

Reactive Oxygen Species Are Required for the Rapid Reactivation of the Sodium-Calcium Exchanger in Hypoxic/Reoxygenated Guinea Pig Ventricular Myocytes

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Running Head: ROS are Required for Rapid NCX Reactivation

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ABSTRACT

The cardiac Na^+ - Ca^{2+} exchanger (NCX) contributes to cellular injury during hypoxia, as its altered function is largely responsible for a rise in cytosolic $[\text{Ca}^{2+}]$. In addition, the NCX in guinea-pig ventricular myocytes undergoes profound inhibition during hypoxia, and rapid reactivation during reoxygenation. The mechanisms underlying these changes in NCX activity are likely complex due to the participation of multiple inhibitory factors, including altered cytosolic $[\text{Na}^+]$, pH and ATP. Our main hypothesis is that oxidative stress is an essential trigger for rapid NCX reactivation in guinea-pig ventricular myocytes, and is thus a critical factor in determining the timing and magnitude of calcium overload. This hypothesis was evaluated in cardiac myocytes using fluorescent indicators to measure cytosolic $[\text{Ca}^{2+}]$ and oxidative stress. An NCX antisense oligonucleotide was used to decrease NCX protein expression in some experiments. Our results indicate that NCX activity is profoundly inhibited in hypoxic guinea-pig ventricular myocytes, but is reactivated within 1-2 minutes of reoxygenation, at a time of rising oxidative stress. We also found that several interventions to decrease oxidative stress, including antioxidants and diazoxide, prevented NCX reactivation and Ca^{2+} overload during reoxygenation. Furthermore, application of exogenous H_2O_2 was sufficient by itself to reactivate the NCX during sustained hypoxia, and could reverse the suppression of reoxygenation-mediated NCX reactivation by diazoxide. These data suggest that elevated oxidative stress in reoxygenated guinea-pig ventricular myocytes is required for rapid NCX reactivation, and thus reactivation should be viewed as an active process, rather than being due to the simple decline of NCX inhibition.

Keywords: antioxidants, diazoxide, heart, hypoxia, ischemia

INTRODUCTION

Dangerously elevated levels of cytosolic $[Ca^{2+}]$, or Ca^{2+} overload, occurs during ischemia or hypoxia in both intact heart and isolated cardiac myocytes (32). The important contribution of Ca^{2+} overload to rapid necrotic death and arrhythmogenesis following ischemia has long been appreciated (24, 32), and it is also likely that Ca^{2+} overload contributes to the induction of myocardial apoptosis (9, 35). A number of studies have shown that the NCX is involved in mediating Ca^{2+} overload following ischemia or hypoxia (11, 21, 30, 33). It is thought that under hypoxic conditions, there is a substantial rise in cytosolic $[Na^+]$ that can promote reverse-mode NCX transport, where the NCX mediates Na^+ efflux and Ca^{2+} influx. We have previously reported that the NCX is the predominant route of Ca^{2+} entry following hypoxia/reoxygenation in adult guinea-pig ventricular myocytes, as cultured myocytes pre-treated with a NCX antisense oligonucleotide showed little rise in cytosolic $[Ca^{2+}]$ when subjected to hypoxia/reoxygenation (7). Therefore, a more complete understanding of the factors that regulate NCX activity, and thus influence Ca^{2+} overload during hypoxia/reoxygenation, would be of great interest.

Previous studies have reported evidence that the NCX is likely inhibited to some degree during hypoxia, and becomes reactivated at some point during reoxygenation (20, 31). However, a full understanding of NCX activity during hypoxia is complicated by the potential involvement of several factors known to regulate NCX activity, including decreased ATP levels (4, 13), cytosolic acidification (5), and increased cytosolic $[Na^+]$ (13). Na^+ -dependent inactivation may be of special interest, as it could serve as a nexus for several NCX regulatory signaling pathways (5, 12).

Reactive oxygen species (ROS) could also modulate NCX activity during ischemia or

hypoxia. ROS can be generated in cardiac myocytes during hypoxia, although a much larger burst of ROS often occurs during reoxygenation (34). The effects of ROS on NCX activity seem complex, and may vary with cell type, species of ROS, and experimental model (1, 10, 27). Perhaps the most interesting effects of ROS on NCX were reported by Santacruz-Toloza et al., who concluded that oxidizing conditions could stimulate NCX activity through removal of Na⁺-dependent inactivation (29). This suggests the possibility that increased ROS levels might be able to overcome some of the inhibitory effects of hypoxia.

We hypothesize that increased oxidative stress during reoxygenation is crucial to initiating both NCX reactivation and the subsequent appearance of calcium overload in guinea-pig ventricular myocytes. Thus, NCX reactivation would be viewed as resulting from the rapid stimulation of NCX activity during reoxygenation, rather than simply due to the slow waning of inhibition as the cell restores more physiological levels of Na⁺, pH and ATP. This hypothesis also implies that NCX reactivation could be induced even during sustained hypoxia, by applying reactive oxygen species to the cardiac myocyte. Here we present data in support of this hypothesis, obtained in guinea-pig ventricular myocyte preparations where we can largely suppress NCX protein expression, and using measurements of cytosolic [Ca²⁺], oxidative stress, and reverse-mode NCX activity.

MATERIALS AND METHODS

Cell Preparations

Experiments were conducted in single adult guinea pig ventricular myocytes. Experiments that involved long-term treatment of the myocytes with antisense oligonucleotides (Figures 2 and 9) required the use of cultured adult guinea-pig ventricular myocytes, while the other experiments used freshly isolated myocytes. Female Hartley guinea pigs were anesthetized with an intraperitoneal injection of sodium pentobarbital before excising the heart. Cells were isolated using an established collagenase dispersion technique (26). A total of 40 animals were used to prepare myocytes for this study, with each figure (except Figure 1) containing data obtained using 3-9 isolations. All procedures using animals were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Cultured adult myocytes were prepared using a sterile technique described previously (7). Isolated myocytes were suspended in serum-free Medium-199 which was supplemented with (mmol/L): 25 HEPES, 5 creatine, 2 L-carnitine, 5 taurine, and 10^{-4} insulin. 0.2% BSA, 100 I.U. penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin were also included. MatTek glass bottom 35mm microwell dishes were coated with 6 μg Cell-Tak™ Cell Adhesive (BD Biosciences, Bedford MA). Myocytes were plated at a density of 10^4 cells/cm² and allowed to attach for four hours, after which the medium was removed and replaced with fresh medium. Myocytes were kept under sterile conditions in a 5% CO₂ incubator at 37°C.

The response of freshly isolated or cultured guinea-pig ventricular myocytes to either the reverse-mode NCX activity assay (7) or hypoxia/reoxygenation (Figure 2B) were very similar. However, to account for potential qualitative differences between the two types of myocyte

preparations, statistical comparisons were only made between treatment groups within a given preparation.

Experimental Protocols

Hypoxia/reoxygenation experiments used a nitrogen-bubbled extracellular solution containing the following (mmol/L): 140 NaCl, 4 KCl, 2.5 CaCl₂, 1 MgCl₂, and 10 HEPES (pH 7.2, 22°C). Experiments were carried out at 22°C to ensure compatibility with earlier studies (6,7). Quiescent myocytes were made hypoxic in a glass, gas-tight Petri dish (6, 26). Hypoxia was maintained for 20 minutes post-rigor at which time the hypoxic solution was removed and replaced with oxygenated solution containing 11 mmol/L glucose. This protocol produces a robust rise in cytosolic [Ca²⁺] that is almost entirely due to reverse-mode NCX activity (7). Experiments using a shorter hypoxic interval produced a smaller rise in cytosolic [Ca²⁺], but which was also NCX-dependent (see also Figure 2B).

The K⁺-free solution used in the reverse-mode NCX activity assay contained the following (in mmol/L): 144 NaCl, 2.5 CaCl₂, 1 MgCl₂, and 10 HEPES (pH 7.2). The Na⁺-free solution contained 140 LiCl, 4 KCl, 2.5 CaCl₂, 1 MgCl₂, 10 HEPES (pH 7.2). Glucose (11 mmol/L) was added unless the solutions were made hypoxic.

Fluorescence measurements of cytosolic [Ca²⁺] and oxidative stress

Oxidative stress was measured in ventricular myocytes that had been loaded with 1 μmol/L of either dihydrorhodamine 123 (DHR-123) or 5-(and 6-)chloromethyl-2',7'-dichlorodihydrofluorescein diacetate ethyl ester (CM-DCFDA) for 30 minutes at 22°C. Indicator fluorescence was measured on a Nikon RCM 8000 laser-scanning confocal microscope using

either the 543 nm output of a HeNe laser, or the 488 nm output of an Ar laser for excitation.

