At least three clonal cell lines expressing each CA IX form were analyzed to eliminate the effect of clonal variation. The cells cotransfected with empty pSG5C and pSV2 neo were used as negative controls.

2.4. Indirect immunofluorescence and immunoblotting

Cells grown on glass coverslips were fixed in ice-cold methanol at −20 °C for 5 min and stained with CA IX-specific monoclonal antibody (MAb) M75 directed to the PG domain or V/10 directed to the CA domain followed by incubation with FITC-labeled secondary antibodies [20,21]. For immunoblotting, cells were rinsed with PBS and extracted in RIPA buffer for 30 min on ice. Protein concentrations were quantified using the BCA kit (Pierce). The proteins (50 μg/lane)

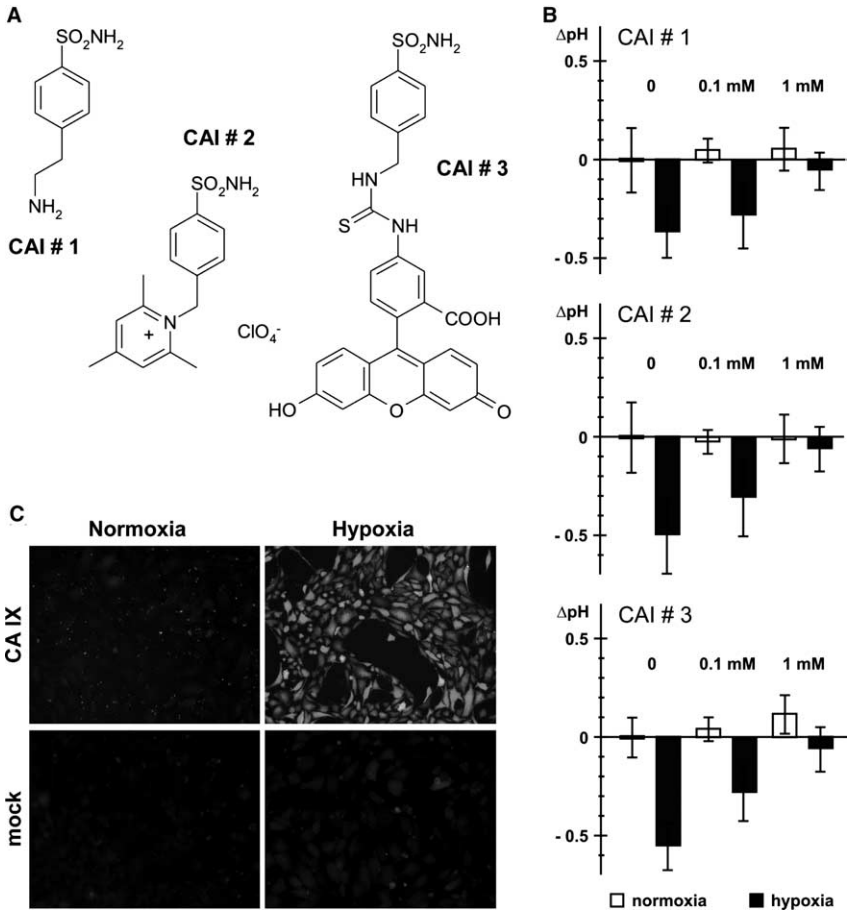

Fig. 2. Sulfonamide inhibition and binding to hypoxic MDCK-CA IX cells. (A) Chemical structures of the CA IX-selective inhibitors used in this study: CAI#1 [4-(2-aminoethyl)-benzenesulfonamide], CAI# 2 [4-(2,4,6-trimethylpyridinium-*N*-methylcarboxamido)-benzenesulfonamide perchlorate], CAI#3 [fluorescein-thioureido-homosulfanilamide]. (B) The sulfonamides were added to MDCK-CA IX cells just before their transfer to hypoxia and pHe was measured 48 h later. At least three independent experiments with three parallel dishes per sample were performed for each inhibitor. Data are expressed as differences between the pH values (ΔpH) measured in the untreated versus treated cells and include the standard deviations. (C) Fluorescence analysis of the transfected MDCK cells plated on the glass coverslips. The cells were treated with the FITC-labeled CAI#3 throughout the 48 h incubation in normoxia and hypoxia, respectively.

were resolved in 10% SDS–PAGE under reducing and non-reducing conditions, respectively, transferred to PVDF membrane and CA IX was detected with the specific MAbs as described [21].

