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Chronic Mild Hypoxia Protects Heart-derived H9c2 Cells against Acute Hypoxia/Reoxygenation by Regulating Expression of the SUR2A Subunit of the ATP-sensitive K^+ Channel*

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Abstract

Chronic exposure to lower oxygen tension may increase cellular resistance to different types of acute metabolic stress. Here, we show that 24-h-long exposure to slightly decreased oxygen tension (partial pressure of oxygen (PO_2) of 100 mm Hg instead of normal 144 mm Hg) confers resistance against acute hypoxia/reoxygenation-induced Ca^{2+} loading in heart-derived H9c2 cells. The number of ATP-sensitive K^+ (K_{ATP}) channels were increased in cells exposed to PO_2 = 100 mm Hg relative to cells exposed to PO_2 = 144 mm Hg. This was due to an increase in transcription of SUR2A, a K_{ATP} channel regulatory subunit, but not Kir6.2, a K_{ATP} channel pore-forming subunit. PO_2 = 100 mm Hg also increased the SUR2 gene promoter activity. Experiments with cells overexpressing wild type of hypoxia-inducible factor (HIF)-1α and dominant negative HIF-1β suggested that the HIF-1-signaling pathway did not participate in observed PO_2-mediated regulation of SUR2A expression. On the other hand, NADH inhibited the effect of PO_2 = 100 mm Hg but not the effect of PO_2 = 20 mm Hg. LY 294002 and PD 184 352 prevented PO_2-mediated regulation of K_{ATP} channels, whereas rapamycin was without any effect. HMR 1098 inhibited the cytoprotective effect of PO_2 = 100 mm Hg, and a decrease of PO_2 from 144 to 100 mm Hg did not change the expression of any other gene, including those involved in stress and hypoxic response, as revealed by Affymetrix high density oligonucleotide arrays. We conclude that slight hypoxia activates HIF-1α-independent signaling cascade leading to an increase in SUR2A protein, a higher density of K_{ATP} channels, and a cellular phenotype more resistant to acute metabolic stress.

A chronic lack of oxygen has been implicated in variety of diseases including atherosclerosis, diabetes, pulmonary fibrosis, neurodegenerative disorders, arthritis, and aging. At the cellular level, hypoxia activates numerous major signaling pathways, resulting in changes in gene expression, which influence the cellular ability to survive or die. These pathways exert their phenotypic influences largely through modulation of transcription factor activities that effect changes in the pattern of gene expression, and some of these pathways are linked to enhanced

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survival, whereas others are associated with cell death. Severe hypoxia, occurring at partial pressure of oxygen (PO_2)[1] below 20 mm Hg, impairs cellular energy production and ion homeostasis, leading to cell injury and cell death. In contrast, a lower degree of hypoxia, defined as PO_2 between 50 and 100 mm Hg, may activate mechanisms that could produce cellular phenotype more resistant to acute severe oxidative stress (1,2). This phenomenon was in particular described in the heart, where acute severe oxidative stress is one of the most important components of different forms of ischemic heart diseases, including myocardial infarction. At the single cell level, it has been shown that isolated cardiomyocytes when cultured at lower oxygen tension acquire resistance against acute severe oxidative stress (3). Such single cell reports have been strongly supported by clinical studies showing that the incidence of myocardial infarction complications and the mortality rate are much lower in populations living at lower PO_2 than those of the rest of the world (4,5).

How moderate hypoxia induces increased cellular resistance to acute severe oxidative stress is yet unknown. In this regard, the present study was undertaken to address this question and to define the molecular basis of chronic mild hypoxia regulation of cellular resistance to acute metabolic challenges. To achieve this, we applied a set of different techniques on heart-derived H9c2 cells, cells that have been previously successfully implemented to study mechanisms of cellular and cardiac protection (6,7). Using this approach we found that chronic minimal hypoxia up-regulates SUR2A subunit of the ATP-sensitive K^+ channel without affecting expression of any other gene. This effect is not associated with activation of HIF-1α-dependent signaling pathway, whereas changes in the NAD/NADH ratio and activation of

[1]The abbreviations used are:

PO_2 partial pressure of oxygen

GFP green fluorescent protein

HIF hypoxia-inducible factor

HPLC high performance liquid chromatography

ERK extracellular signal-regulated kinase

MAPK mitogen-activated protein kinase

MEK1 mitogen-activated protein kinase kinase

PI 3-kinase phosphatidylinositol 3-kinase

RT reverse transcription

GAPDH glyceraldehyde-3-phosphate dehydrogenase

C/EBP CCAAT/enhancer binding protein

AP-1 activator protein-1

mTOR mammalian target of rapamycin

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phosphatidylinositol (PI) 3-kinase and extracellular signal-regulated kinases (ERKs) seem to be crucial for the cytoprotective effect of chronic mild hypoxia. A sole increase in SUR2A protein is sufficient to generate more sarcolemmal ATP-sensitive K^+ (K_{ATP}) channels and create a cellular phenotype resistant to acute severe oxidative stress.

MATERIALS AND METHODS

Heart H9c2 Cells and Gene Transfection

Rat embryonic heart H9c2 cells (ECACC, Salisbury, UK) were cultured in a tissue flask (at 5% CO_2) containing Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM glutamine. For some experiments in culture media NADH (20 mM), NAD (20 mM), PD 184352 (10 μM), or rapamycin (1 μM) or LY294002 (50 μM) would be added. For electrophysiological and imaging experiments, the cells were plated on a 35 × 10- or 60 × 15-mm culture dish containing 12- or 25-mm glass coverslips. The cells were cultured in incubators (Galaxy, oxygen control model, RS Biotech, Irvine, UK) where PO_2 was either 144 mm Hg (21% O_2, v/v), 100 mm Hg (13% O_2), or 20 mm Hg (3%) for 24 h before the experiment. For some experiments H9c2 cells were transfected with HIF-1α (pCMVhHIF-1α) plus HIF-1β/ARNT (pCMVhARNT) or dominant negative ARNT/HIF-1β mutant (pCMVβARNT) or with a ~1200-bp SUR2 gene promoter fragment flanking the 5′ end of SUR2 gene subcloned in TOPO-Glow vector (Invitrogen; for details see below) and then cultured for 24 h (HIF-1α/HIF-1β or HIF-1β mutant transfected cells were cultured on PO_2 = 144 mm Hg and PO_2 = 100 mm Hg, respectively, whereas promoter-transfected cells were cultured under both conditions). With HIF-1 subunits and HIF-1β dominant negative subunit, green fluorescent protein (GFP) subcloned into the mammalian expression vector pcDNA3.1$^+$ was routinely cotransfected to enable cell selection for electrophysiology. The cells were transfected with total of 1-2 μg of plasmid DNA at 60-80% confluence using Superfect reagent (Qiagen) according to the manufacturer's instructions.

Digital Epifluorescent Microscopy

H9c2 cells were superfused with Tyrode solution and loaded with the esterified form of the Ca^{2+}-sensitive fluorescent probe Fura-2 (Fura-2AM, dissolved in dimethyl sulfoxide plus pluronic acid; Molecular Probes, Eugene, OR). The cells were imaged using a digital epifluorescence imaging system coupled to an inverted microscope (Image Solutions, Standish, UK). A mercury lamp served as a source of light to excite Fura-2AM at 340 and 380 nm. Fluorescence emitted at 520 nm was captured, after crossing dichroic mirrors, by an intensified charge coupled device camera and digitized using an imaging software. An estimate of the cytosolic Ca^{2+} concentration, as a function of Fura-2 fluorescence, was calculated according to the equation: $[Ca^{2+}] = (R - R_{min}/R_{max} - R)K_d\beta$, where R is the fluorescence ratio recorded from the cell, R_{min} and R_{max} are the minimal and maximal fluorescence ratios, K_d is the dissociation constant of the dye (236 nM), and β is the ratio of minimum to maximum fluorescence at 380 nm. Hypoxia/reoxygenation was induced in the absence and presence of 100 μM HMR 1098 (Avis Pharma, Frankfurt, Germany) as follows. Single field-stimulated (30 mV, 5 ms, 0.5 Hz) cells were perfused with Tyrode solution containing 136.5 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.53 mM $MgCl_2$, 5.5 mM glucose, 5.5 mM HEPES-NaOH (pH 7.4) at a rate of 1 ml/min. Under these conditions the PO_2 in perfusate was ~140 mm Hg. For hypoxia the solution was continuously bubbled with 100% argon, whereas the exchange of O_2 between solution in the chamber and air was prevented by nitrogen jet. The PO_2 under these conditions was ~20 mm Hg. The duration of hypoxia was 10 min, followed by reoxygenation with Tyrode solution for 10 min.

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Electrophysiology

The cells were superfused with Tyrode solution (136.5 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.53 mM $MgCl_2$, 5.5 mM glucose, 5.5 mM HEPES-NaOH, pH 7.4). Pipettes (resistance 3-5 MΩ) were filled with 140 mM KCl, 1 mM $MgCl_2$, 3 mM ATP, 5 mM HEPES-KOH (pH 7.3). The recordings were made at room temperature (22 °C). During each experiment, the membrane potential was normally held at -40 mV, and the currents evoked by a series of 400-ms current steps (-100mV to +80 mV in 20-mV steps) were recorded directly to hard disk using an Axopatch-200B amplifier, Digidata-1321 interface, and pClamp8 software (Axon Instruments, Inc., Forster City, CA). The capacitance compensation was adjusted to null the additional whole cell capacitative current. The slow capacitance component measured by this procedure was used as an approximation of the cell surface area and allowed normalization of current amplitude (*i.e.* current density). The currents were low pass filtered at 2 kHz and digitized.