Fluorescence images were acquired at wavelengths greater than 545 nm, and subsequently analyzed with the software program MetaMorph[®] (Universal Imaging, West Chester PA). Both indicators were well retained in the myocytes. Significant photo-oxidation of the indicator was avoided by limiting the number of images acquired during an experiment.

Cytosolic $[Ca^{2+}]$ was measured in myocytes loaded with the fluorescent indicators indo-1 AM (2.5 $\mu\text{mol/L}$ for 20 minutes at 22°C), calcium orange AM (10 $\mu\text{mol/L}$ for 60 minutes), or calcium green AM (1 $\mu\text{mol/L}$ for 30 minutes). Indo-1 could not be used in experiments where H_2O_2 was applied, as H_2O_2 readily bleached indo-1. Images were acquired on the confocal microscope using excitation wavelengths of 351-364 nm for indo-1, 488 nm for calcium green, and 543 nm for calcium orange. Emitted fluorescence was measured at wavelengths greater than 545 nm for calcium green or orange, while dual emission images at wavelengths greater or less than 445 nm were acquired for indo-1. Standard ratiometric analysis procedures were applied to the indo-1 images, as previously described (26). Cytosolic $[Ca^{2+}]$ is reported as relative changes in calcium green or orange fluorescence, or as relative changes in the indo-1 fluorescence ratio. Absolute $[Ca^{2+}]$ was not calculated, due to the non-ratiometric nature of calcium green and orange, and because hypoxia alters cytosolic pH, which affects indo-1 calibration (26).

Fluorescence measurements of cytosolic $[Na^+]$ or pH

Cytosolic $[Na^+]$ was measured in myocytes loaded with sodium-binding benzofuran isophthalate (SBFI). Myocytes were loaded with 10 $\mu\text{mol/L}$ of the acetoxymethyl (AM) ester form of SBFI for 50 minutes at 22°C.

Cytosolic pH was measured in myocytes loaded with 2',7'-bis(carboxyethyl)-5(6)-

carboxyfluorescein (BCECF). Myocytes were loaded for 30 minutes at 22°C with 1 µmol/L BCECF-AM. pH measurements were calibrated by comparing BCECF fluorescence to that obtained in myocytes exposed to various calibration solutions containing in (mmol/L) 145 KCl, 2 MgCl₂, 0.05 CaCl₂, 0.025 nigericin, and 11 glucose. The pH of each solution was adjusted between 4.0-9.0 using appropriate pH buffers (MES, HEPES, or Tris).

SBF1 or BCECF fluorescence measurements were made using an inverted fluorescence microscope with a 75 W Xe arc lamp serving as an excitation source. Ratiometric SBF1 measurements were made using interference filters centered at 340 and 380 nm on the excitation side, and 540 nm on the emission side. BCECF measurements were made using 440 and 490 nm excitation filters, and a 535 nm emission filter. Fluorescence was measured using photomultiplier tubes. The output was filtered with a time constant of 50 ms and then digitized on a microcomputer at 1-2 kHz. Standard background subtraction and ratiometric analysis methods were used.

Inhibition of NCX protein expression by an antisense oligonucleotide

An antisense oligonucleotide (5'-**TCGCAGCATGTTGTACAATG**-3') was targeted to a region around the start codon of the cardiac guinea pig NCX (-11 to +9). The oligonucleotide had eight phosphorothioate-modified nucleotides (boldface). A nonsense oligonucleotide (5'-**TCTCGAACGTGTTCAAGATG**-3') was used to control for non-specific effects of the antisense oligonucleotide. Cultured myocytes were treated with antisense (2 µmol/L), nonsense (2 µmol/L), or no oligonucleotide as needed. Fresh oligonucleotide and Medium-199 were added every 48 hours. All cultured myocytes were maintained in culture for 5-7 days. This protocol is very effective at inhibiting NCX protein expression, as there is almost complete

suppression of both evoked reverse-mode NCX activity and sarcolemmal NCX immunofluorescence (7). Oligonucleotides were synthesized at the University of Kentucky Macromolecular Structure Analysis Facility or Integrated DNA Technologies (Coralville IA).

Statistical Analysis

Differences between means were analyzed using paired t-tests when two groups were under comparison, or a one-way analysis of variance for more than two groups. Student-Newman-Keuls multiple comparison testing was used for post hoc significance testing between appropriate groups if warranted following analysis of variance testing. Variance is described as the standard errors of the mean.

RESULTS

We have previously shown that in guinea-pig ventricular myocytes Ca^{2+} overload during hypoxia/reoxygenation is primarily due to reverse-mode NCX activity (7). Figure 1 shows an example of the response of cytosolic $[\text{Ca}^{2+}]$ to hypoxia/reoxygenation in this model. Cytosolic $[\text{Ca}^{2+}]$ was measured in a freshly isolated guinea-pig ventricular myocyte loaded with the fluorescent Ca^{2+} indicator indo-1 AM. The myocyte was exposed to hypoxic conditions (see Methods) for 20 minutes post-rigor, when it was then reoxygenated. It can be seen from the data that the majority of the rise in $[\text{Ca}^{2+}]$ in this model occurs after reoxygenation (also see Figures 3, 4, 7 and 8, and reference 7), despite the presence of substantially elevated $[\text{Na}^+]$ during the prolonged hypoxic period (6, 26).

The possibility that cardiac NCX is profoundly inhibited during hypoxia, and rapidly reactivated by reoxygenation of these cells, was evaluated in experiments summarized in Figure 2. Figure 2A shows typical results from an indo-1 AM loaded isolated myocyte undergoing a functional assay of reverse-mode NCX activity. The myocyte was first exposed to a K^+ -free solution, which is known to inhibit the Na^+/K^+ ATPase and elevate cytosolic $[\text{Na}^+]$. The solution was then switched to a Na^+ -free one, which stimulates the NCX to exchange intracellular Na^+ for extracellular Ca^{2+} . The result of this reverse-mode NCX activity is seen as an increase in the indo-1 fluorescence ratio, and is not seen in guinea-pig ventricular myocytes pre-treated with a NCX antisense oligonucleotide (7).

Figure 2B shows a similar protocol, except that either freshly isolated or cultured myocytes were made hypoxic for 15 minutes before the K^+ -free period, and hypoxia was maintained until the final five minutes of Na^+ -free conditions. The responses of both isolated and cultured myocytes were very similar. In either set of control myocytes, there was no

significant increase in cytosolic $[Ca^{2+}]$ during Na^+ -free hypoxia, in contrast to the large rise in $[Ca^{2+}]$ that occurred under normoxic conditions (Figure 2A). However, when control myocytes were subjected to Na^+ -free reoxygenation, there was a significant increase in cytosolic $[Ca^{2+}]$. These data suggest that NCX activity was strongly inhibited during hypoxia and this inhibition was overcome during reoxygenation. Furthermore, the inability of reoxygenation to induce a significant rise in cytosolic $[Ca^{2+}]$ in cultured myocytes pre-treated with 2 μ mol/L NCX antisense oligonucleotide is evidence that the rise in cytosolic $[Ca^{2+}]$ can be attributed to reverse-mode NCX activity. The maximum indo-1 ratio of nonsense-treated myocytes during reoxygenation was indistinguishable from control cultured myocytes (1.32 ± 0.09 , N=3 versus 1.36 ± 0.09 , N=6).

Figure 2B also shows our first data evaluating whether elevated ROS levels are important to NCX reactivation during reoxygenation. It can be seen that either isolated or cultured myocytes treated with 40 μ mol/L manganese tetrakis (4-benzoic acid)porphyrin chloride (MnTBAP), a mimetic of superoxide dismutase, also failed to show a significant rise in cytosolic $[Ca^{2+}]$ during Na^+ -free reoxygenation.

In order to further examine the role of ROS in our model, three different antioxidants were used to test whether reduced oxidative stress altered cytosolic Ca^{2+} overload during reoxygenation (Figure 3). As shown, cytosolic $[Ca^{2+}]$ rose significantly during reoxygenation in control myocytes, but not when either in MnTBAP, resveratrol, or trolox was present throughout hypoxia/reoxygenation. These data suggest that reducing ROS during hypoxia/reoxygenation can largely prevent Ca^{2+} overload during reoxygenation. Furthermore, since the NCX is the predominant route of Ca^{2+} entry during reoxygenation in this model (7), these data also suggest that ROS are involved in the reactivation of NCX activity during reoxygenation.