2.5. Cell biotinylation and immunoprecipitation

Cells were washed with ice-cold buffer A (20 mM sodium hydrogen carbonate, 0.15 M NaCl, pH 8.0), incubated for 60 min at 4 °C with buffer A containing 1 mg of NHS-LC-Biotin (Pierce), then washed 5 times with buffer A and extracted in RIPA as described above. MAb V/10 in 1 ml of hybridoma medium was bound to 25 μl of 50% suspension of Protein-A Sepharose (Pharmacia) for 2 h at RT. Biotinylated extract (200 μl) was pre-cleared with 20 μl of 50% suspension of Protein-A Sepharose and then added to the bound MAb. Immunocomplexes collected on the Protein-A Sepharose were separated by SDS–PAGE, transferred to a PVDF membrane and revealed with peroxidase-conjugated streptavidin (1/1000, Pierce) followed by enhanced chemoluminescence.

3. Results and discussion

3.1. Ectopic expression of CA IX leads to increased acidification of pHe in hypoxia

Expression of CA IX in tumor cells is induced by hypoxia simultaneously with various components of anaerobic metabolism and acid extrusion pathways. This could complicate the determination of the contribution of CA IX to the overall change in pHe. Therefore, we used MDCK immortalized canine kidney epithelial cells that do not express endogenous CA IX, but were stably transfected to express the human CA IX protein in a constitutive manner. Levels of CA IX in MDCK-CA IX transfectants were comparable between the hypoxic cells maintained for 48 h in 2% O_2 and the normoxic cells incubated in 21% O_2 (Fig. 1A). CA IX was predominantly localized at the cell surface (Fig. 1B), although the membrane staining in hypoxic cells was less pronounced due to a hypoxia-induced perturbation of intercellular contacts [17]. Hypoxic incubation led to expected extracellular acidification in the CA IX-positive as well as CA IX negative cell cultures when compared to their normoxic counterparts (Fig. 1C). However, upon mutual comparison of the hypoxic cells it was evident that pHe was significantly decreased in the cells containing CA IX. Taking into account a steady, hypoxia-independent level of CA IX in MDCK-CA IX cells, this finding indicated that hypoxia activated the catalytic performance of CA IX, which resulted in enhanced pHe acidification.

To exclude the possibility that hypoxia-induced acidification was caused by an increased production of lactic acid, we determined corresponding lactate concentrations in the media

