Effect of extracellular Mg\textsuperscript{2+} on ROS and Ca\textsuperscript{2+} accumulation during reoxygenation of rat cardiomyocytes

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Sharikabad, Mohammad N., Kirsten M. Østbye, Torstein Lyberg, and Odd Brørs. Effect of extracellular Mg\textsuperscript{2+} on ROS and Ca\textsuperscript{2+} accumulation during reoxygenation of rat cardiomyocytes. Am J Physiol Heart Circ Physiol 280: H344–H353, 2001.—The effects of Mg\textsuperscript{2+} on reactive oxygen species (ROS) and cell Ca\textsuperscript{2+} during reoxygenation of hypoxic rat cardiomyocytes were studied. Oxidation of 2',7'-dichlorodihydrofluorescein (DCHF) to dichlorofluorescein (DCF) and of dihydroethidium (DHE) to ethidium (ETH) were used as markers for intracellular ROS levels and were determined by flow cytometry. DCDHF/DCF is sensitive to H\textsubscript{2}O\textsubscript{2} and nitric oxide (NO), and DHE/ETH is sensitive to the superoxide anion (O\textsubscript{2}⁻), respectively. Rapidly exchangeable cell Ca\textsuperscript{2+} was determined by \textsuperscript{45}Ca\textsuperscript{2+} uptake. Cells were exposed to hypoxia for 1 h and reoxygenation for 2 h. ROS levels, determined as DCF fluorescence, were increased 100–130% during reoxygenation alone and further increased 60% by increasing extracellular Mg\textsuperscript{2+} concentration to 5 mM at reoxygenation. ROS levels, measured as ETH fluorescence, were increased 16–24% during reoxygenation but were not affected by Mg\textsuperscript{2+}. Cell Ca\textsuperscript{2+} increased three- to fourfold during reoxygenation. This increase was reduced 40% by 5 mM Mg\textsuperscript{2+}, 57% by 10 μM 3,4-dichlorobenzamil (DCB) (inhibitor of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange), and 75% by combining Mg\textsuperscript{2+} and DCB. H\textsubscript{2}O\textsubscript{2} (25 and 500 μM) reduced Ca\textsuperscript{2+} accumulation by 38 and 43%, respectively, whereas the NO donor S-nitroso-N-acetyl-penicillamine (1 mM) had no effect. Mg\textsuperscript{2+} reduced hypoxia/reoxygenation-induced lactate dehydrogenase (LDH) release by 90%. In conclusion, elevation of extracellular Mg\textsuperscript{2+} to 5 mM increased the fluorescence of the H\textsubscript{2}O\textsubscript{2}/NO-sensitive probe DCF without increasing that of the O\textsubscript{2}⁻-sensitive probe ETH, reduced Ca\textsuperscript{2+} accumulation, and decreased LDH release during reoxygenation of hypoxic cardiomyocytes. The reduction in LDH release, reflecting the protective effect of Mg\textsuperscript{2+}, may be linked to the effect of Mg\textsuperscript{2+} on Ca\textsuperscript{2+} accumulation and/or ROS levels.

Hypoxia; magnesium; calcium; hydrogen peroxide; flow cytometry

Reoxygenation of the myocardium after coronary artery occlusion is essential to prevent or limit infarction but appears to cause damage by itself (17, 22). Many strategies have been tested to further reduce tissue damage during reperfusion (36). Magnesium (Mg\textsuperscript{2+}) given early in the reperfusion period has shown promising results in both clinical (40, 45, 52) and experimental studies (6, 16, 31, 46). There is, however, no consensus regarding the beneficial effects of Mg\textsuperscript{2+} on infarct size and mortality or how such actions should be explained. Two factors assumed to be of importance in ischemia-reperfusion-induced cardiomyocyte damage and death are cellular calcium (Ca\textsuperscript{2+}) overload and oxidative stress. Both these factors may be possible targets for the protective effect of Mg\textsuperscript{2+}.

The levels of reactive oxygen species (ROS) increase during reperfusion of the ischemic myocardium (12, 54). Increased ROS levels have been associated with tissue damage, and strategies used to reduce oxidative stress have been shown to be protective (4, 5, 23). Garcia et al. (11) showed that Mg\textsuperscript{2+} reduced oxidative stress, measured as ascorbate free radical signal in an in vivo coronary occlusion-reperfusion model. Mg\textsuperscript{2+} deficiency has also been associated with an increased nitric oxide (NO) level in rat plasma (38) and red blood cells (33), reduced superoxide dismutase (SOD) and catalase activity in cardiac tissue (29), and increased oxidant levels in endothelial cells (51). It is not known how Mg\textsuperscript{2+} affects ROS in cardiomyocytes. Therefore, we investigated the effect of extracellular Mg\textsuperscript{2+} on ROS levels in isolated rat cardiomyocytes subjected to hypoxia and reoxygenation.