The results of Figure 3 demonstrated that three chemically diverse antioxidants could inhibit NCX-mediated Ca^{2+} overload when present throughout hypoxia/reoxygenation. We also carried out additional experiments to see if antioxidants were still effective when they were applied right at reoxygenation, immediately before the time of maximum oxidative stress and NCX reactivation. Figure 4A shows cytosolic Ca^{2+} measurements demonstrating that resveratrol and MnTBAP were ineffective inhibitors when applied at reoxygenation. Figure 4B summarizes the results of additional experiments in myocytes loaded with CM-DCFDA, where we evaluated the effectiveness of the antioxidants at inhibiting the increase in ROS levels seen during reoxygenation, either when the antioxidants were present throughout hypoxia/reoxygenation or when applied only at reoxygenation. When MnTBAP or resveratrol were present throughout hypoxia and reoxygenation, ROS levels were significantly reduced compared to control. However, when the antioxidants were present only at reoxygenation there was no significant difference in CM-DCFDA fluorescence between control and antioxidant-treated myocytes. It is possible that there was insufficient time for the antioxidants to equilibrate within the myocytes when only applied at reoxygenation.

MnTBAP, resveratrol, and trolox were used for their antioxidant properties. However, NCX-mediated Ca^{2+} entry could also be altered by non-specific effects on cytosolic $[\text{Na}^+]$ or pH. Therefore, we examined the effects of one of the antioxidants, resveratrol, in more detail. Figure 5 shows measurements of cytosolic $[\text{Na}^+]$ and pH on hypoxic myocytes loaded with either the Na^+ indicator SBF1 or the pH indicator BCECF. Hypoxia induced both cytosolic acidification and a rise in cytosolic $[\text{Na}^+]$, seen here as a rise in the SBF1 fluorescence ratio. Resveratrol (10 $\mu\text{mol/L}$) had no significant effect on either parameter. This establishes that resveratrol is not inhibiting the NCX-mediated rise in $[\text{Ca}^{2+}]$ (Figure 3) through a non-specific

cytoprotective effect that would decrease the driving force underlying reverse-mode NCX activity.

Figures 6 and 7 show data from a complimentary set of experiments also designed to test whether decreasing oxidative stress inhibits NCX reactivation by reoxygenation, but using an independent pharmacological approach distinct from antioxidants.

Diazoxide, an activator of some subtypes of ATP-dependent K^+ channels, has been consistently reported to decrease ROS levels in cardiac myocytes when the drug is present *throughout* ischemia/reperfusion (22, 34). The data in Figures 6A and 6B show the results of experiments where we used either the fluorescent indicator DHR-123 or CM-DCFDA to confirm that diazoxide decreased oxidative stress in guinea-pig ventricular myocytes when the drug was present throughout hypoxia/reoxygenation. The data show the change in either DHR-123 or CM-DCFDA fluorescence measured at eight minutes post-reoxygenation, relative to a measurement taken in the same cell just prior to reoxygenation. The measurement is thus relevant to reoxygenation-induced oxidative stress. Diazoxide (30-100 $\mu\text{mol/L}$) had the expected result, strongly inhibiting the increase in both DHR-123 and CM-DCFDA fluorescence. This effect of diazoxide could be prevented by administering 5-hydroxydecanoic acid (5-HD), a drug frequently used to block diazoxide-sensitive ATP-dependent K^+ channels (16). These measurements were taken at eight minutes post-reoxygenation to ensure that any drug effect on oxidative stress had to be sustained over the entire period of time that intracellular Ca^{2+} is accumulating during reoxygenation (see Figures 1 and 3). However, increased oxidative stress in this model is evident as early as two minutes after reoxygenation, as reoxygenation significantly increased DHR-123 fluorescence by $87 \pm 22\%$ (N=6).

Figure 6C shows data from additional CM-DCFDA experiments which confirmed that in

this model essentially all of the increase in oxidative stress occurs following reoxygenation. CM-DCFDA fluorescence was measured in untreated myocytes at four time points during hypoxia/reoxygenation: at the start of hypoxia, at the time of onset of rigor, immediately before reoxygenation (end hypoxia), and eight minutes post-reoxygenation. In this model, hypoxia induced no increase in CM-DCFDA fluorescence (relative to the initial measurement), but a significant increase was seen upon reoxygenation. This suggests that the inhibitory effect of the antioxidants on NCX reactivation must have been manifested during reoxygenation, as that is the only time that there is increased oxidative stress in this model.

The experiments summarized in Figure 7 demonstrate that diazoxide, at concentrations that decrease oxidative stress in guinea-pig ventricular myocytes during reoxygenation, also inhibits NCX-mediated Ca^{2+} overload. Again, cytosolic $[\text{Ca}^{2+}]$ was measured using a fluorescent indicator while freshly isolated guinea-pig ventricular myocytes were subjected to 20 minutes of post-rigor hypoxia followed by reoxygenation.

Figure 7A plots the average change in Ca^{2+} indicator fluorescence, under control (drug-free) conditions, or in the presence of 100 $\mu\text{mol/L}$ diazoxide, or diazoxide plus 100 $\mu\text{mol/L}$ 5-HD, or diazoxide plus 2.5 $\mu\text{mol/L}$ glibenclamide, another ATP-dependent K^+ channel blocker. It can be seen that 100 $\mu\text{mol/L}$ diazoxide profoundly inhibited the NCX-mediated rise in $[\text{Ca}^{2+}]$, suggesting that diazoxide interferes with NCX reactivation during reoxygenation, likely by keeping ROS levels low.

It is of course possible that the inhibitory effect of diazoxide on the rise in $[\text{Ca}^{2+}]$ during reoxygenation could be due to a direct inhibitory effect on the NCX, rather than being mediated through altered oxidative stress. Figure 7A also shows our first data evaluating this possibility. 5-HD and glibenclamide often can prevent or reverse the effects of diazoxide on cardiac muscle

(8, 16), and it can be seen in Figure 7A that both drugs could also prevent the inhibitory effect of diazoxide on the rise in $[Ca^{2+}]$ in reoxygenated myocytes. These observations argue against diazoxide having any direct inhibitory effect on the NCX, and are consistent with a previous report that high concentrations of diazoxide did not alter NCX activity in rat ventricular myocytes (18). It is notable that 5-HD could suppress the inhibitory effects of diazoxide on both oxidative stress (Figure 6) and $[Ca^{2+}]$ during reoxygenation.

Figure 7B summarizes cytosolic $[Ca^{2+}]$ data obtained at a single time-point, ten minutes following reoxygenation, using a wider range of diazoxide concentrations. Either 30 or 100 $\mu\text{mol/L}$ was found to prevent the rise in cytosolic $[Ca^{2+}]$ during reoxygenation.

Figures 6 and 7 demonstrated that diazoxide was a pharmacological tool that could decrease both oxidative stress and the NCX-mediated rise in $[Ca^{2+}]$ during hypoxia/reoxygenation in guinea-pig ventricular myocytes. These observations are consistent with our hypothesis that NCX reactivation during reoxygenation is linked to the generation of ROS. However, an additional implication of our hypothesis is that if diazoxide is suppressing NCX-mediated Ca^{2+} overload during reoxygenation by inhibiting ROS generation, application of exogenous ROS should be able to reverse the inhibitory effects of diazoxide on $[Ca^{2+}]$. Figure 8 summarizes data from a set of experiments designed to test this idea.

Cytosolic $[Ca^{2+}]$ was measured in guinea-pig ventricular myocytes subjected to our standard hypoxia/reoxygenation protocol under control (drug-free) conditions, or in the presence of 100 $\mu\text{mol/L}$ diazoxide. Once again, it can be seen that diazoxide has a strong inhibitory effect on the NCX-mediated rise in $[Ca^{2+}]$ during reoxygenation. In a third set of experiments, cells treated with 100 $\mu\text{mol/L}$ diazoxide were also exposed to 20 $\mu\text{mol/L}$ H_2O_2 seven minutes after reoxygenation. Consistent with the prediction of our hypothesis, the administration of

exogenous ROS reversed the inhibitory effect of diazoxide, as a large rise in cytosolic $[Ca^{2+}]$ occurred after H_2O_2 administration. It should also be noted that when normoxic myocytes were exposed to $20 \mu\text{mol/L } H_2O_2$ there was no significant change in resting $[Ca^{2+}]$ (data not shown).

A final prediction of our hypothesis is that the application of exogenous ROS during hypoxia should be sufficient to trigger a NCX-mediated rise in cytosolic $[Ca^{2+}]$, independent of reoxygenation or the presence of diazoxide. Figure 9 shows data obtained from experiments in cultured myocytes that tested this prediction. The filled circles show data from control cells exposed to hypoxia. $20 \mu\text{mol/L } H_2O_2$ was applied to the cells 15 minutes after the start of hypoxia, and induced a rapid and significant rise in cytosolic $[Ca^{2+}]$, which is consistent with our prediction. The unfilled circles in Figure 9 represent data obtained from myocytes that had been pre-treated with the NCX antisense oligonucleotide. H_2O_2 failed to induce a significant rise in $[Ca^{2+}]$ in the oligonucleotide-treated myocytes, which strongly supports the idea that H_2O_2 is inducing Ca^{2+} overload in this experiment by rapidly stimulating reverse-mode NCX transport, despite the sustained hypoxia. Corresponding $[Ca^{2+}]$ measurements in myocytes pre-treated with a nonsense oligonucleotide (1.53 ± 0.04 , $N=2$) were not statistically different from control myocytes (1.45 ± 0.11 , $N=6$), after five minutes of exposure to H_2O_2 .

