Altering CO_2 during reperfusion of ischemic cardiomyocytes modifies mitochondrial oxidant injury*

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Objective: **Acute changes in tissue CO_2 and pH during reperfusion of the ischemic heart may affect ischemia/reperfusion injury. We tested whether gradual vs. acute decreases in CO_2 after cardiomyocyte ischemia affect reperfusion oxidants and injury.**

Design: **Comparative laboratory investigation.**

Setting: **Institutional laboratory.**

Subjects: **Embryonic chick cardiomyocytes.**

Interventions: **Microscope fields of approximately 500 chick cardiomyocytes were monitored throughout 1 hr of simulated ischemia (Po_2 of 3–5 torr, Pco_2 of 144 torr, pH 6.8), followed by 3 hrs of reperfusion (Po_2 of 149 torr, Pco_2 of 36 torr, pH 7.4), and compared with cells reperfused with relative hypercarbia (Pco_2 of 71 torr, pH 6.8) or hypocarbia (Pco_2 of 7 torr, pH 7.9).**

Measurements and Main Results: **The measured outcomes included cell viability (via propidium iodide) and oxidant generation (reactive oxygen species via 2',7'-dichlorofluorescin oxidation and nitric oxide [NO] via 4,5-diaminofluorescein diacetate oxidation). Compared with normocarbic reperfusion, hypercarbia significantly reduced cell death from 54.8% ± 4.0% to 26.3% ± 2.8% ($p < .001$), significantly decreased reperfusion reactive oxygen species ($p < .05$), and increased NO at a later phase of reperfusion ($p < .01$). The NO synthase inhibitor N-nitro-L-arginine methyl ester (200 μM) reversed this oxidant attenuation ($p < .05$), NO increase ($p < .05$), and the cardioprotection conferred by hypercarbic reperfusion (increasing death to 54.3% ± 6.0% [$p < .05$]). Conversely, hypocarbic reperfusion increased cell death to 80.4% ± 4.5% ($p < .01$). It also increased reactive oxygen species by almost two-fold ($p = .052$), without affecting the NO level thereafter. Increased reactive oxygen species was attenuated by the mitochondrial complex III inhibitor stigmatellin (20 nM) when given at reperfusion ($p < .05$). Cell death also decreased from 85.9% ± 4.5% to 52.2% ± 6.5% ($p < .01$). The nicotinamide adenine dinucleotide phosphate oxidase inhibitor apocynin (300 μM) had no effect on reperfusion reactive oxygen species.**

Conclusions: **Altering CO_2 content during reperfusion can significantly affect myocardial postresuscitation injury, in part by modifying mitochondrial oxidants and NO synthase-induced NO production. (Crit Care Med 2007; 35:1709–1716)**

KEY WORDS: hypercarbia; hypocarbia; oxidants; reperfusion injury; mitochondria

Although much interest has been devoted to the role of reoxygenation in reperfusion injury, the optimal change in tissue carbon dioxide levels after cardiac ischemia that improves function and survival after resuscitation remains unclear. After only 4 mins of ventricular fibrillation cardiac arrest, myocardial Pco_2 may increase to >200 torr and intracellular pH may decrease to ≤6.0 (1).

Evidence suggests that increased myocardial CO_2 due to ischemia during cardiac arrest may adversely affect the heart's ability to be successfully defibrillated and achieve the return of spontaneous circulation (ROSC) (1). Accordingly, it seems that cardiopulmonary resuscitation can attenuate (2) or even reverse (3) this increase in myocardial Pco_2 in animals that eventually achieve ROSC. Such changes in tissue Pco_2 status during cardiopulmonary resuscitation are thought to reflect myocardial perfusion status and may be relatively independent of changes in arterial Pco_2 levels resulting from different ventilation strategies (2, 4).

A somewhat more abrupt change in tissue Pco_2 and pH status occurs after ROSC as full perfusion rapidly normalizes these intracellular indices to prearrest levels (2, 3); however, it is unclear how such an abrupt transition might affect oxidant-mediated postresuscitation injury. Such postresuscitation injury in patients with initial ROSC can be severe, with as high as 90% mortality, most often due to early cardiovascular collapse or persistent severe neurologic dysfunction

*See also p. 1788.

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Drs. Lavani and Chang made equal contributions to this study.

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(5). As cardiac arrest patients are routinely intubated and mechanically ventilated, there is currently potential for wide variation in, and manipulation of, overall CO_2 and acid-base status during the post-ROSC period. A greater understanding of the effect of CO_2 and pH changes on oxidant production during this critical transition could lead to improved postresuscitation ventilation strategies.

Recent work demonstrates the existence of a "pH paradox," that is, rapid normalization of pH during normoxic reperfusion of previously hypoxic/acidotic cardiomyocytes results in decreased cell survival as compared with acidotic reperfusion through hypercarbia (6); however, the mechanism of this protective effect is not entirely clear. In addition, the possible effect of more extreme drops of tissue CO_2 after ischemia to tissue normocarbia or hypocarbia (alkalosis) is less studied. Our own work studying the timing of cell death during simulated ischemia/reperfusion (I/R) in chick cardiomyocytes suggests that after prolonged hypercarbic ischemia, significant injury and cell death occurs at an accelerated rate immediately after reperfusion when oxygen and CO_2 are normalized and substrates reintroduced. Specifically, rapid normalization of perfusion conditions precipitates reactive oxygen species (ROS) generation within 5 mins of reperfusion, leading to H_2O_2 and hydroxyl radical formation, cytochrome c release, caspase activation, and accelerated reperfusion injury and death (7–9). In contrast, these cardiomyocytes can remain remarkably intact without membrane damage or caspase activation if allowed to remain hypercarbic and ischemic for a much longer duration without reperfusion (8, 10). Thus, the conditions (e.g., oxygen, CO_2, and pH) used to resuscitate ischemic cells may be critical for outcome in this model of cardiomyocyte I/R injury. We compared the effect of relative hypercarbic, normocarbic, and hypocarbic reperfusion on re-oxygenation injury, studying outcomes of cardiomyocyte oxidant generation and viability.