Intracellular Ca\textsuperscript{2+} and Ca\textsuperscript{2+} uptake increases markedly during ischemia-reperfusion (27, 42, 44) and during reoxygenation of hypoxic cardiomyocytes (14, 34, 35, 39). These changes, which are often referred to as Ca\textsuperscript{2+} overload, have been associated with cell damage. Inhibition of Ca\textsuperscript{2+} uptake was shown to reduce damage and improve recovery during reperfusion of the intact heart and reoxygenation of cardiomyocytes (30, 50). Mg\textsuperscript{2+} has been observed to inhibit both Ca\textsuperscript{2+} channels (1, 15) and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange across cell membranes (28, 32, 47). Thus the beneficial effect of Mg\textsuperscript{2+} on functional and metabolic recovery of the postischemic myocardium may be related to reduced Ca\textsuperscript{2+} accumulation. Tsukube et al. (48) showed that K\textsuperscript{+}/Mg\textsuperscript{2+} cardioplegia (20 mM of each) enhanced functional recovery.
ery and preserved high-energy phosphates in correlation with a reduction in cytosolic Ca\(^{2+}\) accumulation after surgically induced global ischemia in the aged myocardium. The results of Ichiba et al. (20) showed a cardioprotective effect of Mg\(^{2+}\) by regulating intracellular Ca\(^{2+}\) concentration in a simulated ischemia model with cultured neonatal rat cardiomyocytes. To our knowledge, the effect of Mg\(^{2+}\) on Ca\(^{2+}\) accumulation during reoxygenation of hypoxic adult cardiomyocytes has not been investigated.

The aim of the present study was to investigate the effect of high extracellular Mg\(^{2+}\) on ROS and Ca\(^{2+}\) accumulation during reoxygenation of hypoxic adult rat cardiomyocytes. For this purpose, we chose a previously established model of hypoxia/reoxygenation in isolated rat cardiomyocytes (39). We report here that increasing extracellular Mg\(^{2+}\) to 5 mM at the onset of reoxygenation increased ROS levels, measured as dichlorofluorescein (DCF) fluorescence, a probe sensitive to H\(_2\)O\(_2\) and NO/NO-based radicals. Furthermore, Mg\(^{2+}\)-reduced Ca\(^{2+}\) accumulation and reduced cell injury, measured as lactate dehydrogenase (LDH) release.

**MATERIALS AND METHODS**

**Chemicals and materials.** The following chemicals were obtained from Sigma Chemical (St. Louis, MO): BSA (essentially fatty acid-free), 2',7'-dichlorodihydrofluorescein (DCDHF) diacetate (DCDHF-DA), dihydroethidium (DHE), DL-carnitine, dibutyl phthalate, ouabain, trypsin, menadione, S-nitroso-N-acetyl-pencillamine (SNAP), and SDS. Joklik’s minimum essential medium (MEM) was obtained from Life Technologies (Paisley, UK). Collagenase and deoxyribonuclease were obtained from Worthington Biochemical (Lakewood, NJ). 44Ca\(^{2+}\) was obtained from DuPont de Nemours, NEN Division (Dreieich, Germany). Micro bichrominic acid (BCA) protein assay reagent was obtained from Pierce (Rockford, IL), 3,4-Dichlorobenzamil (DCB) was obtained from Molecular Probes (Eugene, OR). Diisonyl phthalate was obtained from Fluka Chemie (AG Buchs, Switzerland). Opti-Fluor was obtained from Packard (Groningen, The Netherlands). H\(_2\)O\(_2\) (30%) was obtained from Norwegian Medicinal Depot (Oslo, Norway).

**Buffers.** Normal physiological buffer (NPB) contained the following (in mM): 120.0 NaCl, 3.3 KCl, 1.2 KH\(_2\)PO\(_4\), and 24.0 NaHCO\(_3\); pH 7.4. NPB-1 contained NPB with the addition of 0.5 mM CaCl\(_2\), 0.8 mM MgSO\(_4\), and 1% BSA (wt/vol). NPB-2 contained NPB with the addition of 1.0 mM CaCl\(_2\), 0.8 mM MgSO\(_4\), and 0.1% BSA (wt/vol).

**Isolation of cardiomyocytes.** Adult male Wistar rats (200–400 g) were obtained from Mellegaard (Skensved, Denmark) and housed in accordance with the conditions set by the Norwegian Council for Animal Research. The investigation conformed with the guidelines of the Norwegian National Institute for Public Health. Cardiomyocytes were isolated by trypsin-collagenase perfusion according to a slightly modified protocol of Stokke et al. (43). Briefly, the rats were anaesthetized by injection of pentobarbital (100 mg/kg ip). The hearts were excised, and the aortas were cannulated. Initially, the hearts were perfused at room temperature (25°C) for 10 min with nominally Ca\(^{2+}\)-free Joklik’s MEM supplemented with 1.0 mM MgSO\(_4\), 23.8 mM NaHCO\(_3\), 1 mM DL-carnitine, and 62.1 U/ml trypsin (solution A) and continuously gassed with 95% O\(_2\)-5% CO\(_2\). The hearts were then perfused in a recirculated system for 25 min at 37°C with solution B (solution A supplemented with 200 U/ml collagenase and 0.1% BSA) at a rate of ~6–7 ml/min. The ventricles were then excised from the rest of the hearts, opened, rinsed, cut in small pieces in solution C (solution A without trypsin supplemented with 0.5 mM CaCl\(_2\) and 1% BSA), and incubated in a shaking water bath (150 rpm at 37°C) for 10 min. Cells were then incubated in solution B supplemented with 20 μg/ml DNAase in the shaking water bath for 15 min. Cells were washed with NPB-1, and Ca\(^{2+}\) was adjusted to 1 mM. The cell suspension was filtered through a 250-μm nylon mesh, and the cells were resuspended in NPB-2. Cell suspensions used for experiments contained at least 70% viable cells (trypan blue exclusion), of which at least 95% had an elongated shape. LDH release was determined using the quantitative kinetic method (at 340-nm absorption wavelength) of Wroblewski and LaDue (1955) (53) in incubation buffer and normalized to cell protein measured according to Smith et al. (41) by micro BCA protein assay reagent using BSA as standard.

