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MOLECULAR AND CELLULAR MECHANISMS OF DISEASE

Carbonic anhydrase IX and hypoxia-inducible factor 1 attenuate cardiac dysfunction after myocardial infarction

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Abstract

Myocardial infarction (MI) is one of the leading causes of death worldwide. Prognosis and mortality rate are directly related to infarct size and post-infarction pathological heart remodeling, which can lead to heart failure. Hypoxic MI-affected areas increase the expression of hypoxia-inducible factor (HIF-1), inducing infarct size reduction and improving cardiac function. Hypoxia translocates HIF-1 to the nucleus, activating carbonic anhydrase IX (CAIX) transcription. CAIX regulates myocardial intracellular pH, critical for heart performance. Our objective was to investigate CAIX participation and relation with sodium bicarbonate transporters 1 (NBC1) and HIF-1 in cardiac remodeling after MI. We analyzed this pathway in an "in vivo" rat coronary artery ligation model and isolated cardiomyocytes maintained under hypoxia. Immunohistochemical studies revealed an increase in HIF-1 levels after 2 h of infarction. Similar results were observed in 2-h infarcted cardiac tissue (immunoblotting) and in hypoxic cardiomyocytes with a nuclear distribution (confocal microscopy). Immunohistochemical studies showed an increase CAIX in the infarcted area at 2 h, mainly distributed throughout the cell and localized in the plasma membrane at 24 h. Similar results were observed in 2 h in infarcted cardiac tissue (immunoblotting) and in hypoxic cardiomyocytes (confocal microscopy). NBC1 expression increased in cardiac tissue after 2 h of infarction (immunoblotting). CAIX and NBC1 interaction increases in cardiac tissue subjected to MI for 2h when CAIX is present (immunoprecipitation). These results suggest that CAIX interacts with NBC1 in our infarct model as a mechanism to prevent acidic damage in hypoxic tissue, making it a promising therapeutic target.

Keywords CAIX · HIF-1 · NBC1 · Myocardial infarction · Hypoxia

Mariela Beatriz Nolly, Lorena Alejandra Vargas, and María Verónica Correa equally contributed as first authors to the realization of this paper. Maria Teresa Damiani and Bernardo Victor Alvarez equally contributed as last authors to the realization of this paper.

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Introduction

1Myocardial infarction (MI) is one of the leading causes of morbidity and mortality worldwide [74]. Survival and mortality rates are directly related to the severity of infarct size and its progression to heart failure [46], a condition with no existing cure. A characteristic finding in MI is that the affected areas become sufficiently hypoxic to require anaerobic metabolism with, much greater acid (carbon dioxide and lactic acid) production and the need to activate intracellular pH (pH_i) regulatory mechanisms. Myocardial pH_i plays a central role in the regulation of different cellular events such as Ca^{2+} homeostasis, cell metabolism, gene expression, membrane ion conductance, and myofilament sensitivity to Ca^{2+}; all critical for the contractile function of cardiomyocytes [16, 35]. pH_i mostly depends on bicarbonate (HCO_3^-) concentration regulation, which is the main buffer in cells and the extracellular space. The concentration of HCO_3^- is controlled by carbonic anhydrases (CA)-facilitated transmembrane HCO_3^- transport proteins (bicarbonate transporters, BT) [1, 14, 47, 55] which are responsible for at least 50% of pH_i regulation in the heart [61, 62]. BTs are grouped in the SLC4 and SLC26 families. Within the SLC4 family, the Cl^-/HCO_3^- exchangers (anion exchangers, AE) and the Na^+/HCO_3^- co-transporters (NBC) catalyze the movement of HCO_3^- across cardiomyocyte membranes. Both AE and some members of the SLC26 family, namely, SLC26A3 and SLC26A6, exchange Cl^- for HCO_3^- to acidify the cell. The NBCs inwardly transport Na^+ and HCO_3^-, causing intracellular alkalinization. In addition, other membrane proteins responsible for controlling pH_i in the myocardium include the Na^+/H^+ exchanger-1 (NHE1) and the monocarboxylate (lactate/H^+) co-transporter (MCT), which do not require HCO_3^-.

Over many years, it has been found that AEs (AE1, AE2, and AE3), NHE1, and the NBCs (NBC1, NBC3) physically and functionally interact with intracellular carbonic anhydrase II (CAII) [2, 3, 28, 29, 31, 42, 56]. This interaction between CAII and BTs has been called the "bicarbonate transport metabolon" (BTM) [31, 55, 66]. In this manner, CA catalyzes bicarbonate production, more rapidly providing the substrate to the metabolon for its function, thus maximizing the gradient across the plasma membrane. This type of direct interaction of AE/CAII as a BTM is essential for maximizing AE-mediated transport activity since a decrease of 40 to 60% in its activity is found when CAII is free in the cytoplasm and not associated with the AEs [56]. Carbonic anhydrase IX (CAIX) can interact through its extracellular catalytic domain with the AE1, AE2, and AE3 transporters [32]. Also, in line with this, CAIX can interact with the NBC1 (the electrogenic NBCe1, encoded by the SLC4A4 gene) cotransporter in rat myocardium to form another BTM [36].

Cardiomyocytes have developed mechanisms that allow them to survive under hypoxic conditions. Hypoxia with or without ischemia initiates molecular changes and diverse phenotypic alterations [40]. During ischemia, the responses vary with the duration of hypoxia; seconds or minutes of hypoxia mainly induce protein molecular modification while hours of hypoxia induce changes in mRNA and protein expression. Hypoxia-inducible factor 1 (HIF-1), consisting of two subunits, is an important transcription factor and regulator of hypoxic responses after MI [51, 54, 67]. HIF-1α (regulatory subunit) under normoxic situations is rapidly recognized by oxygen-dependent prolyl-asparaginyl-hydroxylases (PHD) [4, 12], allowing its degradation by the Von Hippel-Lindau protein (VHL)/ubiquitin/proteasome pathway [21, 22]. In hypoxic conditions, PHDs are inhibited allowing HIF-1α to remain stable and thus capable of reaching the nucleus where it heterodimerizes with HIF-1β (constitutive subunit) to transcribe hypoxic adaptation genes [23, 40, 50, 52, 53]. Among them are those involved in pH_i regulation (CAIX, NHE1), angiogenic vascular endothelial growth factor (VEGF), proliferation and survival proteins, metabolic enzymes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [8, 13, 15, 25, 50], and enzymes that favor glycolytic over oxidative metabolism to maintain efficient ATP production [38, 51]. In this manner, HIF-1 functions as a master regulator that controls gene and protein expression, that mediate vascular responses to hypoxia and ischemia to promote an increase in capillary density, tissue perfusion, oxygen supply, and cell survival.