MATERIALS AND METHODS

Chemicals. Propidium iodide and 2',7'-dichlorofluorescin diacetate were obtained from Molecular Probes (Eugene, OR); 4,5-diaminofluorescein diacetate (DAF-2 DA) was obtained from EMD Biosciences (San Diego, CA). N-nitro-L-arginine methyl ester (L-NAME), apocynin, and stigmatellin were obtained from Sigma (St. Louis, MO).

Cardiomyocyte Culture. Ventricular embryonic chick cardiomyocytes were prepared as previously described (10, 11). In brief, hearts of the 10-day-old chick embryos were removed, and the ventricles were minced and enzymatically digested with 0.025% trypsin (Invitrogen, Grand Island, NY) for 4–5 cycles with gentle agitation at 37°C. Cell suspension was centrifuged and resuspended. Fibroblast contamination was reduced by preplating, and the purity of cardiomyocytes was confirmed by anti–myosin heavy-chain monoclonal antibodies. Cells (0.7×10^6) were plated onto glass coverslips and cultured in a humidified incubator for 3 days. Experiments were performed on 3- to 5-day-old cardiomyocytes, on which a synchronously contracting layer of cells could be visualized and viability exceeded 95%.

All studies met the requirements of our Institutional Animal Care and Use Committee (IACUC). This work uses embryonic avian cardiomyocytes isolated from fertilized chicken eggs and so is exempt from need for approval of vertebrate animal use.

Perfusion System and I/R Perfusates. Synchronously contracting cells on coverslips were placed in a 1.2-mL Sykes-Moore chamber (Bellco Glass, Vineland, NJ) as previously described (7). Standard perfusate consisted of balanced salt solution with 4.0 mM $[K^+]$, 5.6 mM glucose, and 18 mM $NaHCO_3$ that was bubbled with 21% oxygen, 5% CO_2, and 74% N_2 to produce a P_{O_2} of 149 torr, P_{CO_2} of 36 torr, and pH of 7.4. For simulated ischemia, balanced salt solution was glucose free and contained 8.0 mM $[K^+]$ and 21.35 mM $NaHCO_3$ and was bubbled with 80% N_2 and 20% CO_2 to produce a final P_{O_2} of 3–5 torr, P_{CO_2} of 144 torr, and pH of 6.8. In addition, we added 20 mM of the glycolytic inhibitor 2-deoxyglucose to better model the adenosine triphosphate depletion seen in the fibrillating heart subjected to ischemia due to the highly glycolytic nature of cultured embryonic cardiomyocytes. Compared with standard normocarbic perfusate used for reperfusion (P_{CO_2} of 36 torr, pH 7.4), two other reperfusion solutions were used: the *hypocarbic* reperfusion perfusate and *hypercarbic* reperfusion perfusate. Hypocarbic perfusate consisted of standard balanced salt solution equilibrated with 78% N_2, 1% CO_2, and 21% oxygen to achieve a P_{O_2} of 149 torr, P_{CO_2} of 7 torr, and pH of 7.9. Hypercarbic perfusate consisted of balanced salt solution with 10.7 mM $NaHCO_3$ that was equilibrated with 69% N_2, 10% CO_2, and 21% oxygen to yield a P_{O_2} of 149 torr, P_{CO_2} of 71 torr, and pH of 6.8. Cells were exposed to 1 hr of simulated ischemia and 3 hrs of reperfusion (normocarbia, hypocarbia, and hypercarbia). All perfusates were pumped through the modified Sykes-Moore chamber (0.25 mL/min) by a roller pump and temperature controlled at 37°C. Tubing to the chamber was made of stainless steel to minimize diffusion of ambient oxygen. The low P_{O_2} conditions of simulated ischemia (3–5 torr) were verified using an optical phosphorescence quenching technique (Oxyspot, Medical Systems, Greenvale, NY) (11) or the spectrometer-coupled chemical sensor probe (Ocean Optics, Dunedin, FL).

Light and Fluorescent Microscopy. A Nikon TE 2000-U inverted-phase/epifluorescent microscope was used for cell imaging. A charged-coupled device camera was used to monitor contractions and membrane changes over time in the same field of cells (approximately 70×90 μm). Fluorescent images were acquired from a cooled *Cool*-SNAP-ES camera (Photometrics, Tucson, AZ), and changes in fluorescent intensity over time were quantified with MetaMorph software (Molecular Devices, Downington, PA).

Viability Assay. The exclusion dye, propidium iodide (5 μM), was used as before to assess cell viability (10). It is excluded from viable cells but binds to chromatin after loss of cell membrane integrity (excitation/emission, 540 nm/590 nm). To calculate percentage of cell death after 3 hrs of reperfusion, all cells in the field were permeabilized with digitonin (300 μM). Cell death was calculated as propidium iodide fluorescence at any given time point relative to the maximal value seen after digitonin exposure (100%).

Measure of Intracellular ROS and Nitric Oxide. Oxidant stress was monitored with intracellular probe 2',7'-dichlorofluorescin diacetate (5 μM). On entry, esterases cleave and trap the nonfluorescent 2',7'-dichlorofluorescin inside. Hydrogen peroxide and hydroxyl radical oxidize 2',7'-dichlorofluorescin to generate the fluorescent product dichlorofluorescein (DCF; excitation/emission, 480 nm/520 nm) (12, 13). Intracellular nitric oxide (NO) production was determined using DAF-2 DA (1 μM). DAF-2 DA is an NO indicator that can penetrate rapidly into the cell, where it is hydrolyzed to DAF-2 by intracellular esterases (14). DAF-2 traps NO, yielding fluorescent triazolofluorescein (excitation/emission, 480 nm/520 nm) (15). Of note, this probe may also have some sensitivity to peroxynitrite formation (16) but remains one of the best described indicators of reactive nitrogen species, particularly NO (17). DAF-2 DA has proven useful in our cardiomyocyte model of I/R, helping in previous work to detect NO signaling during preconditioning adaptation (18). Because the excitation/emission wavelengths for these two fluorophores (DCF and DAF) are the same, we measured ROS and NO independently.

Cell Contraction. Cell contractions were assessed as part of functional viability. This was done by observing movement within the same field of cells (13). A return of contraction was denoted when contractions could be seen throughout the field after 3 hrs of reperfusion and marks successful postresuscitation and the return of function in our cardiomyocyte model.