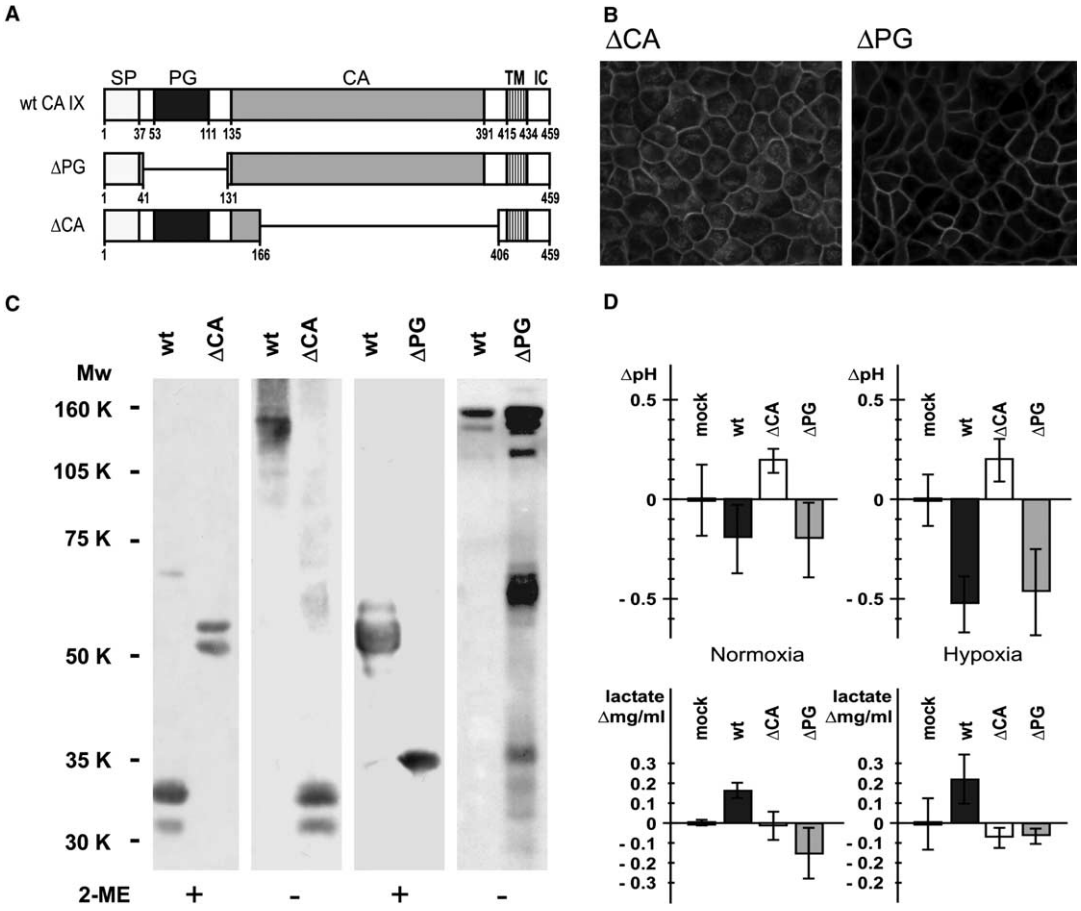

Fig. 3. Expression and acidification capability of the CA IX deletion mutants. (A) Schematic drawing of the domain composition of the wild-type (wt) CA IX with the amino acid positions indicating the extent of the deletions in the N-terminal PG domain (ΔPG) and the central CA domain (ΔCA): SP, signal peptide; PG, proteoglycan-like region; CA, carbonic anhydrase domain; TM, transmembrane anchor; IC, intracytoplasmic tail. (B) Immunofluorescence analysis with M75 and V/10 MAbs specific for different extracellular parts of CA IX. (C) Immunoblotting of ΔCA and ΔPG proteins. The proteins were analyzed under both reducing (2ME+) and non-reducing (2ME−) conditions for their Mw and their capacity to form oligomers. (D) Extracellular pH and lactate in the transfected MDCK cells. At least three independent experiments were performed using three clonal cell lines for each transfected variant with at least three parallel dishes. Data are expressed as mean differences in the pH values (ΔpH) and in the lactate concentrations (Δmg/ml), respectively.

from both CA IX-negative and CA IX-positive transfectants (Fig. 1D). In accord with the literature, production of lactic acid was significantly higher in the cells maintained in hypoxia than in the normoxic cells. However, there were practically no differences between the lactate production in cultures of CA IX-positive and CA IX-negative cells, suggesting that the excessive pHe decrease observed in hypoxia could be explained by the activation of CA IX.

3.2. *Sulfonamides inhibit CA IX-mediated acidification of pHe and bind to hypoxic MDCK-CA IX cells*

Sulfonamides efficiently inhibit CAs by a well-understood mechanism [10]. Despite a principally non-specific action against different CA isoenzymes, sulfonamide structure and properties can be modified to introduce certain preferences for the particular isoforms. We tested three CA IX-selective inhibitors (CAI, Fig. 2A). CAI#1 is a strong inhibitor of CA IX, whereas it is less efficient against the widely distributed cytoplasmic CA II and the plasma membrane-anchored CA IV [22], CAI#2 is practically membrane-impermeable [18] and CAI#3 has a big moiety favoring its interaction with the CA IX active site, which is assumed to form a larger cavity than in CA II [23]. All three sulfonamides were able to reduce the extracellular acidification of MDCK-CA IX cells in hypoxia and their effect on the normoxic pHe was negligible (Fig. 2B). Moreover, FITC-labeled CAI#3 was detected only in hypoxic MDCK-CA IX cells, but was absent from their normoxic counterparts and from the mock-transfected controls (Fig. 2C). Cytoplasmic accumulation of CAI#3 was possibly related to the hypoxia-induced internalization of CA IX described earlier [17]. Lack of the fluorescence signal in the CA IX-negative MDCK cells confirmed the selectivity of the inhibitor, which did not bind to other potentially present CA isoforms and indicated that only the hypoxic MDCK-CA IX cells contain the catalytically active CA IX with the enzyme center accessible to an inhibitor. The latter claim is supported by the generally accepted fact that the reaction between CA and an inhibitor occurs principally via a coordination of the ionized inhibitor to the zinc ion through the network of hydrogen bonds with amino acid residues of the active site, which effectively means that the inhibitor can efficiently bind only to active CA isoforms [10]. It cannot be excluded that hypoxia influences the conformation and hence the accessibility of the active site of CA IX, but this assumption warrants further studies.