Immunoprecipitation and Western Blotting Analysis

Sheep antipeptide antibodies were raised against synthetic peptides comprised of residues 33-47 in the Kir6.2 protein (ARFVSKKGNCNVAHK) and residues 311-32 in the SUR2A protein (CIVQRVNETQNGTNN), conjugated to a carrier protein, keyhole limpet hemocyanin, and used for immunoprecipitation and Western blotting. To obtain the membrane fraction, H9c2 cardiac cells were homogenized in buffer I (10 mM Tris, 20 mM NaH_2PO_4, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml leupeptin, pH 7.8) and incubated for 20 min (at 4 °C). The osmolarity was restored with KCl, NaCl, and sucrose, and the obtained mixture was centrifuged at 500 × *g*. The supernatant was diluted in buffer II (30 mM imidazole, 120 mM KCl, 30 mM NaCl, 20 mM NaH_2PO_4, 250 mM sucrose, 10 μg/ml pepstatin, 10 μg/ml leupeptin, pH 6.8) and centrifuged at 7000 × *g*, the pellet was removed, and the supernatant was centrifuged at 30,000 × *g*. The obtained pellet contains membrane fraction. The protein concentration was determined using the method of Bradford. 10 μg of the epitope-specific Kir6.2 antibody or 40 μg of the epitope-specific SUR2A antibody was prebound to protein G-Sepharose beads and used to immunoprecipitate from 50 μg of membrane fraction protein extract. The pellets of this precipitation were run on SDS-polyacrylamide gels for Western analysis. Western blot probing was performed using 1:1000 dilutions of anti-SUR2A and anti-Kir6.2 antibody, respectively, and detection was achieved using protein G horseradish peroxidase and ECL reagents. Essentially the same protocol was used when Western blot was done with phospho-AP-1 (Abcam, Cambridge), phospho-c-Jun (Abcam), and phospho-C/EBP (Santa Cruz) antibodies on untransfected cells or with anti-GFP antibody (Invitrogen) on cells transfected with the GFP construct containing putative SUR2 promoter fragments (see below); just the total proteins, instead of immunoprecipitates, obtained from H9c2 cells were used, and dilutions of these antibodies were 1:300 to 1:500.

RT-PCR

Total RNA was isolated using a commercial kit (RNeasy, Mini Kit, Qiagen) according to the manufacturer's instructions. First strand cDNA was synthesized with random hexanucleotides from 1 mg of total RNA using a reverse transcription system kit (Promega, Southampton, UK). PCR was done using ReadyMix Red Tag from Sigma in a thermal cycler Model Phoenix (Helena Biosciences, Sunderland, UK) under the following conditions: for Kir6.2, 94 °C for 3 min, 34 cycles of 94 °C for 0.5 min, 66.1 °C for 0.5 min, and 70 °C for 1 min, and the final extension at 70 °C for 5 min; for SUR2A, the conditions were the same as for Kir6.2 except that number of cycles was 37-49, and the annealing temperature was 66.1 °C. Two different sets of primers were used to verify any found differences. The primers had the following sequences; for the 387-base-long product for rat Kir6.2 (primer 1), sense, 5′-ATGCGCAAGACCACCAGC-3′, and antisense, 5′-TGGCGGGCTGTGCAGAG-3′; for the 255-base-long product for rat Kir6.2 (primer 2), sense, 5′-GCACCAATGTGCCCTGCGTC-3′,

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and antisense, 5′-CGGGGTGATCACGGCATGCT-3′; for the 375-base-long product for rat SUR2A (primer 1), sense, 5′-CTAGACGCCACTGTCAC-3′, and antisense, 5′-AGAGAACGAGACACTTGG-3′; and for the 251-base-long product for rat SUR2A (primer 2), sense, 5′-GAGTGTCAGACCTGCGCTTCT-3′, and antisense, 5′-GCTGCTCAGCAGGATTGGTCTC-3′. The levels of GAPDH mRNA was also tested using human GAPDH-primers: sense, 5′-CATCACCATCTTCCAGGAGCGA-3′, and antisense, 5′-GTCTTCTGGGTGGCAGTGATGG-3′, the size of the GAPDH product was 341 bp). There were no significant differences in intensity of GAPDH levels between experimental groups. The nature of the PCR product was confirmed by DNA sequencing. These conditions were set based on our preliminary studies that have demonstrated that under these conditions intensity of the PCR product bend is ~50% of its maximum. The PCR product band intensities were analyzed using Quantiscan software.

DNA Microarray Analysis

Total RNA was isolated from cells cultured at PO_2 = 144 mm Hg and PO_2 = 100 mm Hg as described for the use of Affymetrix microarrays (8). Target RNA was prepared by converting 1 μg of RNA into double-stranded cDNA (Superscript Choice System; Invitrogen) with a T7-$(dT)_{24}$ primer incorporating a T7 RNA polymerase promoter. Biotin-labeled cRNA was synthesized from cDNA by using an RNA transcript labeling kit (Enzo Biochem). After complementary RNA had been fragmented to sizes ranging from 35 to 200 bases by heating (35 min at 95 °C), 10 μg of RNA fragments were hybridized (16 h at 45 °C) to a Rat Genome U34A array (Affymetrix, Santa Clara, CA). After hybridization, chips were automatically washed and stained with streptavidin-phycoerythrin by using a fluidics system. The chips were scanned with a Hewlett Packard GeneArray Scanner. The Affymetrix RG-U34A array contained ~7,000 rat genes and expressed ~1,000 sequence tags from UniGene, GenBank™, and the Institute for Genomic Research data bases. Each gene was presented in the array by 20 perfectly matched oligonucleotides and 20 mismatched control probes that contain a single central-base mismatch. Fluorescence intensity was read for each nucleotide to calculate the average signal intensity for each gene by subtracting the intensities of ~20 perfectly matched oligonucleotides from the intensity of the mismatched nucleotides. All of the calculations were performed using Affymetrix MAS 4.0 algorithm, *i.e.* all of the arrays were scaled to an overall target intensity of 100 prior to comparative analysis. Groups (PO_2 = 144 mm Hg and PO_2 = 100 mm Hg) were compared with each other by pair-wise comparison. Using this method, genes that were present and changed in expression by at least 1.4-fold were meant to be identified.

High Performance Liquid Chromatography

The cells were rapidly frozen, and 0.73 M trichloroacetic acid was added. The solution was then homogenized and centrifuged. The supernatant was removed and placed in tri-*n*-octylamine and FREON (50:50, v/v), vortex-mixed, and centrifuged. The supernatant was taken and used for HPLC. A NovaPak (Reading, UK) C_{18} 4-μm spherical radii bead, dimension 300 × 3.9 mm inner diameter column was used. A mobile phase consisting of 12% methanol, 1.47 mM tetrabutylammonium phosphate, 73.5 mM KH_2PO_4, adjusted to pH 4.0. The flow rate was 1.0 ml/min. NADH and NAD were detected at 254 nm (under these conditions retention times were 9.3 min for NAD and 24.0 min for NADH) using a Varien ProStar HPLC work station (Kinesis, Epping, UK).

Cloning, Subcloning, and Use of Human SUR2 Gene Promoter

For the human SUR2 promoter analysis, a fragment extending 1200 bases downwards from the translation initiation triplet was cloned from human genomic DNA (Promega). The sense primer was 5′-GACCTTTGCTCATCTCCCATC-3′, and the antisense primer was 5′-

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TTTCTTCTTATATGGTTTACTCTAA-3′; PCR was done using Promega PCR core system components. In these reactions the magnesium concentration was 1.87 mM, and the PCR mixtures were run at 94 °C for 5 min; 35 cycles of 94 °C for 45 s, 55 °C for 1 min, and 70 °C for 2 min; and a final extension of 70 °C for 5 min. The PCR product was cloned into TOPO-Glow vector using the manufacturer's instructions (Invitrogen). To create a 380-base-long fragment, a 1200-base-long fragment of putative SUR2 promoter PCR product was fragmented by restriction digestion using the internal *Bgl*II site (380 bases from the start codon). 380-bp fragment was blunt end-ligated into TOPO vector (Invitrogen). The plasmids were transiently transfected into H9c2 cells using Qiagen Superfect reagent and the manufacturer's protocol (see above), the cells were then incubated for 24 h at 37 °C (at 5% CO_2)on PO_2 = 144 mm Hg or PO_2 = 100 mm Hg, and the RNA was extracted. 1 μg of RNA was treated with 0.5 Kunitz units of DNase (Qiagen RNase-free DNase kit). DNase-treated RNA was used to make cDNA in a total volume of 20 μl. ~10% of the cDNA was used in a single PCR to analyze the promoter activity using GFP-specific primers, sense primer was 5′-GGTGATGCTACATACGGAA-3′, and the antisense primer was 5′-TACCTGTCGACACAATCTG-3′. The PCR-run conditions for GFP-primers were 94 °C for 3 min; 35 cycles of 94 °C for 0.5 min, 52 °C for 0.5 min, and 70 °C for 1 min; and a final extension of 70 °C for 5 min. The cDNA was also analyzed for the presence of the vector using promoter-specific primers. The obtained data have been analyzed as described under "RT-PCR."