**Incubation.** Cells were incubated in 25-ml Erlenmeyer flasks in NPB-2 supplemented with trace amounts of 44Ca\(^{2+}\) (=5·10\(^6\) disintegrations per min per ml) in a 37°C water bath.

In normoxia (control cells), the cell suspension was gassed with 95% air-5% CO\(_2\) (P\(_{O_2}\) ~12–14 kPa) and supplied with 5.5 mM glucose. In hypoxia, cells were added to NPB-2 (no glucose added) equilibrated with 95% N\(_2\)-5% CO\(_2\) (P\(_{O_2}\) ~1 kPa). At reoxygenation, gas was changed to 95% air-5% CO\(_2\), and buffer was supplied with 5.5 mM glucose. Oxygen tension in the test flasks was checked using an oxygen electrode (WTW Microprocessor Oximeter Oxi 96, Wissenschaftliche Technische Werkstätten, Weilheim, Germany) near the bottom of the Erlenmeyer flask where the cells were added. Each Erlenmeyer flask was supplied with an inlet and outlet for gas and continuously gassed for the indicated time.

**Flow cytometric determination of ROS and light scatter.** Levels of ROS were measured by flow cytometry as the fluorescence of DCF and ethidium (ETH), which are the oxidation products of DCDHF and DHE with a sensitivity for H\(_2\)O\(_2\)/NO-based radicals and O\(_2\)\(^{•-}\), respectively. DCDHF-DA is an ester that is freely membrane permeable and enters the cells. After entering the cells, DCDHF-DA loses its diacetate group (becoming DCDHF) by esterase action and can be oxidized to highly fluorescent DCF. DCDHF has been shown to be oxidized by H\(_2\)O\(_2\) in cardiomyocytes (49) as well as endothelial cells (8) to highly fluorescent DCF. Other ROS than H\(_2\)O\(_2\), like NO and its reaction product with O\(_2\)\(^{•-}\), which is the highly reactive peroxynitrite (ONOO\(^{-}\)), can oxidize DCDHF (21, 37). Vanden Hoek et al. (49) showed that DHE is relatively more sensitive to O\(_2\)\(^{•-}\) than to H\(_2\)O\(_2\) in cardiomyocytes and that DHE can also be oxidized to ETH by the hydroxyl radical (•OH). DHE reacts with ROS and forms red fluorescent ETH. ETH binds to DNA, causing amplification of the red fluorescence signal.

Cells were incubated for 10 min with the probe (5 or 30 μM, 0.15% DMSO) at 37°C. Cell samples were then either analyzed at once (nonfixed cell samples) or fixed (1% paraformaldehyde), cooled (~4°C), and protected from light for later analysis (cold-fixed cell samples). A FACSsort (Becton-Dickinson, Rutherford, NJ) flow cytometer, equipped with a 488-nm argon ion laser and supplied with the Cell Quest software, was applied to measure ROS levels in the cells. Signals were obtained using a 585-nm bandpass filter (FL-2 channel) for ETH and a 530-nm bandpass filter (FL-1 channel) for DCF. Each determination is based on mean fluorescence intensity of 5,000 cells.
Determination of cell Ca$^{2+}$. Cell Ca$^{2+}$ was determined by $^{45}$Ca$^{2+}$ uptake, as previously described (39). Briefly, cell Ca$^{2+}$ was determined as rapidly exchangeable Ca$^{2+}$ by uptake of $^{45}$Ca$^{2+}$. A 20-ml Falcon tube (Oxnard, CA) containing 0.5 ml of oil mixture (dibutyl phthalate and diisononyl phthalate, 45–55% wt/wt) below 4.8 ml NPB-2 was kept in ice-water (0–5°C). A sample of cell suspension (200 μl) was added to the buffer phase, and the tube was centrifuged (2,000 g for 2 min) within 5 min, allowing cardiomyocytes to pass through the oil to the bottom of the tube. The tip of the tube (containing the cell pellet) was cut off, and the pellet was dissolved in 1 ml of 1% SDS. Radioactivity was determined by liquid scintillation counting (liquid scintillation cocktail, Opti-Fluor from Packard), and protein content was measured in each cell pellet. The extracellular fluid accompanying cells through the oil layer was determined by [14C]mannitol-occupying space and was 1.14 μl/mg cell protein (corresponding to 1.14 nmol Ca$^{2+}$/mg cell protein when incubating in buffer containing 1 mM Ca$^{2+}$). All measurements of cell Ca$^{2+}$ were corrected for extracellular Ca$^{2+}$.

Statistics. All experiments were performed as paired comparisons, and the data was given as means ± SE. Statistical analysis was performed using Statgraphics Plus software (version 4.0, Manugistics, Rockville, MD). Statistical analysis of multigroup comparisons was performed by one-way analysis of variance (ANOVA), and the method to discriminate among the means was Fisher’s least significant difference (LSD) method. Statistical analysis of two-sample comparisons was performed by Student’s t-test (2-sided) on paired data. P < 0.05 was considered to be significant.