MI is a pathological condition characterized by low tissue O_2 content, which increases HIF-1 expression [26]. Infarct size reduction, as well as improved cardiac function, has been shown when HIF-1 is overexpressed [24]. Studies indicate that CAIX is expressed in the normal heart and enhances pH_i control through its interaction with BTs [36]. CAIX has unique distinctive properties; however, much is unknown about this enzyme. Apart from being a transmembrane protein (along with CAIV and CAXII) with an extracellular catalytic domain, it has a hypoxia-related expression pattern and an acidic pKa optimum [39]. CAIX is an isoform heavily expressed in solid tumors characterized by a high metabolic rate, elevated acid production, and a hypoxic microenvironment [19, 38, 58]. Because the important function of CAIX in hypoxic tumors, this led us to hypothesize that it could be similarly involved in myocardial infarction. To our knowledge, no studies have analyzed the role of CAIX in the context of hypoxia that takes place during MI. Our main goal thus was to investigate the contribution of CAIX and its relationship with BTs and HIF-1 in the remodeling that takes place in cardiac tissue after MI.

Materials and methods

"In vivo" infarction by ligation of the left anterior descending coronary artery

Two groups of adults (5-month-old) male Wistar rats underwent an "in vivo" infarction by occlusion of the left

anterior descending coronary artery (LAD) with a 6-0 nylon ligature. Animals were anesthetized with sevoflurane (5% induction and 3% maintenance), and endotracheal intubation was performed to allow mechanical ventilation [5, 17]. To analyze possible variations caused by cardiac infarction on HIF-1 and CAIX, two time points were selected: 2 hours (2 h) and 24 hours (24 h). The animals were then sacrificed for histological and biochemical studies at these time points. All procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH, Publication No. 85–23, revised 1996), and the experimental protocol was approved by the Animal Welfare Committee of La Plata School of Medicine. After coronary ligation, animals were kept according to the Post-Operative Recovery protocol describe by Wu et al. [71], Olivares et al. [34] and also by the guidelines approved by the Institutional Animal Use and Care Committee (IACUC) and Central Michigan University and the Guidelines for Rodent Survival Surgery [7]. Once the surgery was complete, the sterile drape over the animal was removed. The body temperature (by a rectal thermometer), respiratory rate (by visual inspection), heart rate (by palpitation), and abnormal signs of pain were monitored until the animal becomes ambulatory. The rats were removed from the ventilator and then placed in animal cage for anesthesia recovering. One end of the cage was be warmed by a heating pad to allow the rat to seek warmth. Also, water was available to the rat during the observation period. Tramadol (5mg/kg) was administered immediately after surgery and during post-operative recovery.

Cardiomyocyte isolation

Adult (5-month-old) male Wistar rats (n = 3–5 rats for each experimental condition) were anesthetized with sodium pentobarbital to isolate their hearts and through the ascending aorta, connect it to the Langendorff apparatus [6]. Retrograde perfusion was performed using different sequential solutions: (1) 5 min with Hepes solution or 4-(2-hydroxyethyl)-1-piperazine ethanesulfonate (146.2 mM NaCl, 4.7 mM KCl, 11 mM glucose, 10 mM Hepes, 0.35 mM $NaH_2PO_4 \cdot H_2O$, 1.05 mM $MgSO_4 7 \cdot H_2O$, pH 7.4 NaOH) plus 1.35 mM $CaCl_2$; (2) 5 min with Hepes plus 0.1 mM EGTA or ethylenebis(oxyethylenenitrilo)tetraacetic acid; (3) 15 min with enzyme digestion solution (Hepes with 0.06 mM $CaCl_2$, 0.5 mg/ml collagenase type IV Worthington Biochemical Corp.—USA, 1 mg/ml bovine serum albumin, 0.1 mg/ml protease type XIV—Sigma Aldrich). All solutions were bubbled with 100% O_2 and maintained at 37 °C. Finally, the heart was disconnected and placed in the enzymatic digestion solution. Tissue disintegration was accelerated by making mechanical cuts and pipetting. The isolated cardiomyocytes were resuspended in 0.06 mM Hepes solution with $CaCl_2$ and sequentially washed with solutions of increasing calcium concentration (Hepes solution with 0.12 mM, 0.24 mM, and 0.48 mM $CaCl_2$) to avoid cell death by rapid recalcification. The final resuspension was in Hepes buffer 1.35 mM $CaCl_2$. After using this standardized isolation technique, myocytes underwent 2 h and 24 h of hypoxia in an appropriate chamber. Chamber components are made of clear polycarbonate. Dimensions of units were as follows: width 20 cm, length 20 cm, and height 6.5 cm. Units can be stacked to conserve space. Hypoxic conditions were created by maintaining cells in a sealed chamber flooded with 94% N_2, 5% CO_2, and 1–1.5% O_2 incubated at 37 °C in a humidified atmosphere, as previously described by Triantafyllou et al. [60]. Finally, myocytes were processed differently, according to the objectives (immunolocalization and confocal microscopy).

Histological techniques

After performing the "in vivo" infarction, the hearts were removed and cut in three cross-sections (base, medium, apex) and then were submerged in 10% formaldehyde (in phosphate buffer) for 24 h (n = 3–5 rats for each experimental condition). Subsequently they were preserved in 70% ethanol for histological examination using the protein staining reagent diaminobenzidine (DAB, Rochem Biocare) to identify HIF-1, CAIX, and NBC1.

Protein expression

Briefly, lysates from isolated cardiomyocytes were prepared following standardized protocols (n = 3–4 rats for each experimental condition). Samples with the same protein concentration (measured by the Bradford technique) were analyzed by electrophoresis (SDS-PAGE) and subsequently transferred to PVDF membranes for subsequent immunodetection with specific antibodies for HIF-1 (mouse polyclonal anti-HIF1α antibody, 1:1000, Santa Cruz), CAIX (mouse monoclonal anti-CAIX antibody which recognizes the proteoglycan-like attachment domain M75, 1:1000, a kind gift from Professor Silvia Pastoreková, Bratislava, Slovakia), and NBC1 (rabbit polyclonal anti-NBCe1antibody, 1:1000, Millipore, Temecula, CA). Samples were randomly placed in the immunoblot gel to create internal controls for analysis purposes. Images were cropped to facilitate visual comprehension.

Coimmunoprecipitation

Another group of adult Wistar rats (n = 7 rats for each experimental condition) were used for coimmunoprecipitation analysis. Tissue was homogenized with immunoprecipitation buffer supplemented with protease inhibitors (Mini Complete; Roche Molecular Biochemical). Ventricular lysates were centrifuged at 1440 ×*g* for 5 min in a Beckman G5-6K centrifuge. Supernatants (3.5 mL) were removed and

applied to 50 μL protein G Sepharose for 3 h at 4 °C. After centrifugation (5 min, 8000 ×*g*), lysates were incubated with the anti-CAIX monoclonal antibody and 100 μL protein G Sepharose overnight at 4 °C. The resin was washed and resuspended in SDS-PAGE sample buffer. Samples were electrophoresed on 10% acrylamide gels. Immunoblots were probed with an anti-CAIX antibody or anti-NBC1 antibody, previously mentioned at a 1:1000 dilution. Samples were randomly placed in the immunoblot gel to create internal controls for analysis purposes. Images were cropped to facilitate visual comprehension.

