Oxidant Stress during Simulated Ischemia Primes Cardiomyocytes for Cell Death during Reperfusion*

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Ischemia-reperfusion injury induces oxidant stress, and the burst of reactive oxygen species (ROS) production after reperfusion of ischemic myocardium is sufficient to induce cell death. Mitochondrial oxidant production may begin during ischemia prior to reperfusion because reducing equivalents accumulate and promote superoxide production. We utilized a ratiometric redox-sensitive protein sensor (heat shock protein 33 fluorescence resonance energy transfer (HSP-FRET)) to assess oxidant stress in cardiomyocytes during simulated ischemia. HSP-FRET consists of the cyan and yellow fluorescent protein fluorophores linked by the cysteine-containing regulatory domain from bacterial HSP-33. During ischemia, ROS-mediated oxidation of HSP-FRET was observed, along with a decrease in cellular reduced glutathione levels. These findings were corroborated by measurements using redox-sensitive green fluorescent protein, another protein thiol ratiometric sensor, which became 93% oxidized by the end of simulated ischemia. However, cell death did not occur during ischemia, indicating that this oxidant stress is not sufficient to induce death before reperfusion. However, interventions that attenuate ischemic oxidant stress, including pre- and post-conditioning, therapeutic hypothermia during reperfusion, and chemical antioxidants, all confer protection against subsequent cell death (7–9); overexpression of antioxidant enzymes confers protection against ischemia-reperfusion injury (10, 11). Multiple studies have demonstrated that a burst of oxidant stress is generated upon reperfusion of ischemic cardiomyocytes (5, 12, 13). Interventions that attenuate this reperfusion burst, including pre- and post-conditioning, therapeutic hypothermia during reperfusion, and chemical antioxidants, all confer protection against cell death (14–16). This indicates that the ROS burst at reperfusion is both necessary and sufficient to induce cell death in cardiomyocytes. However, there is still uncertainty about the events occurring during ischemia that prime the cell to undergo this lethal burst in oxidant stress within the first few minutes of reperfusion.

Previous studies indicate that mitochondrial oxidant generation increases during cellular hypoxia and suggest that ROS generation may occur during ischemia prior to reperfusion (17–19). These observations led us to hypothesize that ROS production begins during ischemia and to investigate whether this oxidant stress contributes to the cell death during reperfusion.

The assessment of intracellular oxidant stress in real time during ischemia and reperfusion is technically challenging. Spin trap probes coupled with EPR spectroscopy can potentially identify the radical species involved, but high concentrations of spin trap are required, and the radicals detected frequently represent secondary products generated by the

of blood flow relieves the ischemic stress, although morphological evidence of tissue injury appears only after the oxygen supply has been restored (3). This has led to the widely accepted notion that reperfusion is responsible for the cellular damage in ischemia-reperfusion injury (4). However, the events during ischemia that prime the cell for death during reperfusion are not fully understood.

Oxidant stress arising from excessive production of reactive oxygen species (ROS) has long been associated with ischemia-reperfusion injury (4, 5). In this regard, animals with genetic impairment of antioxidant systems exhibit increased susceptibility to myocardial ischemia-reperfusion injury (6); antioxidants administered during ischemia-reperfusion confer protection against subsequent cell death (7–9); and overexpression of antioxidant enzymes confers protection against ischemia-reperfusion injury (10, 11). Multiple studies have demonstrated that a burst of oxidant stress is generated upon reperfusion of ischemic cardiomyocytes (5, 12, 13). Interventions that attenuate this reperfusion burst, including pre- and post-conditioning, therapeutic hypothermia during reperfusion, and chemical antioxidants, all confer protection against cell death (14–16). This indicates that the ROS burst at reperfusion is both necessary and sufficient to induce cell death in cardiomyocytes. However, there is still uncertainty about the events occurring during ischemia that prime the cell to undergo this lethal burst in oxidant stress within the first few minutes of reperfusion.

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3 The abbreviations used are: ROS, reactive oxygen species; FRET, fluorescence resonance energy transfer; HSP, heat shock protein; roGFP, redox-sensitive green fluorescent protein; Cu,Zn-SOD, copper-zinc superoxide dismutase; Mn-SOD, manganese superoxide dismutase; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; ANOVA, analysis of variance.
Oxidant Stress during Ischemia in Cardiomyocytes

