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Impact of hypoxia, simulated ischemia and reperfusion in HL-1 cells on the expression of FKBP12/FKBP12.6 and intracellular calcium dynamics

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ABSTRACT

Aims: To establish a cardiac cell culture model for simulated ischemia and reperfusion and in this model investigate the impact of simulated ischemia and reperfusion on expression of the calcium handling proteins FKBP12 and FKBP12.6, and intracellular calcium dynamics.
Methods: HL-1 cell cultures were exposed to normoxia (as control), hypoxia, simulated ischemia (HEDA) or HEDA + reactive oxygen species (ROS) for up to 24 h and after HEDA, with or without ROS, followed or not by simulated reperfusion (REPH) for 6 h. Viability was analyzed with a trypan blue exclusion method. Cell lysates were analyzed with real-time PCR and Western blot (WB) for FKBP12 and FKBP12.6. Intracellular Ca^{2+}measurements were performed using dual-wavelength ratio imaging in fura-2 loaded cells.
Results: A time-dependent drop in viability was shown after HEDA ($P < 0.001$). Viability was not further influenced by addition of ROS or REPH. The general patterns of FKBP12 and FKBP12.6 mRNA expression showed upregulation after hypoxia, downregulation after ischemia and normalization after reperfusion, which was partially attenuated if ROS was added during HEDA. The protein contents were unaffected after hypoxia, tended to increase after ischemia and, for FKBP12.6, a further increase after reperfusion was shown. Hypoxia or HEDA, with or without REPH, resulted in a decreased amplitude of the Ca^{2+} peak in response to caffeine. In addition, cells subjected to HEDA for 3 h or HEDA for 3 h followed by 6 h of REPH displayed irregular Ca^{2+} oscillations with a decreased frequency.
Conclusion: A threshold for cell survival with respect to duration of ischemia was established in our cell line model. Furthermore, we could demonstrate disturbances of calcium handling in the sarcoplasmic reticulum as well as alterations in the expressions of the calcium handling proteins FKBP12 and FKBP12.6, why this model may be suitable for further studies on ischemia and reperfusion with respect to calcium handling of the sarcoplasmic reticulum.

1. Introduction

Reperfusion therapy for acute myocardial infarction has been shown to reduce infarct size and improve left ventricular function and survival [1], but may also contribute to myocardial reperfusion injury [2]. Among the possible mechanisms of myocardial reperfusion injury are intracellular Ca^{2+}overload and reactive oxygen species (ROS) activation mentioned [3]. Overall, disturbances in intracellular Ca^{2+} handling are key phenomena contributing to both irreversible and reversible myocardial injury associated with hypoxia/ischemia and reoxygenation/reperfusion [4,5].

In a previous porcine study using the microdialysis technique, ischemia and reperfusion resulted in release into the interstitium and increased myocardial expression of FKBP12/FKBP12.6. This was verified in a cell culture model with HL-1 cells and hypoxia where we could document increased mRNA expression of FKBP12.6 and a trend towards an increase of FKBP12 after exposure to 6 h of hypoxia [6]. These findings indicated that FKBP12/FKBP12.6 may play a role in the cellular response to hypoxia/ischemia and reperfusion. Ischemia is, however, not only characterized by hypoxia but also by energy depletion, acidosis and the accumulation of toxic degradation products which initiate inflammation and the activation of reactive oxygen species (ROS); factors contributing to tissue injury. The release and production of FKBP12 and FKBP12.6 during ischemic-like conditions at the edge of cell survival is, hitherto, not fully known.

The aim of this study was to establish a cell culture model with HL-1 cells simulating ischemia and reperfusion and, also in this model, study the impact of ischemia and reperfusion on cell viability, the expression of FKBP12 and FKBP12.6, and intracellular Ca^{2+} handling.

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2. Materials and methods

2.1. Cell culture experiments

HL-1 mouse cardiomyocytes were a kind gift from Dr. W. Claycomb (Louisiana State University Sciences Center, New Orleans, Louisiana, USA) and were maintained in Claycomb medium (Sigma–Aldrich, Stockholm, Sweden) [7,8]. Every third day, the cells were trypsinated for passage with trypsin/EDTA following Andersen's protocol for HL-1 cell culture [8].

