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Forum Review

Role of Oxygen in Postischemic Myocardial Injury

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ABSTRACT

Myocardial function is dependent on a constant supply of oxygen from the coronary circulation. A reduction of oxygen supply due to coronary obstruction results in myocardial ischemia, which leads to cardiac dysfunction. Reperfusion of the ischemic myocardium is required for tissue survival. Thrombolytic therapy, coronary artery bypass surgery and coronary angioplasty are some of the treatments available for the restoration of blood flow to the ischemic myocardium. However, the restoration of blood flow may also lead to reperfusion injury, resulting in myocyte death. Thus, any imbalance between oxygen supply and metabolic demand leads to functional, metabolic, morphologic, and electrophysiologic alterations, causing cell death. Myocardial ischemia reperfusion (IR) injury is a multifactorial process that is mediated by oxygen free radicals, neutrophil activation and infiltration, calcium overload, and apoptosis. Controlled reperfusion of the ischemic myocardium has been advocated to prevent the IR injury. Studies have shown that reperfusion injury and postischemic cardiac function are related to the quantity and delivery of oxygen during reperfusion. Substantial evidence suggests that controlled reoxygenation may ameliorate postischemic organ dysfunction. In this review, we discuss the role of oxygenation during reperfusion and subsequent biochemical and pathologic alterations in reperfused myocardium and recovery of heart function. *Antioxid. Redox Signal.* 9, 1193–1206.

INTRODUCTION

MYOCARDIAL ISCHEMIA caused by obstruction to coronary blood flow is one of the most important factors inducing heart failure. A severe and sustained reduction in blood flow to the myocardium leads to ischemia with loss of ATP, loss of ion homeostasis, acidosis, depletion of glutathione, defective ATP synthesis, structural disorganization, and swelling of myocytes (96). Ischemia inactivates oxidative phosphorylation, leading to loss of adenine nucleotides and cytochrome *c*; accumulation of free phosphate, fatty acids, and lactic acid; increased cellular calcium; and a decrease in cellular pH (34). During the period of no-flow ischemia, cells are metabolically compromised by hypoxic and hypoglycemic conditions that cause cellular dysfunction, eventually leading to cell death. Myocardial ischemia can develop as a consequence of either an increase in oxygen demand or a shortage of oxygen supply. Reperfusion to restore blood supply may require interventions such as thrombolysis, coronary artery bypass surgery (CABG), and percutaneous transluminal coronary angioplasty (PTCA). Reperfusion may reverse the ischemic process and lead to almost complete restoration of oxygen supply and high-energy phosphate stores (117). However, substantial data from animal and human studies suggest that reperfusion of ischemic areas, in particular readmission of oxygen, may exaggerate or cause additional injuries not present at the end of ischemia (10, 82, 97, 98). Thus, the injury that is observed as a result of reperfusion is commonly referred to as ischemia–reperfusion (IR) injury.

Several mechanisms have been proposed to explain the IR injury. These include a robust release of oxygen free radicals, calcium overload, opening of mitochondrial permeability tran-

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sition pores (mPTPs), neutrophil-mediated myocardial and endothelial injury, depletion of high-energy phosphate stores, and progressive decline in microvascular flow to the reperfused myocardium (50, 52, 154). Reactive oxygen-derived free radicals are known to play important roles in the pathogenesis of IR injury (42, 167). Numerous studies have identified the role of oxidative damage mediated by the activation of xanthine oxidase with subsequent formation of reactive oxygen species (ROS), including superoxide ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), and hydroxyl ($^{\cdot}OH$) radicals. Other prominent mechanisms responsible for IR injury include the formation of reactive nitrogen species (RNS) including nitric oxide (NO) and peroxynitrite ($OONO^-$), lipid peroxidation of myocardial membranes, the ROS interaction with iron, a decrease in glutathione (GSH) redox state, depletion of high-energy phosphates including ATP, and altered calcium homeostasis (56). Reintroduction of oxygen to the ischemic tissue at the onset of reperfusion induces a burst of potent free radicals such as O_2^-, $^{\cdot}OH$, NO, and $ONOO^-$ within seconds to minutes. This phenomenon has been demonstrated in experimental settings as well as in humans with acute myocardial infarction (MI) undergoing thrombolytic therapy (9) or PTCA (122). A similar phenomenon has been reported in patients undergoing open heart surgery (71). Studies in isolated hearts suggest that ROS production peaks within the first few minutes of reperfusion (170). The oxygen paradox describes the contradictory need to deliver oxygen to ischemic tissue and the resultant unwanted reduction of oxygen to form ROS that are involved in macromolecule oxidation, membrane dysfunction, and cell signaling for mitochondrially triggered apoptosis. The burst of ROS at the onset of reperfusion increases Ca^{2+} influx through L-type Ca^{2+} channels and leads to cytosolic Ca^{2+} accumulation (8, 91). In addition, ROS have been reported to increase the activity of the Na^+/K^+ exchanger and thereby lead to an increase in cytosolic Ca^{2+} via reversal of Na^+/Ca^{2+} antiporter (126). The accumulation of Ca^{2+} in both the cytosolic and mitochondrial compartments by multiple pathways has been linked to the pathogenesis of necrosis (145). Increases in Ca^{2+} and ROS generation have been shown to be the primary stimuli for opening the mPTP (52, 54). Opening of mPTP may play a role in the transition from reversible to irreversible injury during reperfusion (51, 65, 83). The actions of neutrophils have been particularly emphasized in the pathophysiology or postischemic injury (82, 98). Neutrophils accumulate in the microcirculation, release inflammatory mediators, and contribute to microvascular obstruction and the "no-reflow" phenomenon in the reperfused myocardium (67).

Several strategies have been used in which reperfusion injury can be significantly reduced by modifying the conditions of reperfusion, as well as the composition of the initial perfusate (21, 22). The prophylactic infusion of HBOC-201 (hemoglobin-based oxygen carriers) has been shown to reduce myocardial infarct size in the rat model (24). Controlled oxygen delivery during reperfusion by restricting blood flow has been shown to be beneficial in protecting against IR injury (114). It has been observed that postischemic cardiac function can be significantly improved by restricting coronary flow during reperfusion (107, 114). Studies have shown that the amount of oxygen delivered during reperfusion is an important determinant of postischemic cardiac dysfunction and IR injury. (114). Alterations in the hemodynamic conditions (*i.e.*, blood flow and intracoronary perfusion pressure) during early reperfusion have been shown to attenuate IR injury (62, 107, 121). The restriction of both coronary flow and oxygen delivery during reperfusion improves postischemic cardiac function (94). Modifying the blood and intracoronary perfusion pressure during the early reperfusion period has also been reported to reduce the myocardial reperfusion injury (62, 107, 114, 131). Conversely, hyperbaric oxygen has been shown to reduce ischemic myocardial injury in experimental animal models; it had mixed results in clinical studies (27, 67, 140, 146). Hence, modifications in the early phase of reperfusion conditions may provide a potential opportunity for ameliorating the IR injury to the heart. The objective of this article is to discuss the significance of oxygen delivery during reperfusion of the ischemic myocardium and possible pathologic and biochemical alterations in the reperfused heart.