interaction of primary species with iron or copper ions (for review, see Refs. 15). A number of EPR studies suggest that a burst of radicals is produced at the onset of reperfusion based on the appearance of radical adducts in the coronary sinus during the initial reperfusion period (5, 13, 15, 20–23). However, because blood flow is absent during ischemia, it is difficult to assess ROS production prior to reperfusion using spin trap probes and EPR. Moreover, many spin trap probes are primarily sensitive to oxidants released to the extracellular space (24), which confounds their ability to assess intracellular oxidant stress. Alternative methods using commercially available oxidant-sensitive probes such as dichlorofluorescin are subject to nonspecific oxidation (25, 26), and the fluorescence of the probes decreases under the conditions of low pH that accompany ischemia. Furthermore, oxidant-sensitive probes can potentially access multiple intracellular compartments, making it difficult to identify the intracellular sites affected by oxidative stress (27). Finally, ROS-sensitive fluorophores do not provide ratiometric measures of oxidant stress, making it difficult to ascertain whether changes in fluorescence intensity are due to changes in oxidant production, to changes in pH, or to a change in the steady-state levels of intracellular dye. In this study, we sought to assess cellular oxidant stress during ischemia using a novel redox-sensitive fluorescence resonance energy transfer (FRET) protein expressed in the cytosol of cardiomyocytes. This probe consists of cyan and yellow fluorescent protein fluorophores connected by a redox-sensitive hinge peptide. The hinge region consists of the cysteine-containing regulatory domain of HSP-33, a bacterial heat shock protein with chaperone activity that is activated by the oxidation of thiols in the regulatory domain (28). Using the regulatory domain of HSP-33 for the HSP-FRET sensor therefore yields a ratiometric sensor of protein thiol oxidation state. When expressed in the cytosol, this sensor (HSP-FRET) permits optical interrogation of the reduction state of the thiol pool in that compartment (29). These measurements were confirmed using a second redox-sensitive ratiometric protein redox sensor (roGFP) (30, 31). This mutant of green fluorescent protein is capable of undergoing reversible changes in cysteine dithiol formation, permitting its calibration in vivo and allowing quantification of oxidant stress during ischemia. We applied these approach in cultured cells subjected to simulated ischemia in a flow-through chamber to assess the extent of oxidant stress during ischemia-reperfusion and the significance of this stress for cell death during reperfusion.

EXPERIMENTAL PROCEDURES

Reagents—Adenoviruses expressing catalase or mitochondrial-targeted catalase were obtained as a gift from Drs. J. Bai and A. I. Cederbaum (32); adenoviruses expressing copper-zinc superoxide dismutase (Cu,Zn-SOD) and manganese superoxide dismutase (Mn-SOD) were obtained from the Gene Transfer Vector Core Facility of the University of Iowa (33, 34). Cells were transiently transfected with cDNA encoding HSP-FRET using GenePORTER transfection reagent (Gene Therapy Systems, Inc.). Digitonin was obtained from Aldrich. Human 143B osteosarcoma cells were obtained from American Type Culture Collection and cultured according to the manufacturer’s recommendations. Other chemicals and reagents were obtained from Sigma.

Cultured Cardiomyocytes—Cardiomyocytes were obtained by trypsin digestion of 10–12-day embryonic chick hearts as described previously (19). Cells were plated in the center quadrant of 25-mm glass coverslips and maintained in a tissue culture incubator for 3–7 days. After 3 days in culture, cells reached 70–90% confluence and exhibited spontaneous contraction.

HSP-FRET—The HSP-FRET redox sensor was constructed by cloning cDNA encoding yellow fluorescent protein (YFP) between the Nhe1 and BglIII restriction sites in pECFP-N1 (Clontech) and the redox-regulated domain from HSP-33 (positions 218–287) via EcoRI and BamHI sites. Proper orientation of the construct was verified by bidirectional sequencing. Plasmids were amplified by overnight growth in transformed DH5α cells (Invitrogen) and isolated by column purification (EndoFree plasmid maxi kit, Qiagen Inc.). Cardiomyocytes were transfected with the HSP-FRET construct using GenePORTER transfection reagent according to the manufacturer’s recommendations and 3 μg of DNA and 15 μl of GenePORTER per coverslip. Intracellular fluorescence was detectable within 24 h, and cell studies were carried out within 48 h.

roGFP Redox Sensor—roGFP was generated by introducing the C485/Q80/R/S147/C/Q204 mutations into the pEGFP-N1 vector (Clontech) using the Stratagene QuikChange kit. This construct was then subcloned into the Ad5CMV K-Npa vector between the KpnI and NotI sites (ViraQuest Inc.), re-sequenced, and used to generate a recombinant adenovirus. Coverslips containing 10⁵ cells were incubated with virus for 2 h in serum-free medium and allowed to express the protein for 24–36 h.