DISCUSSION

In this study we evaluated the hypothesis that oxidative stress is an essential trigger for rapid NCX reactivation in hypoxic guinea-pig ventricular myocytes, and is thus a critical factor in determining the timing and magnitude of calcium overload during hypoxia/reoxygenation.

The experiments described here have provided considerable support for this hypothesis, including the following new observations. 1) Hypoxia inhibited, while reoxygenation reactivated, evoked reverse-mode NCX activity in both cultured and isolated myocytes. 2) Antioxidants could prevent reoxygenation from reactivating NCX activity in either the evoked reverse-mode assay or our standard hypoxia/reoxygenation protocol. 3) Diazoxide inhibited both the generation of ROS and NCX-dependent Ca^{2+} accumulation in the hypoxia/reoxygenation protocol. 4) Application of exogenous ROS during reoxygenation rapidly reversed the inhibitory effect of diazoxide on NCX activity. 5) Application of exogenous ROS caused immediate NCX reactivation, even in the normally inhibitory condition of sustained hypoxia.

These results support our view of reoxygenation-induced NCX reactivation as resulting from active stimulation of cardiac NCX, rather than simply being due to the slow removal of inhibitory influences such as altered cytosolic $[\text{Na}^+]$, pH, or ATP.

NCX Regulation in Hypoxic Cardiac Myocytes

Other investigators have reported evidence that the NCX is substantially inhibited in guinea-pig cardiac myocytes during hypoxia and becomes reactivated during reoxygenation (20, 31). However, a full understanding of the role of the NCX during hypoxia is complicated by the potential participation of several known modulators of NCX. Decreased levels of ATP could

potentially inhibit NCX in cardiac myocytes (4, 13). Cytosolic acidification is known to inhibit cardiac NCX (5), and cytosolic pH falls by nearly 0.4 pH units in hypoxic guinea-pig ventricular myocytes (Figure 5). Finally, elevation of $[\text{Na}^+]$ at the cytoplasmic surface of the cardiac NCX to levels known to occur during hypoxia (6, 26) induces an inactivated state of the NCX (15).

A large burst of ROS generation also seems to occur during reoxygenation in this (Figure 6) and similar cardiac myocyte models (34). ROS are another potential modulator of NCX function during hypoxia/reoxygenation, but much less is known about their role, and so much of this study has focused on ROS modulation of cardiac NCX. The effects of ROS on NCX have been previously studied, but their effects seem to vary both with cell type, and the type of oxidative stress. Nitric oxide has been reported to stimulate NCX activity in a glioma-derived cell line (1), while exogenous H_2O_2 has been reported to either stimulate (10) or to have little effect (27) on native cardiac NCX activity. Finally, it was recently reported that NCX activity was stimulated through removal of Na^+ -dependent inactivation when the cardiac isoform NCX 1.1 was expressed in oocytes and exposed to both FeSO_4 and dithiothreitol (29). Interestingly, the molecular mechanisms of several NCX modulators (e.g. ATP, pH, Na^+) could be at least partially dependent on this one aspect of NCX modulation, Na^+ -dependent inactivation (5, 12, 14).

NCX reactivation might be attributed simply to the reversal of the inhibitory events listed above. However, NCX reactivation upon reoxygenation appears to be both rapid and profound, and can be prevented by drugs that decrease oxidative stress (Figures 3 and 7), or promoted using exogenous ROS (Figures 8 and 9), even during sustained hypoxia. These results support the idea that the generation of endogenous ROS plays a critical role in modulating NCX activity

during hypoxia/reoxygenation in guinea-pig ventricular myocytes, controlling the timing and magnitude of NCX-mediated Ca^{2+} overload and cellular injury.

Limitations and Implications

This study was carried out in hypoxic isolated or cultured adult guinea-pig ventricular myocytes. This cellular model has numerous experimental advantages, including the capability of profoundly inhibiting NCX protein expression with antisense oligonucleotides, and a robust response of the NCX to hypoxia/reoxygenation. However, the primary limitation of the model is its relative simplicity. We expect that NCX regulation in intact myocardium during ischemia/reperfusion would be further complicated by additional factors as extracellular acidification, as well as a more graded and variable degree of hypoxia and substrate depletion.

NCX regulation in guinea-pig ventricular myocytes during hypoxia/reoxygenation is a particularly dramatic model, with a profound inhibition of NCX during hypoxia and rapid NCX reactivation during reoxygenation, leading to most Ca^{2+} overload occurring during reoxygenation. However, as described above, multiple factors may be involved in NCX modulation during hypoxia or ischemia, and quantitative differences in these modulatory factors could produce different temporal patterns of NCX inhibition/reactivation and Ca^{2+} overload. We would still expect all the qualitative elements involved in NCX regulation during hypoxia/reoxygenation in guinea-pig ventricular myocytes to be involved in other cardiac models, but model-specific quantitative differences may cause differences in the timing and magnitude of the onset of Ca^{2+} overload. A possible example may be rat ventricular myocytes, where NCX-mediated Ca^{2+} accumulation can occur earlier, during hypoxia (25). In future experiments, it would be interesting to evaluate whether model-specific differences in NCX-

mediated Ca^{2+} accumulation can be explained by differences in the timing and magnitude of ROS generation.

The identification of oxidative stress as critical to NCX reactivation and Ca^{2+} overload during reoxygenation implies that drugs or other treatments that decrease oxidative stress would be effective at decreasing Ca^{2+} -mediated injury in hypoxic or ischemic myocytes. We used antioxidants and diazoxide to decrease oxidative stress and NCX reactivation in our studies, and previous studies have demonstrated that trolox (28), resveratrol (17), and diazoxide (8) are cardioprotective.

We would like to point out that diazoxide has also been reported to increase ROS levels in cardiac myocytes (3, 23), however those studies are not directly comparable to our experimental conditions, as diazoxide was used in pharmacological preconditioning protocols, where the drug was applied and washed out before the onset of hypoxia. Therefore, it seems likely that diazoxide's inhibitory effect on NCX reactivation and NCX-mediated Ca^{2+} overload could contribute to diazoxide's cardioprotective effects, *but most likely only when diazoxide is present throughout hypoxia and reoxygenation.*

There is an ongoing controversy concerning whether diazoxide's cardioprotective effects (such as altered ROS generation) are initiated through ATP-dependent K^+ channel activation or through other mechanisms (19). Here we simply want to point out that the usefulness of diazoxide to our study of NCX reactivation depends only on the ability of diazoxide to decrease oxidative stress (Figure 6), and not on the specific mechanism by which it does this. It is clear that diazoxide's ability to inhibit NCX activity in these experiments depended on the drug's ability to decrease oxidative stress, rather than on other mechanisms such as direct NCX

inhibition, or altering the diastolic membrane potential (2), since the drug's effects were mimicked by antioxidants and reversed by H₂O₂, glibenclamide, and 5-HD.

Finally, we would like to point out that the conclusion that NCX reactivation during reoxygenation and the subsequent rise in cytoplasmic [Ca²⁺] is mediated by oxidative stress is supported by multiple, parallel experimental approaches that both increased and decreased ROS levels. Although some of the experiments used cytoprotective agents such as diazoxide or antioxidants, it is not at all likely that their ability to decrease cytoplasmic [Ca²⁺] during reoxygenation could be attributed to a non-specific reduction of hypoxic injury. The cytosolic [Na⁺] measurements shown in Figure 5, and the immediate reversibility of diazoxide's inhibitory effects shown in Figure 9, demonstrate that hypoxic injury still occurred in myocytes treated with these agents, but that the agents had uncoupled hypoxic injury from NCX-mediated Ca²⁺ entry. Furthermore, the data shown in Figure 9 also support our conclusion, and no cytoprotective agents were used in the experiment.

These studies have contributed valuable evidence supporting a critical role for oxidative stress in modulating NCX activity during hypoxia/reoxygenation in guinea-pig ventricular myocytes. Consideration of these results also identifies possible new experimental directions. This includes identifying the specific source of the oxidative stress (e.g. H₂O₂ generated secondary to mitochondrial dysfunction) most relevant to NCX reactivation during reoxygenation. Another likely future direction is identifying the mechanism by which oxidative stress, such as H₂O₂, induces NCX reactivation in hypoxic myocytes.