ROLE OF OXYGEN IN MYOCARDIAL ISCHEMIA–REPERFUSION INJURY

Controlled oxygen delivery during reperfusion

Oxygen is required to meet the constant energy demands for heart contractility and also plays an important role in the regulation of heart function (106, 153). However, the reoxygenation of the ischemic myocardium may lead to injury and dysfunction. Controlled oxygen delivery during reperfusion has been advocated to prevent IR injury (107, 114). Gradually increasing the perfusion rate and intracoronary pressure during the early minutes of reperfusion has been associated with a reduction in necrosis and tissue edema (114, 131, 155). In pigs, reperfusion of ischemic myocardium after experimental coronary artery occlusion demonstrated that the extent of damage was significantly reduced in hearts with controlled reperfusion when compared with hearts with uncontrolled reperfusion (114). Studies have shown that restricted blood flow and low or hyperbaric oxygen treatment during reperfusion could attenuate IR injury.

Hyperbaric oxygen treatment

The concept of hyperbaric oxygen (HBO) treatment in the prevention of postischemic injury has been pursued for many years (142). Experimental studies have suggested that hyperoxemia provided by HBO may be beneficial in the treatment of reperfusion injury of the myocardium (70, 136, 142). Exposure to HBO before reperfusion was capable of protecting IR injury compared with normobaric oxygen treatment in rabbits. HBO pretreatment conditions the heart, by enhanced enzyme activity and genetic expression of catalase, thereby significantly reducing infarct size after coronary occlusion (70). HBO has been shown to induce the expression of a number of genes in tissues, including heme oxygenase, and to increase glutathione levels (53, 109). HBO delivery by different modes, such as blood-free oxygenated perfluorocarbon (75) or crystalloid so-

lution at a pressure of 3–10 MPa, has also been shown to reduce the infarct size (139). Johnson *et al.* (61) showed that treatment with HBO solution during reperfusion provided higher myocardial blood flow, higher tissue pO_2, lower myeloperoxidase (MPO) (a marker for neutrophil) levels, and attenuated IR injury (61). The proposed mechanisms of myocardial salvage by HBO include improvement of microvascular flow, inhibition of leukocyte adherence, reduction of edema, restoration of oxygen to mitochondria, and maintenance of oxidative metabolism (23). Through the use of HBO, the prevention of reperfusion injury has been successfully demonstrated in animal models, as well as in humans (27, 67).

Hypoxic oxygen delivery during reperfusion

Studies have shown that the extent of reperfusion injury can be reduced by modifying coronary blood flow during early period reperfusion (107, 121, 131). Isolated rat hearts reperfused with 47.5% oxygen-equilibrated perfusate showed better functional recovery when compared with 95% O_2 (94). In this study, an inverse correlation of oxygen consumption and recovery of heart function was found. Low oxygen consumption due to restricted oxygen delivery resulted in a significantly enhanced recovery of heart function in the ischemic reperfused hearts (94). Kaneda *et al*. (67) perfused hearts with different oxygen tensions in an isolated heart ischemia–reperfusion protocol, and found that hearts perfused with a partial pressure of oxygen (pO_2) = 500 ± 50 mm Hg showed significantly higher recovery of heart function when compared with pO_2 = 700 ± 50 mm Hg treatment (67). It was also observed that excessive hypoxia during reperfusion resulted in poor recovery from ischemic injury (57).

Ischemic preconditioning was introduced as a potent means of cardioprotection against IR injury, reducing the incidence of postischemic arrhythmias, enhancing the recovery of cardiac function, and reducing the infarct size (32, 88). Repetitive cycles of short ischemia during early reperfusion significantly reduced infarct size in a canine model (164). Ihnken and co-workers (58) demonstrated that the oxidative myocardial damage occurring during human hyperoxic cardiopulmonary bypass could be limited by reducing the oxygen tension to normoxic levels (58, 111). Subsequent studies have demonstrated that manipulation of this early reperfusion phase can reduce the downstream consequences of IR injury (73, 164). Serviddio *et al.* (135) showed that hearts exposed to 3 min of hypoxia with pO_2 of 150 mm Hg (instead of 600 mm Hg) before reperfusion were able to attenuate the mitochondrial free radical generation and preserved GSH levels (135). In another study, lowering of oxygen tension at the beginning (first 3 min) of reperfusion from 600 to 150 mm Hg resulted in an attenuation of mitochondrial dysfunction induced by normoxic reperfusion of the heart (116). The IR-associated defect in complex I and complex III activity and enhanced production of H_2O_2 in mitochondria was attenuated by hypoxic reperfusion (116). However, in our own study (5), we observed further deterioration of myocardial function when hearts were reperfused with low oxygen (2% O_2 for 5 min of reperfusion) for the first 5 min. These findings clearly suggest that the timing and concentration of oxygen delivery during reperfusion is crucial for the prevention of IR injury.

Myocardial pO_2 during IR

Many methods have been used to measure local myocardial tissue oxygen tension. These methods include Clarke electrode, NADH fluorescence, phosphorescence-quenching method, myoglobin saturation, ^{1}H-NMR, and EPR oximetry (12, 30, 147, 171). EPR oximetry has several significant advantages for *ex vivo* as well as *in vivo* applications. EPR oximetry uses oxygen-sensitive spin probes whose spectral properties can be measured by using EPR spectroscopy. During the last two decades, several new probes have become available for EPR oximetry (45, 59, 110). The most commonly used EPR oximetry probes are lithium phthalocyanine (LiPc) and lithium octa-*n*-butoxy-naphthalocyanine (LiNc-BuO). LiPc or LiNc-BuO probes yield a single sharp EPR line, the width of which is highly sensitive to oxygen tension. Decreased oxygen tension results in a sharpening of the EPR spectrum (44, 45, 59). The strong EPR signal permits the use of small amounts, usually a few micrograms, of the probe implanted in tissue to measure the pO_2 noninvasively for several months. In a typical *ex vivo* setup, the isolated perfused hearts are placed in the active volume of a reentrant or surface-coil resonator of a L-band (1.2 GHz) EPR spectrometer. This gives a continuous on-line measurement of localized myocardial oxygen tension throughout ischemia and reperfusion. This technique allows measurement of the absolute tissue pO_2 values ranging from very low oxygen levels in the millitorr range (1 Torr = 1 mm Hg) to normal physiologic levels (>100 mm Hg in the crystalloid perfused hearts). Friedman *et al.* (44) used EPR oximetry in isolated perfused rat hearts and showed recovery from repetitive ischemia with significantly increased myocardial pO_2. The observed increase in myocardial pO_2 could be due to decreased oxygen consumption or increased local oxygen delivery or both.