Simulated Ischemia-Reperfusion—Studies were carried out as the cells were perfused under controlled oxygen and CO₂ conditions. The base-line perfusate containing NaCl (117 mM), KCl (4 mM), NaHCO₃ (18 mM), MgSO₄ (0.76 mM), NaH₂PO₄ (1 mM), CaCl₂ (1.21 mM), and glucose (5.6 mM) was bubbled with 5% CO₂, 21% O₂, and 74% N₂. The same solution was used during simulated reperfusion. To simulate ischemia, cells superfused with buffer containing NaCl (108.9 mM), KCl (8 mM), NaHCO₃ (21.4 mM), MgSO₄ (0.76 mM), NaH₂PO₄ (1 mM), and CaCl₂ (1.21 mM) without glucose was bubbled with 20% CO₂, 0% O₂, and 80% N₂ (pH 6.8). The O₂/CO₂ environment of the cells was controlled by mounting the coverslip carrying the cells into a flow-through chamber composed of two coverslips separated by a stainless steel spacer ring (Penn-Century, Inc., Philadelphia, PA). The chamber was filled and perfused with medium that had been continuously bubbled with O₂/CO₂/N₂ mixtures gases in a heated water-jacketed gas equilibration column (Radnoti Glass Technology, Inc.), mounted above the stage of an inverted microscope (Nikon), and connected to the flow-through chamber with stainless steel tubing to minimize contamination by room air. Using this system, cells were superfused with medium supplied from the equilibration column, where it was equilibrated with different gas mixtures corresponding to base-line or ischemic conditions. The PO₂ within the flow-through chamber was measured under simulated
ischemic conditions using a porphyrin-based phosphorescence quenching method (35).

Anoxic Ischemia—Anoxic ischemia was identical to the standard simulated ischemic procedure, except that residual O₂ was scavenged from the perfusate by adding sodium lactate (5 mM) and the oxygen scavenger EC-Oxyrase/H₂O₂. EC-Oxyrase is a commercially available enzymatic preparation used to produce anoxic conditions in cell culture by reducing molecular oxygen to water. Control ischemic studies were carried out using the standard ischemic protocol containing sodium lactate (5 mM) without EC-Oxyrase.

Fluorescence Imaging—Fluorescent images of cells expressing HSP-FRET were obtained while the cells were perfused at 37 °C using an oil immersion objective (1.3 numerical aperture, 100×). Cyan fluorescent protein (CFP) images were obtained using an emission filter at 470/430 nm, and YFP emission was measured at 535/530 nm. In both cases, excitation was achieved using a 430/425-nm filter. Images of cells expressing roGFP were acquired similarly using excitation at 400 and 490 nm and emission at 535 nm. Images were acquired every minute using a 16-bit cooled CCD camera (Cascade 650); ratiometric images were calculated using MetaFluor imaging software (Universal Imaging). Microscopic fields generally contained one to four cells, the responses of which were individually tracked as regions of interest during the experiment. Each experiment consisted of the averaged response from one to four cells in a single microscopic field. Ratiometric values were normalized to the values in the same cell at the end of the baseline, with 100% corresponding to the value at the end of the baseline.

Cellular GSH/GSSG Measurements—Reduced and oxidized glutathione levels were measured in cell lysates using the BIOXYTECH[reg] GSH/GSSG-412™ kit (Oxis International, Inc., Portland, OR) according to the manufacturer’s recommendations.

Cell Viability—Cell death was assessed in separate experiments using propidium iodide (5 μM), which was added to the perfusate. This probe is excluded from live cells, but enters cells upon disruption of plasma membrane integrity. Fluorescent images were obtained using a ×10 objective (excitation at 555 nm and emission at 605 nm) at the baseline; at the end of ischemia; and at 1, 2, and 3 h into reperfusion. Following the 3-h measurement, all cells were permeabilized by adding digitonin (300 μM) to the perfusate for 45 min. Cell death during the experiment was calculated as the percent of the post-digitonin value.

Statistical Analysis—Data were analyzed by analysis of variance (ANOVA) for repeated measures. When a statistically significant difference was detected, individual differences were then explored using Student’s t test or Newman-Keuls multiple comparison tests. Statistical significance was determined at the p = 0.05 level.

RESULTS

Ventricular cardiomyocytes isolated from 10–12-day embryonic chick hearts were grown on glass coverslips at a density of 1 × 10⁵ cells. After 3–4 days in culture, cells were 85–95% confluent and exhibited spontaneous contraction. Coverslips were placed in a flow-through chamber and perfused with balanced salt solutions at 37 °C under controlled oxygen conditions.