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FIGURE LEGENDS

Figure 1. Cytosolic $[Ca^{2+}]$ increases during reoxygenation in adult guinea pig ventricular myocytes. Cytosolic $[Ca^{2+}]$ was measured in a freshly isolated myocyte loaded with the fluorescent Ca^{2+} indicator indo-1 AM. The myocyte was subjected to 20 minutes of post-rigor hypoxia, followed by 10 minutes of reoxygenation. An increase in the indo-1 fluorescence ratio represents an increase in cytosolic $[Ca^{2+}]$. The ratio at the start of the experiment is equal to 1.0. The break in the x-axis in this, and similar plots, is due to variability between myocytes in the time to onset of rigor (mean of 25 ± 10 minutes).

Figure 2. Evoked reverse-mode NCX activity in guinea-pig ventricular myocytes is suppressed by hypoxia and reactivated by reoxygenation. Cytosolic $[Ca^{2+}]$ was measured in indo-1 AM-loaded myocytes, both cultured and freshly isolated. A. Under normoxic conditions, reverse-mode NCX activity, seen as a rise in the indo-1 fluorescence ratio, was evoked by first exposing an isolated myocyte to a K^+ -free solution, followed by exposure to a Na^+ -free solution. B. Freshly isolated or cultured myocytes were placed in a hypoxic solution for 15 minutes, followed by hypoxic K^+ -free and then hypoxic Na^+ -free solution, after which myocytes were reoxygenated. Cytosolic $[Ca^{2+}]$ did not increase significantly during the hypoxic Na^+ -free period, but did increase in both sets of control myocytes after reoxygenation with Na^+ -free solution. However, cytosolic $[Ca^{2+}]$ did not increase in myocytes treated with 40 $\mu\text{mol/L}$ MnTBAP, or in cultured myocytes pre-treated with 2 $\mu\text{mol/L}$ antisense oligonucleotide. * $P < 0.05$ versus the initial indo-1 ratio. Each data point represents the mean \pm SE of 6 myocytes.

Figure 3. Antioxidants applied throughout hypoxia/reoxygenation significantly reduce NCX-mediated cytosolic $[Ca^{2+}]$ overload during reoxygenation. Isolated myocytes loaded with calcium orange AM were subjected to the standard hypoxia/reoxygenation protocol. One of three different antioxidants (40 $\mu\text{mol/L}$ MnTBAP, 10 $\mu\text{mol/L}$ resveratrol, or 1 mmol/L trolox) were used throughout hypoxia/reoxygenation to reduce oxidative stress. * $P < 0.05$ when compared to initial Ca^{2+} indicator fluorescence (1.0). Each data point represents the mean \pm SE of 6 myocytes.

Figure 4. Antioxidants applied at reoxygenation do not significantly reduce ROS levels

or cytosolic Ca²⁺ overload during reoxygenation. A. Isolated myocytes loaded with calcium orange AM were subjected to the standard hypoxia/reoxygenation protocol. Either MnTBAP (40 μmol/L) or resveratrol (10 μmol/L) was applied at reoxygenation. There was no significant difference in either antioxidant-treated group versus control. * P < 0.05 when compared to initial Ca²⁺ indicator fluorescence (1.0). Each data point represents the mean ± SE of 3 myocytes. B. The increase in oxidative stress induced by reoxygenation was measured by comparing CM-DCFDA fluorescence eight minutes post-reoxygenation to measured fluorescence just prior to reoxygenation. Oxidative stress increased significantly during reoxygenation in control, untreated myocytes. When MnTBAP (40 μmol/L) or resveratrol (10 μmol/L) were applied at reoxygenation there was no significant difference in CM-DCFDA fluorescence versus control. However, when the antioxidants were present throughout hypoxia/reoxygenation both MnTBAP and resveratrol significantly reduced CM-DCFDA fluorescence versus control, untreated myocytes. * P < 0.05 versus CM-DCFDA fluorescence at the end of hypoxia (1.0). Each bar represents the mean ± SE of 3 myocytes.

Figure 5. Resveratrol does not affect either cytosolic acidification or the rise in cytosolic [Na⁺] during hypoxia. Cytosolic pH or cytosolic [Na⁺] were measured in freshly isolated myocytes loaded with either BCECF-AM or SBFI-AM. Measurements were made in either untreated control myocytes (circles) or in myocytes exposed to 10 μmol/L resveratrol (triangles). The data are plotted as changes in either cytosolic pH (unfilled symbols) or the SBFI fluorescence ratio (filled symbols). An increase in cytosolic [Na⁺] is seen as an increase in the SBFI ratio. Each data point represents the mean ± SE of four myocytes.

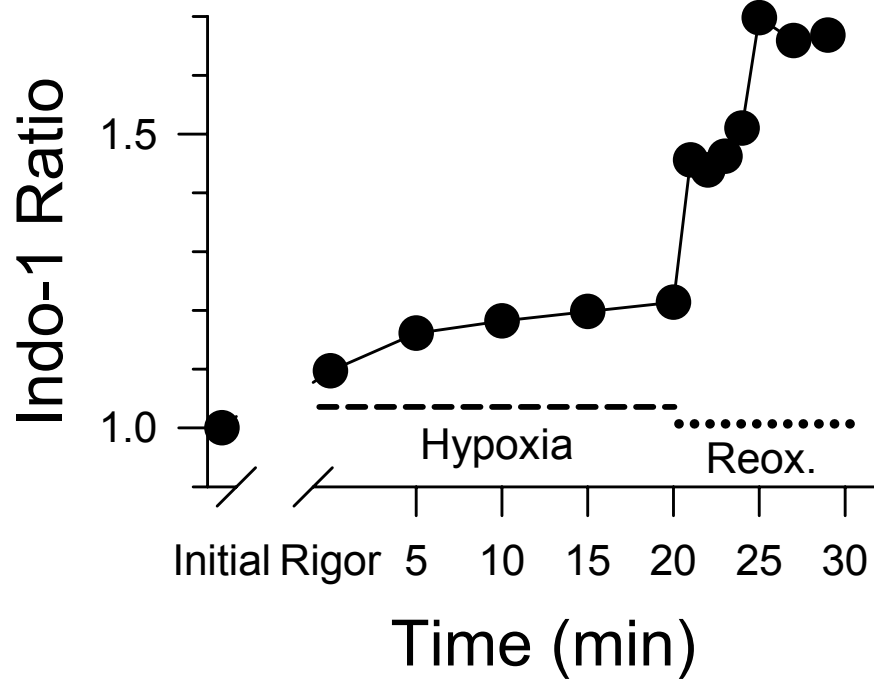
Figure 6. Diazoxide significantly reduces oxidative stress during reoxygenation. A. The increase in oxidative stress induced by reoxygenation was measured by comparing DHR-123 fluorescence at eight minutes post-reoxygenation to DHR-123 fluorescence just prior to reoxygenation. Oxidative stress increased significantly during reoxygenation in control, untreated myocytes. However, no significant increase was seen in isolated adult myocytes treated with either 30 $\mu\text{mol/L}$ or 100 $\mu\text{mol/L}$ diazoxide. * $P < 0.05$ versus DHR-123 fluorescence at the end of hypoxia (1.0). B. Similar experiments and analysis were carried out on myocytes loaded with CM-DCFDA. C. Oxidative stress in this model occurs primarily during reoxygenation. CM-DCFDA fluorescence was measured at various time points during hypoxia/reoxygenation. The fluorescence was normalized to the fluorescence seen at the start of hypoxia. * $P < 0.05$ versus initial fluorescence. Each bar represents the mean \pm SE. N=6 in panel A, 8 in panel B, and 5 in panel C.

Figure 7. Diazoxide inhibits the NCX-mediated rise in cytosolic $[Ca^{2+}]$ during reoxygenation. A. Cytosolic $[Ca^{2+}]$ was measured in isolated myocytes loaded with calcium orange AM. Treatment with either 100 $\mu\text{mol/L}$ 5-HD or 2.5 $\mu\text{mol/L}$ glibenclamide prevented the effect of diazoxide. Drugs were present throughout hypoxia and reoxygenation. B. Dose-response of three concentrations of diazoxide present throughout hypoxia/reoxygenation. Cytosolic $[Ca^{2+}]$ is significantly increased at the end of reoxygenation in all treatment groups, except when myocytes were treated with either 30 $\mu\text{mol/L}$ or 100 $\mu\text{mol/L}$ diazoxide. * $P < 0.05$ versus initial Ca^{2+} indicator fluorescence (1.0). Each data point represents the mean \pm SE of 6 myocytes.