RESULTS

Selectivity of DCDHF and DHE toward different ROS types. To determine selectivity of the probes toward different ROS in this particular test model, we measured sensitivity of cells loaded with DCDHF or DHE to the oxidants H$_2$O$_2$, menadione (O$_2$- donor), and SNAP (NO donor). The effect of diethyldithiocarbamic acid (DDC, 10 mM), an inhibitor of SOD (the enzyme responsible for converting O$_2^•$ to H$_2$O$_2$), alone and in combination with menadione was also tested. Normoxic cells were loaded for 5 min with the probe before exposure to the oxidants. DCDHF-loaded cells dose dependently responded to exposure (5 min) of H$_2$O$_2$ and menadione with increase in DCF fluorescence (23- and 18.5-fold increase with 1 mM H$_2$O$_2$ or menadione, respectively). The increase in DCF fluorescence caused by menadione was almost abolished in the presence of DDC added at the same time as the probe (Fig. 1A). DHE-loaded cells responded to higher concentrations of H$_2$O$_2$ (0.5 and 1 mM) and not to the NO donor SNAP (Fig. 1B). Detection of the ETH signal in the presence of menadione was totally abolished and/or reduced in the presence of menadione in cold-fixed cell samples and/or nonfixed cell samples, respectively. This suggests that the ETH fluorescence signal is quenched by menadione. The ETH signal was increased 240% by DDC (10 min incubation) compared with 50% increase in DCF fluorescence by DDC (Fig. 1C).

Effect of Mg$^{2+}$ on ROS during reoxygenation. ROS levels, as detected by DCF fluorescence, were increased by −55% at the end of hypoxia, and the increase was maintained (60–90% compared with normoxic control, Fig. 2A) during reoxygenation in cold-fixed cell samples. The DCF signal was further increased −60% (at 120 min) by Mg$^{2+}$ added to give a final concentration of 5 mM (from 1 M MgSO$_4$ solution) at the onset of reoxygenation (Fig. 2A). Because of high background-to-signal ratio in these DCF data (50%), we also measured the DCF signal in nonfixed cells where the background was reduced to 10–20% of the DCF signal. ROS levels were unchanged, as detected by DCF fluorescence, at the end of hypoxia but increased −100%
during reoxygenation. The DCF signal was further increased ~50% (at 120 min) by increasing Mg$^{2+}$ to 5 mM at reoxygenation (Fig. 2B). ROS levels, measured as ETH fluorescence (cold-fixed cell samples), were reduced by 15–25% at the end of hypoxia, increased significantly by 16–24% compared with control after reoxygenation, and were not affected by Mg$^{2+}$ (Fig. 3).

**Effect of Mg$^{2+}$ and DCB on cell Ca$^{2+}$ during reoxygenation.** After 1 h of hypoxia, cell Ca$^{2+}$ was unchanged compared with controls. At the onset of reoxygenation, a marked increase in cell Ca$^{2+}$ occurred, which at 120 min had reached about four times control values. Mg$^{2+}$ was added to give a final concentration of 5 and 15 mM (from 1 M MgSO$_4$ solution) at the onset of reoxygenation. After 30 min of reoxygenation, both 5 and 15 mM Mg$^{2+}$ significantly and equally reduced the increase in cell Ca$^{2+}$ by ~40–50%. Increasing extracellular Mg$^{2+}$ from 0.8 to 5 or 15 mM by addition of Mg$^{2+}$ from 1 mM MgSO$_4$ solution would increase osmolarity of incubation buffer by about 8 and 28 mosM. To exclude the possible effect of sulfate and/or osmolarity change, isotonic solutions of MgSO$_4$ or MgCl$_2$ (290 mosM) were added to incubation buffer to yield 5 mM Mg$^{2+}$ concentration at the onset of reoxygenation in a separate set of experiments. Both Mg$^{2+}$ salts reduced to a similar degree the increase in cell Ca$^{2+}$ by ~60% at 120 min (Fig. 5). The effect of Mg$^{2+}$ and DCB alone and in combination on cell Ca$^{2+}$ during reoxygenation was tested. Mg$^{2+}$ (5 mM) significantly reduced the elevation in cell Ca$^{2+}$ by 40%, DCB by 57%, and their combination by 75% (1 h after reoxy-

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Fig. 2. Effect of Mg$^{2+}$ on reactive oxygen species (ROS) levels determined as dichlorofluorescein (DCF) fluorescence during reoxygenation of hypoxic cardiomyocytes. ROS levels in cells exposed to 1 h of hypoxia and 2 h of reoxygenation (■) and cells exposed to 1 h of hypoxia and 2 h of reoxygenation with 5 mM Mg$^{2+}$ (▲) are shown. Values are given as a percentage of control cells at the respective incubation times (●). A: cold-fixed cell preparations. B: nonfixed cells. *P < 0.05 and †P < 0.05 vs. normoxic control cells (●) and ‡P < 0.05 vs. hypoxia/reoxygenation (▲) at the respective incubation times by ANOVA and the least significant difference (LSD) method in both A and B. Each point represents the means ± SE of 9–12 and 7–11 determinations from 4 and 5 different cell isolations in A and B, respectively. Each determination is the mean DCF fluorescence intensity of 5,000 cells.

Fig. 3. Effect of Mg$^{2+}$ on ROS levels determined as ethidium (ETH) fluorescence during reoxygenation of hypoxic cardiomyocytes. ROS levels in cells exposed to 1 h of hypoxia and 2 h of reoxygenation (■) and cells exposed to 1 h of hypoxia and 2 h of reoxygenation with 5 (▲) and 15 mM Mg$^{2+}$ (●) are shown. Values are given as a percentage of control cells at the respective incubation times (●). Each point represents the means ± SE of 8 determinations from 4 different cell isolations. Each determination is the mean ETH fluorescence intensity of 5,000 cells. *P < 0.05 and †P < 0.05 vs. normoxic control cells (●). There was no significant difference between the means of groups exposed to hypoxia/reoxygenation with or without Mg$^{2+}$ at the respective incubation times by ANOVA and the LSD method.