Cardiomyocytes transiently transfected with the HSP-FRET sensor exhibited a uniform pattern of confocal fluorescence (Fig. 1B) that was indistinguishable in appearance from the pattern observed in cells transfected with a commercially obtained, non-targeted, GFP expression vector (pEGFP-N1) (data not shown). Confocal analysis of the spatial distribution of CFP and YFP fluorescence in cells expressing HSP-FRET revealed uniform correspondence between the two fluorophores, indicating that expression of the intact protein was achieved (data not shown). By individually photobleaching the CFP and YFP moieties, we determined that the FRET transfer ratio for this probe is 4.3%, where a value of zero corresponds to an absence of
Oxidant Stress during Ischemia in Cardiomyocytes

Förster energy exchange and a value of 100% indicates that emission from the acceptor fluorophore was completely dependent on energy transfer from the donor (data not shown).

During base-line perfusion with normoxic medium (21% O2 and 5% CO2), a stable HSP-FRET ratio in the range of 0.6–0.8 was typically observed. To assess the sensitivity of HSP-FRET to oxidant stress, fluorescent images were collected every 20 s for 30 min as the cells were perfused with normoxic medium containing H2O2 or medium alone (time controls) (Fig. 1D). A dose-dependent increase in the fluorescence ratio was detected in cells subjected to exogenous oxidants, indicating that the probe is sensitive to exogenous H2O2.

To assess oxidant stress during ischemia, cardiomyocytes transiently expressing HSP-FRET were subjected to base-line normoxic perfusion (20 min) followed by simulated ischemia (30 min) while fluorescent images were collected every 60 s. Within 6 min after switching to ischemia, significant increases in the HSP-FRET ratio were detected (Fig. 2A), consistent with the presence of oxidative stress. The HSP-FRET ratio increased progressively during the first 15 min of ischemia, reaching a maximal change by that time. The increase in the HSP-FRET ratio observed during ischemia was comparable with the increase seen during treatment with 100 μM H2O2, which likely reflected complete oxidation of the sensor. At the end of ischemia, reperfusion with normoxic medium was associated with a marked loss of fluorescence intensity for both CFP and YFP within the first 10 min of reperfusion (data not shown). This loss of fluorescence intensity was not observed in cells treated with antioxidants, suggesting that it reflected hyperoxidation of the sensor protein during the reperfusion oxidant burst.

The conditions of ischemia include hypercapnia, induced by equilibration with 20% CO2 that readily enters the cells by diffusion. This creates intracellular acidosis (pH ~ 6.7), which alone could have altered the structure of HSP-FRET, leading to changes in proximity between YFP and CFP, or altered the fluorescent properties of CFP and/or YFP directly. In either case, this could have influenced the change in the fluorescence ratio that was observed during ischemia. To determine whether these changes in pH could have influenced the response measured during ischemia, control studies were conducted using the same acidic perfusate used for ischemia, except that O2 levels were not decreased (21% O2 and 20% CO2, mock ischemia). Mock ischemia produced a small shift in the fluorescence ratio, but this was significantly less than that observed during hypoxic ischemia. YFP is known to exhibit pH-dependent fluorescent behavior (36), which may have contributed to this response. To test whether the pH sensitivity of YFP contributed to the ischemic response, the YFP mutant citrine, which exhibits a lesser sensitivity to pH compared with YFP, was subcloned into the HSP-FRET sensor, replacing YFP (36). Significant increases in the citrine HSP-FRET ratio were still observed during ischemia, indicating that the pH sensitivity of YFP was not responsible (data not shown).

To determine the extent to which ROS were responsible for the changes in the fluorescence ratio during ischemia, experiments were repeated in the presence of the chemical antioxidant N-acetylcysteine (500 μM). Antioxidant administration was initiated during the base line and was maintained for the remainder of the experiment. When cardiomyocytes expressing HSP-FRET were perfused with medium containing N-acetylcysteine, the ischemia-induced changes in the fluorescence ratio were abrogated (Fig. 2B). This compound acts by increasing the reduction state of thiols in the cell, suggesting that ROS production during ischemia was responsible for the changes in the fluorescence ratio.

H2O2 can be generated during ischemia by the dismutation of superoxide by SOD in the cytosol or mitochondria. To determine the contribution of H2O2 to the observed changes in the HSP-FRET fluorescence ratio, cardiomyocytes were transduced with an adenovirus expressing catalase (32), which degrades H2O2. The dose of adenovirus was determined from Western blot studies demonstrating a dose-dependent increase in catalase expression with increasing concentrations of adenovirus (Fig. 2D), and increases in catalase expression throughout the cytosol were confirmed by confocal immunofluorescence microscopy (Fig. 2C). Compared with control cells infected with an adenovirus expressing LacZ, cells overexpressing catalase demonstrated a significant attenuation in HSP-FRET oxidation during ischemia (Fig. 2F). Because the HSP-FRET sensor was expressed throughout the cytosol, these results indicate that significant oxidant stress arising from H2O2 occurs in the cytosol during ischemia.