In our study (5), the mean myocardial tissue oxygen tension in the isolated perfused preischemic heart was mean 217 ± 5 mm Hg. With a similar technique, the baseline myocardial pO_2 of 198 ± 12 mm Hg was reported by Friedman *et al*. (45). With the onset of global ischemia, tissue oxygen tension rapidly declined to <30 mm Hg within 1 min of ischemia ($p < 0.001$ *vs.* baseline for all groups). At the end of the 20-min global ischemia, the tissue oxygen tension was 7.10 ± 2.45 mm Hg. With the onset of reperfusion, tissue oxygen tension was significantly higher in the 95% versus 2% O_2 reperfusion groups (Fig. 1). At 5 min reperfusion, perfusate was changed to 95% O_2–saturated perfusate. During the reperfusion period, the tissue pO_2 gradually increased, although it remained significantly depressed when compared with preischemic baseline values. The decrease in oxygenation in hearts reperfused with 2% O_2 compared with 95% O_2 paralleled the extent of myocardial impairment assessed from the recovered hemodynamic and contractile functions. In an *in vivo* mouse model of MI, Zhao *et al.* (163) showed that the myocardial pO_2 measured by EPR oximetry was markedly increased after reperfusion. The pO_2 remained elevated for up to 48 h after IR and then returned to baseline values. The inhibition of myocardial oxygen consumption by the elevated NO during reperfusion was proposed as a possible mechanism of increased myocardial pO_2 during reperfusion. The decrease in oxygen consumption after IR was directly correlated with the decreased myocardial function (59).

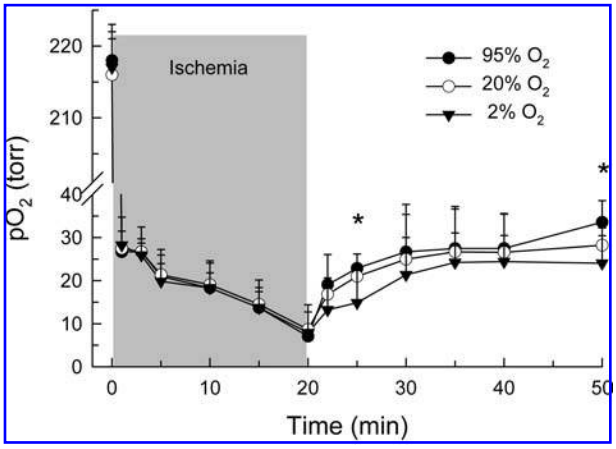

FIG. 1. Myocardial tissue oxygen tension during global ischemia and early reperfusion. The tissue oxygenation was measured by EPR spectroscopy by using an implanted oxygen-sensing probe. A rapid decline in myocardial tissue O_2 tension is noted with the onset of global ischemia. A significant depression in myocardial oxygen tension is observed during the first 5 min of reperfusion with 2% O_2 (*$p < 0.05$ 95% O_2 *vs.* 2% O_2, $n = 4$/group).

ROS in IR injury

During ischemia, a strong reductive pressure, with the reintroduction of oxygen, results in the burst of reactive oxygen species (ROS). This ROS burst is thought primarily to be responsible for the postischemic myocardial contractile dysfunction (13, 16, 17). This phenomenon of contractile dysfunction, often referred to as *stunning* (19), is seen after a number of clinical syndromes including both regional (acute MI) and global (cardiac arrest) myocardial ischemia (68, 99). The IR-induced ROS generation may occur from multiple sources and mechanisms, depending on the duration of preceding ischemia. Earlier studies have identified endothelial cells as a major source of ROS that can then react with iron to form hydroxyl radicals (169). These reactions can be inhibited by xanthine oxidase blockers (168). After prolonged periods of ischemia sufficient to result in necrosis, a major source of ROS appears to be neutrophils, which can be blocked with antineutrophil interventions, causing a reduction of infarct size (36). However, after shorter periods of ischemia, resulting in myocardial stunning but not necrosis, the major source of the ROS burst at reperfusion appears to be non–neutrophil mediated (14). Other sources of the ROS burst at reperfusion include NAD(P)H oxidases, mitochondria (particularly complexes I and III), cyclooxygenase/lipoxygenase, and cytochrome P450 (2, 3, 20, 74, 78). Because of abundance of mitochondria in cardiomyocytes, the mitochondrial electron-transport chain may also be an important subcellular source of ROS and contribute to myocardial IR injury (42). Mitochondria consume >90% of the oxygen used by the cells, of which 5–10% of oxygen is reduced *via* a univalent pathway in which oxygen free radicals, particularly superoxide radicals, are produced. The production of ROS can be greatly enhanced when mitochondrial respiration is stimulated under conditions of altered redox state, a condition that may occur in ischemic-reperfused hearts. The IR-associated alterations in complex I and complex III activity may also contribute toward H_2O_2 generation in mitochondria (116). Xanthine oxidase is a significant source of oxygen free radicals in the reperfused heart (150). Several reports describe the efficacy of antioxidants and free radical scavengers such as superoxide dismutase (SOD), catalase, melatonin, and vitamin E in minimizing IR injury (9). Overexpression of MnSOD, CuZnSOD, or glutathione peroxidase has been reported to protect the heart from IR injury, further supporting the involvement of oxidative stress in IR injury (28, 29). The inhibition of rac-dependent pathways have been shown to attenuate IR injury (72).