2.1.1. Experimental protocols regarding the viability and expression of FKBP12/FKBP12.6

All experiments were performed after cultivating the cells until confluent and contracting (3–4 days after seeding). All given conditions were tested on three consecutive cell cultures. The cells were incubated in a closed incubator (New Brunswick, Scientific Innova CO-48) under the following conditions for 6, 12, 18 and 24 h, respectively:

(1) *Normoxia:* temperature at 37 °C, 95% humidity, 5% CO_2 and 21% O_2 on Claycomb medium (Sigma–Aldrich), pH 7.5.
(2) *Hypoxia:* as in (1) above but with 94% CO_2 and 1% O_2.
(3) *Hypoxia, energy depletion and acidosis* (*HEDA*): as in (2) but with phosphate buffer saline without Mg^{2+} and Ca^{2+} but with hydrochloric acid added (PBS–HCl; pH 6.4) instead of Claycomb medium (Sigma–Aldrich), achieving an acidic and energy-depleted environment.
(4) *ROS-activation:* as in (3) above but with the addition of 30 μM hydrogen peroxide (PBS–HCl–H_2O_2) (pH 6.4).
(5) HEDA + re-establishment of physiological conditions (REPH): as in (3) followed by 6 h of REPH by restitution with Claycomb medium (Sigma–Aldrich), 21% of O_2 saturation and normalization of pH to 7.5.
(6) HEDA + ROS + REPH: as in (4) followed by REPH for 6 h.

Acidic and ROS simulation was modified after Cicconi et al. [9].

To evaluate further the time influence on the viability of the HL-1 cells subjected to HEDA for 1–6 h, a separate staged study was performed (Fig. 1A).

For the intracellular Ca^{2+} measurements, HL-1 cell cultures were subjected to 3 h of normoxia, hypoxia, HEDA, and HEDA with 6 h of REPH.

2.1.2. Cell viability

At the end of each stage in the above mentioned protocol, the cells were trypsinated for analysis of cell viability.

Cell viability was immediately estimated with a trypan blue dye exclusion method in a Vi-Cell XR 2.03 (Beckman Coulter Vi-Cell XR™) [10].

2.1.3. Real-time polymerase chain reaction

Total RNA was isolated using the RNeasy kit (Qiagen, GmbH, Germany). Reverse transcription of RNA was performed with a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed to detect mRNA expression of FKBP12, FKBP12.6 and 18S (as house-keeping gene) [11] (human 18S rRNA Part No. 4319423E) using TaqMan gene expression assays (Applied Biosystems).

2.1.4. Western blotting

FKBP12 in cell lysates was analyzed by Western blot (WB) using a specific mouse anti-FKBP12 (Ab58072; Abcam, Cambridge, UK) and FKBP12.6 with a specific goat antibody (Goat α-FKBP12.6 R&D Systems, # AF4174). The antibody used for FKBP12 was shown to be highly specific for recombinant FKBP12 and did not detect any recombinant FKBP12.6, while the antibody used for FKBP12.6 detected recombinant FKBP12.6, but also minor levels of recombinant FKBP12.

A mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Ab 8245-100, Abcam) was used as an internal loading control [12].

Immunoreactive bands were visualized with peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Uppsala, Sweden). Bands were densitometrically analyzed with ImageQuant 5.0 Software (Academic Computing Health Sciences, University of Virginia, USA). Recombinant FKBP12 (FKBP1A No. H00002280-P01, Abnova Heidelberg, Germany) and recombinant FKBP12.6 (FKBP1B No. H00002281-P01, Abnova) were used as controls.

2.1.5. Intracellular Ca^{2+}-measurements

Changes in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) were recorded using dual-wavelength ratio imaging in fura-2 loaded HL-1 cells [13]. Cells were prepared and measurements performed as previously described [14], with minor modifications. Briefly, HL-1 cells were plated on glass Petri dishes (MatTek Corporation, USA). The membrane-permeable acetooxymethylester of fura-2 was dissolved in dimethyl sulfoxide at 1 μg/μl. After interventions mentioned above (experimental protocol section), cells were loaded for 30 min at room temperature using 2 μl fura-2 stock solution added to 1 ml extracellular solution consisting of (in mM) 140 NaCl, 3.6 KCl, 2.6 $CaCl_2$, 0.5 $MgSO_4$, 0.5 NaH_2PO_4, 2 $NaHCO_3$, 5 HEPES, 5 glucose (pH 7.4 with NaOH). During this procedure all cells were subjected to