Figure 8. Application of H₂O₂ reverses the inhibitory effect of diazoxide on the reoxygenation-induced rise in [Ca²⁺]. Cytosolic [Ca²⁺] was measured using isolated myocytes loaded with calcium green AM. Diazoxide (100 μmol/L) significantly reduced cytosolic [Ca²⁺] as seen in Figure 6A. However, when 20 μmol/L H₂O₂ was added to diazoxide-treated myocytes after seven minutes reoxygenation, cytosolic [Ca²⁺] significantly increased. * P < 0.05 versus the initial Ca²⁺ indicator fluorescence (1.0). Each data point represents the mean ± SE of 6 myocytes.

Figure 9. Hydrogen peroxide increases cytosolic [Ca²⁺] in hypoxic control myocytes, but not in NCX antisense-treated myocytes. Adult guinea pig ventricular myocytes were maintained in culture for 5-7 days. Cytosolic [Ca²⁺] was measured in myocytes loaded with calcium green AM. Myocytes were subjected to hypoxia, and 20 μmol/L H₂O₂ was added 15 minutes later, if required. After application of H₂O₂, cytosolic [Ca²⁺] increased in control myocytes but not in antisense-treated myocytes. * P < 0.05 versus initial Ca²⁺ indicator fluorescence (1.0). Each data point represents the mean ± SE of 6 myocytes.

Figure 1



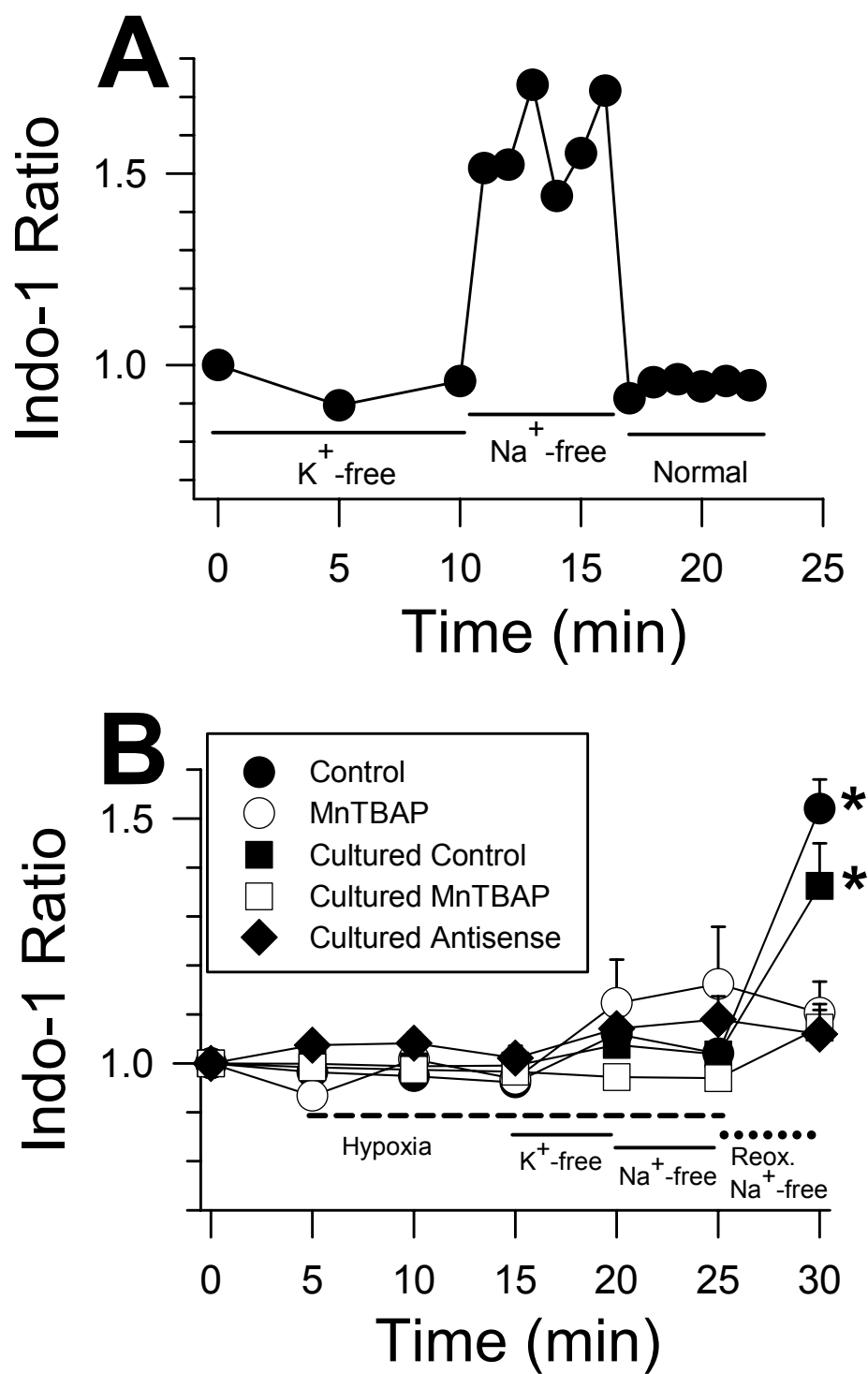
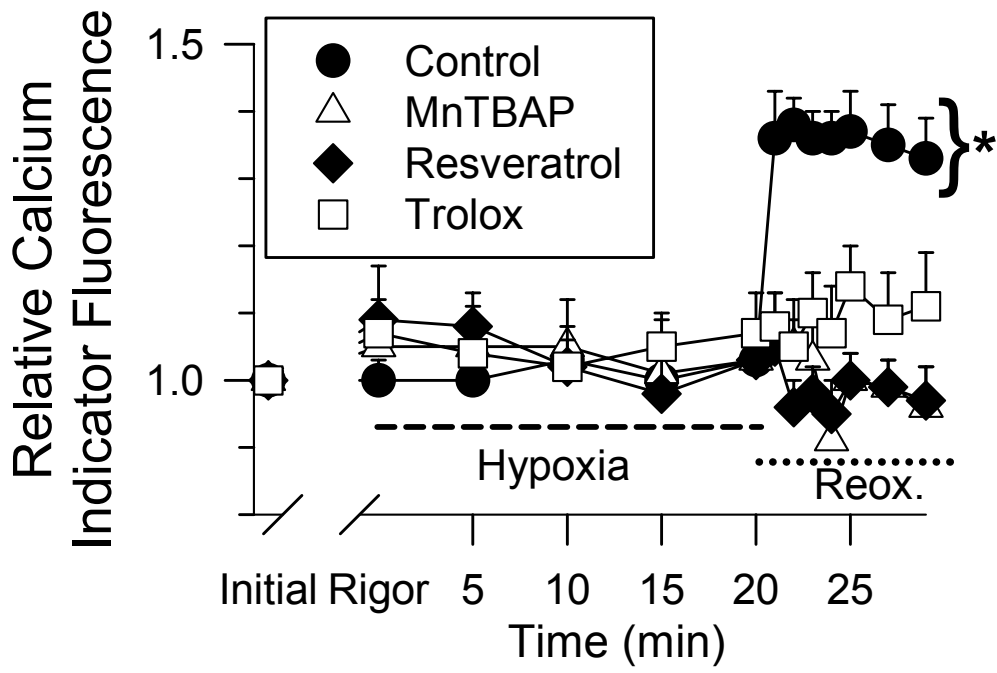
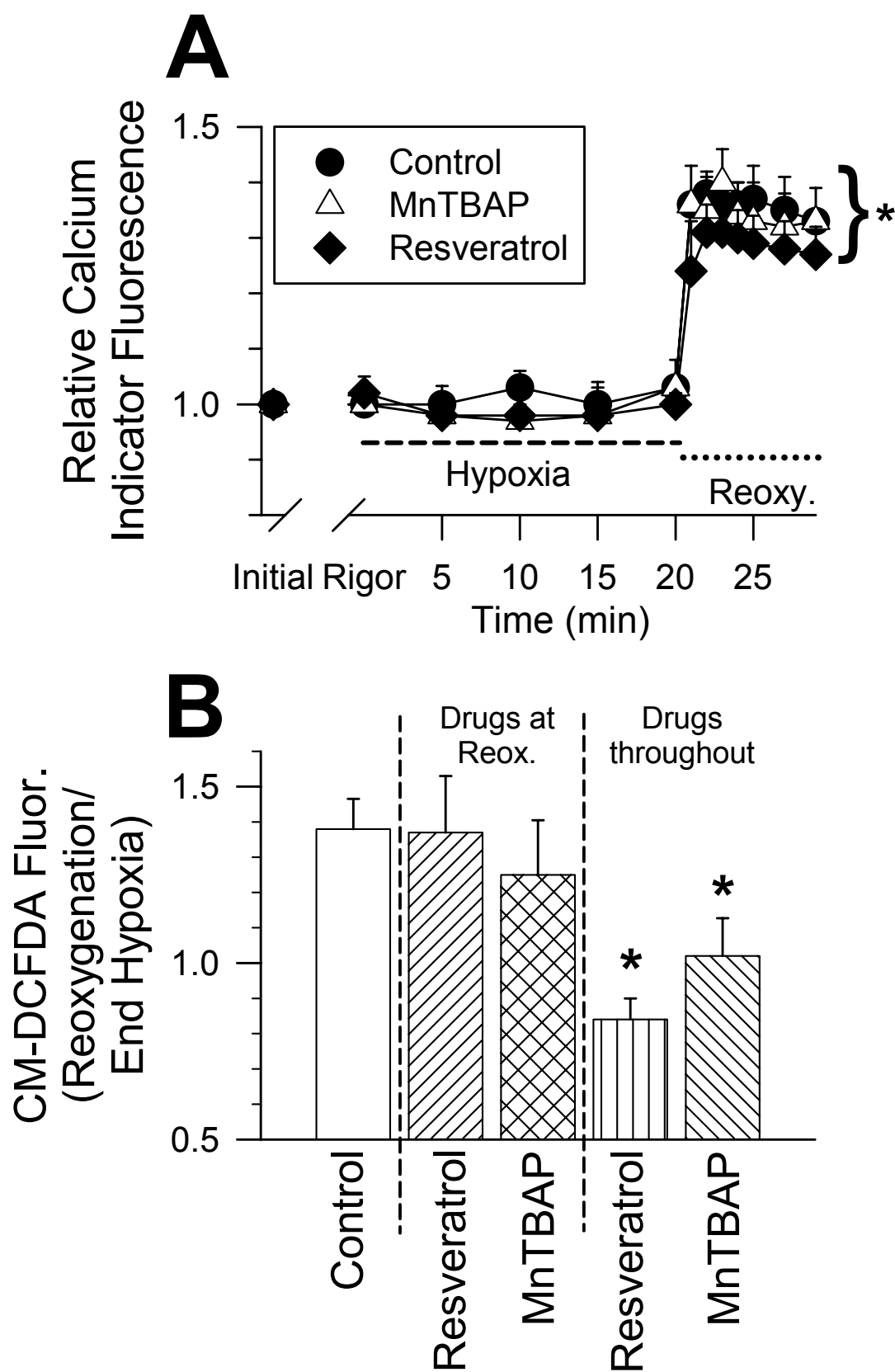