Reperfusion of the isolated rat heart with oxygenated buffer has been shown to generate free radicals, as detected by EPR spectroscopy (47, 170). In our study (5), we directly demonstrated the generation of ·OH and R· or RO· adducts of 5,5-dimethyl-1-pyrroline N-oxide (DMPO) in hearts reperfused with 95% O_2. A burst of free radical generation was observed during the early seconds of reperfusion, with peaking occurring within the first 30 s of reperfusion (Fig. 2). The reperfusion-induced ROS generation was markedly decreased by SOD, suggesting that ·OH and R· are generated *via* O_2-dependent Fenton chemistry. This study focused on the first few minutes of reperfusion and the implications of ROS formation on early return

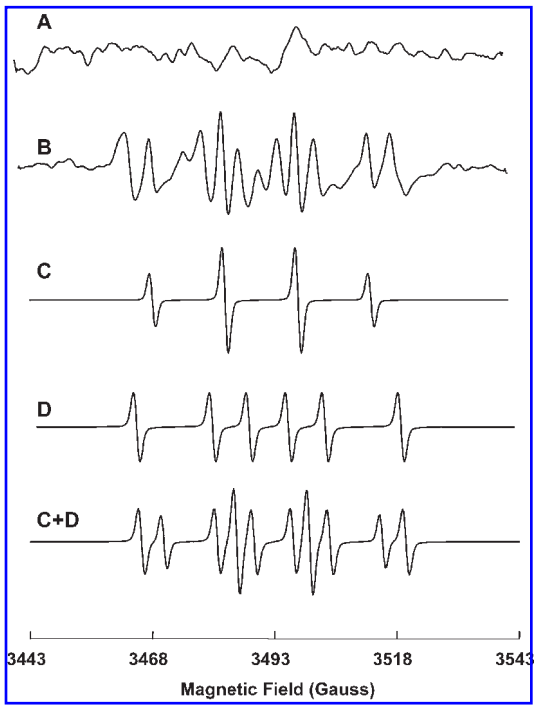

FIG. 2. EPR spectra of effluent of heart perfusate. (**A**) Preischemia. (**B**) Effluent collected 2 min after reperfusion with 40 m*M* DMPO, showing hydroxyl adduct and alkyl adduct. (**C**) Simulation of DMPO-OH adduct. (**D**) Simulation of DMPO-alkyl adduct. *E*, C + D simulation of both DMPO-OH adduct + stimulation of DMPO-alkyl adduct (compare with 4B). Microwave frequency, 9.786 GHz; microwave power, 10 mW; modulation amplitude, 1 G. Scan time, 30 s; number of scans, 10.

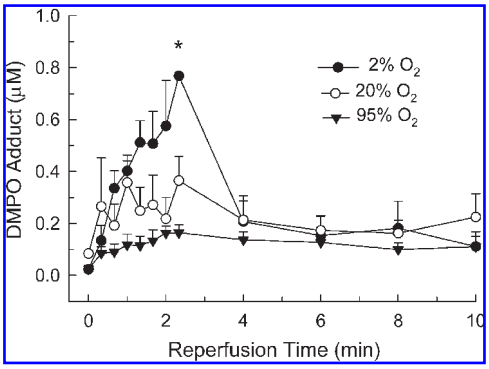

FIG. 3. Myocardial oxygen free radical formation at reperfusion with different O_2 concentrations. The free radicals were measured as DMPO adducts in the effluent by using spin-trapping EPR spectroscopy. The DMPO adduct peaked in the first 2 min in all groups and was significantly elevated in the most hypoxic reperfusion group (2% O_2) compared with the 20% and 95% O_2 reperfusion groups (*$p < 0.001$ *vs.* 95% O_2 and 20% O_2; $n = 4$/group).

of contractile function. Early reperfusion of the ischemic myocardium is a critical period for successful resuscitation in humans from cardiac arrest. Most of the current resuscitation failures from cardiac arrest occur within minutes. In humans, the early death after the initial successful reperfusion often occurs in the first minutes of reperfusion. This is likely the time period most affected by the initial ROS generation of reperfusion.

In hearts reperfused with 2% O_2 for the first 5 min, followed by 95% O_2, the oxygen free radical generation peaked in the first 2 min of reperfusion. Furthermore, the magnitude of oxygen radical generation was significantly higher in the 2% O_2 group compared with 95% O_2 or 20% O_2 (Fig. 3). In the same hearts, aconitase activity, which is inhibited by oxidative stress, was significantly depressed after 30-min reperfusion in the 2% O_2 group compared with the 95% O_2 group. In another study from our group using an ROS-detecting fluorescent probe, we observed that hearts reperfused with 20% O_2 perfusate during the first 5 min of reperfusion had significant attenuation of the intracellular ROS burst but increased extracellular ROS formation (Fig. 4). Recovery of left-ventricular (LV) function, measured as dP/dt_{max}, during the 30-min reperfusion period, was significantly depressed in the 2% O_2 group compared with the 95% group (Fig. 5). At the end of 30-min reperfusion, tissue ATP, phosphocreatine, and phosphorylation potential were all significantly reduced in the 2% and 20% O_2 groups when compared with 95% O_2. Hearts reperfused with low (2% O_2) oxygen for the first 5 min and then with 95% O_2 in the presence of ROS inhibitors, exhibited a significant reduction in the radical generation (Fig. 6A). In these groups of hearts, the recovery of dP/dt_{max} during reperfusion was significantly improved in hearts with low and high O_2 reperfusion treated with Tiron (Fig. 6B). An inverse correlation between the recovery of contractility (percentage recovery, dP/dt_{max}) and ROS (DMPO adduct) production early in reperfusion was noted across all groups ($R = -0.709$; $p < 0.05$). Results of our study clearly demonstrated the key role of tissue oxygen tension in mediating the ROS generation during reperfusion in the whole heart and the association of early ROS generation with initial depression of contractile function. We noted significantly higher ROS production in the more-hypoxic tissue, in contrast with higher myocardial tissue oxygen tension, which yielded lower ROS production. Modulating the ROS burst at reperfusion has functional significance, as indicated by the association of ROS production during early reperfusion with the return of myocardial contractile function in the early reperfusion period. In our study, an inverse relation was noted between radical production in the first 10 min, most of which occurred in the first 2–4 min, and the return of contractile function. These data confirmed our study hypothesis that tissue oxygen tension is a primary determinant of the initial ROS generation at reperfusion. By using EPR oximetry, we measured tissue oxygen tension in the heart rather than perfusate pO_2, to determine more reliably the oxygen available to the cells during the period of IR. We demonstrated the crucial role of tissue oxygen tension during early minutes of reperfusion when the burst of ROS production peaked.