Statistics

The data are presented as the means ± S.E., with *n* representing the number of experiments. The mean values between two groups were compared by the paired or unpaired Student's *t* test or Rank tests where appropriate. The results for Kir6.2 and SUR2A obtained with RT-PCR for each sample were normalized taking into account that the mean values between more then two groups were compared by the one-way or one-way Rank analysis of variance. All of the statistical tests were done using the SigmaStat program (Jandel Scientific). $p < 0.05$ was considered statistically significant.

RESULTS

Chronic Mild Hypoxia Confers Resistance to Acute Hypoxia/Reoxygenation-induced Ca^{2+} Loading in H9c2 Cells

Intracellular Ca^{2+} loading is a reliable on-line parameter of a metabolic condition in mammalian cells, including heart-derived H9c2 cells (7). Under control conditions (cells cultured at PO_2 = 140mm Hg), hypoxia/reoxygenation-induced Ca^{2+} loading in all cells tested, suggesting that this cellular phenotype is sensitive to such an insult (Fig. 1, *A* and *C*). In contrast, the same insult produced intracellular Ca^{2+} loading only in 8.3% of cells chronically exposed to PO_2 = 100 mm Hg (Fig. 1, *B* and *C*).

Chronic Mild Hypoxia Increased the Number of K_{ATP} Channels in Plasma Membrane by Regulating Expression of SUR2A Subunit

It has been previously shown that the density of K_{ATP} channels may regulate cellular resistance to oxidative stress (7,9). To assess the relative number of functional K_{ATP} channels composed of Kir6.2 and SUR2A subunits (7,10) that are present in plasmalemma, H9c2 membrane fraction was immunoprecipitated with anti-Kir6.2 antibody and probed with the anti-SUR2A antibody and vice versa (immunoprecipitated with anti-SUR2A antibody and probed with anti-Kir6.2 antibody; using this approach only those subunits physically associated with each other were measured) (7,9). This strategy revealed more than 2-fold higher levels of Kir6.2 and SUR2A proteins in cells cultured at mild hypoxia compared with those cultured at normoxia (Fig. 2; band density, for Kir6.2 11 ± 3 and 19 ± 2 under control conditions and hypoxia, respectively, and for SUR2A 18 ± 2 and 35 ± 5 under control conditions and hypoxia,

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respectively; $p < 0.01$ in both cases; $n = 5$ for each), although at the same time no significant changes were observed in amounts of secondary antibody heavy chain (for Kir6.2 21.6 ± 2.5 and 20.6 ± 3.2 under control conditions and hypoxia, respectively, and for SUR2A 16.2 ± 1.1 and 16.8 ± 1 under control conditions and hypoxia, respectively; $p = 0.59$ and 0.37 for Kir6.2 and SUR2A, respectively; $n = 5$ for each). To determine whether changes in the transcriptional activity of Kir6.2 and SUR2 genes underlie changes in number of plasmalemmal K_{ATP} channels, we measured Kir6.2 and SUR2A mRNAs using RT-PCR. We designed two separate sets of primers (see the methods), and we tested whether the primers that we designed and RT-PCR could detect differences in mRNA levels. Therefore, we applied RT-PCR with two sets of Kir6.2 and SUR2A primers on slightly different amounts of DNA template using the same number of cycles. These experiments have demonstrated that the primers and conditions we used are capable of detecting less then 2-fold differences in mRNA (Fig. 3) and that >30 amplification cycles used in this study would not lose difference in initial message. Thus, RT-PCR analysis with two different sets of primers for each subunit demonstrated that levels of Kir6.2 mRNA did not change by mild hypoxia (Fig. 4, *B* and *D*). In contrast, >2-fold higher levels of SUR2A mRNA in cells exposed to PO_2 = 100 mm Hg relative to those exposed to PO_2 = 144 mm Hg were found (Fig. 4, *A* and *C*). The applied degree of hypoxia did not affect levels of GAPDH mRNA (Fig. 4*E*).

SUR2 Gene Promoter Is Regulated by Chronic Mild Hypoxia

To examine whether a slight decrease of PO_2 would activate transcription of SUR2A, we cloned the putative human SUR2 gene promoter and measured the promoter-driven expression of a reporter gene (GFP). H9c2 cells were transfected with the 1200-bp fragment of the putative human SUR2 promoter subcloned into the GFP promoterless vector. Under these conditions the transcription of GFP was directly dependent upon activity of SUR2 promoter. RT-PCR analysis demonstrated a >2-fold higher level of GFP mRNA in cells cultured at PO_2 = 100 mm Hg compared with those cultured at PO_2 = 144 mm Hg (Fig. 5, *A* and *B*). No difference was observed between GAPDH levels in transfected cells irrespective of PO_2 (Fig. 5*C*). A computer-assisted search using putative human SUR2 gene promoter sequence revealed the presence of binding sites for CCAAT/enhancer binding protein (C/EBP) and activator protein-1 (AP-1) transcription factors. Mild hypoxia increased phosphorylation of AP-1 transcription factors and in particular c-Jun, whereas phosphorylation of C/EBP was not increased (Fig. 5*D*). The obtained RT-PCR results with promoter were further confirmed at the level of expressed GFP protein. In H9c2 cells transfected with GFP promoterless vector, no signal for GFP on a Western blot using anti-GFP antibody was visualized (Fig. 5*E*). On the other hand, when constructs containing a 1200- or 380-bp putative promoter fragment were introduced into H9c2 cells, GFP was detected in total protein extract (Fig. 5*E*). Exposure to mild hypoxia increased the level of expressed GFP regardless of whether cells were transfected with the 1200- or 380-bp putative promoter fragment (Fig. 5*E*).

Chronic Mild Hypoxia-induced Increase in Number of Sarcolemmal K_{ATP} Channels Is Independent on HIF-1α

It is well established that chronic hypoxia regulates genes expression primarily via HIF-1 (11). To determine whether HIF-1 mediates mild hypoxia-induced increase in the number of K_{ATP} channels, we employed patch clamp electrophysiology on untransfected and transfected H9c2 cells exposed to different oxygen tensions. Pinacidil, a prototype K_{ATP} channel opener, induces whole cell K^+ current proportionally to the number of K_{ATP} channels in membrane (7,9,12). Pinacidil-sensitive component of current was approximately two times higher in cells cultured at PO_2 = 100 mm Hg compared with those at PO_2 = 144 mm Hg (Fig. 6). It has been reported that overexpression of HIF-1α/HIF-1β activates HIF-1-mediated signaling even under normoxic conditions (13). In cells cotransfected with HIF-1α/HIF-1β and cultured in normoxia (PO_2 = 144 mm Hg), the pinacidil-sensitive component of the whole cell K^+ current did not

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differ when compared with untransfected cells exposed to the same PO_2 (Fig. 7*A*). In addition, transfection of the HIF-1β dominant negative mutant did not interfere with increase in pinacidil-sensitive K^+ current component induced by PO_2 = 100 mm Hg (Fig. 7*A*). Cadmium, in low concentrations, triggers a redox/proteasome-dependent degradation of HIF-1α protein, reducing HIF-1 activity and suppressing the hypoxic induction of hypoxia-inducible genes (14). The addition of cadmium (5 μM) did not change the effect of PO_2 = 100 mm Hg on SUR2A mRNA levels (Fig. 7*B*).

Chronic Mild Hypoxia-mediated Increase in Number of K_{ATP} Channels Depends on NADH/NAD Ratio and MKK1 Signaling Pathway

One of the main features of cells exposed to mild hypoxia was the ~3-fold increase in the NADH/NAD ratio as revealed by HPLC (data not shown). The addition of 20 mM NADH in cell culture media inhibited hypoxia-mediated increase in sarcolemmal K_{ATP} channel proteins (Fig. 8*A*). The inhibitory effect of NADH was not observed in cells kept at higher grade of hypoxia (PO_2 = 20 mm Hg) (Fig. 8*A*). Adding 20 mM NAD to culture media increased the level of sarcolemmal K_{ATP} channel proteins in cells cultured under normoxic conditions (Fig. 8*A*). To assess the involvement of major cytoprotective pathways signaling in mild hypoxia-mediated effects, we tested the involvement of PI 3-kinase, mitogen-activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR) (15-17). Rapamycin (1 μM), a selective inhibitor of mTOR (18), did not affect the PO_2-mediated regulation of K_{ATP} channels (Fig. 8*B*). In contrast, LY 294002 (50 μM) and PD 184 352 (10 μM), inhibitors of PI 3-kinase and MAPK-kinase (MKK1) (18), prevented an increase in SUR2A subunit induced by chronic exposure of H9c2 cells to PO_2 = 100 mm Hg (Fig. 8*B*).

Chronic Mild Hypoxia-mediated Increase in Cellular Resistance to Acute Hypoxia/Reoxygenation Seems to Be Mediated Solely by Increase in Sarcolemmal K_{ATP} Channel Levels

If the increased resistance toward acute hypoxia/reoxygenation is primarily due to an increased number in sarcolemmal K_{ATP} channels, an antagonist of these channels opening should inhibit mild hypoxia-induced cytoprotection. We tested the effect of HMR 1098, a compound that specifically targets K_{ATP} channel subunits expressed in sarcolemma (7,19). In the presence of HMR 1098 (100 μM), 84% of cells chronically exposed to normoxia responded to acute hypoxia/reoxygenation with intracellular Ca^{2+} loading (Fig. 9, *A* and *C*). This was not significantly different from cells maintained under mild hypoxia and exposed to the same acute challenge in the presence of HMR 1098 (p = 0.41; hypoxia/reoxygenation induced Ca^{2+} loading in 65% of these cells; Fig. 9, *B* and *C*). At the same time, the presence of 100 μM HMR 1098 abolished the cytoprotective effect of chronic mild hypoxia (from only 8 to 65% of cells affected by hypoxia/reoxygenation in the absence and presence of HMR 1098, respectively; p = 0.009; see Figs. 1 and 9). To test whether expression of other genes, apart of SUR2A, is affected by mild hypoxia, we performed DNA microarray analysis using Affymetrix microarrays (8). Comparison of hybridization patterns from the cells exposed to PO_2 = 144 mm Hg and PO_2 = 100 mm Hg did not reveal any differences in gene expression between the two groups, including differences in genes known to participate in cellular stress response and/or cardio/cytoprotection (data not shown).