Data Analysis. For each individual experiment, a field of ~500 cells on a 25-mm coverslip was observed over time throughout conditions of simulated I/R. All data are presented as mean ± SEM of n observations, where n

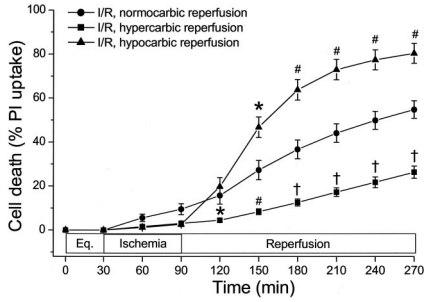

Figure 1. Comparison of ischemia/reperfusion (*I/R*) cell death with reperfusion conditions of hypocarbia (Pco_2 of 7 torr, pH 7.9), normocarbia (Pco_2 of 36 torr, pH 7.4), and hypercarbia (Pco_2 of 71 torr, pH 6.8). All cells were equilibrated with normoxic balanced salt solution for 30 mins and subjected to 1 hr of simulated ischemia and 3 hrs of reperfusion with adjusted CO_2 levels. Hypocarbic reperfusion (n = 5) yielded significantly increased cell death as compared with the normocarbic group, and hypercarbic reperfusion (n = 15) showed significant reduction of cell death (analysis of variance: group main effect, $p < .001$; group-by-time interaction, $p < .001$). *$p < .05$, #$p < .01$, †$p < .001$ vs. normocarbic control.

represents the number of individual coverslips studied. Cell death and fluorescence data were analyzed by two-way repeated measures analysis of variance with one between-groups factor (type of reperfusion) and one repeated-measures factor (time). Both the group main effect and the group-by-time interaction were tested for significance, and the latter incorporated a Greenhouse-Geisser correction to the degrees of freedom to allow for lack of sphericity (i.e., unequal variances and serial correlation over time). If either the main group effect or the group-by-time interaction term was statistically significant, subsequent comparisons were performed at each time point using a two-sample Student's *t*-test allowing for unequal variances. Cell contraction data were analyzed by Fisher's exact test. A *p* value of <.05 was considered statistically significant.

RESULTS

Effects of Hypocarbia/Hypercarbia on Cell Viability. As seen in Figure 1, the cell death seen during the 1 hr of ischemia before hypocarbic, normocarbic, or hypercarbic reperfusion was not significantly different. After ischemia, percentage cell death in hypocarbic reperfusion was significantly higher (80.4% ± 4.5% at 270 mins, n = 5) compared with normocarbic reperfusion (54.8% ± 4.0%, n = 10, $p < .01$). By contrast, hypercarbic reperfusion resulted in significantly lower cell death (26.3% ± 2.8% at 270 mins, n = 15, $p < .001$ vs. normocarbic reperfusion). The return of spontaneous cell contraction was seen in zero of five hypocarbia experiments, as compared with three of ten normocarbia ($p = .50$) and 10 of 15 hypercarbia experiments ($p = .033$). Thus, all groups demonstrated some component of postresuscitation (i.e., reperfusion) injury as previously reported (7–10, 19), but this component was significantly modulated by altering the drop of tissue CO_2 induced at reperfusion.

Effects of Hypocarbia/Hypercarbia on Oxidant Generation. In our model of I/R injury, an oxidant burst (as measured by the intracellular probe 2',7'-dichlorofluorescin diacetate) was observed within the first 10 mins of reperfusion. We studied whether hypocarbic and hypercarbic reperfusion affected these reperfusion oxidants. As seen in Figure 2*A*, hypocarbic reperfusion resulted in an increase of the oxidant burst (6.0 ± 1.0 arbitrary units [a.u.] at 96 mins, n = 6, vs. 3.4 ± 0.4 a.u. in the normocarbia group, n = 4, which was of borderline significance [$p = .052$]). By contrast, hypercarbic reperfusion decreased the maximal DCF fluorescence seen from a control value of 3.4 ± 0 .4 a.u. to 1.8 ± 0.1 a.u. (n = 4, $p < .05$).

One pathway described previously in this cardiomyocyte model for attenuating ROS generation at reperfusion involves preconditioning (7), an adaptive response associated in this same model with increased NO generation (18). Thus, we studied whether the decreased reperfusion ROS associated with hypercarbia were also related to NO generation. Indeed, cardiomyocytes contain constitutive NO synthase (NOS) isoforms, including a neuronal-NOS isoform (20–22), that is activated by hypercarbic acidosis (23, 24). Furthermore, NO can inhibit mitochondrial ROS generation (25). As seen in Figure 2*B*, the decreased DCF fluorescence induced by hypercarbic reperfusion was reversed by the NOS inhibitor, L-NAME (200 μM), when given as previously described (18) during a 2-hr preincubation period and during equilibration and I/R (n = 5). The hypercarbia burst of DCF fluorescence was increased from 1.7 ± 0.1 a.u. to 3.8 ± 0.7 a.u. by L-NAME at 8 mins of reperfusion ($p < .05$), suggesting indirectly that NOS-mediated NO generation may help mediate the hypercarbia effect on the reperfusion ROS burst.

Effects of Hypocarbia/Hypercarbia on NO Generation. Endogenous NO generation has been implicated to have both protective and detrimental roles in various *in vitro* and *in vivo* models of I/R injury (for review, see Jones and Bolli [26]). To test whether endogenous NO was affected by hypocarbic or hypercarbic reperfusion, we continuously monitored the entire course of I/R using the NO indicator DAF-2 DA. As shown in Figure 3*A*, the NO generation was greater in the hypocarbia group during the first minutes of reperfusion, resulting in a significantly higher peak level of NO at 15 mins of reperfusion (n = 8, $p < .05$ compared with normocarbic control). The NO level thereafter declined gradually, as in the normocarbia group, exhibiting no differences by 1 hr of reperfusion. In contrast, during the initial moments of reperfusion, no significant difference in DAF-2 fluorescence was noted between hypercarbia and normocarbia groups. After the peaking of NO at around 15 mins of reperfusion, however, hypercarbic reperfusion resulted in sustained generation of NO compared with the gradual decline seen in normocarbic reperfusion, leading to a significantly higher level of NO in the later phase of reperfusion (n = 8, $p < .01$) (Fig. 3*B*). Given the reported sensitivity of DAF-2 for both NO and peroxynitrite (16, 17), it is possible that the initial burst of DAF-2 oxidation seen in hypocarbic reperfusion reflects increased peroxynitrite formation (with increased cell death). The later, sustained DAF-2 oxidation may be a result of NO generated via NOS (leading to the associated cardioprotection).