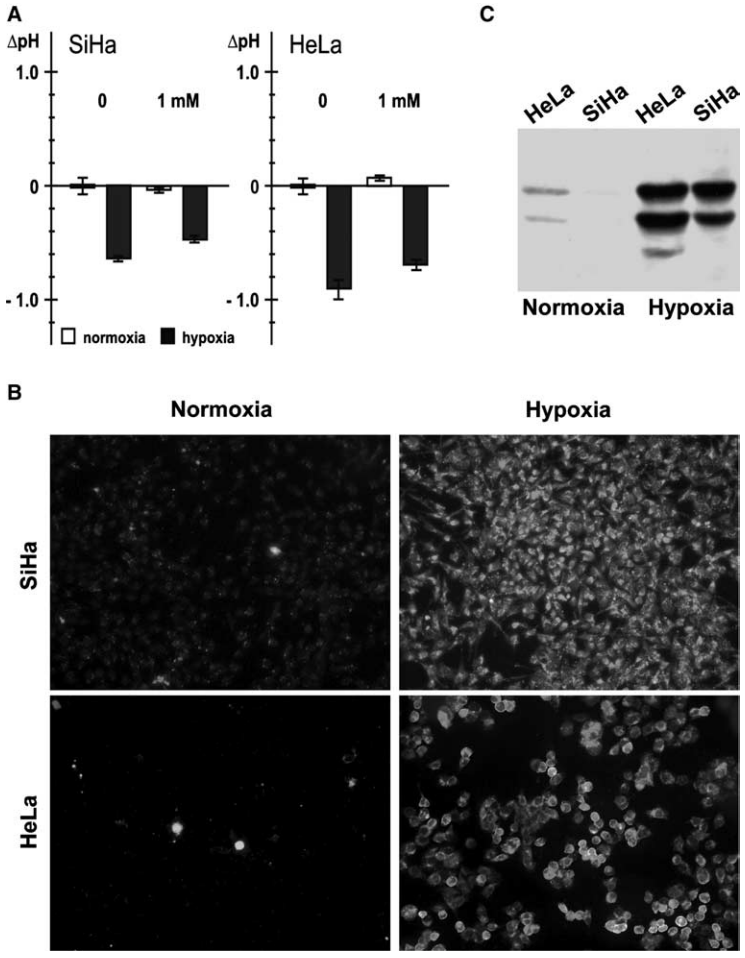

Fig. 4. Treatment of the tumor cells by CAI#3 sulfonamide. (A) HeLa and SiHa cervical carcinoma cells were incubated for 48 h in normoxia and hypoxia, respectively, either in the absence or in the presence of 1 mM CAI#3 sulfonamide. Mean differences in the pH values determined in the treated versus control dishes are shown on the histogram with indicated standard deviations. The experiment was repeated three times using at least three parallel dishes for each sample. (B) HeLa and SiHa cells plated on the coverslips were treated with FITC-labeled CAI#3 sulfonamide during 48 h incubation in normoxia and hypoxia, washed with PBS and inspected under the fluorescence microscope. (C) Immunoblotting analysis of CA IX expression in the normoxic and hypoxic carcinoma cells with M75 MAb.

3.3. Intact CA IX catalytic domain is required for the extracellular acidification in hypoxia

In addition to the enzyme domain (CA), the extracellular part of CA IX contains an N-terminal proteoglycan-related region (PG) that is absent from the other CAs and seems implicated in cell adhesion [24]. To examine involvement of these domains in the pHe control, we produced deletion variants of CA IX, in which we removed either the PG region (ΔPG) or a large portion of the CA domain (ΔCA), Fig. 3A. Immunofluorescence analysis using two MAbs, namely PG-specific M75 for ΔCA and CA-specific V/10 for ΔPG, has shown that both deleted proteins were transported to the plasma membrane (Fig. 3B). The mutants were expressed at levels comparable with the wild-type CA IX (Fig. 3C). Interestingly, ΔCA was unable to form oligomers possibly due to the absence of two out of four cysteines (C174 and C336) required for the proper S-S bonding. As judged from the molecular weights, ΔPG mutant appeared to assemble into dimeric and tetrameric complexes, rather than into trimers. Elimination of a large part of the CA domain perturbed the acidification capacity of CA IX, whereas removal of the PG region had no such effect (Fig. 3D). This differential behavior could be reasonably attributed to the absence versus presence of the catalytic activity of CA IX, because the cells expressing these variants produced similar levels of lactic acid (Fig. 3D). It also indicates that the CA domain is both necessary and sufficient for the enzyme activity and that PG and CA portions of CA IX molecule can be functionally separated, although they may still cooperate in response to diverse physiological factors. Based on the knowledge that the extracellular acidosis interferes with the cell adhesion, the enzyme activity carried out by CA domain might influence the adhesion-related properties of PG region and *vice versa*. Indeed, CA IX was shown to destabilize E cadherin-mediated intercellular adhesion in transfected MDCK cells, which was particularly dramatic in the hypoxic monolayer [17] in conditions accompanied by CA IX-mediated extracellular acidosis described here.