Fig. 4. Effect of Mg$^{2+}$ on cell Ca$^{2+}$ during reoxygenation of hypoxic cardiomyocytes. Cell Ca$^{2+}$ in cells exposed to 1 h of hypoxia and 2 h of reoxygenation (■) and cells exposed to 1 h of hypoxia and 2 h of reoxygenation with 5 (▲) and 15 mM Mg$^{2+}$ (●) compared with control cells (●) are shown. Each point represents the means ± SE of 16 determinations from 4 different cell isolations. *P < 0.05 vs. hypoxia/reoxygenation (■) at the respective incubation times by ANOVA and the LSD method.
Combination on cell Ca\(^{2+}\) during reoxygenation. Cell Ca\(^{2+}\) in control cells, cells exposed to 1 h of hypoxia and 1 h of reoxygenation, and cells exposed to 1 h of hypoxia (H) and 1 h of reoxygenation (R) with Mg\(^{2+}\) increased to 5 mM from isotonic solutions of either MgSO\(_4\) or MgCl\(_2\) (290 mosM) to avoid osmolarity changes in the incubation buffer are shown. Each column represents the means ± SE of 6 determinations in duplicates or quadruplicates from 3 different cell isolations. *\(P < 0.005\) vs. hypoxia/reoxygenation by ANOVA and the LSD method.

Fig. 5. Effect of MgSO\(_4\) or MgCl\(_2\) on cell Ca\(^{2+}\) during reoxygenation of hypoxic cardiomyocytes. Cell Ca\(^{2+}\) in control cells, cells exposed to 1 h of hypoxia and 1 h of reoxygenation, and cells exposed to 1 h of hypoxia (H) and 1 h of reoxygenation (R) with Mg\(^{2+}\) increased to 5 mM from isotonic solutions of either MgSO\(_4\) or MgCl\(_2\) (290 mosM) to avoid osmolarity changes in the incubation buffer are shown. Each column represents the means ± SE of 6 determinations in duplicates or quadruplicates from 3 different cell isolations. *\(P < 0.005\) vs. hypoxia/reoxygenation by ANOVA and the LSD method.

The Na\(^+-K\)-ATPase inhibitor ouabain (1 mM), added at the start of hypoxia, amplified the increase in cell Ca\(^{2+}\) sixfold by the end of reoxygenation compared with hypoxia/reoxygenation without ouabain. This large increase in cell Ca\(^{2+}\) was reduced 20% by Mg\(^{2+}\) (5 mM), 55% by DCB (10 \(\mu\)M), and 66% by the combination of Mg\(^{2+}\) and DCB than with Mg\(^{2+}\) alone (Fig. 6).

Fig. 6. Effect of Mg\(^{2+}\), 3,4-dichlorobenzamil (DCB), and their combination on cell Ca\(^{2+}\) during reoxygenation of hypoxic cardiomyocytes. Cell Ca\(^{2+}\) in control cells (●), cells exposed to 1 h of hypoxia and 2 h of reoxygenation with 5 mM Mg\(^{2+}\) (▲), 10 \(\mu\)M DCB (○) and Mg\(^{2+}\) and DCB in combination (●) are shown. All supplements were added at the onset of reoxygenation. Each point represents the means ± SE of 12–20 determinations from 3 different cell isolations. *\(P < 0.05\) vs. hypoxia/reoxygenation (●) and †\(P < 0.05\) vs. hypoxia/reoxygenation with Mg\(^{2+}\) (▲) at the respective incubation times by ANOVA and the LSD method.

Fig. 7. Effect of Mg\(^{2+}\), DCB, and their combination on cell Ca\(^{2+}\) during reoxygenation of hypoxic/ouabain-treated cardiomyocytes. Cell Ca\(^{2+}\) in control cells (●), cells exposed to 1 h of hypoxia and 2 h of reoxygenation (●), cells exposed to 1 h of hypoxia and 2 h of reoxygenation in the presence of ouabain (1 mM) added from start (○), cells exposed to 1 h of hypoxia and 2 h of reoxygenation in the presence of ouabain added from start with 5 mM Mg\(^{2+}\) (▲), 10 \(\mu\)M DCB (○), and Mg\(^{2+}\) and DCB in combination (●) are shown. All supplements were added at the onset of reoxygenation. Each point represents the means ± SE of 12–16 determinations from 3 different cell isolations. *\(P < 0.05\) vs. hypoxia/reoxygenation in the presence of ouabain (○) and †\(P < 0.05\) vs. hypoxia/reoxygenation in the presence of ouabain with Mg\(^{2+}\) (▲) at the respective incubation times by ANOVA and the LSD method.

Effect of H\(_2\)O\(_2\) and NO donor SNAP on cell Ca\(^{2+}\) during reoxygenation. We found that Mg\(^{2+}\) increased the fluorescence signal obtained from the H\(_2\)O\(_2\) and the NO-sensitive probe DCF during reoxygenation and that Mg\(^{2+}\) also reduced Ca\(^{2+}\) accumulation during reoxygenation. We tested whether H\(_2\)O\(_2\) and the NO donor SNAP, added extracellularly, affected cell Ca\(^{2+}\) during reoxygenation. H\(_2\)O\(_2\) (25 and 500 \(\mu\)M) significantly reduced Ca\(^{2+}\) accumulation by 38 and 43%, respectively, at 120 min (1 h of hypoxia and 1 h of reoxygenation) (3 separate experiments, \(P < 0.05\), paired t-test), whereas SNAP (1 mM) had no effect (Fig. 8).