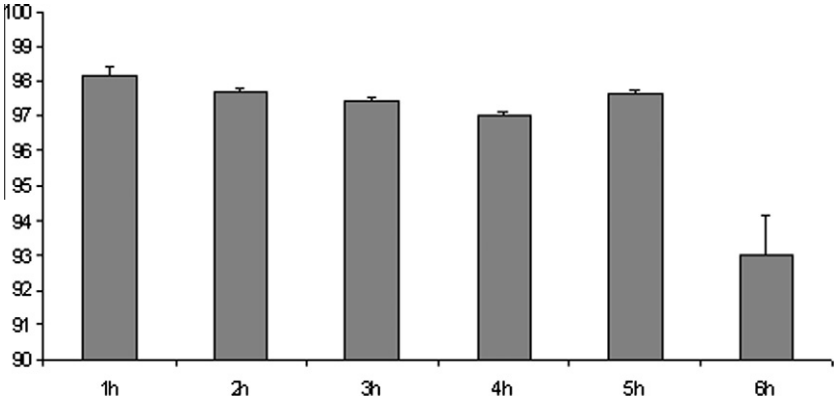

Fig. 1A. The time relationship of simulated ischemia on HL-1 cells for 1–6 h. Values shown are mean ± SEM.

normal oxygen conditions as well as normalization of pH to 7.4. Recordings were obtained using a Nikon Diaphot 300 inverted microscope (Nikon, Japan) and a Lambda DG-4 xenon lamp illumination system (Sutter Instrument Company, USA). Images were captured using a QuantEM 512SC CCD camera (Photometrics, USA) and Metafluor software (Meta imaging series 7.5, Molecular Devices, USA). Cells were excited at 340 and 380 nm and emitted light was collected at 510 nm. The fluorescence ratio ($F340/F380$) was calculated offline and the $[Ca^{2+}]_i$ was determined using Eq. (5) of [13] and a dissociation constant (K_d) of 224 nM. Calibrations were performed using solutions with a high Ca^{2+} concentration (12 mM $CaCl_2$ + 10 mM EGTA) or lacking Ca^{2+} (10 mM EGTA) in order to achieve the maximum (R_{max}) and minimum (R_{min}) ratio, as well as S_{f2}/S_{b2}. During the experiments, cells were continuously superfused with the extracellular solution and 10 mM of caffeine (Sigma) was added once via the perfusion system to the culture dish. All measurements were carried out at 32 °C.

2.2. Analysis

Functional regions of interest (ROIs) were regions of well-synchronized cells and five or six regions were selected for each recording.

2.3. Statistics

Continuous data are expressed as mean ± standard error of mean (SEM) of three independent experiments. The results of real time-PCR for FKBP12 and FKBP12.6 were normalized to the levels of 18S and these values were then, for each exposure time (6, 12, 18 or 24 h), normalized to the mean levels in cell cultures exposed to normoxia. The results of WB for FKBP12 and FKBP12.6 were normalized to the levels of GAPDH and these values were then, for each exposure time (6, 12, 18 or 24 h), normalized to the mean levels in cell cultures exposed to normoxia. All other values are presented as absolute values. A statistical analysis of all groups with respect to time of exposure to intervention, and kind of intervention for viability were made with a two-way ANOVA including post hoc analysis with a test of least square difference (LSD). For the results of real time-PCR and Western blot analysis, within-group comparisons were performed for each exposure time with a one-way ANOVA and also with post hoc analysis according to Dunnett's two-sided t-test in which all interventions were compared with normoxia. The significance level was set at $P < 0.05$.

The significance of differences between groups of the intracellular $[Ca^{2+}]_i$ measurements was evaluated by Student's t-test (unpaired) and two-way ANOVA using OriginPro 8 (OriginLab Corporation, USA).

3. Results

3.1. Cell viability, contents and expression of FKBP12 and FKBP12.6 (Figs. 1 and 2)

3.1.1. Normoxia

During normoxic conditions, viability was constant over-time for up to 24 h of observation (Fig. 1B). Neither the mRNA-expression of FKBP12 or FKBP12.6 nor the cytosolic contents of FKBP12 or FKBP12.6 changed over time during normoxia, (data not shown; in Fig. 2 all data from the intervened groups were normalized to those of the normoxic groups (controls) for each exposure time).