Figure 3





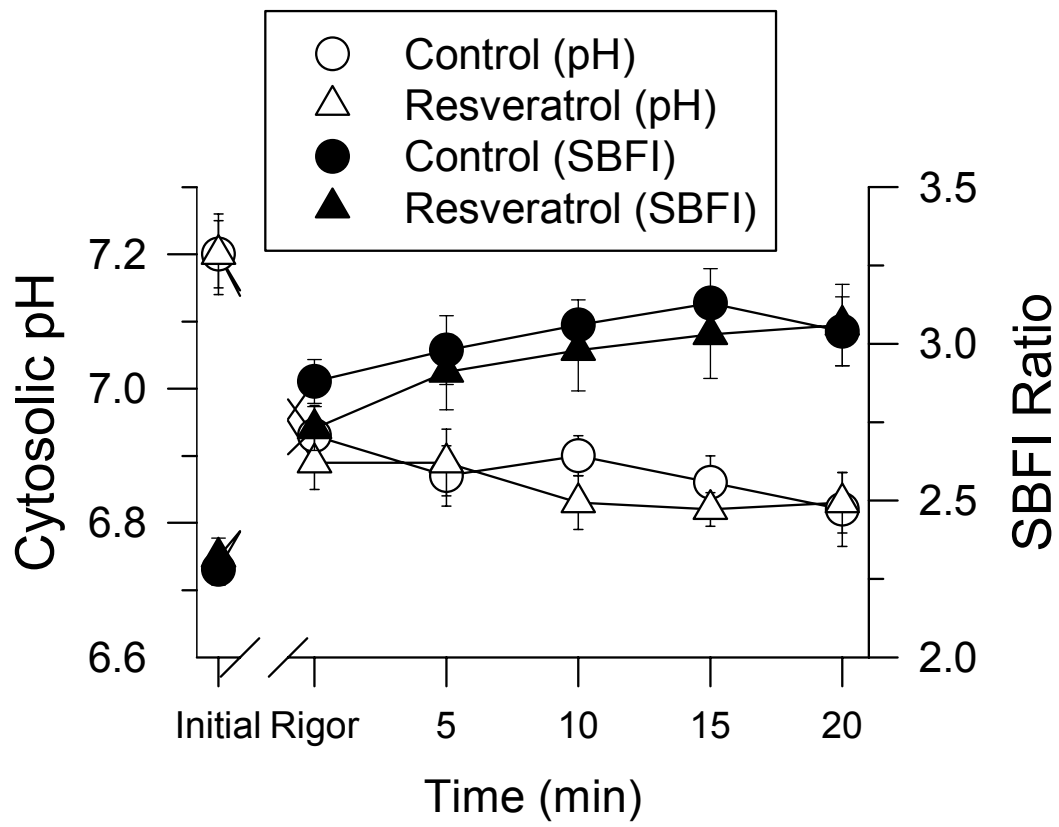
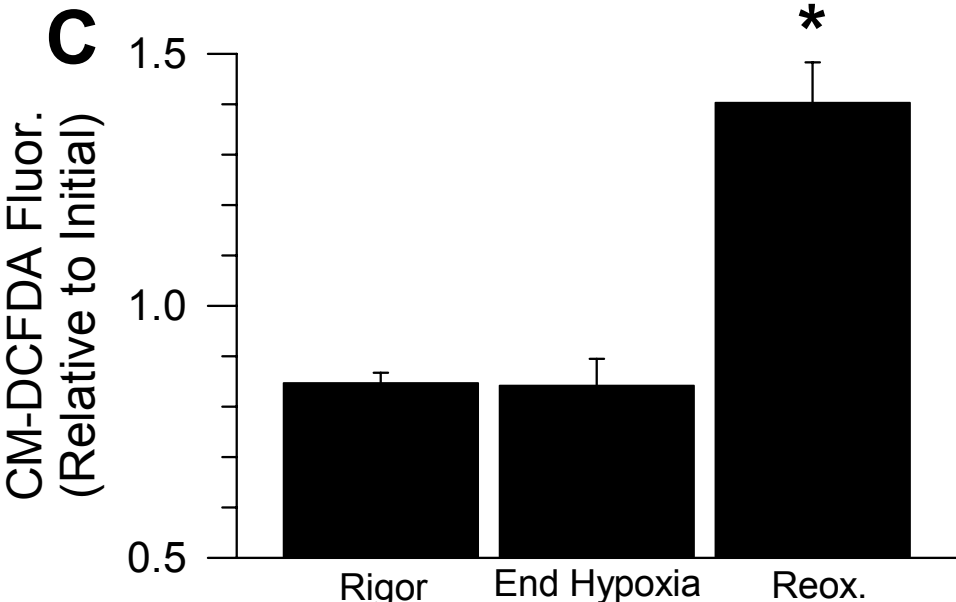
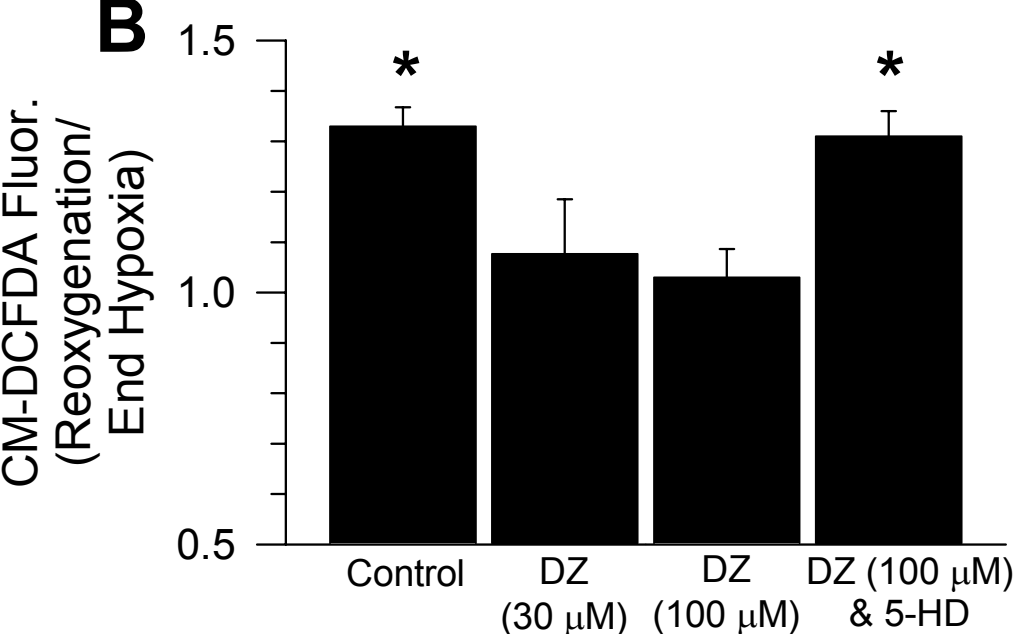
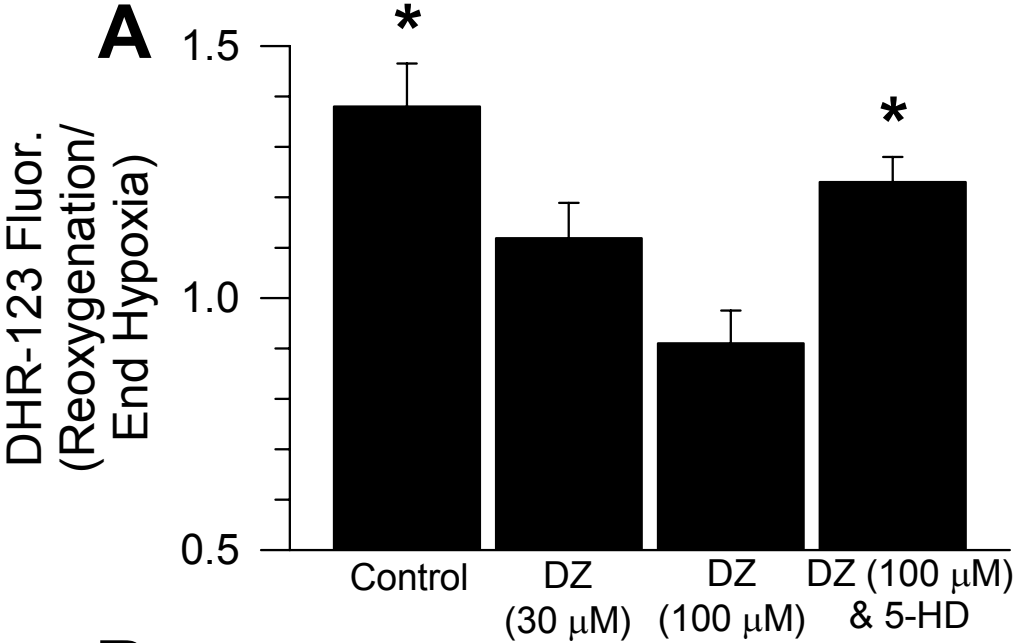