Immunolocalization

The heart was processed to perform immunofluorescence according to pre-established protocols (n = 3 rats for each experimental condition) [36]. Single freshly dissociated myocytes were plated onto 22 × 22 mm laminin (25–50 μg/mL)-coated glass coverslips and incubated at 37 °C for 30 min to allow attachment. Cells were rinsed with PBS 1X, fixed with paraformaldehyde 3.5%, and permeabilized with Triton X-100 (0.1%). Myocytes were incubated with primary anti-CAIX antibody or anti-HIF-1 antibody as previously mentioned. Primary antibodies were used at a 1:100 dilution. Secondary antibodies conjugated to fluorophores Alexa 488 and Alexa 594 were used at a 1:200 dilution. Coverslips were washed three times in PBS containing 0.2% gelatin and two times in PBS and then mounted in Prolong Anti-fade solution containing Dapi for nuclei staining (Thermo Fisher Scientific, USA). Afterwards they were analyzed by fluorescent and confocal microscopy. Images of cells stained with HIF-1 and CAIX were deconvolved using an image-processing technique designed to remove blur and to enhance contrast and resolution. Channel-specific point spread functions were generated and the signal-noise ratio was adjusted until the deconvolved images were free of pixel noise.

Bioinformatic analysis

Public dataset GSE27975 of CAIX and NBC1 mRNA expression in normoxia or hypoxia (1% oxygen) for 8 h in the HL-1 *Mus musculus* cardiomyocyte cell line was analyzed [41]. Total RNA was isolated and hybridized to an Affymetrix gene array. The raw probe intensities were downloaded from the GEO database using the GEO query R package and then analyzed using the Robust Multichip Average (RMA) normalization [20]. Differential gene expression and their associated p-values were assessed with the linear model of the Limma R package was used with the eBayes method [45].

The protein-protein interaction between CAIX (Car9)[#], NBC1 (Slc4a4)[#], and HIF-1 (Hif1a)[#] proteins from *Rattus norvegicus* was analyzed in the STRING database (https://string-db.org) from the Swiss Institute of Bioinformatics and the European Molecular Biology Laboratory (EMBL) [59]. Connections were identified by selecting the string indicative of interaction confidence. The thickness of the lines indicates the strength of the experimental data. A minimum interaction score of 0.15 was used as a filter to detect interaction networks. The gene family in the pathway involved was annotated with different color nodes. The database used a combination of genes and protein names. [#]Since the database displayed a combination of genes and protein names, the correspondence between them is indicated between round brackets.

Statistical analysis

Continuous variables were expressed as mean ± SEM and evaluated either by ANOVA and Student-Newman-Keuls multiple comparison post-test analysis (when comparison among different groups was performed) or by unpaired Student *t*-test (between two groups). A $p < 0.05$ was considered significant (*).

Results

Localization of CAIX and HIF-1 in remote and infarcted heart areas

CAIX and HIF-1 protein expression were analyzed in slices from non-infarcted and infarcted areas of the left ventricle. Immunohistochemical studies using the DAB technique revealed increased HIF-1 expression in the infarcted area compared to a remote non-infarcted area measured at 2 h after LAD, which was no longer different 24 h later (Fig. 1A–E).

Similarly, we found increased CAIX protein expression in the infarcted area in comparison to a remote non-infarcted area. The increased CAIX expression at 2 h after MI was followed by a decrease at 24 h, but it remained increased compared to a remote non-infarcted area (Fig. 1F–J). To explore CAIX protein expression pattern, we incorporated an additional time point at 4 h and observed an increase that was intermediate among 2 and 24 h (Fig. 1 supplemental data). As observed in the micrograph images, HIF-1 is mainly located in the nucleus, whereas CAIX is distributed throughout the cell and localizes at the plasma membrane at 24 h.

Furthermore, we analyzed the expression of NBC1 and found it increased in the infarcted area in comparison to a remote non-infarcted area. Consistent with increased HIF-1 and CAIX expression, NBC1 expression was more prominent 2 h after MI compared to 24 h (Fig. 1K–O). No HIF-1, CAIX, and NBC1 DAB staining were observed in sham infarct rats (Fig. 2 supplemental data).