Duranteau *et al.* (40) showed that ROS production during ischemia was greatest with the most hypoxic perfusion conditions. A notable difference was that the ROS measured during reperfusion was much higher than that during ischemia. Whereas the low-level ROS production during ischemia may

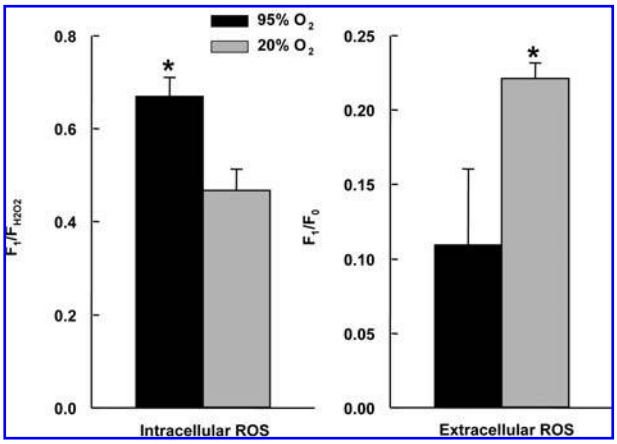

FIG. 4. Intracellular and extracellular ROS burst at reperfusion with 95% and 20% O_2. (*Left*) Hearts were loaded with dihydrofluorescein and reperfused with 95% or 20% O_2-saturated perfusate after 20 min of global ischemia. The ROS burst seen in the first minutes of reperfusion is significantly higher ($p < 0.001$) when compared with 95% O_2 compared with 20% O_2. The signal represents intracellular ROS generation and was normalized to the signal generated by a known bolus of H_2O_2 injected into the heart at the end of each experiment ($F1/FH_2O_2$). FH_2O_2, fluorescent signal from known concentrations of H_2O_2 injected into the heart at the end of the experiment. (*Right*) Hearts loaded with Amplex Red and horseradish peroxidase and reperfused with 95% or 20% O_2-saturated perfusate after 20 min of global ischemia ($n = 4$/group). The signal is expressed as $F1/F_0$ with F_0 defined as the fluorescent signal at baseline. This is a measure of extracellular ROS generation.

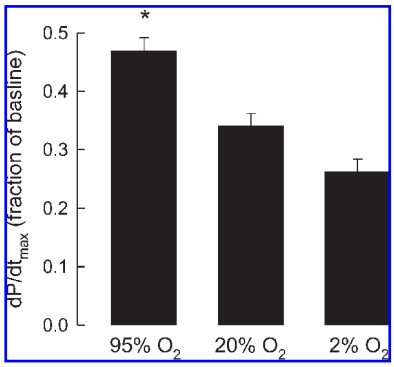

FIG. 5. Recovery of dP/dt_{max} during reperfusion with variable O_2 concentrations. The recovery of dP/dt_{max} is expressed as a fraction of preischemic baseline value. The recovery of dP/dt_{max} was significantly increased in the 95% O_2 group (*$p < 0.001$ *vs.* 20% and 2% O_2; $n = 6$/group).

have important cardioprotective signaling functions, the large ROS burst at the onset of reperfusion still may compromise the recovery of contractile function. Hence, the prevention of ROS generation during early reperfusion by controlling oxygen delivery may attenuate IR injury.

Nitric oxide in IR injury

Nitric oxide (NO), which is produced by a variety of mammalian cells, is an important mediator of both physiologic and pathologic vascular functions (84, 100). NO production is mediated by nitric oxide synthase (NOS). NO is a powerful vasodilator and may improve blood flow during reperfusion (39). NO can preserve ischemic blood flow and attenuate platelet aggregation and neutrophil–endothelial interactions after IR (4). Low concentrations of NO can increase the myocardial function, whereas high concentrations depress myocardial function by mediating inflammatory process after IR. Reperfusion after an ischemic period is associated with impaired bioavailability of NO, most likely due to enhanced inactivation of NO by superoxide and reduced production of NO. Earlier studies on NO and NOS inhibition in models of IR have yielded mixed results. Studies have shown that enhanced NO synthesis during IR (15, 172) and treatment with NOS inhibitors decrease the functional impairment of the heart after IR episodes (35, 105, 159). However, NO has also been shown to play a cardioprotective role in myocardial IR injury (63, 80, 115, 131, 138, 158). Supplementation with L-arginine (substrate for NO production by NOS) and NO donors during reperfusion has been shown to be cardioprotective in regional, as well as in global ischemic models (80, 138, 158).

Several possible mechanisms behind the cardioprotective effect of NO have been proposed. Studies have demonstrated that NO donors and L-arginine attenuate neutrophil accumulation in the reperfused myocardium area, by inhibiting the expression of adhesion molecules such as P-selectin, ICAM, and VCAM (64, 81). Because NO is a vasodilator, it is possible that improved blood flow contributes to the protective actions of NO. Also, NO is known to trigger and mediate ischemic preconditioning and to protect hearts against IR injury (15, 89). The other mechanism may include the inactivation of superoxide radicals. NO rapidly reacts with superoxide, which is formed in large amounts during IR. This reaction yields peroxynitrite, which is highly reactive and cytotoxic in high concentrations but also has been suggested to exert cardioprotective effects at low concentrations (123). Studies performed with isolated rat hearts have shown that L-arginine and several NO donors can attenuate postischemic reperfusion damage (60, 104, 148, 158). Conversely, NO and its reaction products have also been demonstrated to cause detrimental effects on the reperfused heart (118, 119). The treatment of hearts with NO donors such as SNAP or SIN-1 increased the formation of $ONOO^-$ and exacerbated the myocardial damage after IR (8, 162). NCX-4016 [2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester], an

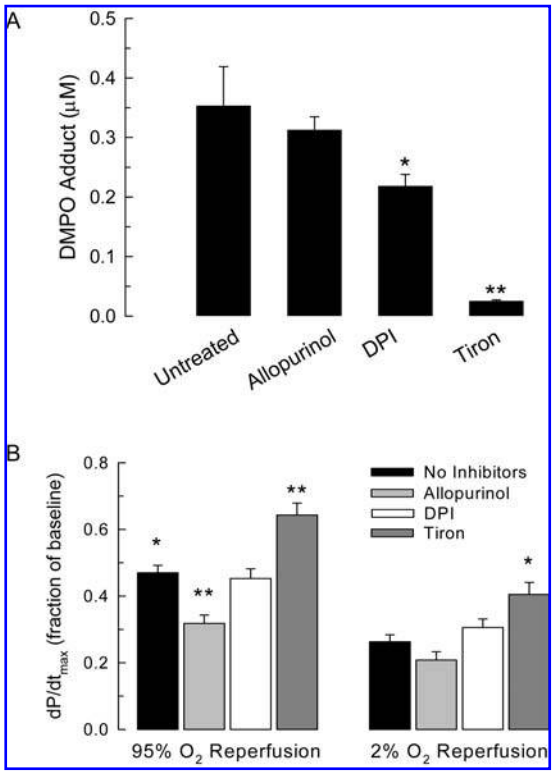

FIG. 6. (A) Effect of scavengers/inhibitors of oxygen free radical formation in hearts reperfused with low O_2. Hearts were perfused with allopurinol (1 m*M*), DPI (1 μM), or Tiron (10 m*M*) before ischemia and reperfused with 2% O_2 concentration. The free radicals were measured as DMPO adducts in the effluent by using spin-trapping EPR spectroscopy. The DMPO adduct concentration was significantly decreased in hearts treated with DPI (*$p < 0.01$) and Tiron (**$p < 0.001$) compared with that in untreated control hearts ($n = 4$/group). (**B**) Effect of scavengers/inhibitors of oxygen free radical formation on the recovery of dP/dt_{max} in hearts reperfused with low (2%) and high (95%) initial O_2. dP/dt_{max} is measured at the end of 30-min reperfusion and is expressed as a fraction of the preischemic baseline. (*$p < 0.001$ *vs.* untreated 2% O_2 group, $n = 6$/group; **$p < 0.05$ *vs.* untreated 95% O_2 group; $n = 6$/group).