Role of NO Synthase in the Protection of Hypercarbic Reperfusion. To further test the role of NOS in the sustained, increased NO production in hypercarbic reperfusion, and its contribution to cell protection, the NO profile and cell viability were assessed in hypercarbic reperfusion compared with hypercarbic reperfusion with L-NAME (200 μM) incubation all course during I/R, as described above. As seen in Figure 4*A*, cotreatment of L-NAME blocked maintenance of the sustained NO levels induced by hypercarbic reperfusion (n = 8, $p < .05$). Meanwhile, L-NAME also reversed the protection of hypercarbic reperfusion (cell death increased from 28.3% ± 3.2% to 54.3% ± 6.0% at 270 mins, n = 3 in each group, $p < .05$) (Fig. 4*B*). These data suggest that the NO increase in the later phase of reperfusion is mediated by NOS activation and that it contributes to the protective effect conferred by hypercarbic reperfusion.

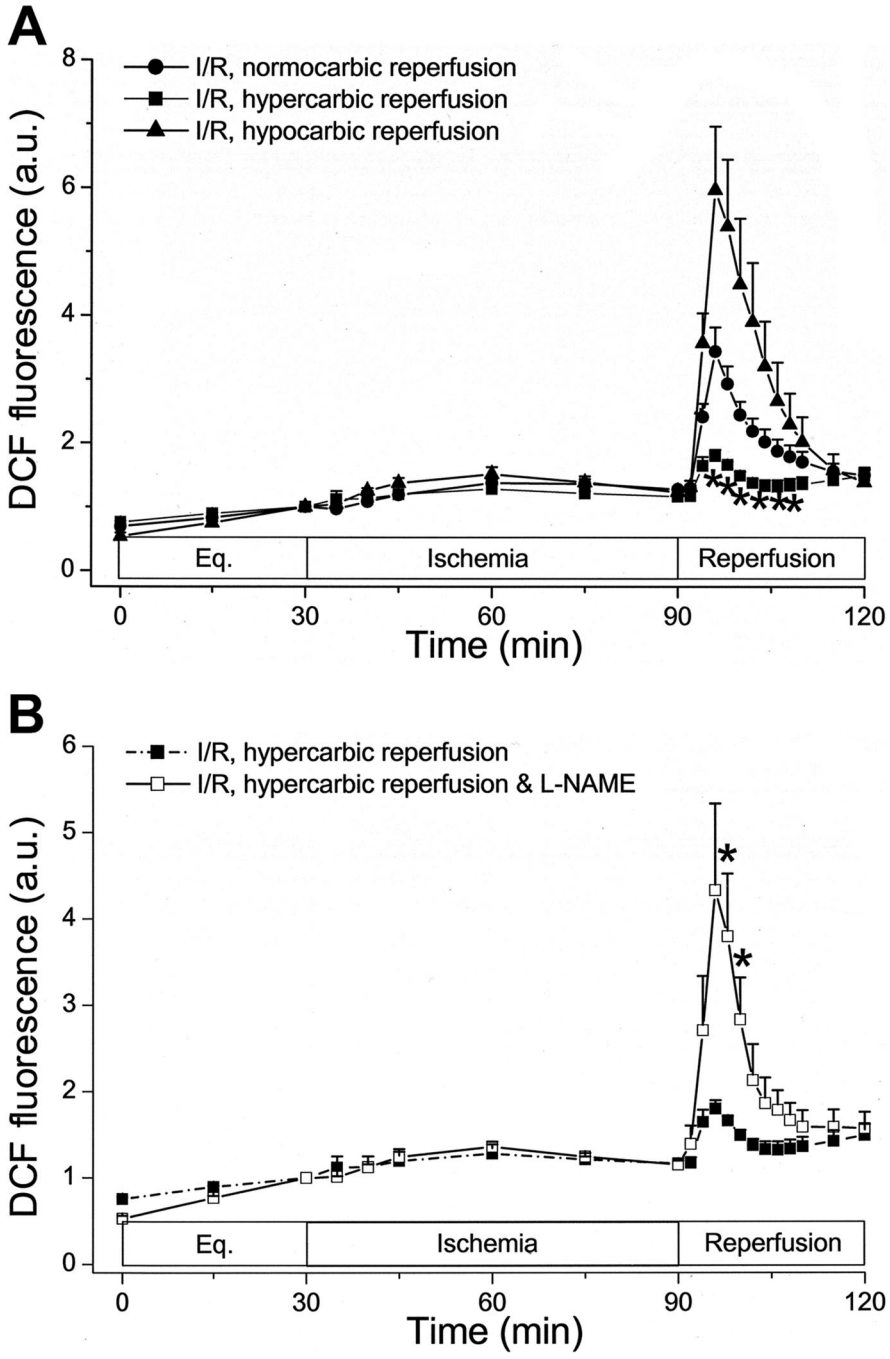

Figure 2. Comparison of dichlorofluorescein (*DCF*) fluorescence, indicator of reactive oxygen species (*ROS*) generation, during conditions of hypocarbic, normocarbic, and hypercarbic reperfusion. *A*, hypocarbic reperfusion demonstrates a two-fold increase in maximal DCF intensity (within first 10 mins of reperfusion) ($p = .052$) over normocarbic control (n = 4), whereas hypercarbic reperfusion shows significant attenuation in ROS generation during this same time period (n = 4) (analysis of variance: group main effect, $p = .045$; group-by-time interaction, $p = .006$). *$p < .05$ vs. normocarbic control. *B*, N-nitro-L-arginine methyl ester (*L-NAME*, 200 μM) incubated 2 hrs before and during the entire experiment significantly reversed the hypercarbia-induced attenuation in reperfusion ROS burst (n = 5) (analysis of variance: group main effect, $p = .068$; group-by-time interaction, $p = .037$). *$p < .05$ vs. hypercarbic group; *a.u.*, arbitrary units. *I/R*, ischemia/reperfusion.