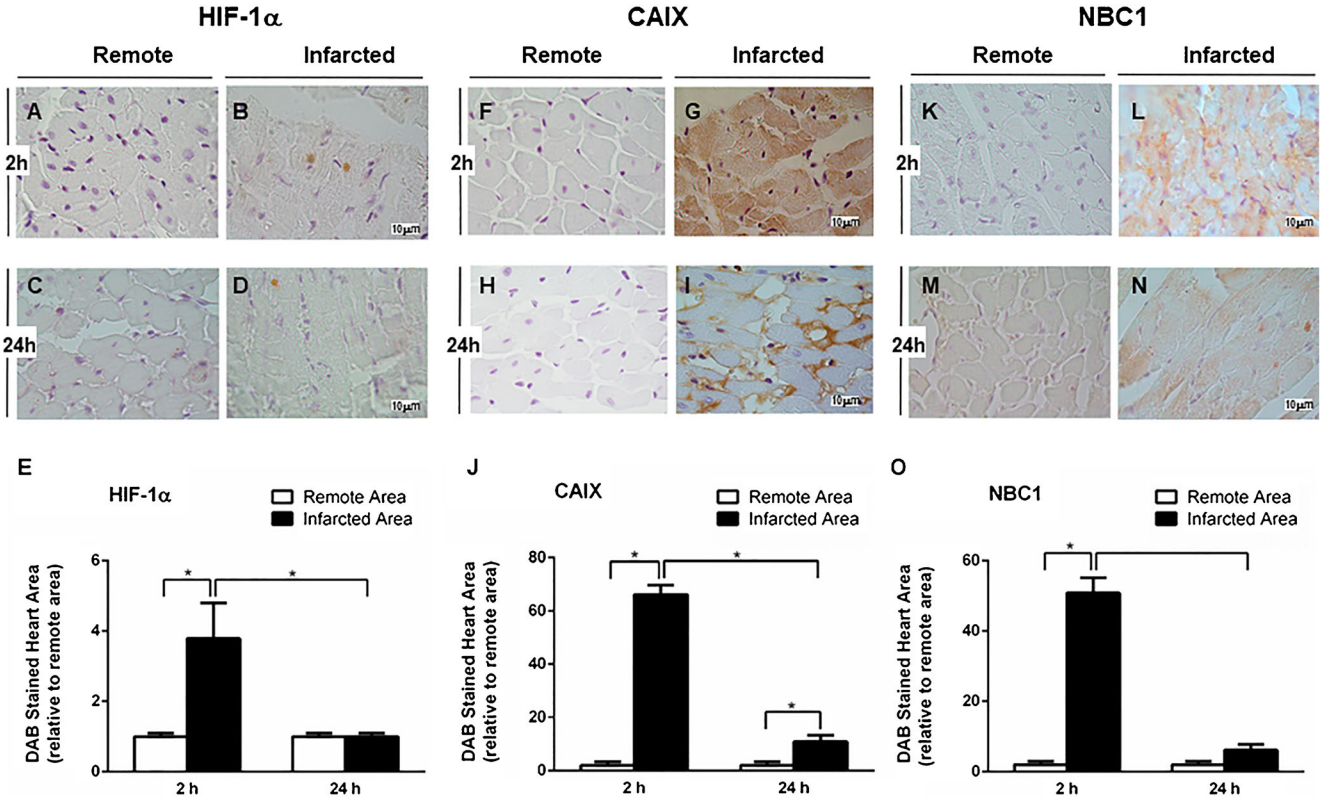

Fig. 1 Location of CAIX and HIF-1 in remote and infarcted areas of the heart. **A**–**E** Micrograph images of high-power view (×100) from a representative section of the rat heart after LAD, using DAB stain to identify HIF-1 expression. **A**, **B** Representative section of the heart left ventricle after 2 h post-infarction in remote and infarcted areas. **C**, **D** Representative section of the heart left ventricle after 24 h post-infarction in remote and infarcted areas. **E** Summary of the normalized stained heart area relative to a remote area. Statistical significance was determined by ANOVA and Student-Newman-Keuls multiple comparison post-test analysis. Values are mean ± SEM, $*p < 0.05$. **F**–**J** Micrograph images of high-power view (×100) from a representative section of the rat heart after LAD, using DAB stain to identify CAIX expression. **F**, **G** Representative section of the heart left ventricle after 2 h post-infarction in remote and infarcted areas. **H**, **I** Representative section of the heart left ventricle after 24 h post-infarction in remote and infarcted areas. **J** Summary of the normalized stained heart area relative to a remote area. Statistical significance was determined by ANOVA and Student-Newman-Keuls multiple comparison post-test analysis. Values are mean ± SEM, $*p < 0.05$. **K**–**O** Micrograph images of high-power view (×100) from a representative section of the rat heart after LAD, using DAB stain to identify NBC1 expression. **K**, **L** Representative section of the heart left ventricle after 2 h post-infarction in remote and infarcted areas. **M**, **N** Representative section of the heart left ventricle after 24 h post-infarction in remote and infarcted areas. **O** Summary of the normalized stained heart area relative to a remote area. Statistical significance was determined by ANOVA and Student-Newman-Keuls multiple comparison post-test analysis. Values are mean ± SEM, $*p < 0.05$

Expression of HIF-1, CAIX, and NBC1 in cardiac tissue and isolated myocytes after myocardial infarction and hypoxia

To examine the modifications of the three proteins (HIF-1, CAIX, and NBC1) after the "in vivo" infarction by the LAD occlusion procedure, we analyzed cardiac tissue and hypoxic isolated myocytes by immunoblotting and confocal microscopy.

We observed increased HIF-1 expression in cardiac tissue undergoing infarction by immunoblotting after 2 h compared to 24 h (Fig. 2A and B). To confirm the distribution and expression of these proteins, we studied isolated cardiomyocytes subjected to hypoxia by confocal microscopy and verified increased HIF-1 expression (Fig. 2C and D). Among other proteins, GAPDH synthesis was stimulated by HIF-1 activation. We confirmed GAPDH expression increase in our hypoxic model by immunoblot (Fig. 3A and 3B supplemental data).

When analyzing CAIX, we observed its expression increase in cardiac tissue undergoing infarction by immunoblotting 2 h after MI compared to 24 h (Fig. 3A and B). Also, we evaluated CAIX expression by Western blotting in isolated cardiomyocytes after hypoxia and observed increased CAIX expression at 2 h compared to 24 h (Fig. 3C and D). To analyze the effect of hypoxia itself on CAIX, isolated myocardial cells were cultured under hypoxia for 2 h and 24 h. The results of Fig. 3E and F show significant increased CAIX expression in cardiomyocytes exposed to hypoxic conditions when compared to normoxia as measured by fluorescence intensity by confocal microscopy.