DISCUSSION

The present study demonstrates that a mild decrease in PO_2 confers resistance in heart-derived H9c2 cells against severe oxidative stress by activating an HIF-1α-independent but PI 3-kinase- and MKK1-dependent pathway, leading to the phosphorylation of c-Jun/AP-1, which up-regulates SUR2A subunit without affecting the expression of any other stress-responsive gene.

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A sole increase of SUR2A subunit protein is sufficient to increase the number of K_{ATP} channels in sarcolemma, generating a phenotype more resistant to metabolic injury.

Both in clinical and basic science reports, it has been suggested that chronic exposure to mildly decreased PO_2 stimulates cardiac resistance to severe oxidative stress (3-5). To reproduce this phenomenon at the single cell level, we chronically exposed H9c2 cells to mild hypoxia and tested their resistance to acute hypoxia/reoxygenation. H9c2 cells are derived from embryonic mouse heart and are generally accepted to be a good model for cardiomyocytes (6,7). Studies in both cardiomyocytes and H9c2 cells have established that hypoxia/reoxygenation induces intracellular Ca^{2+} loading, which represents a major indicator of the degree of cell injury (7, 9). In the present study, cells maintained at PO_2 = 100 mm Hg acquired resistance to hypoxia/reoxygenation, suggesting that mild hypoxia created a phenotype resistant to severe oxidative stress independently of neuronal, vascular, and hormonal outside-of-cell influences.

It has been previously shown that the opening of K_{ATP} channels protects H9c2 cells/cardiomyocytes against severe metabolic stress (20-22). In sarcolemma, Kir6.2, a pore-forming K_{ATP} channel subunit, and SUR2A, a regulatory subunit, physically associate to form K_{ATP} channels (10,23,24). We hypothesize that chronic mild hypoxia may affect the levels of K_{ATP} channel subunits. To secure measuring of only those subunits forming the channel, we immunoprecipitated from a membrane fraction using anti-Kir6.2 antibody and probed the precipitate with anti-SUR2A antibody and vice versa. Both Kir6.2 and SUR2A subunits were found in significantly higher levels in membrane fraction from mild hypoxia-cultured than from normoxia-cultured cells, suggesting that a slight decrease in PO_2 increases the density of K_{ATP} channels in the sarcolemma of H9c2 cells.

RT-PCR with two independent sets of primers demonstrated that PO_2 = 100 mm Hg increased the levels of SUR2A mRNA but not Kir6.2 mRNA. Both in adult cardiomyocytes and H9c2 cells, the level of Kir6.2 is in excess over the level of SUR2A subunit (7,9,12). The biological consequence of this disproportion is that the number of sarcolemmal K_{ATP} channels is primarily controlled by the levels of SUR2A (7,9,12). RT-PCR results suggested that transcriptional control of SUR2A is associated with a mild hypoxia-induced increase in the density of K_{ATP} channels. To further test this hypothesis, we cloned the human SUR2 gene promoter to test whether mild hypoxia would affect its activity. The obtained results showed that chronic exposure to PO_2 = 100 mm Hg does increase the activity of SUR2 gene promoter, suggesting transcriptional regulation of SUR2 gene by mild hypoxia. Examination of the sequence of 1200 bp of the 5′-flanking putative SUR2 promoter region showed that there are binding sites for AP-1 and C/EBP, transcription factors known to be involved in intracellular signaling in the heart (25). The AP-1 family of transcription factors, consisting of Jun, Fos, ATF, and Maf as well as Nrl proteins, are important regulators of immediate-early signals directing cellular proliferation, survival, differentiation, and environmental stress response (26). The proto-oncogene c-*jun* encodes a major component of AP-1 transcription factors, and it has been previously shown to be activated during low oxygen conditions in different cell types including cardiomyocytes (27,28). On the other hand, C/EBP has been also recently implicated in hypoxia-mediated regulation of gene expression (29). Because the phosphorylation state of c-*jun* and C/EBP is the primary determinant of their activity, we used phospho-specific antibodies against these transcription factors. The obtained results, that mild hypoxia activated c-*jun*/AP-1 but not C/EBP, suggest that the increase in SUR2A mRNA is due to activation of SUR2 promoter by c-*jun*/AP-1, which provides further evidence that transcriptional regulation of SUR2 gene underlies increase in number of sarcolemmal K_{ATP} channels.

It is well established that the HIF-1 signaling system transduces chronic hypoxia-mediated regulation of genes expression (30). In addition, it has been recently reported that the response

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of c-*jun*/AP-1 to chronic hypoxia is HIF-1α-dependent (28), and this, as well as the nature of the challenge itself, prompted us to consider the involvement of HIF-1α-mediated signaling in the effect of mild hypoxia. We have previously shown that an increase in sarcolemmal K_{ATP} channels density results in an increase in whole cell K^+ current induced by pinacidil, a K_{ATP} channel opener (7,9,12). In the present study, the magnitude of the response to pinacidil was increased in cells cultured at PO_2 = 100 mm Hg compared with those cultured on PO_2 = 144 mm Hg, further confirming that mild hypoxia increases the density of K_{ATP} channels in H9c2 cell. Our findings that overexpression of HIF-1α/HIF-1β did not change the number of K_{ATP} channels in cells in normoxia and that dominant negative HIF-1β as well as cadmium, an agent that induces degradation of HIF-1α protein (14), did not change the effect of mild hypoxia suggest that a HIF-1 signaling pathway was not involved in the effect of mild hypoxia. It should be also noted that GAPDH, an enzyme known to be up-regulated by hypoxia and HIF-1α (31), was not affected by a small decrease in PO_2 in our study. This suggests that hypoxia-responsive elements were not activated by mild hypoxia, and this would be in accord with the recent report demonstrating that decrease in PO_2 for more then 80 mm Hg is required to activate HIF-1 signaling in the heart (32).

Even under mild hypoxia, we found that intracellular NAD/NADH ratio increased ~3-fold, confirming that this parameter may serve as very sensitive oxygen sensor (see also Ref. 33). Taking together that both NADH/NAD and K_{ATP} channels levels are changed with slight decrease in PO_2, we hypothesize that changes in the NADH/NAD ratio may be a part of the signaling transduction pathway controlling the expression of the SUR2A subunit. There is evidence that NAD and NADH may cross the membranes and alter the NAD/NADH ratio (34-36). Indeed, the addition of NADH prevented a mild hypoxia-induced increase in K_{ATP} channels density, whereas NAD alone mimicked the effect of mild hypoxia, suggesting that the NAD/NADH ratio is crucial for the regulation of SUR2A expression. It should also be noted that the increase of K_{ATP} channels induced by a higher degree of hypoxia was not inhibited by NADH, implying that NAD/NADH is particularly important to activate the signaling cascade that specifically sense slight/mild changes in PO_2. It has been recently proposed that signaling pathways involving protein kinases such as the MAPK family members, may be activated by NADH/NAD (37,38). At the same time, among the protein kinases that target c-*jun*/AP-1 *in vivo*, the MAPK, stress-activated protein kinases/c-Jun N-terminal kinases, and ERKs are activated by hypoxia (39). Here, experiments with selective inhibitors of different kinases (18) suggested that PI 3-kinase (sensitive to LY 294002) and MAPK kinase/ERKs (MEK1/EERKs; sensitive to 184 352) are involved mild hypoxia-induced signaling, whereas this was not the case for mTOR. A finding that mTOR was not involved in up-regulation of SUR2A subunit was not surprising from a perspective that mTOR-mediated response to hypoxia is HIF-1-dependent (40). Thus, a lack of mTOR involvement would be evidence more to support the idea that HIF-1 does not contribute to the effect of PO_2 we observed. On the other hand, the involvement of PI 3-kinase has been recently reported to regulate expression of hypoxia marker MN/carbonic anhydrase IX in carcinoma and sarcoma cell lines exposed to moderate hypoxia in HIF-1-independent manner (41), thus supporting our notion that a slight decrease of PO_2 may activate PI 3-kinase without affecting HIF-1 signaling pathways. In accord with our results is also a recent report that insulin-like growth factor-I induces antiapoptotic signaling in cardiomyocytes that involves activation of both PI 3-kinase and MEK1/ERKs (42). Even more interestingly, it has been recently reported that up-regulation of c-*jun* mRNA in cardiomyocytes requires activation of the MEK1/ERKs cascade (43), which seems to be the cascade involved in the regulation of SUR2A subunit and K_{ATP} channel levels we describe in this study. Taking everything together, the obtained data suggest that mild hypoxia induces moderate changes in the NADH/NAD ratio and activates PI 3-kinase- and MEK1/ERK-dependent signaling, without involving HIF-1, which may up-regulate c-*jun* to target SUR2 promoter to increase the level of SUR2A protein and stimulate formation of sarcolemmal K_{ATP} channels.