Effect of Mg\(^{2+}\) and DCB on LDH release during reoxygenation. Mg\(^{2+}\) reduced the increase in LDH release (measured in the incubation buffer at 120 and 180 min) almost completely in the cells exposed to hypoxia/reoxygenation, whereas DCB had no significant effect (Fig. 9A). In the cells exposed to hypoxia/reoxygenation in the presence of ouabain, LDH release was increased by a factor of 3 compared with normoxic controls. This increase was significantly reduced 33% by Mg\(^{2+}\), 25% by DCB, and 64% by the combination of...
Mg²⁺ and DCB (at 180 min). Thus the combination of DCB and Mg²⁺ is more effective in reducing LDH release than each agent alone in the presence of very high cell Ca²⁺ levels (Fig. 9B). The addition of H₂O₂ (0.5 mM) increased LDH release by 89% compared with cells exposed to hypoxia and reoxygenation alone, whereas 25 μM H₂O₂ reduced LDH release significantly by 15% (three separate experiments, P < 0.05, paired t-test).

DISCUSSION

Reperfusion of the ischemic myocardium causes reperfusion injury, which is associated with and possibly mediated by increased levels of ROS and Ca²⁺ overload. This study was designed to investigate the effect of high extracellular Mg²⁺ on ROS, cell Ca²⁺ accumulation, and LDH release during reoxygenation of hypoxic cardiomyocytes. We found that Mg²⁺ increased oxidation of DCDHF (to fluorescent DCF), a sensitive probe to H₂O₂ and NO/NO-based radicals in this model. Mg²⁺ also reduced Ca²⁺ accumulation and LDH release from hypoxic rat cardiomyocytes during reoxygenation.

Sensitivity of DCDHF and DHE to different ROS types. O₂⁻ is a key radical in generation of ROS. Oxidation of DHE within the cell to the fluorescent ETH has been used previously as an indicator for O₂⁻ (49). Our recent findings (39), that ETH fluorescence increased during reoxygenation was reduced by the antioxidant N-2-mercaptopropionyl-glycine and influenced by inhibitors of mitochondrial electron transport, confirmed the sensitivity of DHE to O₂⁻. The
large increase in ETH fluorescence by the SOD inhibitor DDC, the small increase with \( \text{H}_2\text{O}_2 \), and the lack of sensitivity to the NO donor SNAP indicates that ETH is a probe that is primarily sensitive for \( \text{O}_2^- \) levels. The lack of increase in ETH fluorescence by menadione (\( \text{O}_2^- \)-donor) was unexpected. On the contrary, the ETH signal was totally abolished in the cold-fixed cell preparations and reduced in the nonfixed cell preparations in the presence of menadione. The most likely explanation is that menadione quenches the fluorescence signal of ETH and not that ETH is insensitive to \( \text{O}_2^- \).

Oxidation of DCDHF within the cell to the fluorescent DCF has been used as a probe for \( \text{H}_2\text{O}_2 \) (8, 49), which is produced from \( \text{O}_2^- \) by a reaction catalysed by SOD. The increase in DCF fluorescence with \( \text{H}_2\text{O}_2 \) and the \( \text{O}_2^- \)-donor menadione in the present work suggests that DCDHF oxidation is sensitive to both \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \). However, almost complete abolishment of the menadione-induced increase in DCF fluorescence with DDC convincingly demonstrated that oxidation of DCDHF to DCF is much more sensitive to \( \text{H}_2\text{O}_2 \) than \( \text{O}_2^- \). The increase in DCF fluorescence with the NO donor SNAP indicated that oxidation of DCDHF to DCF can also be related to NO/NO-based radicals, in agreement with observations by Ischiropoulos et al. (21) and Pos sel et al. (37).

**Effect of Mg\(^{2+}\) on ROS levels during reoxygenation of hypoxic cardiomyocytes.** To our knowledge, this is the first report on the effect of elevated extracellular Mg\(^{2+}\) on ROS levels in cardiomyocytes exposed to hypoxia/reoxygenation. We found in the present study significantly increased DCF and ETH fluorescence during reoxygenation, indicating increased ROS levels (\( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \)) and possibly increased levels of NO/NO-based radicals. Elevated ROS (\( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \)) levels during reoxygenation was presumably due to reestablished substrate availability and reduced antioxidant capacity and possibly to damage of the mitochondrial electron transport chain. Such an increase in ROS is in accordance with the established effects of hypoxia/reoxygenation (12, 54). Our results showing that elevated extracellular Mg\(^{2+}\) during reoxygenation further increased DCF fluorescence but not ETH fluorescence indicate that the levels of \( \text{H}_2\text{O}_2 \) and/or NO/NO-based radicals were further increased by Mg\(^{2+}\) during reoxygenation without a corresponding increase in \( \text{O}_2^- \) level. Such an increase in \( \text{H}_2\text{O}_2 \) by Mg\(^{2+}\) might be caused by increased formation (from \( \text{O}_2^- \)), increased SOD activity, and/or reduced elimination of \( \text{H}_2\text{O}_2 \) by antioxidants, catalase and glutathione peroxidase. There are previous reports (10, 55) showing an enhanced myocardial NO synthesis during ischemia-reperfusion. There are no reports on the effect of Mg\(^{2+}\) on NO during hypoxia/reoxygenation to our knowledge. Enhanced NO production has been observed in tissues (red blood cells and rat plasma) from Mg\(^{2+}\)-deficient rats (33, 38). The present results do not allow a firm conclusion as to whether \( \text{H}_2\text{O}_2 \) or NO or both represent the oxidant type that is increased by Mg\(^{2+}\) in this study.