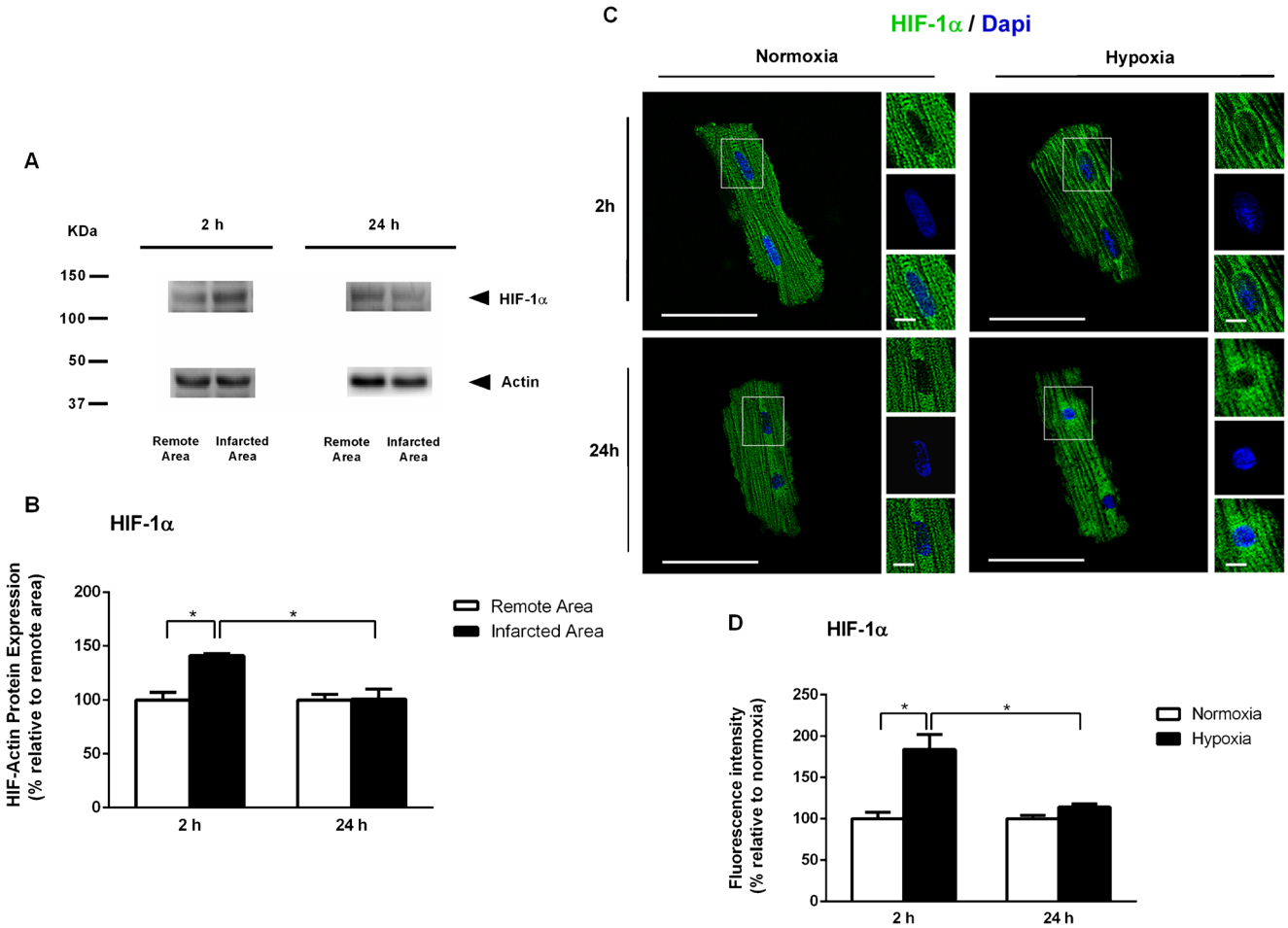

Fig. 2 Expression of HIF-1 in cardiac tissue at 2 h and 24 h post-infarction and isolated myocytes subjected to hypoxia. **A** Expression of HIF-1 protein in adult Wistar rat hearts. Lysates were prepared from adult rat heart ventricles. The protein sample was subjected to SDS-PAGE analysis, transferred to PVDF membrane, and probed with anti-HIF-1 antibodies. Filled arrow indicates the protein. Summary of the normalized HIF-1/actin protein expression values in the infarcted area relative to a remote area of the heart. **B** Confocal immunofluorescence images of rat cardiomyocytes fixed and immunostained with anti-HIF-1α antibody, as described in "Materials and methods" section. **C** Summary of the normalized fluorescence intensity values in hypoxia relative to normoxia. **D** Statistical significance was determined by ANOVA and Student-Newman-Keuls multiple comparison post-test analysis. Values are mean ± SEM, $*p < 0.05$. Scale bar value corresponds to 30 μm and 5 μm for inset

As regards to NBC1, we observed that NBC1 protein expression by immunoblotting was increased at 2 h of infarction compared to 24 h in cardiac tissue after LAD (Fig. 4A and B). Consistently, we observed the same increase tendency with higher expression of NBC1 at 2 h of hypoxia compared to 24 h (Fig. 4C and D) in isolated hypoxic cardiomyocytes using the same technique.

Using a bioinformatics-database analysis, we found that CAIX mRNA expression was significantly higher in hypoxic (8 h) cardiomyocytes in a mouse model, reaffirming our protein findings. NIBC1 mRNA was overexpressed, also in agreement with our protein expression results. HIF-1 mRNA showed a decreased expression at the evaluated time points, in accordance with previous studies [11, 44]. Interestingly, other hypoxia-related mRNAs were upregulated including VEGFA, HIF1β (Arnt), and GAPDH. CAII (Car2) and VEGF receptor 1 (Flt1) mRNA values were not significantly modified (Fig. 3C supplemental data).

CAIX and NBC1 interaction in infarcted cardiac tissue

Our findings prompted us to analyze the possibility of CAIX interacting with NBC1 using co-immunoprecipitation followed by immunoblot analysis. First, we corroborated that CAIX was present in a remote non-infarcted area and that it was not detected when immunoprecipitation was performed without the anti-CAIX antibody bound to protein Sepharose beads, thus confirming the validity of the technique. We observed an increase in the levels of CAIX in cardiac tissue subjected to infarction for 2 h (Fig. 4E and F). Furthermore, we observed an increase in the levels of NBC1 in cardiac tissue subjected to MI for 2 h in the presence of CAIX, indicating that they interact in our infarct model. NBC1 was not detected when immunoprecipitation was performed