NO-releasing aspirin derivative initially designed to overcome the side effects of NSAIDs on the gastric mucosa, provided greater cardiovascular protection than did the parent compound, aspirin (124, 125, 156). *In vivo* studies have shown that NCX-4016 is cardioprotective when used in rabbits subjected to coronary artery ligation (124). NCX-4016 is metabolized *in vitro* and *in vivo,* leading to the release of NO (18, 25, 26, 43). NCX-4016 has been reported to protect hearts from postischemic injury by increasing the bioavailability of NO (26, 124).

Peroxynitrite has recently been shown to cause deleterious effects in the heart after IR (86, 160). Peroxynitrite is formed mainly in the endothelium, myocytes, and neutrophils (11, 76, 101). Wang *et al.* (157) demonstrated that the interaction between NO and superoxide during the early phase of reperfusion forms peroxynitrite, an important determinant of postischemic myocardial function (157). It has been observed that peroxynitrite causes an irreversible inhibition of mitochondrial respiration through modification of the iron–sulfur centers of the electron-transport enzymes (120). In parallel with the increase in formation of superoxide, increased peroxynitrite formation was observed in the coronary effluent of hearts after IR, which was significantly attenuated by the antioxidant Tempol (Fig. 7). Peroxynitrite can nitrate and hydroxylate phenolic compounds, especially tyrosine residues, which in turn alter the activities of essential proteins and enzymes. Peroxynitrite has been reported to produce cellular damage by lipid peroxidation, nitration of tyrosine residues, oxidation of sulfhydryl groups and DNA-strand breakage in the heart, in addition to inducing depletion of antioxidants (11, 85, 86, 90).

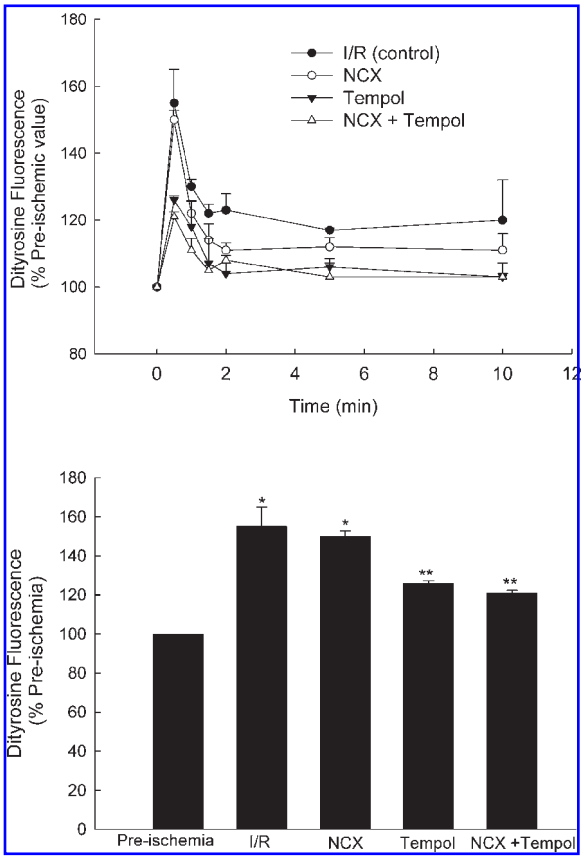

FIG. 7. The concentration of dityrosine (an indicator of peroxynitrite production) in coronary effluents from isolated perfused hearts subjected to IR was measured by using a microplate fluorimeter with excitation/emission filters of 320/410 nm. L-tyrosine (0.3 m*M*) was dissolved in 2 m*L* of 1N NaOH and added to the Krebs buffer saturated with a 95% O_2 and 5% CO_2 gas mixture. The measurement of dityrosine in the heart perfusate, formed by the reaction of peroxynitrate and tyrosine, is used as an estimate of peroxynitrite generation. *Upper,* time course of dityrosine formation in coronary effluent from hearts subjected to IR. *Lower*, Dityrosine formation at 1 min of reperfusion. Data are expressed as percentage of preischemic baseline, represented as mean ± SD ($n = 3$). *$p < 0.001$ *vs.* preischemia; **$p < 0.01$ *vs.* control (IR).

Apoptosis in IR injury

Apoptosis has been recognized as one of the important mechanisms of cell death during IR (48, 95, 133, 141, 165). Prolonged periods of myocardial ischemia are related to an increase in the rate of necrosis, whereas reperfusion leads to an enhancement in apoptosis (6, 37, 38). Reperfusion restores oxygen and also energy that is required for the completion of apoptosis and accelerates the apoptotic process (37, 38, 48). The IR-induced apoptosis is mediated by different signaling cascades that also involve the mitochondria-initiated pathway, which is mediated by free radicals and oxidative stress, resulting in the release of cytochrome *c* from mitochondria, activation of caspase-9, and downregulation of the antiapoptotic protein, Bcl-2 (49). The incidence and extent of apoptosis is dependent on the duration of ischemia and reperfusion (66). Mitochondrial dysfunction leading to leakage of cytochrome *c* and caspase activation after IR lends additional evidence in support of IR-related apoptosis (165). ROS-induced mitochondrial dysfunction after IR appears to be an important mechanism of apoptosis (152). This is further supported by the reported experimental evidence that ROS play a crucial role in apoptotic signaling (95, 112, 130) by downregulating the antioxidant gene Bcl-2, promoting increased DNA fragmentation (95) in the heart subjected to IR.

The signal-transduction pathway leading to IR-induced apoptosis may involve p38 MAP kinase, C-Jun-N-terminal kinase, PI3 kinase/Akt, the mitochondrial death pathway, or a combination of these (77). Other pathways involving p53, nuclear factor-κB, and Fas/fas ligand system have also been suggested (77).