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If our conclusion is right that an increase in K_{ATP} channel density is the only/main event involved in generation of cellular phenotype resistant to oxidative stress, then inhibition of the channel activation should inhibit the protective effect of mild hypoxia. HMR 1098, an antagonist that selectively blocks the Kir6.2/SUR2A subtype of the K_{ATP} channel (19), abolished resistance of H9c2 cells to acute hypoxia/reoxygenation. In the presence of this antagonist no difference in response to hypoxia/reoxygenation was observed between cells cultured under control conditions and under mild hypoxia. These results clearly demonstrate a direct link between increased numbers of K_{ATP} channels and increased resistance to oxidative stress. This conclusion is further supported by our microarray analysis revealing that mild hypoxia does not change the expression of ~7,000 genes, including genes encoding proteins involved in stress response and cytoprotection, probes of which were present on Affymetrix gene chips. A probe specific for SUR2A mRNA was absent from this chip as opposed to Kir6.2-specific probe, which was present on the chip, and the obtained results were further confirmation of the constancy of Kir6.2 mRNA. Thus, based on these results, it seems that an increase in the number of sarcolemmal K_{ATP} channels is the sole factor responsible for chronic mild hypoxia-induced cytoprotection.

The present study explains how chronic slightly lower oxygen tension leads to increased resistance to oxidative stress. This may have long term consequences on numerous aspects of cellular function, and it may influence the outcome of different physiological and pathophysiological processes. As an example, the present study could explain the mechanism underlying the low mortality rate of myocardial infarction and longer life in populations living at high altitudes (44). It seems that a small decrease in PO_2, in a magnitude that does not activate hypoxia and cell stress signaling, selectively induces the SUR2 gene promoter, leading to higher levels of SUR2A protein, more sarcolemmal K_{ATP} channels, and a cellular phenotype more resistant to hypoxia/ischemia-reperfusion/reoxygenation injury because of more efficient regulation of membrane potential and Ca^{2+} homeostasis (22,45-47). Therefore, this study would suggest that: 1) regulation of SUR2A levels as a therapeutic strategy against myocardial infarction deserves to be seriously considered and 2) there is a signaling pathway activated by a small decrease in PO_2 that selectively targets the SUR2 gene promoter that should be identified and exploited as a means to induce SUR2A expression.

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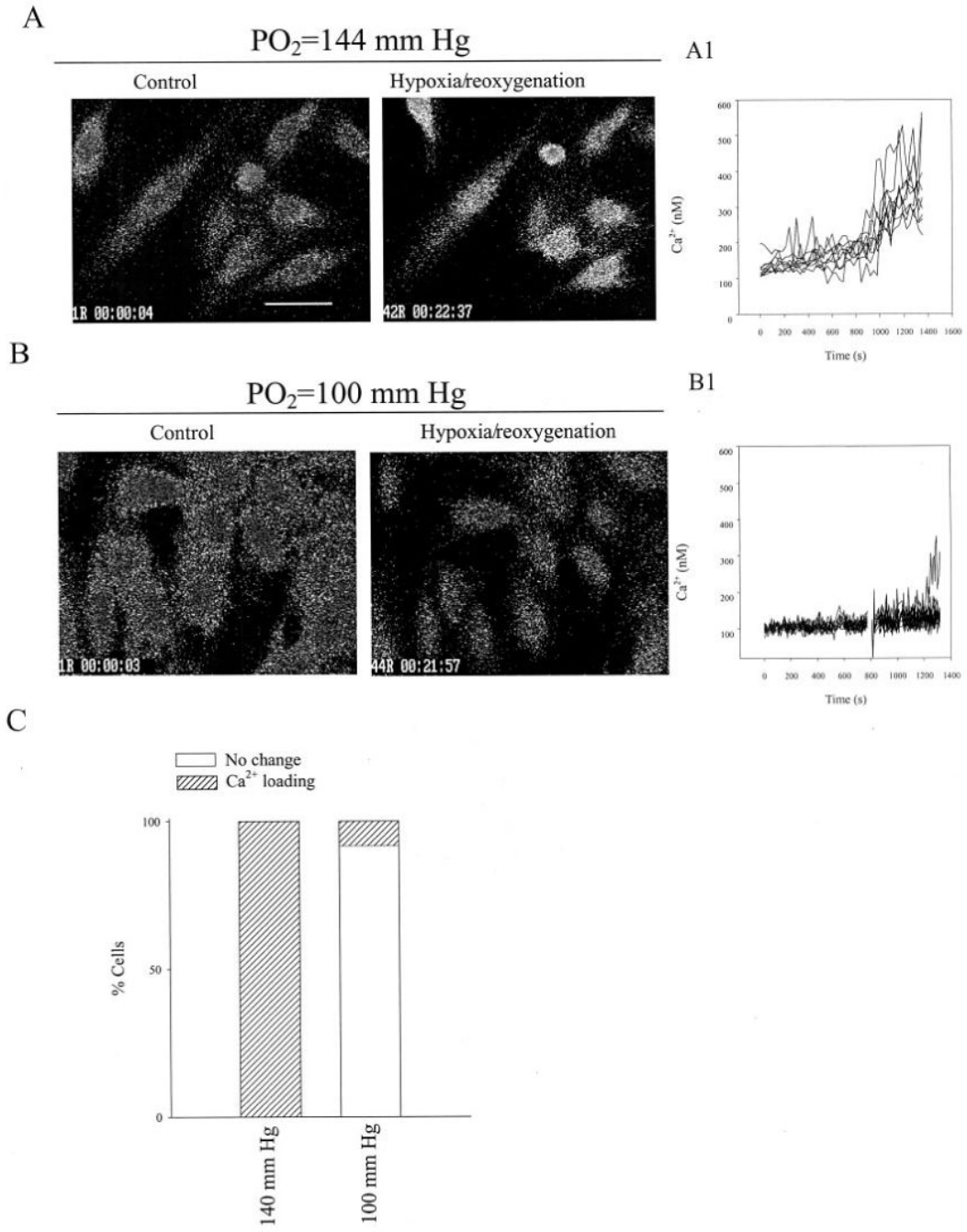

Fig. 1. Chronic mild hypoxia confers resistance to acute hypoxia/reoxygenation in H9c2 cells

A and *B*, epifluorescent digital images of cells cultured at PO_2 = 144 mm Hg (*A*) and PO_2 = 100 mm Hg (*B*) loaded with Fura-2 prior (*Control*) and following hypoxia/reoxygenation (*Hypoxia/reoxygenation*). The *white bar* corresponds to 30 μm. The *graphs* are time courses of intracellular concentration of Ca^{2+} (*A1* and *B1* correspond to *A* and *B*, respectively). Each line on the graphs represents a single cell from the corresponding image field. *C*, percentage of cells cultured at PO_2 = 144 mm Hg and PO_2 = 100 mm Hg that responded (defined as 50% increase in resting Ca^{2+} with the time course pattern of Ca^{2+} increase as depicted in *A*) or did not respond to hypoxia/reoxygenation with Ca^{2+} loading (n = 12-24). *, p = 0.0006.

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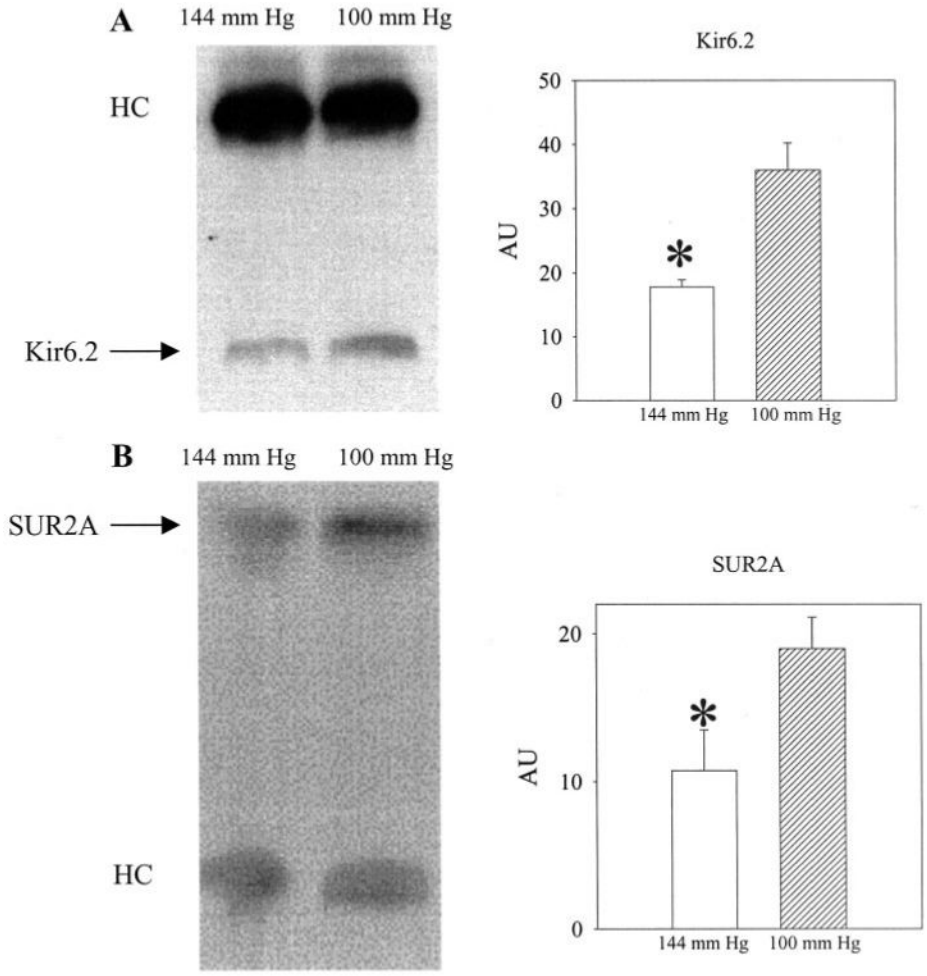

Fig. 2. Chronic mild hypoxia induces increase in sarcolemmal K_{ATP} channels

Shown are Western blots and corresponding graphs with anti-Kir6.2 (*A*) and anti-SUR2A (*B*) antibodies of anti-SUR2A (*A*) and anti-Kir6.2 (*B*) immunoprecipitate from membrane fractions from H9c2 cells cultured at PO_2 = 144mm Hg and PO_2 = 100 mm Hg. The blots were cross-probed (anti-Kir6.2 antibody was used on anti-SUR2A immunoprecipitate, and anti-SUR2A antibody was used on anti-Kir6.2 immunoprecipitate). Each *bar* represents the mean ± S.E. of the mean (n = 5 for each). *, $p < 0.05$. *HC*, heavy chain.