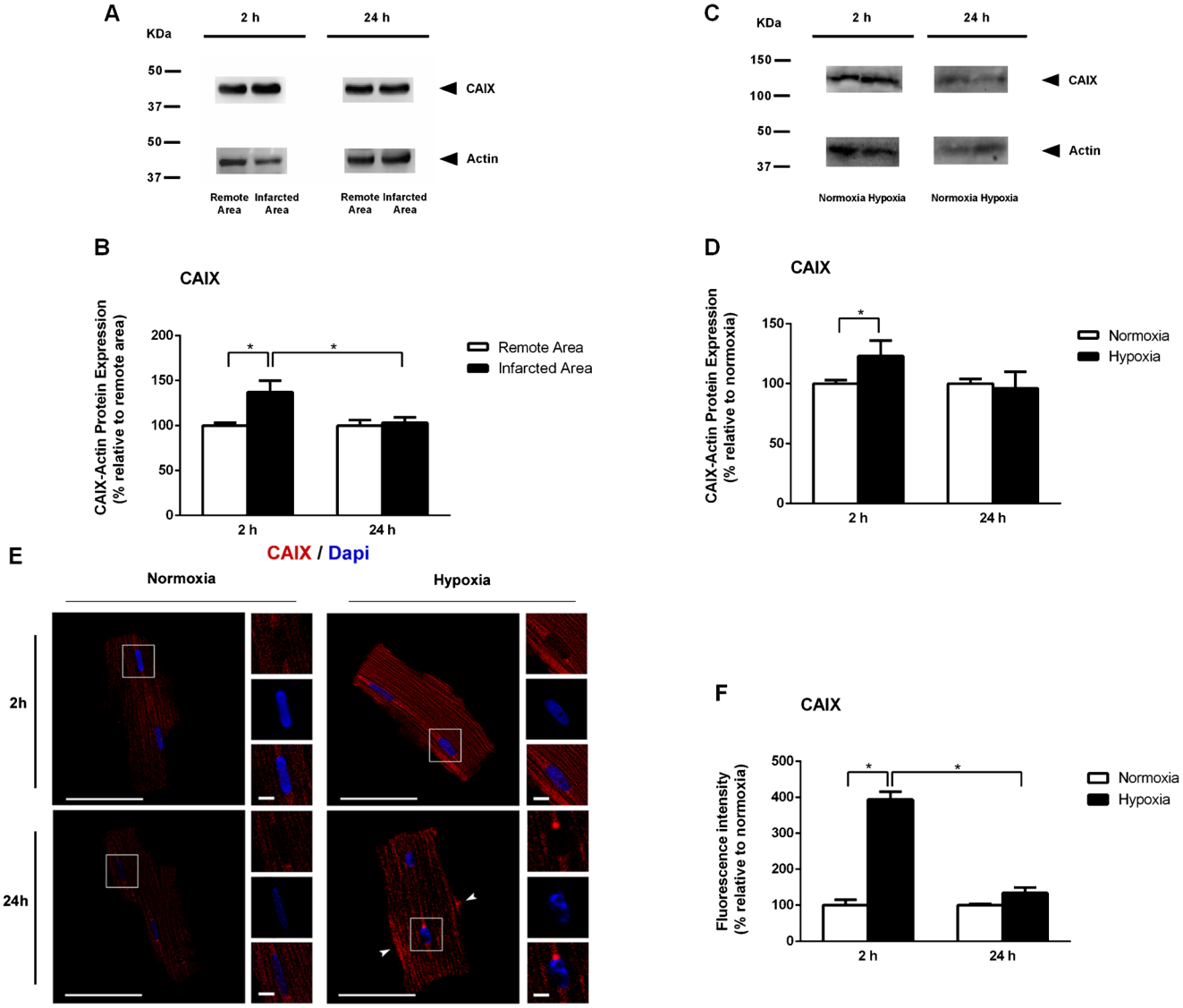

Fig. 3 Expression of CAIX in cardiac tissue at 2 h and 24 h post-infarction and isolated myocytes subjected to hypoxia. **A** Expression of CAIX protein in adult Wistar rat hearts. Lysates were prepared from adult rat heart ventricles. The protein sample was subjected to SDS-PAGE analysis, transferred to PVDF membrane, and probed with anti-CAIX antibodies. Filled arrow indicates the protein. **B** Summary of the normalized CAIX/actin protein expression values in the infarcted area relative to a remote area of the heart. **C** Expression of CAIX protein in adult Wistar rat cardiomyocytes. Lysates were prepared from adult rat cardiomyocytes. The protein sample was subjected to SDS-PAGE analysis, transferred to PVDF membrane, and probed with anti-CAIX antibodies. Filled arrow indicates the protein. **D** Summary of the normalized CAIX/actin protein expression values in hypoxia relative to normoxia. **E** Confocal immunofluorescence images of rat cardiomyocytes fixed and immunostained with anti-CAIX antibody, as described in "Materials and methods" section. **F** Summary of the normalized fluorescence intensity values in hypoxia relative to normoxia. Statistical significance was determined by ANOVA and Student-Newman-Keuls multiple comparison post-test analysis. Values are mean ± SEM, $*p < 0.05$. Filled arrows indicate protein accumulation zones. Scale bar value corresponds to 30 μm and 5 μm for inset

without the anti-CAIX antibody bound to the resin (Fig. 4G and H).

Through STRING database analysis (https://string-db.org, from the Swiss Institute of Bioinformatics and the European Molecular Biology Laboratory—EMBL), we examined HIF-1, CAIX, and NBC1 protein interaction. The interaction among multiple proteins involved in pH_i control such as the NHE and BT arise from the STRING curated database. In this network, CAIX appears as an interaction hub between pH-regulatory-proteins, HIF-1 pathway, and cell adhesion pathway, suggesting CAIX importance in this hypoxic response mechanism (Fig. 4I).

Discussion

The main goal of our study was to identify the participation of HIF-1/CAIX/NBC1 pathway during the early survival stage after MI. Once cellular homeostasis is affected by hypoxia, the