Apoptosis in the myocardium ultimately leads to cardiomyopathy and dysfunction. Recently, several studies have demonstrated the involvement of Akt and mitogen-activated protein kinases (MAPKs) in mediating intracellular signal-transduction events associated with stress conditions including IR (108). Recent studies have shown that brief periods of ischemia may protect the heart against subsequent ischemic episodes and reperfusion injury, possibly by the activation of prosurvival kinases such as Akt and ERK1/2 (55, 102, 113). Conversely, activation of p38 MAPK during transient ischemia and reperfusion resulted in IR injury (92, 144). MAPKs (p38 MAPK, ERK1/2, and JNK) are shown to be activated in different cell systems and hearts subjected to IR (41, 92, 132, 143, 161). The activation of Akt- and MAPK-signaling cascades have been shown to modulate oxidant-mediated tissue injury (7, 137). Previous studies demonstrated that ERK1/2 is activated in the first few

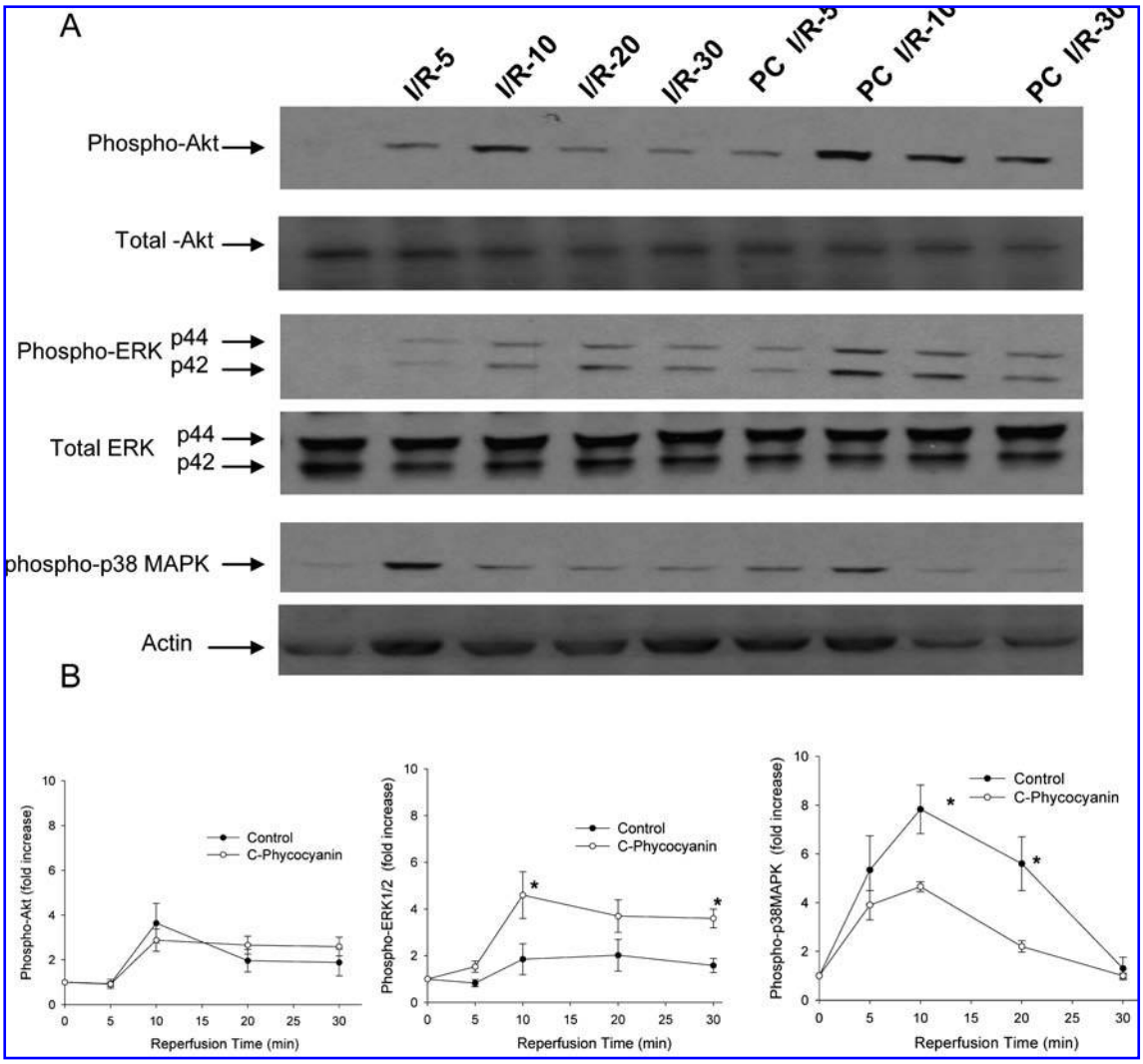

FIG. 8. Akt, ERK1/2, and p38 MAPK phosphorylation in heart subjected to IR. The phosphorylation of Akt, ERK1/2, and p38 MAPK was measured in hearts subjected to ischemia (30 min) and reperfusion time as indicated (5, 10, 20, and 30 min), without and with PC (10 μM). (**A**) Phosphorylated Akt, ERK1/2, and p38 MAPK were detected by Western blot analysis. (**B**) Quantitative analysis of phosphorylated Akt, ERK1/2, and p38 MAPK. Values are from three independent experiments and are expressed as mean ± SEM. $*p < 0.01$ *versus* control. PC treatment significantly attenuated the IR-induced activation of p38 MAPK and enhanced the ERK1/2 activity.

minutes of reperfusion, and it offers cardioprotection against oxidative stress through blocking apoptosis (46, 55, 87, 161). Oxidative stress has also been suggested to play a role in p38 MAPK activation during IR (31). Studies have been shown that ischemia alone or IR activates p38 MAPK in heart and cultured cardiomyocytes. Administration of SB203580 reduces myocardial apoptosis, causing recovery after reperfusion (92, 161). p38 MAPK inhibition has been reported to be cardioprotective, possibly through suppression of apoptosis after a decrease in caspase-3 activity (93). Inhibition of p38 MAPK suppresses cardiomyocyte apoptosis and improves cardiac function after myocardial IR (17). Our study showed a decrease in Akt and ERK1/2 phosphorylation during ischemia but a marked increase in the phosphorylation of both kinases during reperfusion (69). However, p38 MAPK was increased during ischemia, and it remained high during reperfusion (Fig. 8). The IR-induced alterations in p38 MAPK and ERK1/2 were attenuated by an antioxidant, C-phycocyanin. Recent studies have shown the important role of PI3-kinase-Akt signaling pathway in the survival of cardiomyocytes, in addition to protecting against myocardial IR injury in mice (151, 166). The antiapoptotic effect of Akt is mediated by direct phosphorylation and inactivation of proapoptotic proteins, including caspase-9, an upstream activator of caspase-3 (33). Activation of ERKs is important in preventing cardiomyocytes from oxidative stress–induced apoptosis (1). Despite activation of the prosurvival kinases such as ERK1/2 and Akt during IR, the activation of p38 MAPK may surpass that of the prosurvival kinases, thus mediating