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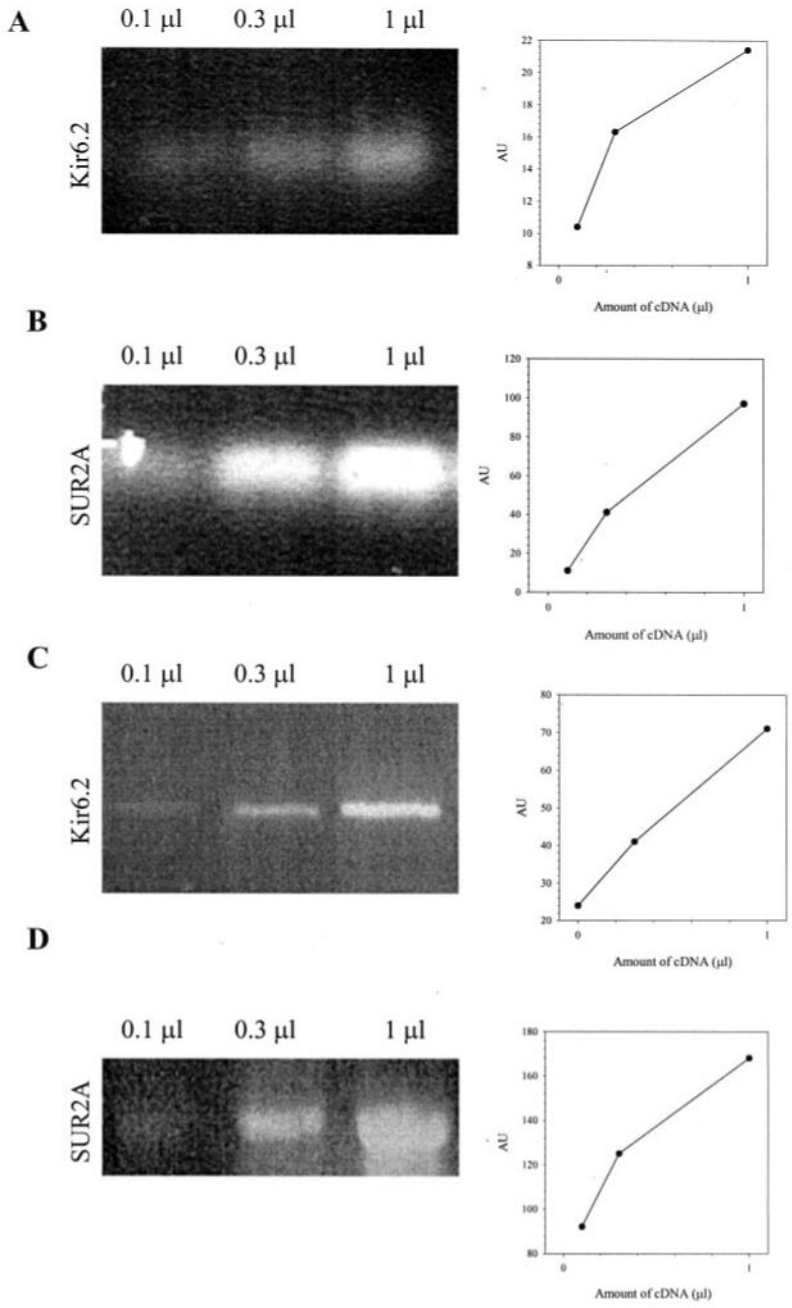

Fig. 3. RT-PCR detects relatively small initial Kir6.2 and SUR2A mRNA differences

Shown are the RT-PCR products obtained with two different, independent sets of Kir6.2- and SUR2A-specific primers on H9c2 cells using different dilutions of the same cDNA pool (*left panels*) and corresponding graphs (*right panels*) showing amount-bend intensity relationship.

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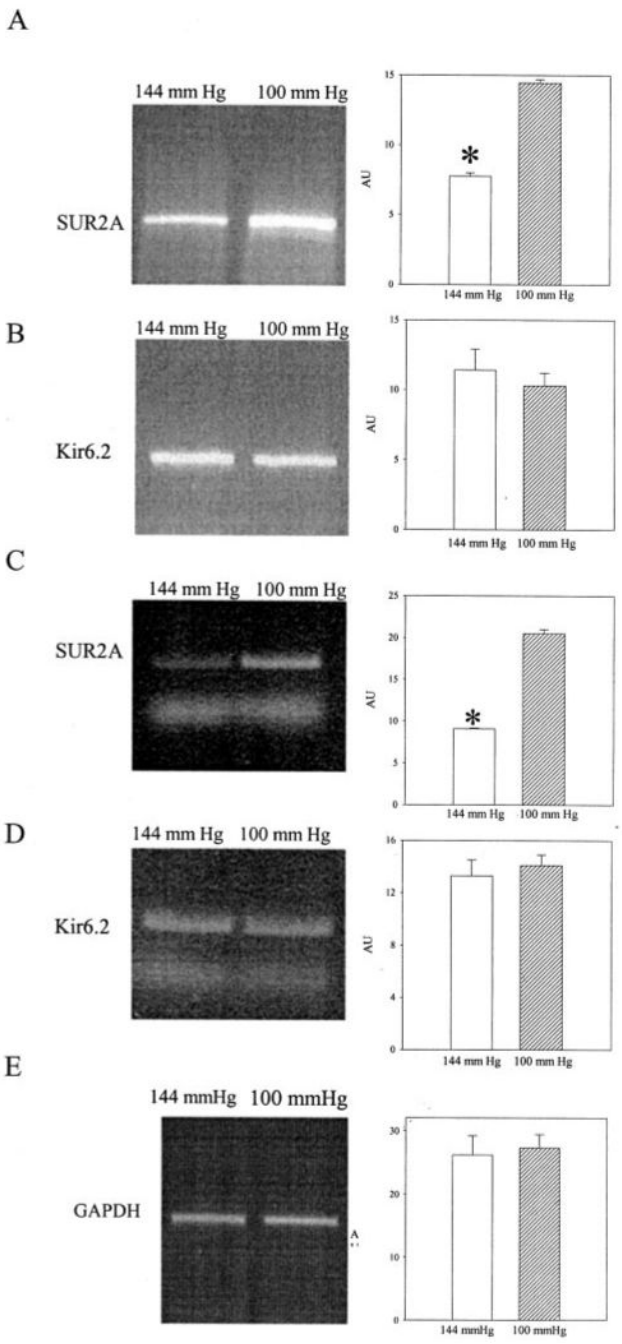

Fig. 4. Chronic mild hypoxia-induced increase in sarcolemmal K_{ATP} channels is solely mediated by increase in SUR2A channel subunit

A-D, RT-PCR products and corresponding graphs obtained with two different sets of Kir6.2-specific (*B* and *D*) and SUR2A-specific (*A* and *C*) primers from H9c2 cells cultured at PO_2 = 144 mm Hg and PO_2 = 100 mm Hg. *E*, RT-PCR products and corresponding graph obtained with GAPDH-specific primers from H9c2 cells cultured at PO_2 = 144 mm Hg and PO_2 = 100 mm Hg. Each *bar* represents the mean ± S.E. (n = 2-4). *, $p < 0.05$.