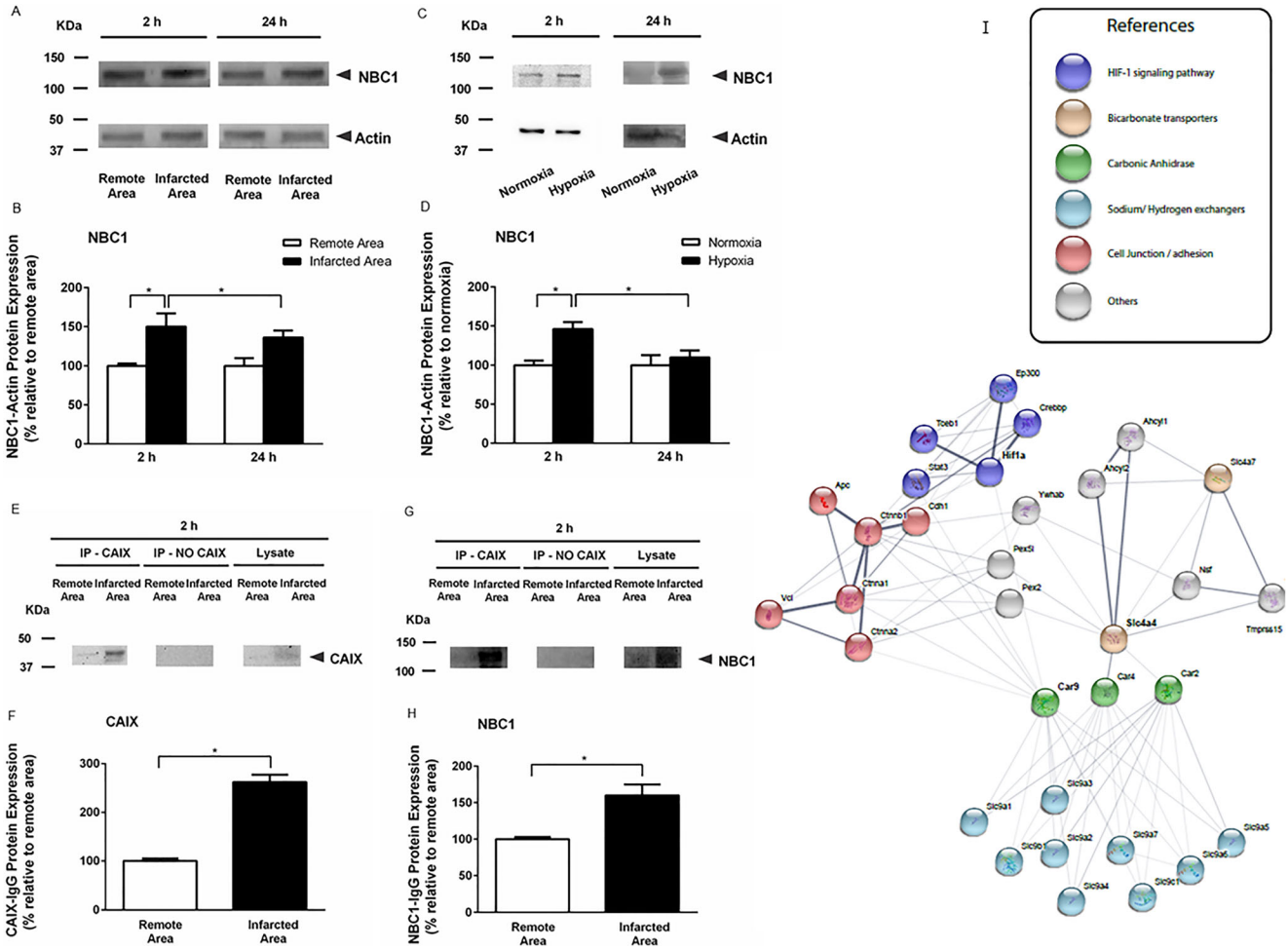

Fig. 4 Interaction between NBC1–CAIX–HIF-1 in cardiac tissue through experimental, biochemical, and curated databases. Expression of NBC1 in cardiac tissue at 2 h and 24 h post-infarction and isolated myocytes subjected to hypoxia (**A**–**D**). **A** Expression of NBC1 protein in adult Wistar rat hearts. Lysates were prepared from adult rat heart ventricles. The protein sample was subjected to SDS-PAGE analysis, transferred to PVDF membrane, and probed with anti-NBC1 antibodies. Filled arrow indicates the protein. **B** Summary of the normalized NBC1/actin protein expression values in the infarcted area relative to a remote area of the heart. **C** Expression of NBC1 protein in adult Wistar rat cardiomyocytes. Lysates were prepared from adult rat cardiomyocytes. The protein sample was subjected to SDS-PAGE analysis, transferred to PVDF membrane, and probed with anti-NBC1 antibodies. Filled arrow indicates the protein. **D** Summary of the normalized NBC1/actin protein expression values in hypoxia relative to normoxia. Statistical significance was determined by ANOVA and Student-Newman-Keuls multiple comparison post-test analysis. Values are mean ± SEM, $*p < 0.05$. CAIX and NBC1 interaction in cardiac tissue at 2 h post-infarction (**E**–**H**). **E** Coimmunoprecipitation of CAIX. Cell lysates were immunoprecipitated (IP) with anti-CAIX, resolved by SDS-PAGE, blotted, and probed with an anti-CAIX antibody to detect CAIX. Samples of the lysate were probed to indicate the total amount of CAIX in each sample (right). Filled arrow indicates the protein. **F** Summary of the normalized protein in the infarcted area relative to a remote area of the heart. **G** Coimmunoprecipitation of NBC1. Cell lysates were immunoprecipitated (IP) with anti-CAIX, resolved by SDS-PAGE, blotted, and probed with an anti-NBC1 antibody to detect NBC1. Samples of the lysate were probed to indicate the total amount of NBC1 in each sample (right). Filled arrow indicates the protein. **H** Summary of the normalized protein in the infarcted area relative to a remote area of the heart. Statistical significance was determined by *t*-test. Values are mean ± SEM, $*p < 0.05$. CAIX, NBC1, and HIF-1 protein-protein interaction network (**I**). Representation of a simplified protein-protein interaction network showing CAIX (Car9), NBC1 (Slc4a4), and HIF-1 (Hif1a) protein physical connection. The nodes network indicates genes with previous experimental interaction evidence. Nodes are connected by edges of varying width representing the strength of the evidence available. Green nodes are code for carbonic anhydrase genes, light blue nodes are NHE exchangers, orange nodes represent BT, dark blue nodes are genes of the HIF-1 signaling pathway, and red nodes are part of the cell junction/adhesion pathway. White nodes represent other genes found to interact with the target genes

expression of certain proteins is modified. It has been described that CAIX participates in pH_i restoration to its normal value after hypoxia in tumoral tissue. This process also activates pH-sensitive transporters such as NBC1, AE, and NHE1 [38]. In this study, we analyzed the location and expression of CAIX, HIF-1, and NBC1 in the cardiac tissue of infarcted "in vivo" rats by ligating the left anterior descending coronary artery. Confocal microscopy showed increased HIF-1 and CAIX expression in isolated myocytes subjected to 2 h of hypoxia. CAIX tended to be principally located at the

cardiomyocyte plasma membrane, while HIF-1 was mainly in the nucleus. The expression pattern of HIF shown in our figures in normoxic conditions has been reported by other authors, such as Watanabe et al. [68], Wikenheiser et al. [69], and Wikenheiser et al. [70]. CAIX can be found in HIF-1 negative areas, likely by HIF-1 degradation due to reoxygenation or the unfolded protein response [38]. Because CAIX can respond to a wide range of hypoxia, its distribution partially overlaps with HIF-1 distribution and other hypoxia-induced proteins [19, 58, 72]. CAIX staining in the perinecrotic area can also be found in moderately hypoxic cells [38]. In addition, even though CAIX is a transmembrane protein mainly found in the plasma membrane, some cytoplasmic staining could be related to newly generated protein and its transport from the endoplasmic reticulum to plasma membrane, as has been shown in tumor cells by Pastorekova et al. [38, 49, 73].

Immunohistochemical studies using the DAB technique revealed an increase expression of HIF-1, CAIX, and NBC1 in the peri-infarct zone in comparison to healthy areas. These results were more noticeable after 2 h of MI compared to those obtained after 24 h. In addition, CAIX expression-curve analysis revealed that this increase at 2 h of MI was followed by a decrease at 4 h and 24 h. Protein concentration normalization at 24 h could be due to CAIX participation that had improved cellular conditions enough to stop new synthesis combined with catabolism of existing enzyme. Our results show that CAIX staining is predominantly localized in cardiomyocytes. Also, no CAIX-DAB-stain was observed in the sham infarct rats. However, minor staining in fibroblasts, below the detection limit of the methods, cannot be excluded.

We observed an increase in HIF-1, CAIX, and NBC1 expression in cardiac tissue after MI by immunoblotting. These results were more evident at 2 h of MI in the peri-infarcted