3.1.2. Hypoxia

Exposure to hypoxia resulted in a slight, but significant reduction in viability after 18 h, which was further pronounced after 24 h (Fig. 1B).

The mRNA-expression of FKBP12 after 6 h of hypoxia was upregulated compared with after normoxia, but for longer exposure times the expression was rather downregulated (Fig. 2A). Hypoxia significantly upregulated mRNA expression of FKBP12.6 compared with normoxia for 6, 12 and 18 h, respectively (Fig. 2C).

The cytosolic contents of neither FKBP12 nor FKBP12.6 changed over time (Fig. 2B and D).

3.1.3. HEDA

Simulated ischemia interfered significantly with viability already after 6 h (Fig. 1B), but detailed analysis of shorter exposures revealed that viability was completely unaffected for up to 5 h of HEDA (Fig. 1A).

The mRNA expressions of both FKBP12 and FKBP12.6 were unaffected after 6 h of exposure to HEDA, but for longer exposure times, the expressions were decreased (Fig. 2A and C).

There was a trend towards an increase in the cytosolic contents of both FKBP12 and FKBP12.6 for longer exposures to HEDA (significant for FKBP12 after 18 h) (Fig. 2B and D).

3.1.4. HEDA with ROS

HEDA with ROS interfered significantly with viability already after 6 h of exposure (Fig. 1B).

The mRNA expressions of both FKBP12 and FKBP12.6 tended to be downregulated without any differences to HEDA alone (Fig. 2A and C).

The cytosolic contents of both FKBP12 and FKBP12.6 tended to increase with longer exposure times, without any difference to HEDA alone (Fig. 2B and D).

3.1.5. HEDA with REPH

HEDA with REPH interfered significantly with viability already after 6 h of exposure (Fig. 1B).

The mRNA expressions of both FKBP12 and FKBP12.6 increased in relation to time of exposure to HEDA before REPH (Fig. 2A and C).

The cytosolic contents of both FKBP12 and FKBP12.6 increased with longer exposure times, for FKBP12.6 already after 12 h (Fig. 2B and D).

While REPH did not change viability compared with HEDA alone, mRNA expression was normalized compared with HEDA alone and even upregulated compared with normoxia.

3.1.6. HEDA with ROS and REPH

HEDA with ROS and REPH interfered significantly with viability already after 6 h (Fig. 1B).

The addition of ROS to HEDA before REPH partly attenuated the normalization of mRNA expression of both FKBP12 and FKBP12.6, observed after HEDA + REPH compared with HEDA alone (Fig. 2A and C).

The cytosolic contents of both FKBP12 and FKBP12.6 measured tended to increase after longer exposure times, more markedly for FKBP12.6 (Fig. 2B and D).

3.1.7. Intracellular Ca^{2+}-measurements

Fig. 3 shows representative examples of recordings of $[Ca^{2+}]_i$ in HL-1 cells after exposure to normoxia, hypoxia, HEDA, and HEDA + REPH, respectively, before, during and after application of 10 mM of caffeine. As summarized in Fig. 4, basal $[Ca^{2+}]_i$ (Fig. 4A) and the oscillation amplitudes (Fig. 4B) did not differ significantly between the groups. After hypoxia, the oscillation frequency was lower compared with after normoxia and tended to be lower also after HEDA with or without REPH (Fig. 4C). After the latter interventions, the oscillations were also noticeably irregular (Fig. 3). The peak $[Ca^{2+}]_i$ response to caffeine was significantly reduced after hypoxia, HEDA, and HEDA + REPH compared with after normoxia, most markedly after HEDA with or without REPH (Fig. 4D).