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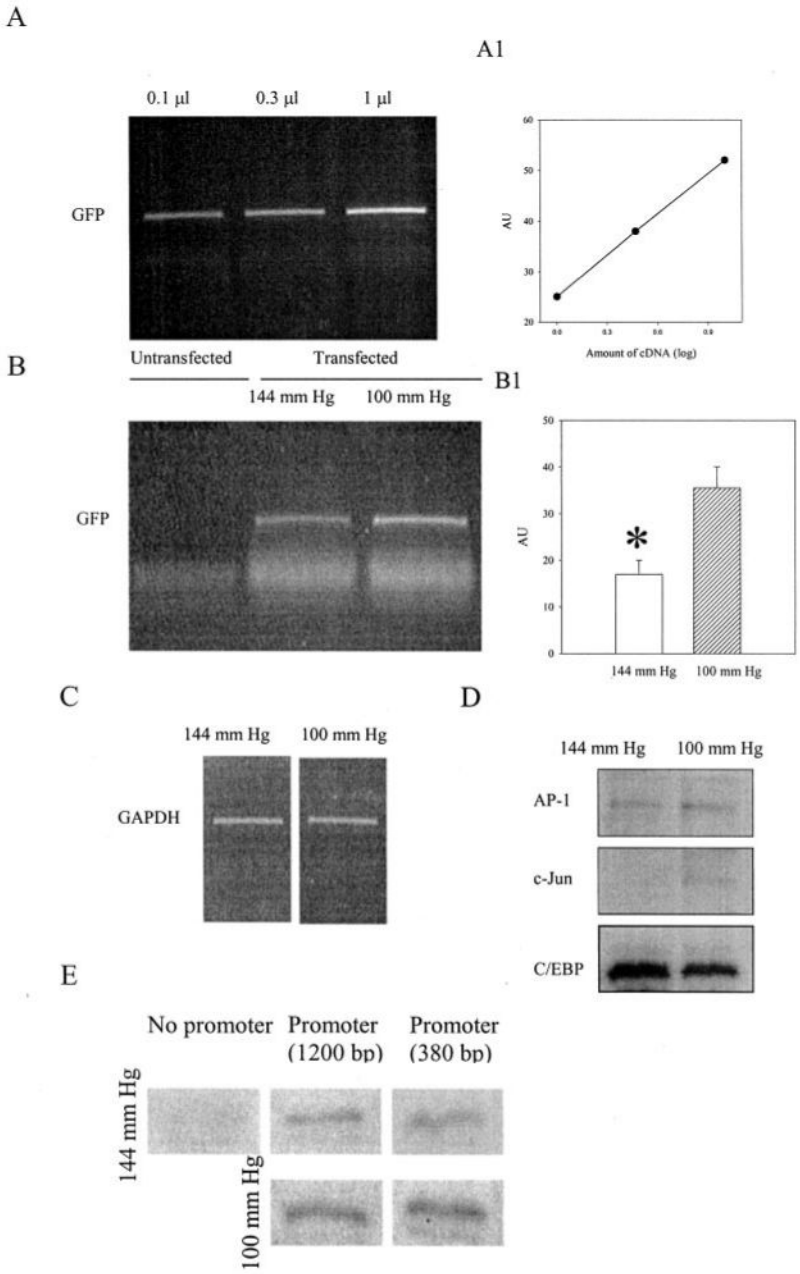

Fig. 5. Chronic mild hypoxia regulates activity of human SUR2 promoter

A and *B*, RT-PCR products and corresponding graphs obtained with GFP-specific primers from H9c2 cells transfected with SUR2 promoter-GFP gene using different amounts of cDNA (*A* and *A1*) or with GFP-specific primers from untransfected H9c2 cells and cells transfected with SUR2 promoter-GFP gene cultured at PO_2 = 144 mm Hg and PO_2 = 100 mm Hg (*B* and *B1*). Each *bar*/*point* represents the mean ± S.E. (n = 2-3). *, $p < 0.05$. *C*, RT-PCR products obtained with GAPDH-specific primers from transfected H9c2 cells cultured at PO_2 = 144 mm Hg and PO_2 = 100 mm Hg. *D*, Western blots with general anti-phospho-AP-1 (*AP-1*), anti-phospho-c-*jun* (*c-Jun*), and anti-phospho-C/EBP (*C/EBP*) antibodies of total proteins from H9c2 cells cultured at PO_2 = 144 mm Hg and PO_2 = 100 mm Hg. *E*, Western blots with anti-GFP antibody of total proteins from H9c2 cells transfected with promotorless TOPO-Glow vector (*No*

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promoter) TOPO-Glow vector containing 1200 (*Promoter (1200 bp)*) and 380 (*Promoter (380 bp)*) bases cultured at PO_2 = 144 mm Hg and PO_2 = 100 mm Hg.

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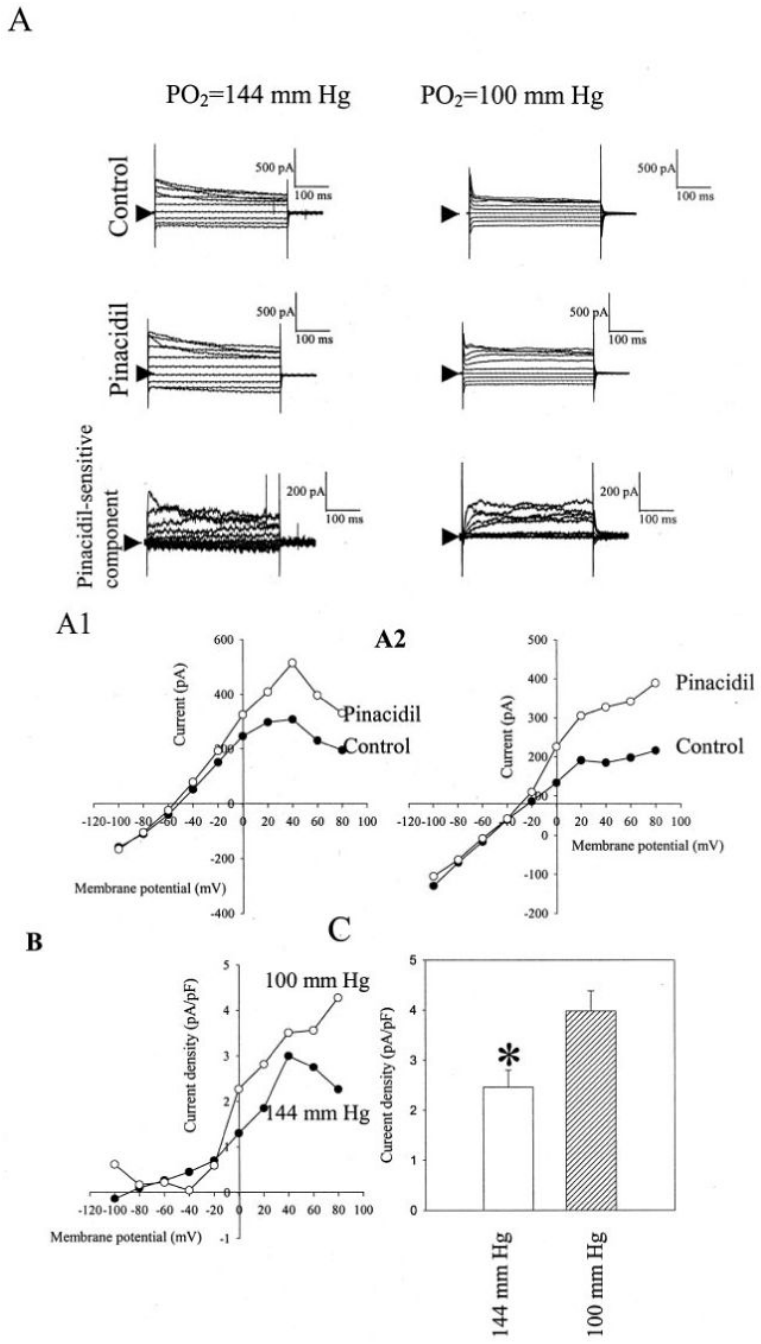

Fig. 6. Chronic mild hypoxia increases pinacidil-induced whole cell membrane current

A, membrane currents evoked by identical families of 400-ms voltage pulses in cells that were first maintained under control conditions and then exposed to 100 μ_M pinacidil for 2 min and in cells cultured at PO_2 = 144mm Hg and PO_2 = 100 mm Hg. *A1* and *A2*, current-voltage relationships for conditions in *A* (graphs are aligned with corresponding experiments above). The pinacidil-sensitive component of current (*B*) for cells in *A* and current density (*C*) at 80 mV. Each *bar* represents the mean ± S.E. (n = 7 for each). *, $p < 0.05$. The *arrowheads* indicate zero current levels.

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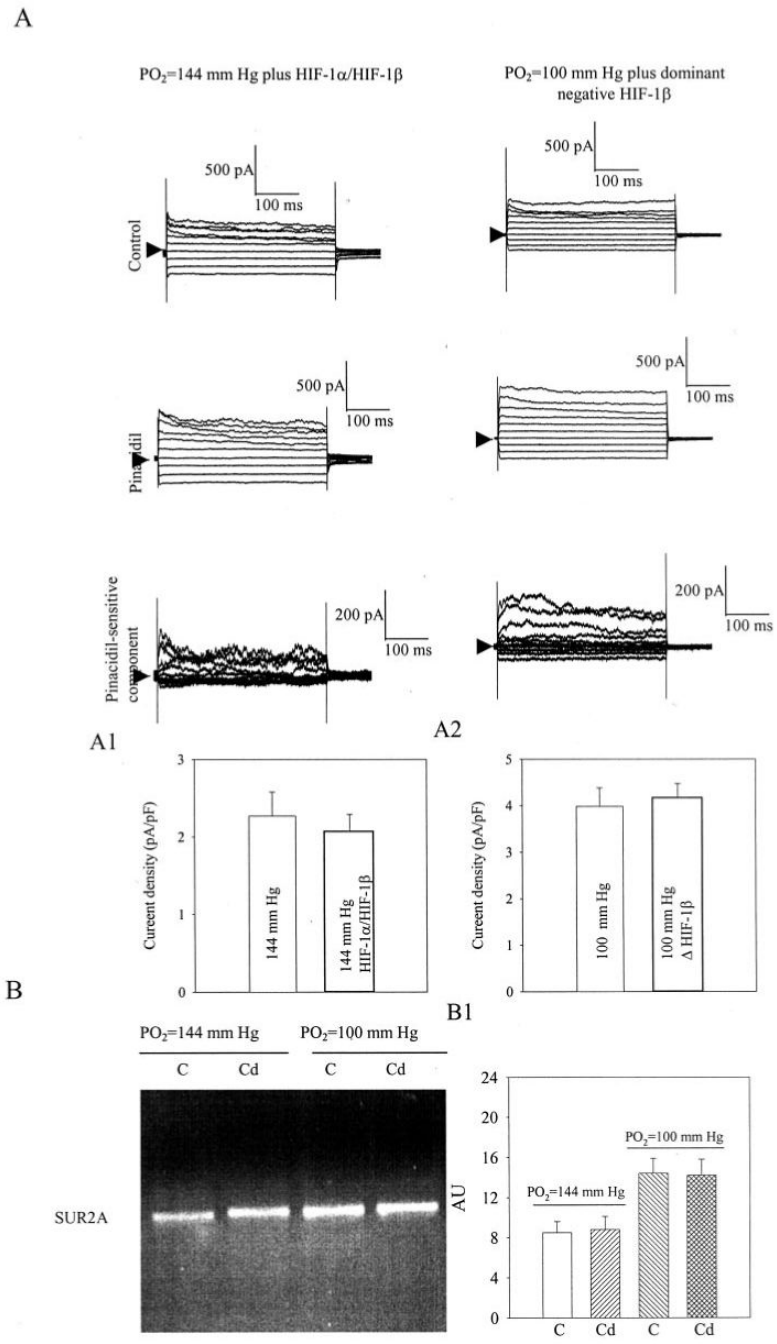