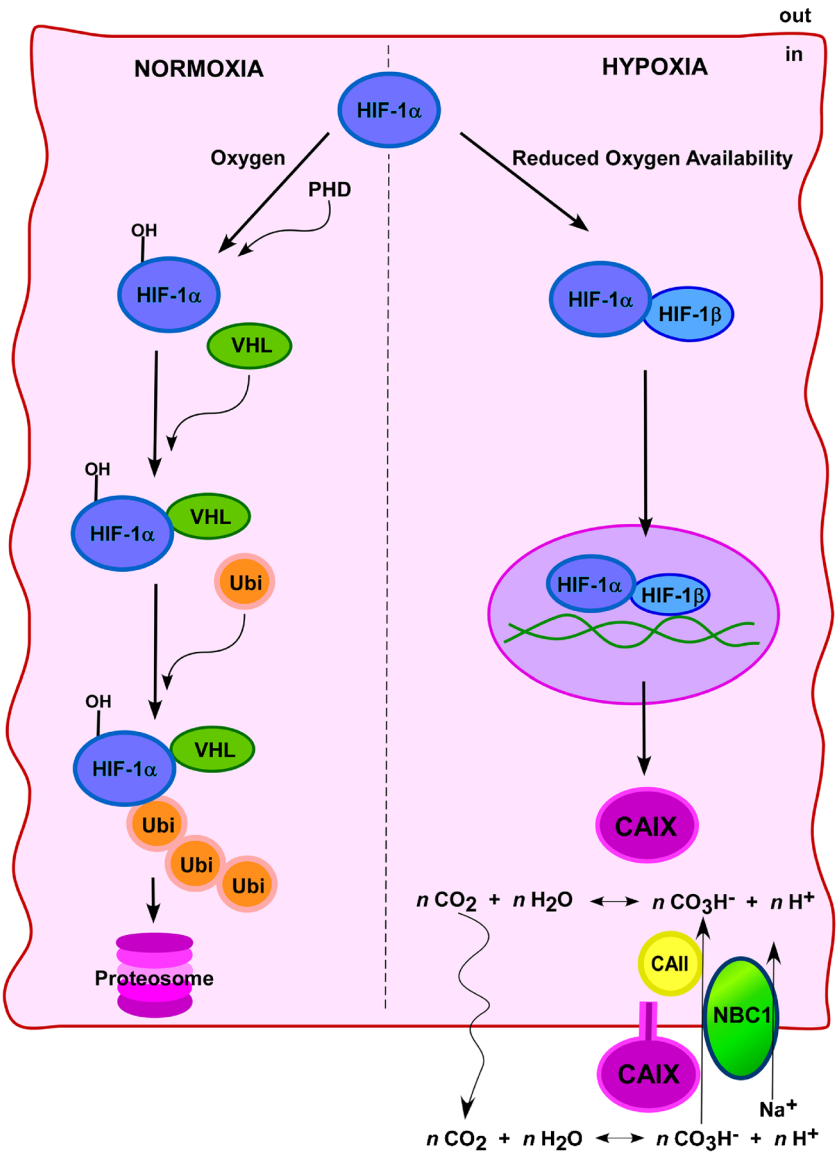

Fig. 5 Schematic model of the effect of HIF-1, CAIX, and NBC1 in the infarcted heart. In normoxic conditions, HIF-1 is hydroxylated by prolyl hydroxylases, favoring the recognition of VHL and targeting this complex for ubiquitin and proteasome degradation. Under hypoxic conditions, hydroxylation is inhibited and HIF-1α remains stable and dimerizes with HIF-1β activating the transcription of several genes including those that are in charge of pH_i regulation, such as CAIX, angiogenesis, proliferation, and survival mechanisms. The extracellular catalytic domain of CAIX regulates pH_i by catalyzing the formation of HCO_3^- that will be incorporated into the cell through the NBC1, helping to restore the cell to its normal conditions

zone compared to the healthy area. We could not detect HIF-1 expression in isolated cardiomyocytes by immunoblot with our methodology, mainly because of rapid oxygen-mediated HIF-1 degradation. Nevertheless, the increased expression of CAIX and GAPDH in hypoxic cardiac tissue is a consequence of HIF-1 activation. HIF-1 binding to the HIF-1 response element in the CAIX gene stimulates CAIX transcription. This event is increased by acidosis and hypoxia [39]. CAIX expression increase induced by hypoxia and mediated by HIF-1α was found using different cancer cell models [72]. On the contrary, CAIX expression decrease correlates with a HIF-1α expression decrease as well [43]. Our CAIX and NBC1 upregulation findings after a hypoxic stimulus were reaffirmed by a bioinformatics-database analysis at the mRNA level in cultured mouse cardiomyocytes. Our protein results were obtained from *Rattus norvegicus* cardiac myocytes, while microarray analysis was performed in *Mus musculus* with different experimental conditions (cultured cell lines). This hypoxic response mechanism may constitute evidence of the relevant function of these genes in cardiomyocytes hypoxic response and should be further analyzed. It has been proposed that the endogenous regenerative properties of the mammalian adult heart can be reactivated by gradual exposure to increasing systemic hypoxemia, highlighting a potential role of therapeutic hypoxia in regenerative medicine [33].

Finally, we found the NBC1-CAIX interaction to be very significant at 2 h in infarcted cardiac tissue that was undetected when CAIX was absent, reinforcing its necessary presence. Increased CAIX and NBC1 expression suggests that CAIX could interact with NBC1, possibly assembling into a BTM, in an attempt to restore cardiac cell function. A HIF-1, CAIX, and NBC network has been demonstrated through experimental, biochemical, and curated databases. Our results show the interaction between CAIX and NBC1 in cardiac tissue and how it correlates with increased protein expression after infarction. A possible protein interaction can be direct or by establishing pH-regulating complexes with other ion transporters channel families. The network built among these interacting proteins suggests that they operate closely related in response to hypoxia.

Plasma membrane CAs have their catalytic site facing the extracellular space. This binding sequence is conserved among all the known membrane bound CAs. To accelerate CO_2 hydration, they interact through their catalytic domain with BT, creating the transport metabolon [36, 39]. CAIX may improve the acid base status of the ischemic zone by interacting with BTPs and participating in other mechanisms restoring pH_i to its physiological value and promote cell survival. This result suggests that CAIX interacts with NBC1 in our infarct model, making it a promising therapeutic target (Fig. 5).

Conclusions

Many efforts are being made to repair or salvage damaged myocardium with MI. Some techniques or procedures established in preclinical cell and animal studies have not been able to be translated into clinical therapies. VEGF is one such example that promotes the growth of new blood vessels in tissues with reduced perfusion as in obstructed blood vessels. Bypassing the blocked vessels allows the tissue to be better reoxygenated and become resistant to ischemia. HIF-1 stimulates the transcription of VEGF in ischemic or infarcted myocardium [27]. However, this response does not always adequately succeed and may worsen the situation in MI. The overexpression of VEGF can lead to complications such as fragile and immature vessels, angioma formation, and systemic hypotension [10, 30]. VEGF treatment is often not sufficient to ameliorate or prevent myocardial damage [18].

While our present results suggest a possible important protective role of CAIX, the final answer will only be determined by selective pharmacologic CAIX inhibition, antisense CAIX mRNA administration, and cardiac specific conditional CAIX knockout experiments. Given that several CA isozymes are present in the heart, the situation is very likely complicated. As a reflection of this, our group has shown that administration of benzolamide, a poorly membrane-permeant CA inhibitor that inhibits all membrane CAs (CAIV, CAXII, CAXIV), is protective in acute MI with subsequent reperfusion, which was not studied in the present work. Thus, even though a possible protective role of CAIX is lost during ischemia, inhibition of the other membrane-bound forms as well as CAIX during the critical injurious reperfusion period may be beneficial by slowing intracellular pH recovery. CA inhibition has other effects in the normal heart and isolated myocytes, such as a small reduction in contractile force, alteration in myocardial energetics, and intracellular acidosis [48, 57, 64, 65]. In line with these, in vivo studies have shown reduced myocardial O_2 delivery and CO_2 elimination, as well as reduced fatty acid uptake [57]. Conversely, CA inhibitors might be useful in post-infarction period by limiting pathological cardiac hypertrophy [2, 9, 37, 63, 64]. It would be interesting to analyze the effect of using not only a selective CAIX inhibitor but also PHD inhibitors that increase HIF-1 response in our in vivo MI experimental model. Our next goal will be to analyze the effect of a CAIX-selective-inhibition in this specific model, for which ongoing experiments are being conducted.

Our findings demonstrate that MI-stimulated HIF-1 activation increases CAIX expression in our in vivo MI hypoxic model and in isolated hypoxic cardiomyocytes. HIF-1 and CAIX may be beneficial in limiting myocardial tissue damage produced during the ischemic phase of MI. Targeting the HIF-1/CAIX/NBC1 axis could open new roads to develop novel therapeutic approaches to prevent MI injury in patients in whom reperfusion cannot be re-established.

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Declarations

Conflict of interest The authors declare no competing interests.

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