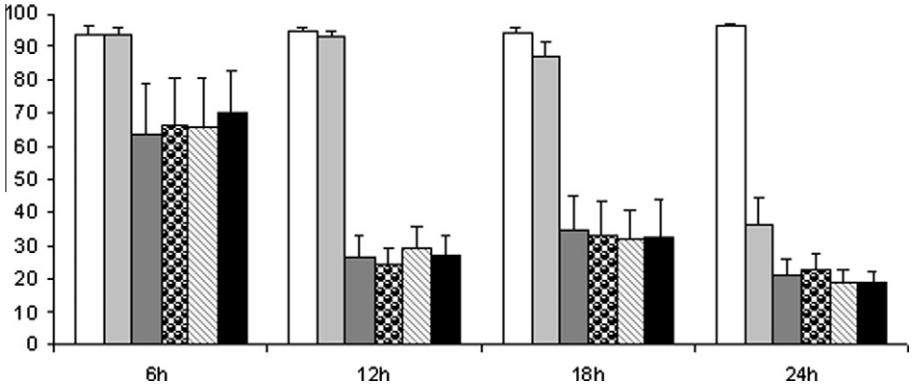

Fig. 1B. The time-dependent decrease of cell viability is shown for all groups ($P < 0.001$). Cells subjected to HEDA showed a marked decrease from 12 to 18 and 24 h, respectively, which was more pronounced than among cells subjected to normoxia or only hypoxia. The cells which underwent REPH after HEDA showed a small but significant increase in viability compared with HEDA alone ($P < 0.001$). The 24 h HEDA + ROS activated group was not exposed to reperfusion. Values shown are mean ± SEM.