Mitochondrial Inhibition with Stigmatellin Blocks the Increased Reperfusion ROS and Reduces Cell Death Caused by Hypocarbic Reperfusion. We have reported previously that mitochondrial oxidants induce significant injury in this cardiomyocyte I/R model (27). Additional work suggests that ROS generation during I/R occurs from complex III (28). Consistent with this, attenuation of reperfusion oxidants using the complex III mitochondrial inhibitor stigmatellin significantly attenuates cell death after reperfusion and improves contractile recovery (19). If the mitochondria are a major source of the reperfusion oxidant burst induced by hypocarbia, then inhibition of the respiratory chain should also attenuate the burst of DCF fluorescence seen at hypocarbic reperfusion. As shown in Figure 5*A*, the addition of stigmatellin (20 nM), given during the first 15 mins of reperfusion only, significantly attenuated the burst of DCF fluorescence seen during hypocarbic reperfusion (2.3 ± 0.3 a.u. vs. 6.0 ± 1.0 a.u., n = 5 in each group, $p < .05$). On the other hand, blockade of cytoplasmic membrane NADPH oxidase (another major source of intracellular ROS) with apocynin (300 μM) failed to attenuate the reperfusion ROS burst, either during hypocarbic (Fig. 5*B*) or normocarbic reperfusion (Fig. 5*C*). In fact, values were significantly increased with apocynin. These again suggest that the reperfusion oxidant burst is predominantly mitochondrial in origin and that the hypocarbia-induced oxidant increase comes from the same source. Nevertheless, it is quite likely that multiple oxidant sources contribute to outcome after reperfusion, and NADPH oxidases could contribute to later reperfusion injury.

As to the contribution of the ROS burst to the extent of I/R injury, we previously reported that stigmatellin (20 nM) given during the first 15 mins of reperfusion remarkably reduced both the reperfusion ROS burst and cell death, decreasing cell death during normocarbic reperfusion from >53% to about 10% cell death (19). Stigmatellin administered at the same dose and timing also significantly attenuated the increase of cell death caused by hypocarbic reperfusion (52.2% ± 6.5% at 270 mins vs. 85.9% ± 4.5% with hypocarbic reperfusion alone, n = 5 in each group, $p < .01$) (Fig. 5*D*). Taken together, these results suggest that a mitochondrial oxidant burst at reperfusion is crucial in the pathogenesis of I/R injury and that this ROS burst and associated injury can be modulated both up and down by CO_2 drop-offs at reperfusion.

DISCUSSION

The results of the current study show that CO_2 and pH changes induced during the re-oxygenation of ischemic chick car-

