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Measurement of endothelial cell free radical generation: Evidence for a central mechanism of free radical injury in postischemic tissues

(electron paramagnetic resonance/anoxia/reperfusion/superoxide/cell injury)

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ABSTRACT **Oxygen free radicals have been demonstrated to be important mediators of postischemic reperfusion injury in a broad variety of tissues; however, the cellular source of free radical generation is still unknown. In this study, electron paramagnetic resonance measurements with the spin trap 5,5′-dimethyl-1-pyrroline-*N*-oxide (DMPO) demonstrate that bovine endothelial cells subjected to anoxia and reoxygenation become potent generators of superoxide and hydroxyl free radicals. A prominent DMPO-OH signal $a_N = a_H = 14.9$ G is observed on reoxygenation after 45 min of anoxic incubation. Quantitative measurements of this free radical generation and the time course of radical generation are performed. Both superoxide dismutase and catalase totally abolish this radical signal, suggesting that O_2 is sequentially reduced from $O_2^{-\cdot}$ to H_2O_2 to $OH\cdot$. Addition of ethanol resulted in trapping of the ethoxy radical, further confirming the generation of $OH\cdot$. Endothelial radical generation was shown to cause cell death, as evidenced by trypan blue uptake. Radical generation was partially inhibited and partially scavenged by the xanthine oxidase inhibitor allopurinol. Marked inhibition of radical generation was observed with the potent xanthine oxidase inhibitor oxypurinol. These studies demonstrate that endothelial cells subjected to anoxia and reoxygenation, conditions observed in ischemic and reperfused tissues, generate a burst of superoxide-derived hydroxyl free radicals that in turn cause cell injury and cell death. Most of this free radical generation appears to be from the enzyme xanthine oxidase. Thus, endothelial cell free radical generation may be a central mechanism of cellular injury in postischemic tissues.**

Over the past decade, increasing evidence has accumulated suggesting that reactive oxygen free radicals are generated in cells and tissues and are important mediators of a variety of important pathologic processes. Oxygen free radicals have been proposed to mediate postischemic reperfusion damage in a variety of tissues, including the heart, lung, kidney, gastrointestinal tract, and brain (1). In all of these tissues, it has been shown that intravascular administration of free radical scavenging enzymes or drugs can prevent reperfusion injury and enhance postischemic functional recovery. Thus, these studies have provided indirect evidence for free radical generation in a wide variety of organs. Free radical generation in postischemic tissues has been measured with electron paramagnetic resonance (EPR) techniques (2). Both direct and spin-trapping EPR techniques have demonstrated that there is a burst of oxygen free radical generation after postischemic reperfusion of the heart (2–7).

While there is a compelling body of literature suggesting that oxygen free radicals are generated in postischemic tissues, the mechanism of this free radical generation is still poorly understood. It is not even known which cell type or types are responsible for the observed radical generation. To date, no direct measurement of intrinsic oxygen free radical generation by any solid tissue cell type has been reported. Previous studies demonstrating that intravascularly administered radical scavenging enzymes prevent reperfusion injury in a variety of tissues have led to the hypothesis that vascular endothelial cells could be the central source of the intrinsic burst of oxygen free radical generation that occurs on reperfusion of ischemic tissues.

In this study, we have applied EPR techniques to determine whether vascular endothelial cells intrinsically generate oxygen free radicals when subjected to anoxia and reoxygenation, conditions observed in ischemic and reperfused tissues. These measurements demonstrate that endothelial cells are potent sources of the reactive superoxide ($O_2^{-\cdot}$) and hydroxyl ($OH\cdot$) free radicals. This free radical generation is shown to cause cell injury and cell death. The enzyme xanthine oxidase is demonstrated to be the source of most of the observed free radical generation.

MATERIALS AND METHODS

Fetal bovine aortic endothelial cells were isolated essentially as described (8) except that 0.1% (wt/vol) trypsin (type III, bovine pancreatic; Sigma) was also present. Primary cultures were maintained on a standard growth medium (MEM_{10}) consisting of minimum essential medium (MEM with Earle's salts; GIBCO) supplemented with 10% fetal bovine serum (Sterile Systems, Logan, UT), L-glutamine (2 mM), and sodium bicarbonate (2.2 mg/ml) with penicillin G (200 units/ml) and streptomycin sulfate (200 μg/ml). Identification as vascular endothelium was accomplished by examination of cellular morphology at confluence and by the presence of factor VIII-related antigen as determined by immunofluorescence localization (9) with rabbit antiserum against bovine factor VIII. This assay showed staining of >99% of the cells, proving that the cultures consisted of pure vascular endothelial cells. Stock cultures were maintained on MEM_{10} growth medium without penicillin or streptomycin and were incubated in 150-cm^2 flasks in an atmosphere of 5% CO_2/95% air at 37°C with 90% relative humidity. The cells were subcultured at split ratios of 1:3 or 1:5 by using 0.1% trypsin in Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium. For the EPR studies, the cell monolayer was gently washed with 10 ml of PBS and then the cells were harvested from the culture flasks by incubation with 2 ml of the 0.1% trypsin solution for 10 min followed by addition of 8 ml of quench solution (MEM alpha medium with 10% fetal bovine serum; GIBCO). The cells were then centrifuged at 100 × *g* for 5 min, washed twice with 10 ml of

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Abbreviations: EPR, electron paramagnetic resonance; DMPO, 5,5′-dimethyl-1-pyrroline-*N*-oxide; SOD, superoxide dismutase.

PBS, and then suspended in the desired final volume of PBS. Cell counts were either performed with a Coulter counter or were done manually with a hemocytometer.