The source of cytosolic oxidant stress during ischemia is not known. Mitochondria represent a potential source of ROS generation through their ability to generate superoxide via univalent electron transfer to O2. Superoxide generated from the mitochondrial electron transport chain can potentially be released into the matrix compartment (37), where Mn-SOD resides, or to the cytosol via the intermembrane space, where Cu,Zn-SOD can be found (38). To determine the role of H2O2 production in the matrix on the magnitude of oxidant stress in the cytosol during ischemia, catalase was overexpressed in the matrix using an adenovirus containing the mitochondrial targeting sequence for Mn-SOD appended to the catalase cDNA (Fig. 2E) (32). Dose-dependent expression was determined by immunoblotting of whole cell protein lysates (Fig. 2D). In cardiomyocytes overexpressing mitochondrial catalase and cytosolic HSP-FRET, there was a significant decrease in oxidant stress detected during ischemia by ANOVA (p = 0.0013), although only sporadic differences were detected by post hoc analysis during ischemia (Fig. 2G). These results indicate that relatively little of the cytosolic oxidant stress appears to originate from the matrix compartment during ischemia.

Superoxide itself can act as an oxidant and could be involved in the oxidant response observed with HSP-FRET. To determine the extent to which superoxide contributes to the oxidant stress detected during ischemia, Mn-SOD overexpression in the mitochondrial matrix was achieved using a recombinant adenovirus (33). Compared with cells infected with a LacZ control adenovirus (Fig. 3A), cells transduced with an Mn-SOD adenovirus (Fig. 3B) demonstrated dose-dependent expression as detected by immunoblotting of whole cell protein lysates (Fig. 3E). Cells overexpressing Mn-SOD tended to show an increase in HSP-FRET oxidation during ischemia compared with control cells infected with a LacZ virus, but this was not statistically significant (p > 0.05 by ANOVA) (Fig. 3F). To
FIGURE 2. Oxidant stress during ischemia as assessed with HSP-FRET in cardiomyocytes. A, HSP-FRET responses to ischemia (0% O2 and 20% CO2; n = 8) or mock ischemia (21% O2 and 20% CO2; n = 10) for 30 min. B, effect of N-acetylcysteine (NAC; 500 μM; n = 7) on HSP-FRET response to ischemia. C, laser scanning confocal image showing overexpression of catalase. D, Western blot showing dose-dependent increases in expression of catalase and mitochondrion (Mito)-targeted catalase expression in cardiomyocytes. pfu, plaque-forming units. E, laser scanning confocal image showing cellular localization of mitochondrion-targeted catalase. F, HSP-FRET response to ischemia in control cells transduced with a LacZ adenovirus (n = 6) in comparison with cells transduced with an adenovirus encoding human catalase (n = 10). G, HSP-FRET response to ischemia in control cells transduced with a LacZ adenovirus (n = 5) in comparison with cells transduced with an adenovirus encoding mitochondrion-targeted catalase (n = 7). All values are the means ± S.E. ANOVA tests revealed significant between-group differences for A (p < 0.00001), B (p < 0.0001), C (p < 0.00001), and D (p = 0.0013). Post hoc differences are indicated (*, p < 0.05).
Oxidant Stress during Ischemia in Cardiomyocytes

FIGURE 3. Effects of SOD on HSP-FRET responses to ischemia in cardiomyocytes. A, laser scanning confocal image of cardiomyocytes infected with an adenovirus expressing LacZ and immunostained with antibodies against human Mn-SOD. B, laser scanning confocal image of cardiomyocytes infected with an adenovirus expressing Mn-SOD and immunostained with antibodies against human Mn-SOD. C, laser scanning confocal image of cardiomyocytes infected with an adenovirus expressing LacZ and immunostained with antibodies against human Cu,Zn-SOD. D, laser scanning confocal image of cardiomyocytes infected with an adenovirus expressing Cu,Zn-SOD and immunostained with antibodies against human Cu,Zn-SOD. E, Western blots showing dose-dependent increases in Mn-SOD and Cu,Zn-SOD in cardiomyocytes transduced with the corresponding adenoviruses. pfu, plaque-forming units. F, HSP-FRET responses to ischemia in control cells transduced with the LacZ adenovirus (n = 5), the Mn-SOD adenovirus (n = 5), or the Cu,Zn-SOD adenovirus (n = 6). No significant differences were detected by ANOVA.

determine whether superoxide in the cytosol is required for HSP-FRET oxidation, Cu,Zn-SOD overexpression was achieved using a recombinant adenovirus (Fig. 3, C and D), and dose-de-
rescence intensity within the first few minutes, similar to the response observed with HSP-FRET studies. Collectively, these results indicate that HSP-FRET is appropriate for quantifying ischemic oxidant stress, but is unsuitable for assessing the reperfusion oxidant burst because it is already extensively oxidized by the end of ischemia.