3.4. FITC-CAI #3 sulfonamide binds to and increases pHe of the hypoxic tumor cells

To see whether the phenomenon of CA IX-mediated acidification is applicable to tumor cells with endogenous

CA IX, we examined the effect of CAI#3 sulfonamide on the pHe of the cervical carcinoma cells HeLa and SiHa, respectively. Under hypoxia, tumor cells coordinately express elevated levels of multiple HIF-1 targets, including CA IX [25]. In addition, activity of many components of the hypoxic pathway and related pH control mechanisms, such as ion transport across the plasma membrane, are abnormally increased in order to maintain the neutral intracellular pH [1]. This explains the considerably decreased pHe of the hypoxic versus normoxic HeLa and SiHa cells (Fig. 4A). The acidosis was partially reduced by CAI#3 inhibitor, in support of the idea that activation of CA IX is just one of the many consequences of hypoxia. Moreover, FITC-CAI#3 accumulated in the hypoxic HeLa and SiHa cells that contained elevated levels of CA IX, but not in the normoxic cells with a diminished CA IX expression (Fig. 4B and C). As indicated by the ability to bind CAI#3 and mediate its accumulation in hypoxia, CA IX expressed in the hypoxic tumor cells was catalytically active. Exclusive binding of the FITC-conjugated CAI#3 sulfonamide to the hypoxic cells that express activated CA IX offers an attractive possibility for the use of similar sulfonamide-based compounds for the imaging purposes in vivo. Moreover, CA IX-selective sulfonamide derivatives may potentially serve as components of the therapeutic strategies designed to increase pHe in the tumor microenvironment and thereby reduce the tumor aggressiveness and the drug uptake [1,6,26,27].

3.5. *Expression of ΔCA mutant in HeLa cells reduces their pHe acidification in hypoxia*

Based on the assumption that the enzyme-dead ΔCA mutant could abolish the function of the endogenous CA IX protein, we generated HeLa-ΔCA transfectants. These cells contained ΔCA but not CA IX under normoxia, expressed both proteins under hypoxia, and in the non-reducing conditions exhibited an atypical band presumably corresponding to mixed oligomers composed of both CA IX and ΔCA (Fig. 5A–C). No significant differences in pHe were observed between the normoxic HeLa-mock and HeLa-ΔCA cells. On the other hand, HeLa-ΔCA transfectants treated by hypoxia produced less acidic medium than the control HeLa-mock cells