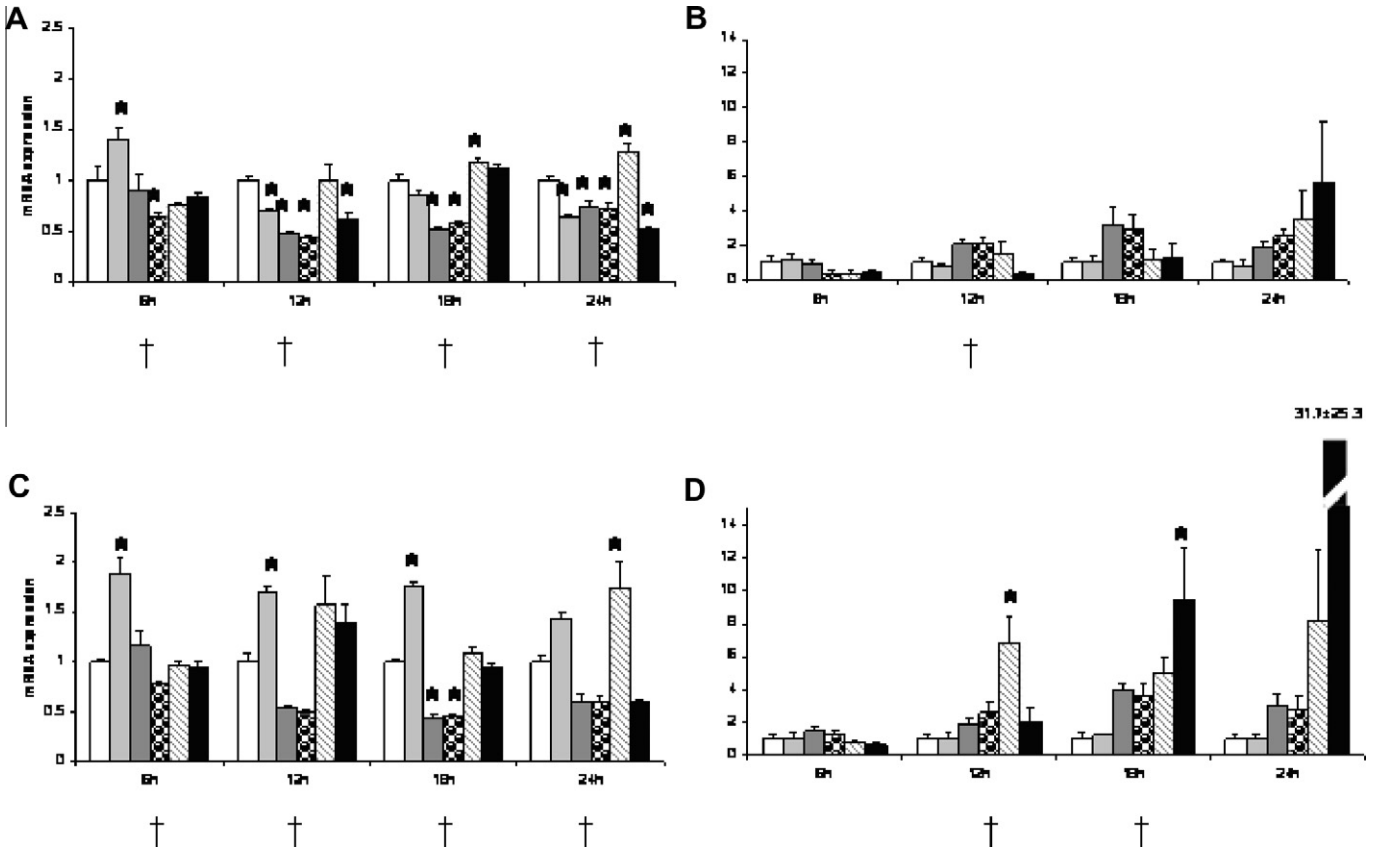

Fig. 2. Bars are represented as white for normoxia, light grey for hypoxia, dark grey for HEDA, grey with dots for HEDA + ROS, striped grey for HEDA + REPH, and black for HEDA + ROS + REPH. (A) Presents the mRNA expression of FKBP12 after exposure described above during the respective periods. ANOVA for between-group differences showed $P < 0.05$ at all times. $^{*}P < 0.05$ in comparison with normoxia for the same period in post hoc analysis is shown with the † below the *x*-axis. (B) Shows FKBP12 in cell lysates as WB contents in cells exposed to normoxia, hypoxia, HEDA, HEDA + ROS, HEDA + REPH, HEDA + ROS + REPH for 6, 12, 18 and 24 h. The FKBP12 content is normalized to GAPDH and, thereafter, to normoxia. ANOVA for between-group differences showed $P < 0.05$ for 12 h, and ns for 6, 18 and 24 h. (C) Presents the mRNA expression of FKBP12.6 after exposures described above during the respective periods. ANOVA for between-group differences showed $P < 0.05$ at all times. $^{*}P < 0.05$ in comparison with normoxia for the same period in post hoc analysis is shown with the † below the *x*-axis. (D) Shows the WB content in cell lysates of FKBP12.6 in cells exposed to normoxia, hypoxia, HEDA, HEDA + ROS, HEDA + REPH, HEDA + ROS + REPH for 6, 12, 18 and 24 h. The FKBP12.6 content is normalized to GAPDH and, thereafter, to normoxia. ANOVA for between group differences showed $p < 0.05$ for 12 and 18 h, and ns for 6, and 24 h. $^{*}P < 0.05$ in comparison with normoxia for the same period in post hoc analysis is shown with the † below the *x*-axis.

4. Discussion

Any intervention performed to rescue myocardial tissue during an acute myocardial infarction will take place in a situation with profound ischemia affecting cells close to the edge of survival. A cellular model used for studies with clinical relevance must then be constructed to mimic similar conditions. Hence, one of the primary aims of the present study was to define the threshold of survival in HL-1 cells after exposure to simulated ischemia and reperfusion.

As has been found by others, HL-1 cells are fairly resistant to hypoxia alone [8]. In our study, this was confirmed as hypoxia alone

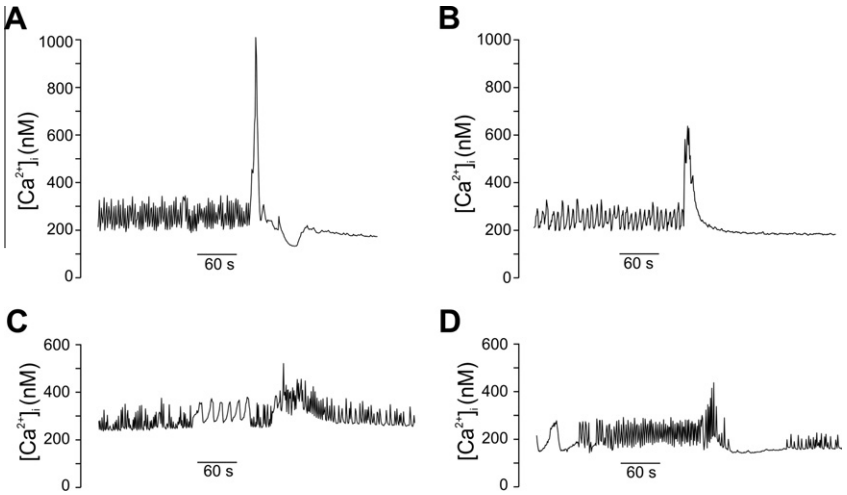

Fig. 3. Time-dependent changes in cytosolic Ca^{2+} upon extracellular application of 10 mM caffeine to cells pre-exposed to (A) normoxia, (B) hypoxia, (C) HEDA or (D) HEDA + REPH. Traces show four representative recordings out of a total of 22–23 analysed ROIs from four separate experiments in each group.