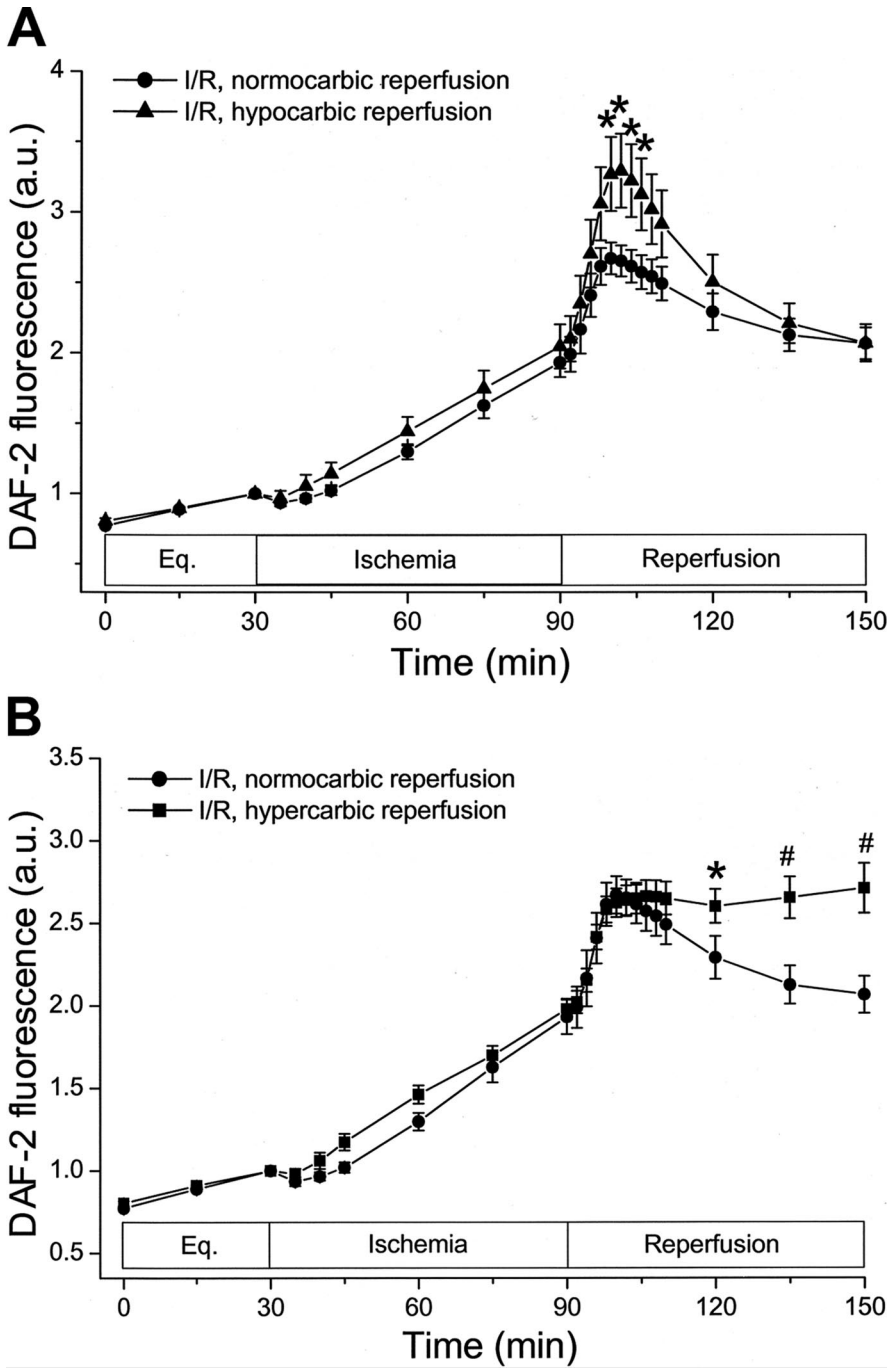

Figure 3. Comparison of nitric oxide (*NO*) generation (indicated by 4,5-diaminofluorescein fluorescence) during conditions of hypocarbic, normocarbic, and hypercarbic reperfusion. *A*, compared with normocarbic control, hypocarbic reperfusion resulted in a significantly higher peak of NO generation early after reperfusion (n = 8) (analysis of variance: group main effect, $p = .076$; group-by-time interaction, $p = .002$). *$p < .05$ vs. normocarbic control. Both conditions resulted in similar levels of NO later in reperfusion. *B*, in hypercarbic reperfusion, no significant difference in NO generation was noted in the early phase of reperfusion as compared with normocarbic control. After the peaking of NO at around 15 mins of reperfusion, hypercarbia resulted in sustained generation of NO, leading to a higher level of NO in the later phase of reperfusion (n = 8) (analysis of variance: group main effect, $p = .13$; group-by-time interaction, $p = .0008$). *$p < .05$, #$p < .01$ vs. normocarbic control; *a.u.*, arbitrary units. *I/R*, ischemia/reperfusion; *DAF*, diaminofluorescein diacetate.

diomyocytes can significantly modify postresuscitation oxidants and injury. Specifically, hypercarbic reperfusion (Pco_2 of 71 torr, pH 6.8) decreased cell death significantly relative to normocarbic reperfusion (Pco_2 of 36 torr, pH 7.4). In contrast, marked increases in cell death occurred with hypocarbic reperfusion (Pco_2 of 7 torr, pH 7.9). In addition, the hyperbaric group showed no functional return of contraction, whereas two thirds of the hypercarbic group recovered synchronous spontaneous contraction. With respect to oxidant generation, it seems that hypercarbic protection is mediated by attenuation of mitochondrial complex III-generated ROS because the ROS burst and postresuscitation injury abrogated by hypercarbia is also attenuated by the mitochondrial complex III inhibitor stigmatellin. Finally, this attenuation in mitochondrial ROS by hypercarbia seems mediated in part via NOS-mediated NO generation. Hypercarbic reperfusion was associated with sustained, increased DAF-2 DA oxidation. In addition, L-NAME reversed hypercarbic reperfusion decreases in ROS, increases in NO generation, and the associated cardioprotection.

The pH Paradox. The acceleration of cell death in this study despite the normalization of pH at reperfusion, the pH paradox, is consistent with work by others (6, 29–31). We used chick cardiomyocytes, similar to the study carried out by Bhatti et al (6). Whereas most studies used chemical means to decrease pH, the present study, following the approach of Bhatti et al. (6), used CO_2 to modify pH. Our results extend this work in a number of ways. First, we studied whether hypercarbia may decrease ROS generation at reperfusion. Major sources of oxidant generation and the overall redox state of the mitochondria may likely be affected by CO_2 and pH. The direct inhibition of plant mitochondrial respiration by hypercarbia has already been reported (32), and mammalian mitochondria seem to be inhibited by hypercarbia (33, 34). If hypercarbia exerts such an "antioxidant" effect on cardiomyocyte mitochondria, it would be consistent with our model, suggesting that antioxidants given in combination just at the point of reperfusion significantly decrease cell death and improve contractile recovery (7) and help prevent the cytochrome c release seen within minutes of reperfusion (8). In addition, the notion that higher CO_2 levels may protect by inducing partial mitochondrial inhibition at reperfusion is consistent with recent work in these cardiomyocytes. For example, the complex III inhibitor stigmatellin given briefly at reperfusion significantly attenuates postresuscitation cell death (19). Other protective effects of CO_2 may include blocking the opening of the mitochondrial permeability transition pore (31). The present study suggests that the rate of drop-off in cardiomyocyte CO_2 from ischemia to reper-