**Effect of Mg\(^{2+}\) on Ca\(^{2+}\) accumulation during reoxygenation of hypoxic cardiomyocytes.** Results from the present work demonstrated that Mg\(^{2+}\) (5 mM) inhibited Ca\(^{2+}\) accumulation during reoxygenation of hypoxic cardiomyocytes (5 mM Mg\(^{2+}\) apparently was the maximally effective Mg\(^{2+}\) concentration because 15 mM Mg\(^{2+}\) did not produce greater inhibition than 5 mM). MgSO\(_4\) and MgCl\(_2\) salts, added from their isotonic solutions, reduced Ca\(^{2+}\) accumulation equally and to the same degree as MgSO\(_4\), added from 1 M solution. This excludes that the inhibitory effect of MgSO\(_4\), added from 1 M solution, on Ca\(^{2+}\) accumulation during reoxygenation was caused by SO\(_4^2^-\) or increased osmolarity. In a recent paper (39), we showed that reoxygenation-induced Ca\(^{2+}\) uptake was not inhibited by the L-type Ca\(^{2+}\) channel inhibitor verapamil (1 or 10 \( \mu \text{M} \)) but was inhibited ~70% by the Na\(^+\)/Ca\(^{2+}\) exchange inhibitor DCB. We concluded that this Ca\(^{2+}\) uptake was probably mediated by Na\(^+\)/Ca\(^{2+}\) exchange. The present results can therefore be explained by inhibition of Na\(^+\)/Ca\(^{2+}\) exchange caused by higher extracellular Mg\(^{2+}\) concentrations during reoxygenation. There is substantial support in the literature for the ability of Mg\(^{2+}\) to inhibit Na\(^+\)/Ca\(^{2+}\) exchange. An inhibitory effect of Mg\(^{2+}\) on Na\(^+\)/Ca\(^{2+}\) exchange has been shown in rat vascular smooth muscle during lowering of extracellular Na\(^+\) (3). In guinea pig cardiac myocytes, the outward Na\(^+\)/Ca\(^{2+}\) exchange current was reduced by increasing extracellular Mg\(^{2+}\) (28). Howarth and Levi (19) showed that, in patch-clamped rabbit ventricular myocytes, internal free Mg\(^{2+}\) might partially inhibit the activity of the Na\(^+\)/Ca\(^{2+}\) exchange. Because intracellular Na\(^+\) accumulation is a prerequisite for Na\(^+\)/Ca\(^{2+}\) exchange to work in reverse mode (beside membrane potential), we also used hypoxic/ouabain-treated cells for testing the effect of Mg\(^{2+}\) under conditions where an amplified Na\(^+\)/Ca\(^{2+}\) exchange takes place. Mg\(^{2+}\) also attenuated Ca\(^{2+}\) accumulation in hypoxic/ouabain-treated cells in which a much larger uptake of Ca\(^{2+}\) occurred at reoxygenation (6 times higher than hypoxia/reoxygenation without ouabain). Relative inhibitory effects of Mg\(^{2+}\) and DCB were somewhat smaller in the presence than absence of ouabain. Because the inhibitory effect of the combination of Mg\(^{2+}\) and DCB was not significantly larger than that of DCB alone (Figs. 6 and 7), it could not be decided whether Mg\(^{2+}\) and DCB exerted their inhibitory effects via the same or different mechanisms.

**Interaction between Ca\(^{2+}\) and ROS.** In the present study, Mg\(^{2+}\) reduced Ca\(^{2+}\) accumulation and increased ROS levels. An interesting question is whether these two effects are interdependent. One possibility is that Mg\(^{2+}\)-induced reduction in cell Ca\(^{2+}\) caused increased ROS. However, there appears to be no evidence in favor of such an effect. On the contrary, we found in a previous study (39) that a decrease in cell Ca\(^{2+}\) (obtained by reducing extracellular Ca\(^{2+}\)) was associated with reduced ROS levels (measured as ETH fluorescence). Moreover, Greene and Paller (13) observed that...
Ca\textsuperscript{2+} derived from extracellular sources was associated with an increase in O\textsubscript{2}\textsuperscript{-} production via a calmodulin-dependent conversion of xanthine dehydrogenase to xanthine oxidase during hypoxia and reoxygenation of cultured renal epithelial cells. Thus inhibition of Ca\textsuperscript{2+} uptake by Mg\textsuperscript{2+}, as demonstrated in the present study, cannot easily explain the increase in ROS levels (measured as DCF fluorescence). On the other hand, there is a possibility that a Mg\textsuperscript{2+}-induced increase in ROS levels caused an inhibition of Ca\textsuperscript{2+} accumulation by inhibiting, for example, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange. Therefore, we tested the effect of \textit{H\textsubscript{2}O\textsubscript{2}} and SNAP (NO donor) on Ca\textsuperscript{2+} accumulation during reoxygenation. The finding that 25 or 500 \textmu{}M \textit{H\textsubscript{2}O\textsubscript{2}} significantly decreased Ca\textsuperscript{2+} accumulation, whereas SNAP had no effect, would fit the hypothesis that Mg\textsuperscript{2+} reduced Ca\textsuperscript{2+} accumulation by increasing \textit{H\textsubscript{2}O\textsubscript{2}}. As discussed in the previous section, in the present model, there is evidence that the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger is responsible for Ca\textsuperscript{2+} accumulation during reoxygenation. The data in the literature on the effects of ROS on Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange and on Ca\textsuperscript{2+} homeostasis in the heart are controversial, as reviewed by Kaneko et al. (26). Ka-minishi et al. (25) also observed an inhibitory effect of \textit{H\textsubscript{2}O\textsubscript{2}} at high concentrations (5–10 mM) on cell Ca\textsuperscript{2+} at room temperature in oxygenated adult rat cardiomyocytes. Our results, obtained at 37°C, are consistent with their findings. These effects of ROS need to be further investigated.