To provide another independent assessment of cellular oxidant stress during ischemia, reduced and oxidized glutathione (GSH/GSSG) levels were measured in cardiomyocytes subjected to simulated ischemic conditions using a glove box (Coy Instruments) maintained at 37 °C (1% O2 and 20% CO2). Using an air lock, cells were transferred into the box, where the medium was immediately replaced with ischemia medium previously equilibrated overnight in the box. After 60 min, some cells were disrupted for analysis within the chamber prior to reoxygenation to provide a measurement of GSH/GSSG levels at the end of ischemia but prior to reperfusion. In other experiments, cells were removed and transferred to a cell culture incubator (5% CO2 in room air) for 15 min prior to disruption to simulate conditions of reperfusion. As a positive control, other cells were subjected to exogenous H2O2 (50 μM for 15 min in serum-free medium) under normoxic conditions. Compared with normoxic controls, cells subjected to 60 min of ischemia without reperfusion exhibited a significant decrease in GSH/GSSG levels (Fig. 6). Reoxygenation tended to produce an even greater depletion of GSH/GSSG, but this was not different from non-reoxygenated cells (p < 0.05). Similar decreases in GSH/GSSG levels were achieved in normoxic cells subjected to exogenous hydrogen peroxide. These findings support the concept that significant oxidant stress occurs during ischemic conditions prior to reoxygenation.

To determine the significance of oxidant stress during ischemia for cell death during ischemia-reperfusion, cardiomyocytes were subjected to simulated ischemia and reperfusion while antioxidants were administered only during the baseline and ischemia. Cell death was assessed after 1, 2, and 3 h of reperfusion, which was carried out in the absence of antioxidants. Significant cell death was observed in control cells during the reperfusion period (Fig. 7). However, paired experiments using cells from the same isolation batches treated with N-(2-mercaptobutyryl)glycine or N-acetylcysteine during the baseline and ischemia demonstrated significantly less cell death.
Generation of ROS during ischemia would seem paradoxical, as O₂ levels are decreased. During regional ischemia in the heart, cellular oxygen levels decrease, but complete tissue anoxia is prevented by the diffusion of O₂ from surrounding tissue regions that remain perfused. To assess the levels of oxygenation in our simulated ischemia model, PO₂ within the flow-through chamber was measured using a porphyrin probe-based phosphorescence quenching method (Oxyspot, Medical Systems Inc.). After initiation of ischemic conditions, a rapid decrease in PO₂ was detected in the flow-through chamber (Fig. 8A). A stable level of hypoxia was achieved within 5 min, reaching a minimal value of 5–7 mm Hg even though the perfusate equilibration column was bubbled with a gas mixture containing 0% oxygen. This residual oxygen in the flow-through chamber was presumably due to the diffusion of O₂ through plastic junctions used to connect the chamber to the column. To achieve anoxic conditions, an enzymatic oxygen scavenger (EC-Oxyrase) was added to the ischemic mixture 5 min prior to the start of ischemia. Anoxic ischemia was associated with a more rapid decrease at the start of ischemia and a greater sustained decrease throughout the ischemic challenge. B, HSP-FRET assessment of oxidant stress in cardiomyocytes during the base line and simulated ischemia. Ischemic cells were subjected to the standard simulated ischemia for 30 min (n = 6). In anoxic ischemia, the oxygen scavenger EC-Oxyrase was added to the ischemic mixture 5 min prior to the start of ischemia (n = 5). Sodium lactate (1 mM), the substrate for EC-Oxyrase activity, was added to the ischemic perfusate in both groups. *, p < 0.05 compared with controls.
Oxidant Stress during Ischemia in Cardiomyocytes

Although the source of these oxidants is not fully understood, multiple therapeutic strategies that attenuate the burst, including pre- and post-conditioning, chemical antioxidants, and therapeutic hypothermia, are highly protective. These observations strongly suggest that the ROS burst at reperfusion is both necessary and sufficient to induce cell death after ischemia.

The results of this study show that a lesser degree of oxidant stress begins during ischemia prior to reoxygenation. The magnitude of this oxidant stress is sufficient to decrease the cellular GSH/GSSG ratio and to cause virtually complete oxidation of two redox-sensitive protein sensors, HSP-FRET and roGFP, but cell death does not increase during the ischemia, indicating that the immediate effects of this oxidant stress are not lethal. Indeed, in a previous study, we found that cell death increased minimally prior to reperfusion even when the ischemia was extended to 3 h (39). Hence, we conclude that the oxidant stress during simulated ischemia is not by itself sufficient to cause cell death.