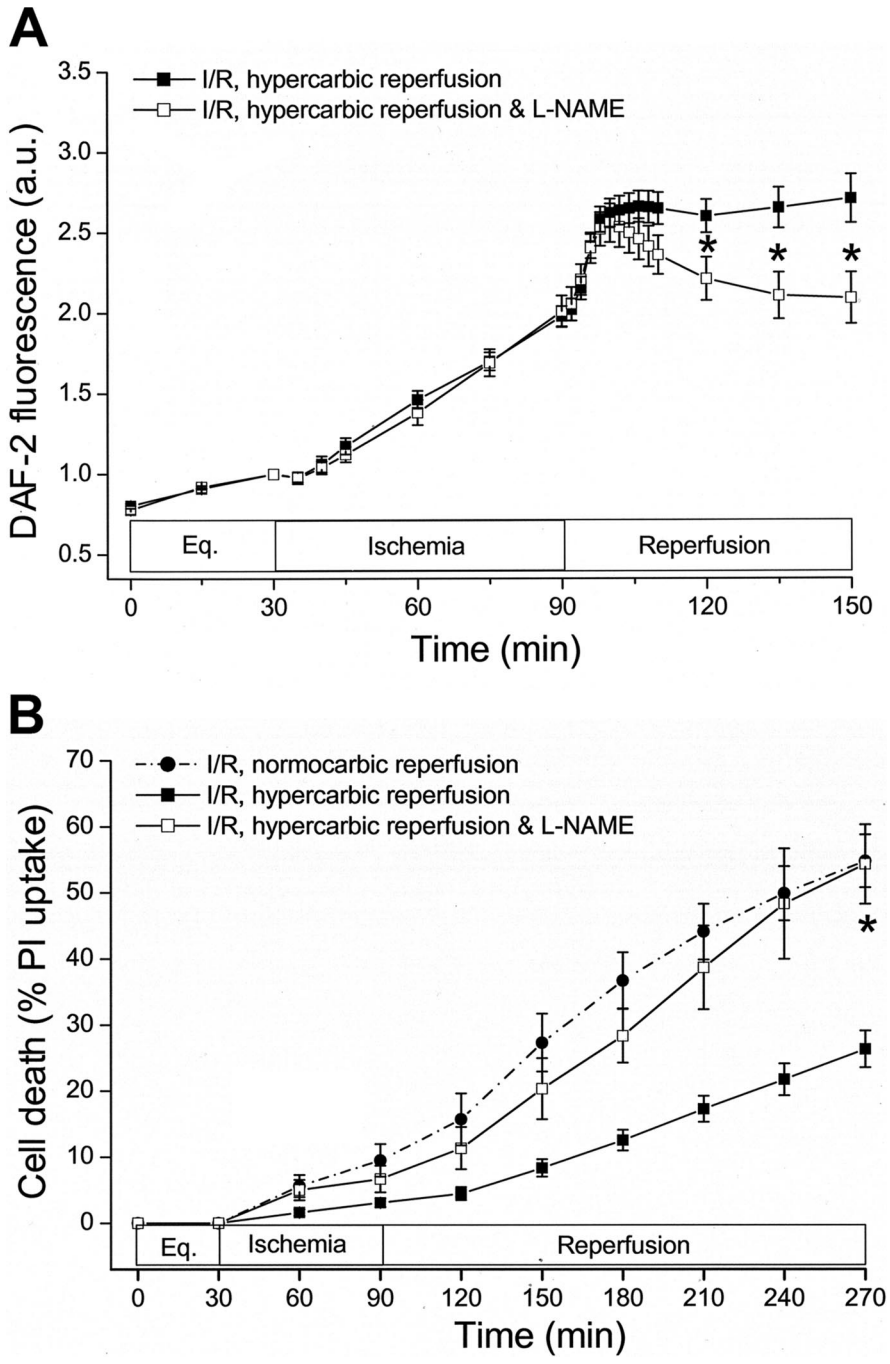

Figure 4. Effect of N-nitro-L-arginine methyl ester (*L-NAME*) on hypercarbia-induced sustained nitric oxide (*NO*) increase and protection. *A*, administration of L-NAME (preincubated and all course at 200 μM) significantly abrogated the sustained increase of NO induced by hypercarbia back to the control level (n = 8) (analysis of variance: group main effect, $p = .24$; group-by-time interaction, $p = .014$). *$p < .05$ vs. hypercarbic group; *a.u.*, arbitrary units. *B*, L-NAME also significantly reverses the protective effect conferred by hypercarbic reperfusion (n = 3) (analysis of variance: group main effect, $p = .052$; group-by-time interaction, $p = .032$). The normocarbic cell death reference was provided as a *dotted line tracing*. *$p < .05$ vs. normocarbic control. *I/R*, ischemia/reperfusion; *DAF*, diaminofluorescein diacetate.

fusion can modulate mitochondrial ROS-mediated postresuscitation injury.

There are likely a number of ROS sources in these cells, including NADPH oxidases. However, as seen in Figure 5, *B* and *C*, apocynin did not attenuate the ROS burst seen in the first minutes of reperfusion in this particular model. In fact, at this early time point and the highest dose tested, apocynin may have increased the ROS burst somewhat. Others have reported that apocynin can actually activate the pentose phosphate pathway and tricarboxylic acid cycle, effects that could increase ROS generation (35). Nevertheless, NADPH oxidases are an important source of oxidants that could play a later role in reperfusion injury in this particular model.

Finally, although hypercarbia has been reported to increase NO generation that can be blocked by NOS inhibition (23), this effect has not been reported in the context of the I/R transition. Given that constitutive NOS activity is both oxygen and pH dependent, it is not surprising that during re-oxygenation, CO_2 and resulting pH changes would affect NO generation. The observation of NO-mediated cardioprotection in the present study is consistent with previous work in these cells describing increased NO signaling during preconditioning (18). Thus, a controlled reperfusion with less abrupt fall-offs of tissue CO_2 may provide some level of ischemic postconditioning protection that is mediated via some of these same NO-signaling pathways.

Hypocarbia Increases Postresuscitation Injury. The current work contrasts the protective effects of hypercarbia with the deleterious effects of hypocarbic reperfusion. We found that hypocarbic alkalosis significantly increased both cardiomyocyte cell death and reperfusion ROS. In contrast to studies of hypercarbic or acidotic reperfusion, there have been relatively few studies on the effects of hypocarbic or alkalotic reperfusion of ischemic cells. Bhatti et al. (6) did study the effects of increased pH during ischemia but not during reperfusion (6). Increasing evidence suggests that hypocarbia seems to induce substantial adverse physiologic and medical effects (36). In addition, case reports of hyperventilation in the clinical setting of intact circulation suggest that P_{CO_2} levels as low as 8 torr with associated arterial pH of 7.8—as modeled in this study—are possible to achieve (37). In regard to the fall-off of tissue CO_2 after resuscitation, the tendency to hyperventilate in the resuscitation setting with ventilation rates 2–3 times the American Heart Association guidelines (4, 38, 39) would make rapidly normalized CO_2 or even relative hypocarbia in the minutes after reperfusion more likely.

Extrapolating the results of our study, post-ROSC hyperventilation with rapid tissue CO_2 drops might increase myocardial oxidant stress and dysfunction. In addition to ventilation, pharmacologic agents that increase perfusion pressure