Fig. 7. Chronic mild hypoxia-mediated increase in K_{ATP} channels is not mediated by HIF-1

A, membrane currents evoked by identical families of 400-ms voltage pulses in cells that were first maintained under control conditions and then exposed to 100 μ_M pinacidil for 2 min, in cells transfected with HIF-1α/HIF-1β and cultured at PO_2 = 144 mm Hg, and in cells transfected with dominant negative HIF-1β cultured at PO_2 = 100 mm Hg. *A1* and *A2*, current density at 80 mV of pinacidil-sensitive component of current for cells in *A* (*graphs* are aligned with corresponding experiments above). Each *bar* represents the mean ± S.E. (n = 7 for each). The *arrowheads* indicate zero current levels. *B*, RT-PCR products obtained with SUR2A-specific primers from H9c2 cells cultured at PO_2 = 144 mm Hg and PO_2 = 100 mm Hg in the absence

(*C lanes*) and presence of 5 μ_M cadmium (*Cd lanes*). *B1*, graph corresponding to RT-PCR products depicted in *B*. Each *bar* represents the mean ± S.E. (n = 3).

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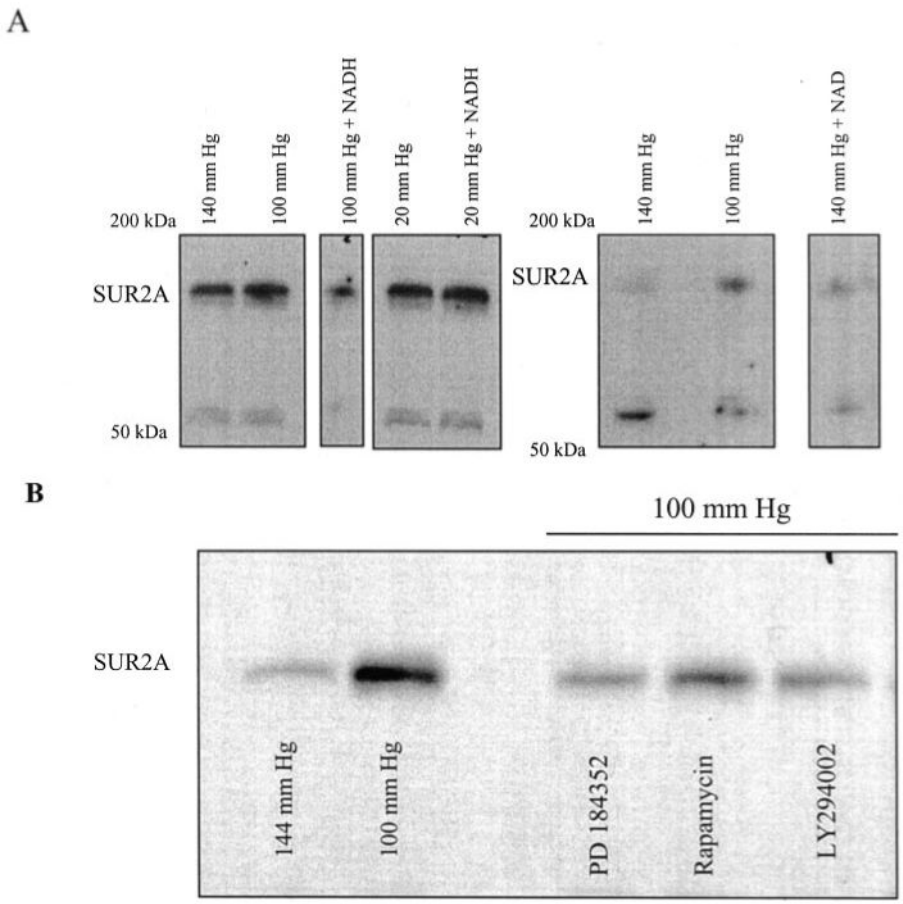

Fig. 8. Changes in NAD/NADH ratio and activation of PI 3-kinase and ERK are involved in the effect of chronic mild hypoxia

Shown are typical Western blots of anti-Kir6.2 immunoprecipitates from membrane fraction obtained from H9c2 cells and probed with anti-SUR2A antibody under depicted conditions (concentrations used were 20 mM NADH, 20 mM NAD, 10 μM PD 184352, 1 μM rapamycin, and 50 μM LY294002).

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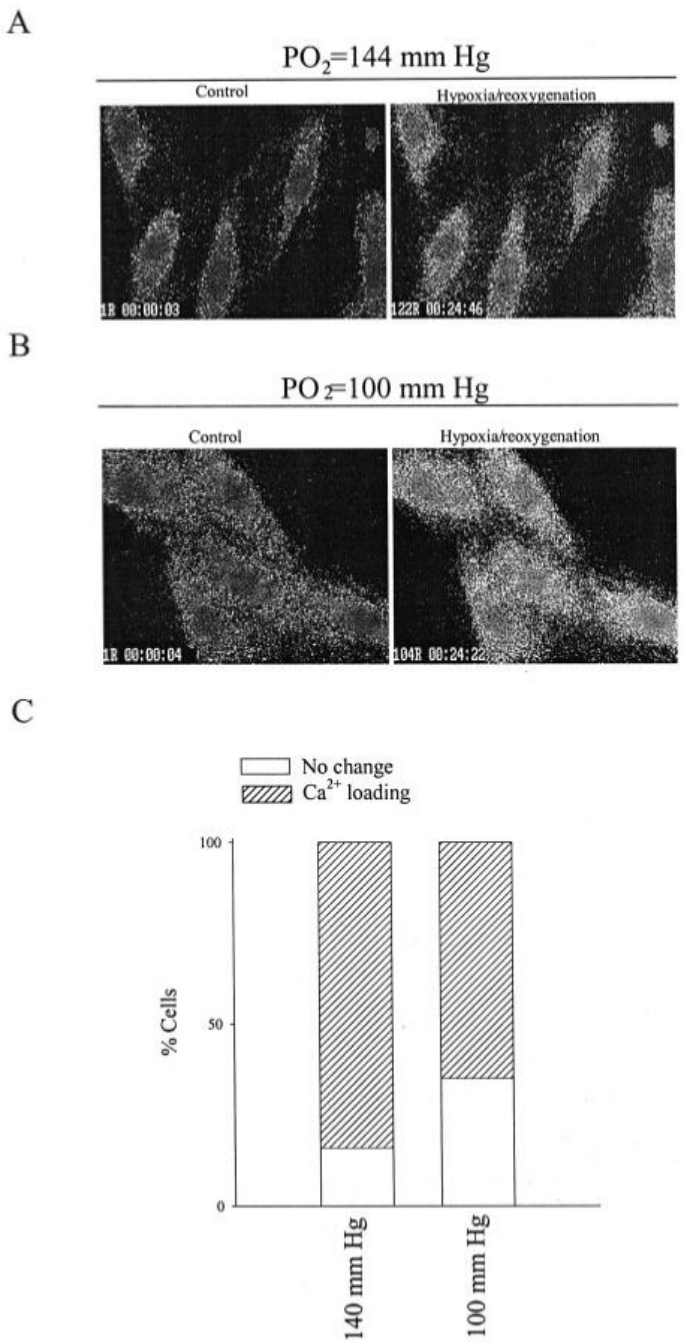

Fig. 9. Chronic mild hypoxia protects H9c2 cells against acute hypoxia/reoxygenation by K_{ATP} channel-dependent mechanism

Shown are epifluorescent digital images of cells cultured at PO_2 = 144 mm Hg (*A*) and PO_2 = 100 mm Hg (*B*) loaded with Fura-2 prior (control), and following hypoxia/reoxygenation in the presence of HMR 1098 (100 μ_M), a selective antagonist of sarcolemmal K_{ATP} channels. Magnification was ×40. *C*, percentage of cells cultured at PO_2 = 144 mm Hg and PO_2 = 100 mm Hg that responded (defined as 50% increase in resting Ca^{2+} with the time course pattern of Ca^{2+} increase as depicted in *A*) or did not respond to hypoxia/reoxygenation with Ca^{2+} loading in the presence of HMR 1098 (*n* = 12-21).