However, two lines of evidence indicate that ROS production during ischemia is important for regulating cell death during reperfusion. First, chemical antioxidants administered only during the base line and ischemia abrogated the ischemic oxidant stress detected by HSP-FRET and significantly decreased cell death during reperfusion. Second, when ROS production during ischemia was prevented by removal of residual O2 with an extracellular scavenger, oxidation of the HSP-FRET sensor was abrogated, and cell death during reperfusion was virtually abolished. These findings indicate that oxidant stress during ischemia is required for oxidant-mediated cell death during reperfusion. It is conceivable that ischemic oxidant stress could prime the cell for reperfusion oxidant stress by eroding cellular antioxidant defenses, thereby leaving the cell more vulnerable to oxidant generation at reperfusion. Alternatively, ischemic ROS could trigger events that enhance the availability of substrates necessary for the reoxygenation burst, effectively priming the cell for ROS generation at the start of reperfusion. In either case, ROS during ischemia appears to be necessary for promoting the reoxygenation burst, but not sufficient by itself to induce cell death. This conclusion is consistent with previous observations that therapeutic interventions applied at reperfusion can still protect the cell, as the reperfusion ROS burst is the mediator of cell death. It is also consistent with the observation that protective strategies applied before ischemia can be protective. Indeed, it is conceivable that preconditioning strategies confer protection by attenuating ROS production during ischemia, thereby lessening the death at reperfusion by preventing the oxidant burst that mediates cell death.

Measurement of ROS during Ischemia—Experimental detection of ROS production during ischemia has been confounded by the lack of suitable tools for assessing intracellular oxidant stress. Previous studies have utilized spin trap probes to assess ROS levels in perfusate in the intact heart (5, 13). However, the absence of perfusion during ischemia precludes the separate assessment of oxidant stress during the ischemic and reperfusion periods. A previous study of simulated ischemia in cultured cardiomyocytes relied on oxidant-sensitive fluorescent probes such as dichlorofluorescein, which are subject to nonspecific oxidation and which are pH-sensitive (40). Despite

### DISCUSSION

**Ischemic ROS Predispose to Reperfusion Injury**—Numerous studies have shown that a burst of oxidant stress occurs during the first few minutes after reperfusion of ischemic myocardium.

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**FIGURE 9.** A, cardiomyocyte morphology during the baseline, simulated ischemia, and reperfusion. Ischemic cells were subjected to the standard ischemia challenge for 30 min (n = 4). In anoxic ischemia, the oxygen scavenger EC-Oxyrase was added to the ischemic mixture 5 min prior to the start of ischemia (n = 4). Sodium lactate (1 mM), the substrate for EC-Oxyrase activity, was added to the ischemic perfusate in both groups. B, cell death in simulated ischemia in comparison with anoxic ischemia. *, p < 0.05 compared with the control group.
technical limitations, previous studies have suggested that ROS production is increased during ischemia (41, 42). To further clarify this response, in this study, we utilized a novel fluorescent FRET sensor to detect changes in thiol redox status in cells subjected to simulated ischemia and reperfusion. Significant oxidant stress was detected using this sensor during the ischemic insult prior to reperfusion.

Some variability in the extent of oxidation during ischemia was observed among different experimental groups. It is likely that this variation reflects differences in the batches of isolated cells or other systematic variations in the behavior of these primary cells. However, all experiments were carried out in treated cells and in contemporary controls to minimize the contribution of such variation to our experimental results.

HSP-FRET is regulated by cysteine thiols, so it provides a measure of protein oxidation, which is an important target of oxidative stress in the cell. These results are corroborated by independent measurements of GSH/GSSG levels in cardiomyocytes during ischemia obtained using a glove box, which permits analysis of cellular glutathione oxidation during simulated ischemia prior to reoxygenation. They are also supported by assessments of oxidant stress obtained using roGFP, a thiol-based redox sensor that permits calibration of the extent of oxidation at the base line and during ischemia. Neither HSP-FRET nor roGFP is suitable for assessing reperfusion oxidant stress after ischemia because the oxidation of both sensors was virtually complete before reperfusion began. The magnitude of the oxidant stress during ischemia is significantly less than the burst seen at reperfusion, so a sensor capable of quantifying the latter may lack the sensitivity to detect the former.