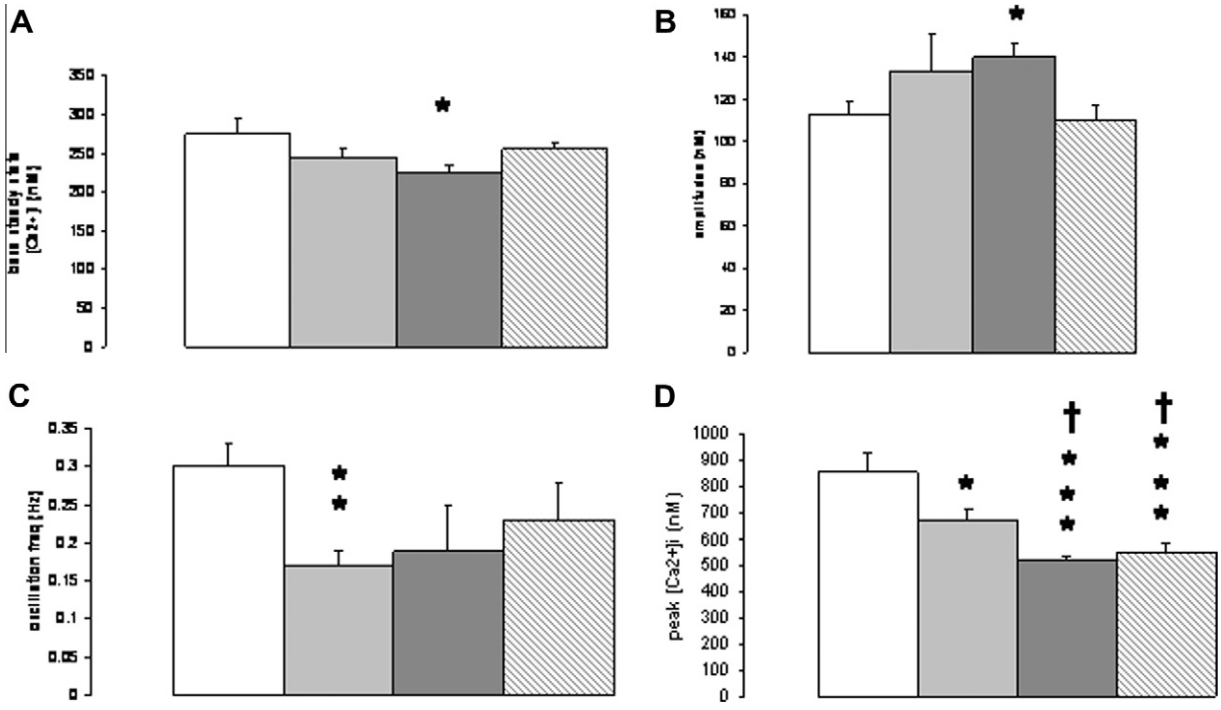

Fig. 4. Data are mean values + SEM of a total of 22–23 ROIs. *indicates $P < 0.05$, **$P < 0.01$, ***$P < 0.0001$, †indicates significance ($P < 0.05$) within groups. Bars are represented as white for normoxia, light grey for hypoxia, dark grey for HEDA, and grey striped for HEDA + REPH. (A) Shows base steady state. (B) Shows the mean amplitudes. (C) Presents the calcium oscillation frequency and (D) displays the peak caffeine response.

did not to any greater extent affect survival for up to 18 h of exposure. Beyond this period, a dramatic drop in survival was observed even with only hypoxic conditions. In contrast, simulated ischemia (HEDA) resulted in increased cell death already after 6 h of exposure, and this cell death was profound after extended exposure periods. Thus, a threshold for survival of approximately 6 h could be defined. Notably, neither the addition of hydrogen peroxide (H_2O_2) simulating the ROS activation in the medium [9], nor the subsequent re-establishment of physiological conditions simulating reperfusion (REPH) further influenced viability at any time of exposure to simulated ischemia.