\textit{Effect of Mg\textsuperscript{2+} on LDH release during reoxygenation of hypoxic cardiomyocytes.} Release of intracellular enzymes is a consequence of cell damage and cell membrane alterations. Our results showing that Mg\textsuperscript{2+} inhibited LDH release in reoxygenated cells suggest a protective role of Mg\textsuperscript{2+} during reoxygenation of cardiomyocytes. Modulation of ROS metabolism and/or reduction of Ca\textsuperscript{2+} accumulation may be the underlying mechanism(s) for the protective action of Mg\textsuperscript{2+}. Previously, ROS in excess of cellular antioxidant capacity have been associated and correlated to cell injury, and antioxidative treatments have shown a protective effect in the posthypoxic myocardium (4, 5, 23). The diversity and manifold of ROS (and NO-based radicals) and their possible effects make it difficult to predict the exact relation of different ROS to cell damage. Byler et al. (7) showed that \textit{H\textsubscript{2}O\textsubscript{2}} cytotoxicity in cultured cardiac myocytes requires reactions catalyzed by intracellular iron necessary for production of highly reactive -OH. Some ROS activate cell signaling cascades with protective effects. Extracellular signal-regulated kinases activation was shown to protect cardiac myocytes from apoptotic cell death during oxidative stress (2). Das et al. (9) showed that ROS function as second messenger during ischemic preconditioning of the heart and are associated with reduced myocardial infarct size on subsequent prolonged ischemia. Direct evidence for the protective effect of \textit{H\textsubscript{2}O\textsubscript{2}} also exists. Hegstad et al. (18) reported that low concentrations of \textit{H\textsubscript{2}O\textsubscript{2}} (25 \textmu{}M) actually improved posts ischemic recovery of the rat heart. Thus our findings indicating that Mg\textsuperscript{2+} increased certain ROS raise the possibility that protective effects of Mg\textsuperscript{2+} might involve one of the following mechanisms: 1) Mg\textsuperscript{2+}-induced increase in \textit{H\textsubscript{2}O\textsubscript{2}}, possibly as a result of less conversion of \textit{H\textsubscript{2}O\textsubscript{2}} to highly reactive -OH; 2) signaling pathways mentioned above may be the underlying mechanism for protection; or 3) NO/NO-based radicals may be involved. Extrapolating data from the effect of exogenously added \textit{H\textsubscript{2}O\textsubscript{2}} on DCF fluorescence in normoxic cells (Fig. 1) and DCF fluorescence in reoxygenated cells (Fig. 2, A and B) indicated that the \textit{H\textsubscript{2}O\textsubscript{2}} level in the presence of increased extracellular Mg\textsuperscript{2+} was ~10–20 \textmu{}M. Because addition of 25 \textmu{}M \textit{H\textsubscript{2}O\textsubscript{2}} reduced the LDH release in posthypoxic cardiomyocytes, the protective effect of Mg\textsuperscript{2+} on LDH release may at least partly be explained by increased \textit{H\textsubscript{2}O\textsubscript{2}}. The increase in LDH release by a higher concentration of \textit{H\textsubscript{2}O\textsubscript{2}} (500 \textmu{}M) shows that this concentration is cytotoxic, which is in agreement with reports from others (7, 24).

On the other hand, a reduction in LDH release by Mg\textsuperscript{2+} was also correlated with reduced cell Ca\textsuperscript{2+} accumulation, an observation in accordance with previous extensive evidence that Ca\textsuperscript{2+} overload has deleterious effects in the posthypoxic myocardium (30, 50). In cells exposed to hypoxia and reoxygenation in the presence of ouabain, the reduction in LDH release was correlated with reduced cell Ca\textsuperscript{2+} by both Mg\textsuperscript{2+} and DCB, and, under these conditions, the combination of Mg\textsuperscript{2+} and DCB was more effective than either agent alone in reducing LDH release. These observations are consistent with a role for Ca\textsuperscript{2+} in cell damage and subsequent LDH release in our model.

In conclusion, elevation of extracellular Mg\textsuperscript{2+} to 5 mM at reoxygenation increased the fluorescence of the \textit{H\textsubscript{2}O\textsubscript{2}}/NO-sensitive probe DCF without increasing that of the O\textsubscript{2}\textsuperscript{-}-sensitive probe ETH, reduced Ca\textsuperscript{2+} accumulation, and decreased LDH release during reoxygenation of hypoxic and hypoxic/Na\textsuperscript{+}-loaded (ouabain treated) cardiomyocytes. The reduction in LDH release, reflecting the protective effect of Mg\textsuperscript{2+}, may be linked to the effect of Mg\textsuperscript{2+} on ROS levels, Ca\textsuperscript{2+} accumulation, or both. Further studies should be performed to clarify the effect of Mg\textsuperscript{2+} on ROS metabolism, for example, the type(s) of radicals/ROS and site(s) of ROS production that are affected. The mechanism behind the action of Mg\textsuperscript{2+} on Ca\textsuperscript{2+} transport systems and accumulation during reoxygenation also needs further investigation.

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