Our findings do not contradict previous studies showing that a burst of oxidant production also occurs after the start of reperfusion (12, 43). Severe oxidant stress likely occurs at reperfusion because many of the electron carriers associated with intermediary metabolism are highly reduced, which would facilitate superoxide generation as O$_2^-$ returns to the cell. The HSP-FRET ratio decreased at the start of reperfusion because of a relative increase in YFP fluorescence and decrease in CFP fluorescence. However, the fluorescence intensity of both fluorophores also decreased markedly at this point. This suggests that the events at reperfusion may have included a further oxidation of the HSP-FRET sensor and/or a loss of plasma membrane permeability, leading to escape of the fluorophore from the cells. The magnitude of the reperfusion-induced decrease in the ratio was attenuated in cells protected by antioxidants or anoxic ischemia, two conditions that would have lessened the overall oxidant stress. We therefore speculate that reperfusion is associated with a marked burst of oxidant production, which is responsible for inducing further oxidation of the HSP-FRET protein, leading to loss of fluorescence and the associated loss of cell viability. Disruption of HSP-FRET function by excessive oxidant stress was also detected in experiments in which exogenous H$_2$O$_2$ was added at 300 $\mu$M (Fig. 1D) or higher concentrations (data not shown).

**Paradox of Increased ROS Levels in Ischemia**—The idea that ROS production increases during ischemia is counterintuitive given that O$_2$ levels are decreased during ischemia. However, the rate of superoxide generation is affected both by the level of O$_2$ and by the availability of reduced flavins and quinones capable of acting as a source for univalent electron transfer. The tendency of reduced flavins to increase superoxide production at lower oxygen concentrations was originally described by Misra and Fridovich (44). During ischemia, mitochondrial electron transport slows, augmenting the reduction state of electron carriers. This favors superoxide generation as long as some oxygen is still available.

The overexpression of catalase in the cells significantly decreased the oxidation of HSP-FRET in cardiomyocytes, indicating that H$_2$O$_2$ is a significant source of cytosolic oxidant stress during ischemia. However, overexpression of catalase in the mitochondrial matrix produced a smaller attenuation of the oxidation of HSP-FRET in the cytosol. This indicates that the escape of mitochondrial matrix-generated H$_2$O$_2$ to the cytosol represents a relatively minor source of ischemic oxidant stress in these cells. However, because ROS can also be released from the outer surface of the mitochondrial membrane (37), our results do not exclude the possibility that some of the oxidant stress may have originated from that organelle.

**Responsiveness of the Redox-sensitive Sensors**—Oxidant stress during ischemia likely begins with the generation of superoxide, which is rapidly dismutated to hydrogen peroxide by SOD. If HSP-FRET were specifically oxidized by superoxide, then overexpression of SOD would have attenuated the oxidant signal by shortening the lifetime of that radical. However, neither Cu,Zn-SOD nor Mn-SOD overexpression decreased the oxidation of HSP-FRET during ischemia. In fact, SOD tended to increase HSP-FRET oxidation, which is consistent with the observed sensitivity of the probe to H$_2$O$_2$. These observations do not refute the conclusion that superoxide is the initial source of ROS during ischemia, but they do indicate that HSP-FRET is not able to detect the oxidant stress until the superoxide is converted to peroxide. Separate studies in which exogenous NO was applied to cells indicated that HSP-FRET fails to respond, suggesting that the HSP-FRET responses during ischemia cannot be due to interaction with NO.

Preliminary studies with HSP-FRET indicated that reduction of the protein and refolding after oxidation are slow processes. By contrast, the roGFP sensor is capable of undergoing reversible changes in the redox state, making it possible to detect increases and/or decreases in oxidative stress and permitting calibration of the oxidation state in live cells. Despite these differences, both sensors yielded qualitatively similar responses to ischemia and revealed that cytosolic oxidant stress occurs during ischemia.

**Relevance to the Intact Heart**—Studies in the intact heart support the conclusion that oxidant stress begins during ischemia. In isolated perfused hearts, Lesnfsky et al. (45) found that mitochondrial cardiolipin levels decreased significantly during ischemia, leading to the inactivation of mitochondrial cytochrome oxidase, which requires cardiolipin for structural integrity (46). Another study documented protein oxidation during ischemia-reperfusion (47), but did not clarify whether reperfusion injury depends on ischemia-induced oxidant stress. Our findings underscore the importance of ischemic oxidant stress in contributing to cell dysfunction and death in ischemia-reperfusion injury. However, caution should be exercised when...
extrapolating the present findings into the intact heart. Future studies will be needed to determine whether the tissue levels of residual oxygen during in vivo ischemia are sufficient to drive the events we observed in our simulated ischemia-reperfusion model.

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Oxidant Stress during Ischemia in Cardiomyocytes