Figure 6



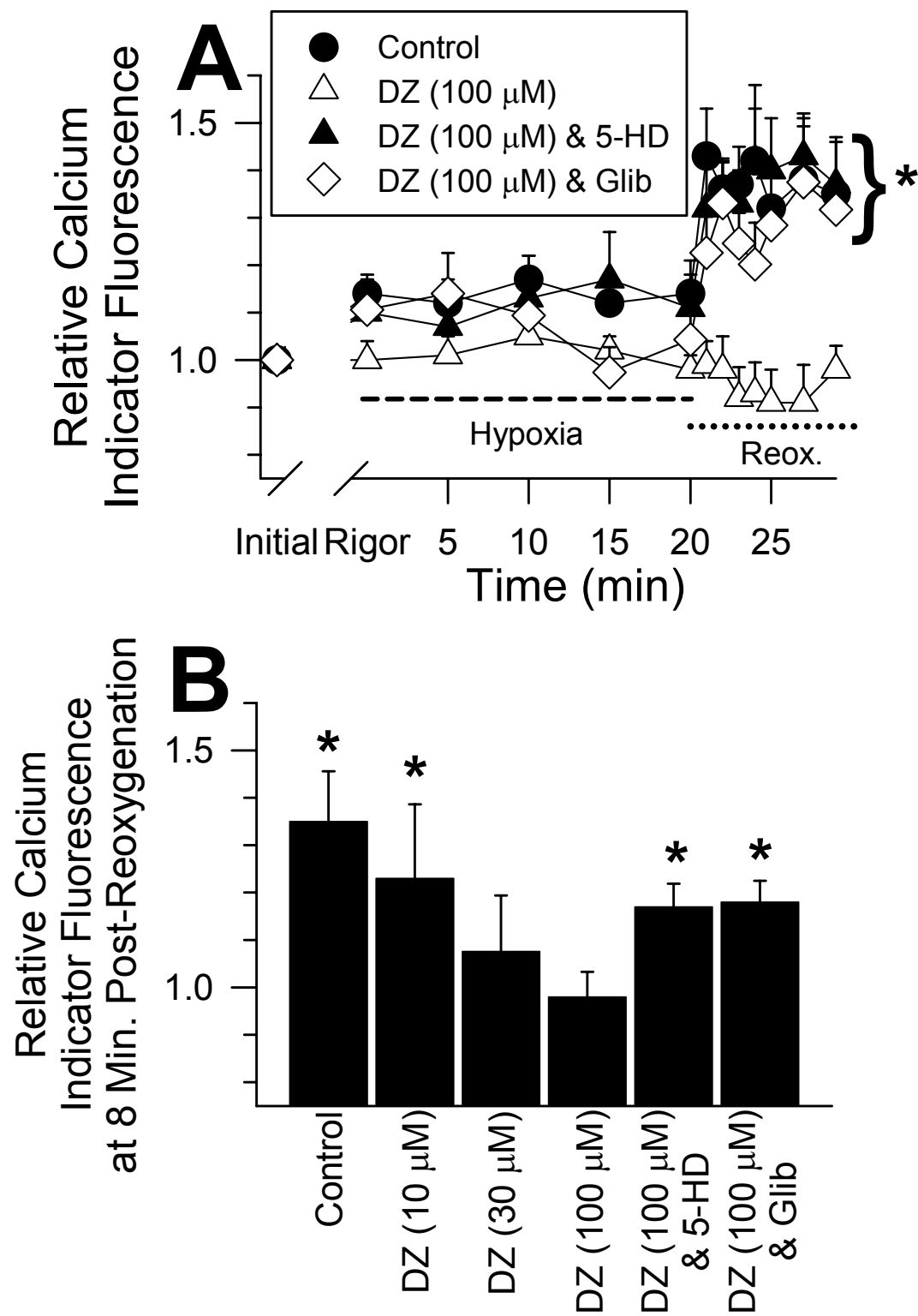


Figure 8

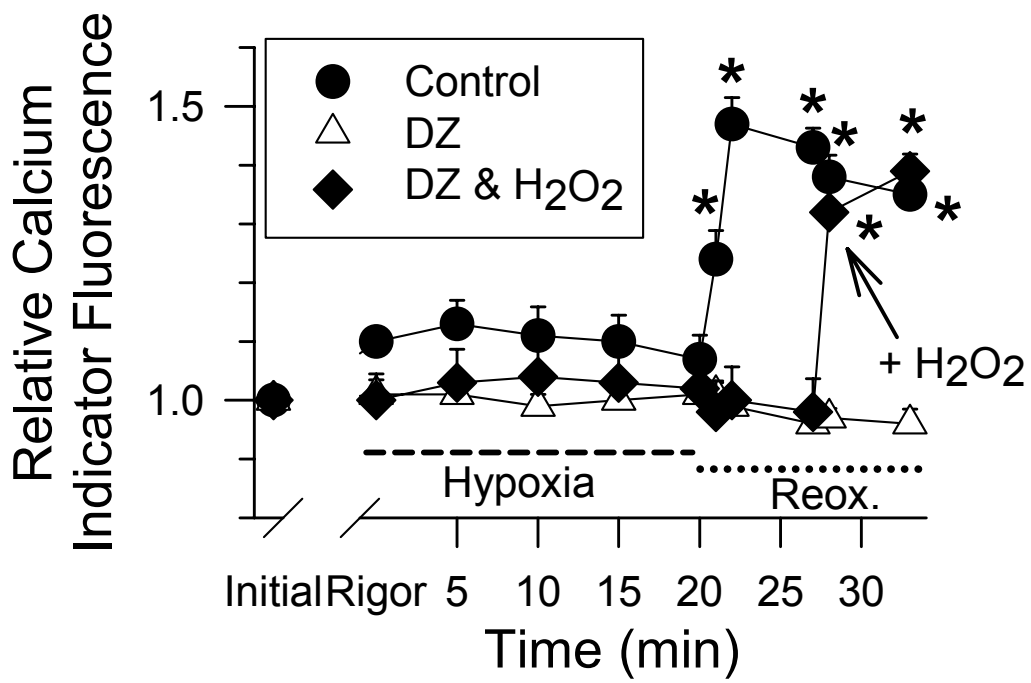


Figure 9