ROS has been considered as one of the damaging agents causing reperfusion injury when restoring coronary artery flow during acute myocardial infarction [5]. ROS originate when free electrons are dissociated due to the electron transport chain uncoupling in several biochemical reactions, e.g. by activated neutrophil leukocytes starting different chain reactions. Neutrophils are absent in our model and any lack of effect on viability by the exogenous addition of hydrogen peroxide must be interpreted with caution. Even if viability seemed to be unaffected, ROS had a significant impact on FKBP12 and FKBP12.6 expressions, as discussed below.

Again, certain properties of the model must be recognized. HL-1 cells are derived from a cell line, and are therefore by definition regarded to be immortal when cultivated in physiological conditions [8]. Furthermore, even if they were contracting at the time of the experiment they were not carrying out any mechanical work load and were, more or less, in a resting condition with reduced metabolic demands.

For measurements of $[Ca^{2+}]_i$ we chose to expose the cells to ischemia during a period below the threshold for cell death, i.e. for 3 h. Importantly, after the interventions, all cells subjected to calcium measurements were, prior to these measurements, incubated similarly, including normoxia and normal pH, why calcium changes during ischemia or early reperfusion were not studied. Previous studies have demonstrated that during ischemia there is a gradual increase in myocyte cytosolic $[Ca^{2+}]_i$, with a brief further increase after reperfusion, where after $[Ca^{2+}]_i$ seems to normalize [15–17]. In our study exposure to ischemia, afterwards reduced the response to caffeine, and the peak $[Ca^{2+}]_i$ upon stimulation was significantly lower compared with responses in normoxia treated cells. This is in agreement with findings in a previous study using a rat cell model exposed to chronic intermittent hypoxia [18]. Our findings with reduced caffeine-induced peaks were further well in line with previous findings of reduced SR calcium contents after ischemia and reperfusion [19]. Additionally, we could observe that the oscillations became more irregular. Thus, our cell model showed signs of a disturbed SR calcium handling function that fits well with the clinical presentation seen during ischemia and reperfusion in humans, contraction disturbances and arrhythmias, and that has been reported by others using other models [17,20].

Acidosis and ischemia may inhibit the ryanodine 2 receptor (RyR2), and previous studies have indicated that the SR depletion of calcium may be caused by a release of this inhibition upon reperfusion, causing a calcium leak [20,21]. FKBP12 and FKBP12.6, of which the homologue FKBP12.6 has the highest affinity for the RyR2, are considered to be the most important regulators of the RyR2 function [22,23]. These proteins have not been extensively studied in the situation of acute ischemia and reperfusion. In our previous study [6], we found that hypoxia tended to upregulate the mRNA expressions of both proteins, especially FKBP12.6, which was confirmed in the present study. Long exposure times to ischemic like conditions, however, downregulated mRNA expressions of both proteins, with normalization after simulated reperfusion. The latter was attenuated in some of the experiments if ischemia was combined with ROS (hydrogen peroxide). FKBP12.6 contents, however, showed a different pattern. Due to low numbers and the dispersion of the results, few of the changes were statistically significant, but the overall pattern was an increased content after ischemia and, for FKBP12.6, this increase seemed to be even more pronounced after reperfusion after longer periods of ischemic exposures. The different associations between mRNA expressions and protein contents indicate that both transcriptional and post transcriptional regulation, as well as reduced degradation due to cell injury, may serve as explanations for an increase in protein contents after ischemia with or without ROS, and with and without reperfusion.

The finding of mRNA upregulation of FKBP12.6 after hypoxia, downregulation after ischemia with normalization, and even upregulation, after reperfusion together with increased protein contents of this protein after longer periods of ischemia and reperfusion, indicate that disturbed RyR2-FKBP12/FKBP12.6 function may be of vital importance during myocardial ischemia and reperfusion. To establish whether these alterations are compensatory or causative and to what extent they may be altered by interventions require further investigations.

We have been able to demonstrate a threshold of survival for HL-1 cells for various periods of stress with hypoxia in combination with energy depletion and acidosis, i.e. simulated ischemia. Furthermore, in this model, we could demonstrate that ischemia may cause severe calcium handling disturbance in the SR, and that RyR2 and its stabilizing proteins FKBP12 and FKBP12.6 may be involved. The cell model may be a useful tool for further investigations in cardiomyocytes exposed to severe ischemic injury at the edge of survival and to further study mechanisms of calcium handling during and after ischemia to search for targets of intervention against ischemic and reperfusion injury in association with acute myocardial infarction.

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