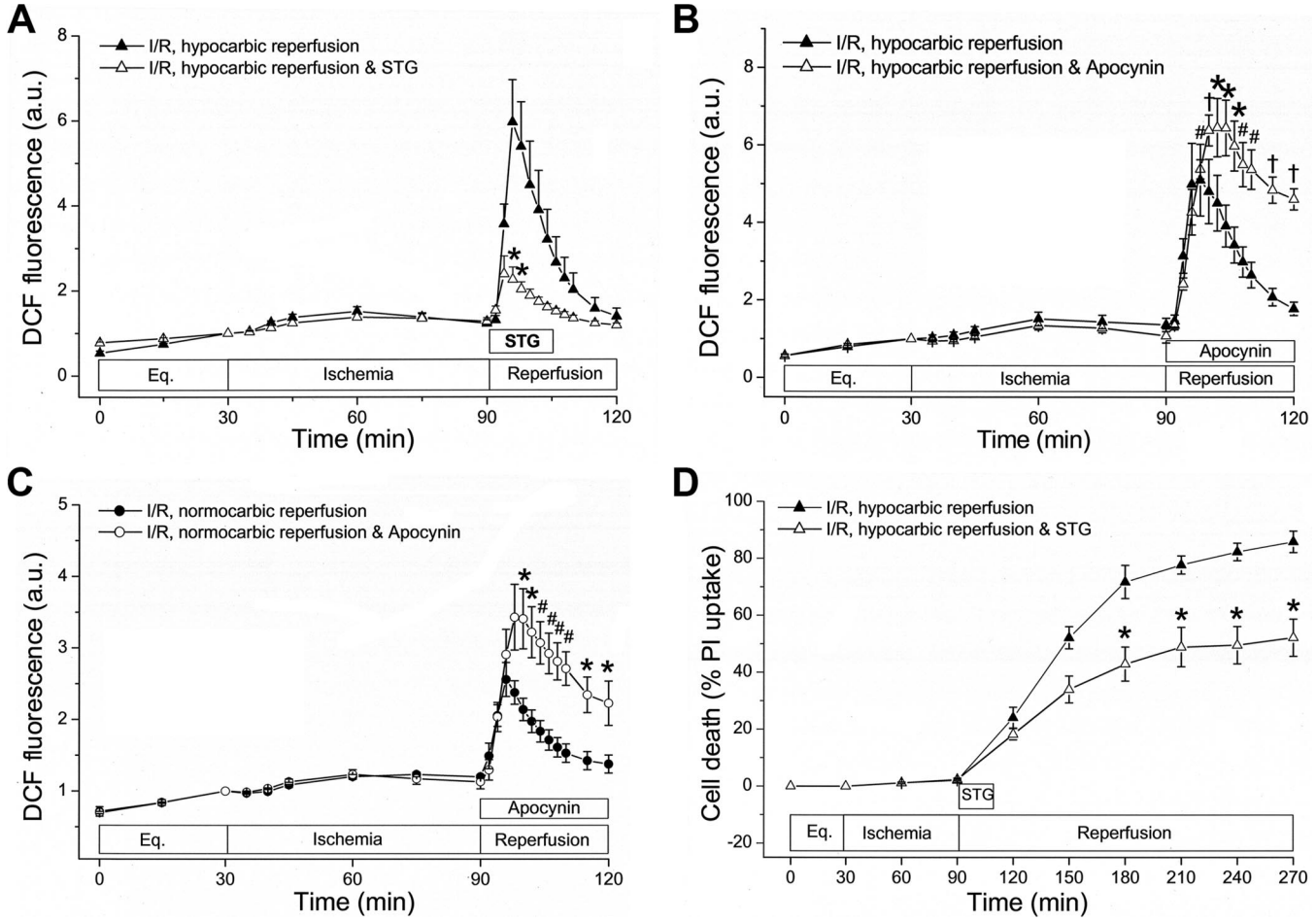

Figure 5. Source of the reperfusion reactive oxygen species (*ROS*) burst induced by hypocarbia and its role in reperfusion injury. *A*, stigmatellin (*STG*, 20 nM) administered during the first 15 mins of reperfusion significantly attenuated the ROS burst caused by hypocarbia (n = 5) (analysis of variance: group main effect, $p = .069$; group-by-time interaction, $p = .020$). *$p < .05$ vs. hypocarbic group. Apocynin (300 μM) given at reperfusion failed to attenuate and, instead, increased the reperfusion ROS burst either in hypocarbic reperfusion (*B*) (analysis of variance: group main effect, $p = .002$; group-by-time interaction, $p = .023$ [*$p < .05$, #$p < .01$, †$p < .001$ vs. hypocarbic group]) or normocarbic reperfusion (*C*) (analysis of variance: group main effect, $p = .011$; group-by-time interaction, $p = .012$ [*$p < .05$, #$p < .01$, †$p < .001$ vs. normocarbic control]). *D*, stigmatellin (20 nM) administered during the first 15 mins of reperfusion significantly reduced cell death caused by hypocarbic reperfusion (n = 5) (analysis of variance: group main effect, $p = .0078$; group-by-time interaction, $p = .0005$). *$p < .05$ vs. hypocarbic group; *a.u.*, arbitrary units. *I/R*, ischemia/reperfusion; *DCF*, dichlorofluorescein.

after ROSC could also alter how quickly tissue CO_2 declines after ROSC. Our recent work in a swine model of cardiac arrest suggests that oxidant stress can peak within 20 mins of ROSC (40). Thus, it is conceivable that changes in CO_2 during this time window could modulate this oxidant stress. Furthermore, in our mouse model of cardiac arrest and in our chick cardiomyocyte model of I/R, optimizing conditions (e.g., temperature) within the first 20 mins of ROSC or reperfusion, respectively, proved critical for determining cardiovascular postresuscitation injury and survival (42). The present study raises the question of what the optimal fall-off of tissue CO_2 should be during this critical time. Although maintaining hypercarbic conditions for prolonged periods of time into reperfusion would be unwise and could itself compromise hemodynamic function of the heart, the stigmatellin data suggest that even brief, transient mitochondrial inhibition in the first minutes of reperfusion can protect against subsequent postresuscitation injury. Thus, simply optimizing the decline of tissue CO_2 for a number of minutes after ROSC from cardiac arrest (using optimized ventilation and controlled perfusion strategies) could be enough to decrease mitochondria-mediated ROS oxidant damage.

Limitations of Study. The chick cardiomyocyte system used for this work has a number of strengths and significant limitations. Such models, though helping to focus on cardiomyocyte responses to redox changes seen during conditions of I/R, cannot model the complexities of the heart—including endothelial, inflammatory, and neurohumoral responses. In addition, it is impossible to model all the conditions of *in vivo* ischemia and reperfusion at the cellular level. Nevertheless, this heart cell model does exhibit a number of features seen in a whole heart and its response to I/R: spontaneous synchronous contractions, ability to be preconditioned, hypothermia protection, oxidant generation, and critical membrane changes at reperfusion that are consistent with accelerated death pathways. These critical membrane changes include cell membrane blebbing, cardiac enzyme release, loss of mitochondrial membrane function, and cytochrome c release. At best, this model can only lend some insight to questions that ultimately need to be answered in more complex biological sys-

tems. This particular heart cell study suggests that more controlled rates of tissue CO_2 washout can affect cardiovascular postresuscitation injury. Further work will be needed to translate this work into our mouse model of cardiac arrest, which also demonstrates lethal cardiovascular postresuscitation injury after ROSC. In this setting, ventilation and use of various gas mixtures can be used to control the fall-off of CO_2 that occurs within 30 mins after ROSC.

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