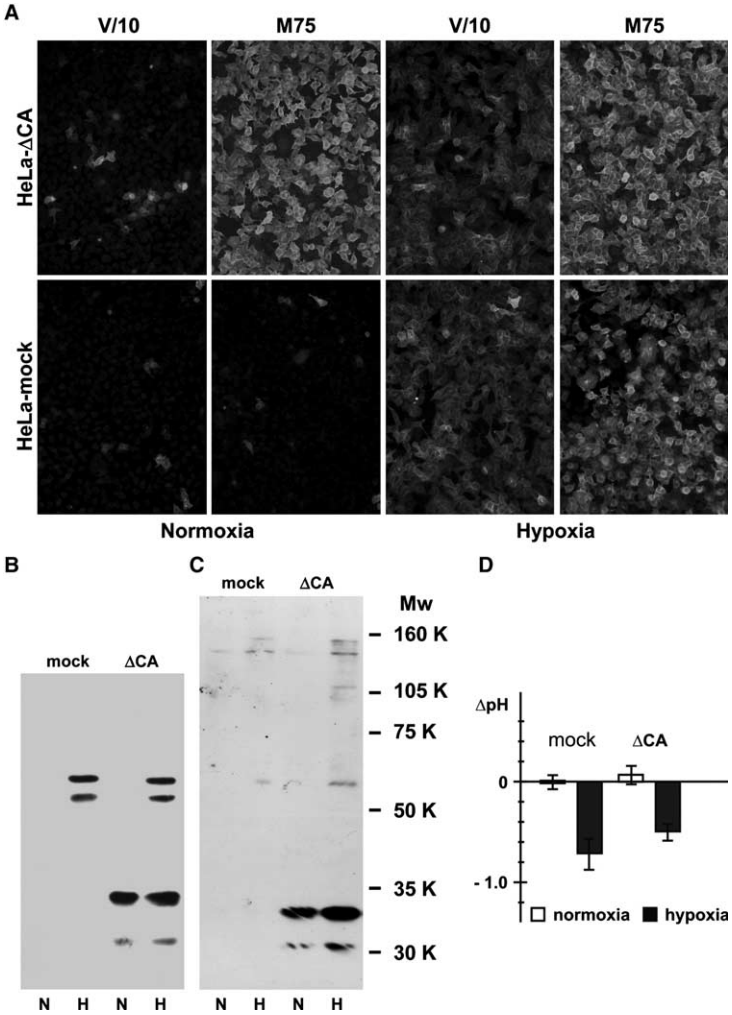

Fig. 5. Ectopic expression of ΔCA mutant in HeLa cells. (A) Immunoflourescence analysis of the mock-transfected and ΔCA-transfected HeLa cells was performed either with V/10 MAb (to detect endogenous CA IX protein) or with M75 MAb (to visualize both CA IX and ΔCA mutants). (B) Immunoblotting analysis of HeLa-ΔCA cells as well as the mock-transfected controls using M75 MAb. The cells were maintained in normoxia (N) and hypoxia (H), and analyzed under reducing (C) and non-reducing conditions (D). Values of pHe in the culture media of HeLa cells transfected with ΔCA in comparison to the mock-transfected controls. Data represent mean differences in the pH values and corresponding standard deviations. The experiment was repeated three times with three different clones of the transfected HeLa, each having at least three parallel dishes.

(Fig. 5D), suggesting that the inactive ΔCA deletion variant interfered with the activity of the wild-type protein, and further supporting the role of CA IX. Altogether, these data strongly imply that the acidification of the extracellular pH in the hypoxic tumor cells does involve CA activity and that CA IX directly participates in this phenomenon.

3.6. How might CA IX contribute to extracellular acidosis?

In the context of the above results that place CA IX among the direct contributors to the hypoxic microenvironment, it is tempting to propose possible means of its action. Data obtained from studies with the physiologically relevant CA isoforms II and IV indicate that these CAs physically interact with anion exchangers (AE) to form a metabolon that facilitates bicarbonate transport in differentiated cells [28,29]. It seems plausible that CA IX could work as an extracellular component of the similar metabolon in tumor cells. Assembly and/or activation of such a metabolon would be especially meaningful in low oxygen conditions, because a highly efficient transport of bicarbonate is required particularly in the hypoxic cells for the buffering of intracellular pH and biosynthetic reactions. According to this model, enhanced conversion of CO_2 to bicarbonate by the hypoxia-activated CA IX would be coupled with the increased production of extracellular protons contributing to acidosis. Data obtained in this work fit well with this proposal. Further supportive hints come from the studies of von Hippel-Lindau tumor suppressor protein (pVHL), the main negative regulator of HIF-1, which can downregulate CA IX (obviously as a direct HIF-1 target) and can also reduce the transport activity of AEs [30,31].

Downstream effects of CA IX can be at least partially anticipated on the basis of the known connections between the acidic pHe and certain features of the tumor phenotype [1–6]. Moreover, as a part of the hypoxic acidification machinery, CA IX might facilitate a nucleolar sequestration of pVHL and activation of HIF, which is a recently described pH-dependent mechanism proposed to serve a protective role in reoxygenated cells [32]. In such case, HIF-mediated increase in the level and activity of CA IX resulting in enhanced acidification might create a feedback loop leading to prolonged HIF activation, which is certainly an attractive possibility requiring experimental proof.

In conclusion, this study provides the first direct evidence for the role of CA IX in acidification of extracellular pH. Our findings significantly improve the view of CA IX as a molecule, whose expression levels as well as catalytic activity are regulated by oxygen availability, and thus open new possibilities for its better understanding and clinical exploitation.

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