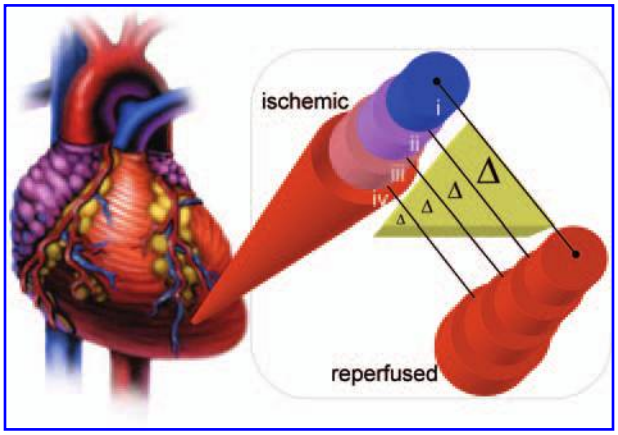

FIG. 9. Cellular responses to reoxygenation after chronic hypoxia. Chronic hypoxia results in cellular adjustments such that reoxygenation causes hyperoxic insult. In the ischemic tissue, the focus of insult is represented by the blue center (region **i**). Focal ischemia is known to be associated with graded oxygenation from near-zero status at focus to levels increasing with distance from the focus. The concentric circles represent regions (**i–iv**) of the tissue, with increasing graded distance from the focus of insult. In the reperfused tissue, the corrected pO_2 state is represented by change of color from a shade of blue (hypoxic) to red. Δ represents ΔpO_2 in response to reoxygenation (ΔpO_2: **I** > **ii** > **iii** > **iv**). Important elements triggered by oxygen during the course of reoxygenation-associated remodeling include the following: in region **I,** cell-death or fatal oxidative injury at the focal point of insult, making room for regenerating tissues; in region **ii,** nonfatal cellular stress, triggering reparative responses; in region, **iii** survival of phenotypically altered cells that favor remodeling (physiologic or pathologic/fibrogenic). Fibrosis denies room to regenerating healthy cells; and in region **iv,** correction of pO_2 of mildly hypoxic cells localized beyond a critical distance from the focus of insult, favoring regeneration and restoration of physiologic functioning of the organ. Whereas a large ΔpO_2 causes ROS-mediated injury in reoxygenated region **i**, perceived hyperoxia supports remodeling in regions **ii** and **iii**. (For interpretation of the references to color in this figure legend, the reader is referred to the web verison of this article at www.liebertonline.com/ars)

myocardial apoptosis and necrosis. Several pharmacologic agents have been shown to be cardioprotective by modulating Akt, p38 MAPK, or ERK1/2 activities (55, 79, 149, 151). Overall, preventing the formation of ROS by restricting the flow during reperfusion may attenuate reperfusion-induced apoptosis.

Oxygen stress: "perceived hyperoxia"

Under normoxic (ambient) conditions, the pO_2 ranges from 90 to <3 mm Hg in mammalian organs with the heart at ~35 mm Hg (5%) and arterial blood at ~100 mm Hg. In response to chronic moderate hypoxia, cells adjust their normoxia setpoint such that reoxygenation-dependent relative elevation of pO_2 results in a "perceived hyperoxia" (Fig. 9) (134). Primary heart cells, isolated from a 5% O_2 environment in the tissue, but maintained in culture at 20% O_2, may be considered exposed to "oxygen stress" that would be sensed by mechanisms responding to supraphysiologic levels of O_2. Recent studies have tested the hypothesis that O_2, even in marginal relative excess of the pO_2 to which cells are adjusted, results in the activation of specific signaling pathways that alter the phenotype and function of cells (127–129). The results from unbiased screening for O_2-sensitive genes have confirmed the p21–p53 axis as being a key target in cardiac fibroblasts exposed to elevated O_2. This finding is consistent with the current notion that many of the signaling pathways that control cellular decisions related to tissue remodeling are regulated by nuclear interactions of cell-cycle proteins (103). Microscopic visualization of cardiac fibroblasts revealed that the nucleus of cells exposed to 20% O_2 clearly stained more prominently for the presence of p21 protein compared with fibroblasts at 3% O_2. Exposure of cardiac fibroblasts to 20% O_2 resulted in a significant increase in p21 promoter-driven luciferase reporter activity (127). Strikingly, p21-deficient cells completely escaped from elevated O_2-induced growth arrest. Thus, p21 has been identified as a key effector of perceived hyperoxia (127).

SUMMARY AND CONCLUSIONS

Oxygen supply during reperfusion is not entirely beneficial for myocardial recovery. The benefit of reperfusion therapy has been attributed to timely reestablishment of blood flow to ischemic myocardium. However, rapid and complete restoration of blood flow in cases of acute myocardial infarction is associated with impaired microvascular perfusion. Robust release of free radicals during early phase of reperfusion may contribute myocardial reperfusion injury. Free radical generation and subsequent myocardial injury after reoxygenation may be dependent on the level of reoxygenation. Hence, controlling the oxygen delivery during the early reperfusion attenuates oxygen free radical generation, mitochondrial dysfunction, and limits myocardial reperfusion injury. Controlling flow or perfusing with low oxygen during early reperfusion, in combination with antioxidants, represents a potential experimental strategy in preventing myocardial ischemia reperfusion injury.

ABBREVIATIONS

CABG, coronary artery bypass surgery; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; EPR, electron paramagnetic resonance; ERK, extracellular-regulated kinase; GSH, glutathione; H_2O_2, hydrogen peroxide; HBO, hyperbaric oxygen; HBOC, hemoglobin-based oxygen carrier; IR, ischemia–reperfusion; LiNc-BuO, lithium octa-*n*-butoxynaphthalocyanine; LiPc, lithium phthalocyanine; LV, left ventricular; MAPK, mitogen-activated protein kinase; MI, myocardial infarction; MPO, myeloperoxidase; mPTP, mitochondrial permeability transition pore; NCX-4016, 2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester; NO, nitric oxide; NOS, nitric oxide synthase; NSAID, nonsteroidal anti-inflammatory drug; $O_2^{-\cdot}$, superoxide; ·OH, hydroxyl; $OONO^-$, peroxynitrite; PTCA, percutaneous transluminal coronary angioplasty; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase.

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