The spin-trapping studies were performed by using the spin trap 5,5'-dimethyl-1-pyrroline-*N*-oxide (DMPO) at a final concentration of 50 or 100 mM. Care was taken to keep the DMPO-containing solutions covered to prevent any light-induced degradation. The DMPO (>97% pure) was purchased from Aldrich and was further purified by double distillation. Anaerobic cellular preparations were achieved by purging under a gentle stream of pure nitrogen gas and reoxygenation was achieved by reexposure to air.

Cell viability was assessed by trypan blue exclusion. Cells were stained with 0.02% trypan blue in PBS and cell counts of 100 cells were performed after 2 min with a Zeiss standard laboratory light microscope.

EPR spectra were recorded in flat cells at room temperature with a Bruker IBM ER 300 spectrometer operating at X-band with 100-kHz modulation frequency and a TM 110 cavity. The microwave frequency and magnetic field were precisely measured with an EIP 575 microwave frequency counter and Bruker ER 035M NMR gaussmeter. Spectral simulations were performed with programs that assume isotropic g and A tensors, written in either basic or asyst as described (10). Quantitation of the free radical signals was performed by comparing the double integral of the observed signal to that of a known concentration of the TEMPO free radical in aqueous solution as described (4). These measurements were performed with the same flat cell and nonsaturating microwave power.

Recombinant human copper–zinc superoxide dismutase (>99% purity; 3000 units/mg) was obtained from Biotechnology General (New York). Purified bovine liver catalase was obtained from Sigma with an assayed activity of 11,000 units per mg of protein. Deferoxamine mesylate was obtained from CIBA–Geigy. Allopurinol was purchased from Aldrich and oxypurinol was obtained from Burroughs Wellcome (Research Triangle Park, NC).

Superoxide dismutase (SOD) was denatured by a modification of the procedure of Hodgson and Fridovich (11) in which the protein was titrated to a pH of 10 and incubated at room temperature for 12 hr in the presence of a 10-fold excess of hydrogen peroxide followed by two-step dialysis. Catalase was denatured by incubation at 100°C for 30 min.

RESULTS

The endothelial cells were harvested from the culture flasks, washed, and then resuspended in PBS. Concentrated suspensions of $\approx 1.6 \times 10^7$ cells in 1 ml were made anoxic by incubation under anaerobic conditions at 37°C for 45 min. The cells were then reoxygenated by addition of an aerobic solution of DMPO and exposure to air. For these initial experiments, the final DMPO concentration was 100 mM. The cells were then immediately transferred to the EPR flat cell and spectra were acquired. The spectra of these reoxygenated cells exhibited a prominent 1:2:2:1 quartet pattern (Fig. 1, spectrum A). Measurement and computer simulation of the spectrum demonstrated that the observed hyperfine splittings were $a_N = a_H = 14.9$ G, indicative of the trapped OH·, DMPO-OH (12). On similar addition of DMPO to identical solutions of endothelial cells that were aerobically incubated at 37°C, no detectable EPR spectrum was observed (curve B). These measurements were repeated many times with different preparations of cells, and each time prominent signals of DMPO-OH were observed in the reoxygenated cells with no significant signal observed in the controls.

Experiments were performed to measure the time course of the endothelial free radical generation. Immediately after reoxygenation, EPR spectra were recorded every 2 min

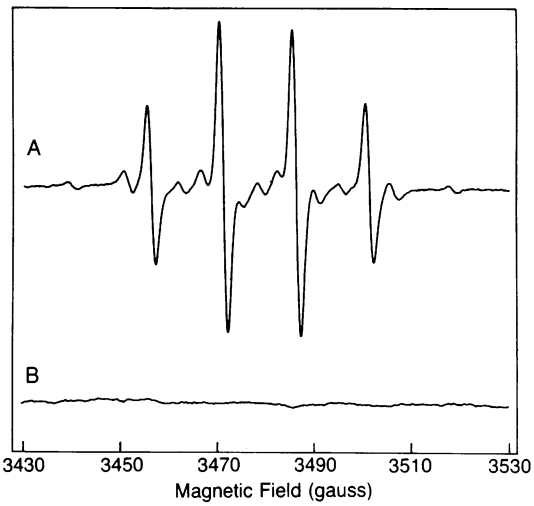

FIG. 1. EPR spectra of preparations of endothelial cells (1.6×10^7 cells in 1 ml) in the presence of 100 mM DMPO. Spectra: A, subjected to 45-min 37°C anoxia and then reoxygenated; B, identical preparation of cells not subjected to anoxia. Spectra were recorded at a microwave frequency of 9.774 GHz with a microwave power of 20 mW and the modulation amplitude of 0.5 G.

(2-min acquisition; 100-G sweep) for a period of 1 hr. These measurements demonstrated that as early as 1 min after reoxygenation the hydroxyl radical signal was observed. This signal rapidly increased to a half-maximum value after only 8 min followed by a further gradual increase over the following 40 min (Fig. 2). After 50 min of reoxygenation, a rapid decrease in signal was observed and after an additional 15 min there was no observable signal. Quantitation of the maximum observed DMPO-OH signal was performed. A maximum radical concentration of 1.2 μM was observed in preparations of 1.6×10^7 cells per ml with 100 mM DMPO.

In ischemic tissues, increased duration of ischemia and maintenance of higher tissue temperatures during ischemia result in increased severity of tissue injury. To determine whether similar modification of the duration of anoxia or the temperature of anoxic incubation would alter the observed free radical generation, similar preparations of cells were anaerobically incubated at 37°C for periods of only 30 min or for 45 min at room temperature, and then they were reoxygenated. In these preparations, similar DMPO-OH signals were observed; however, the radical concentration was reduced by a factor of 2–4 when compared to the preparations of 45-min 37°C anoxic incubation. Thus, increasing length and temperature of anoxic incubation appeared to result in

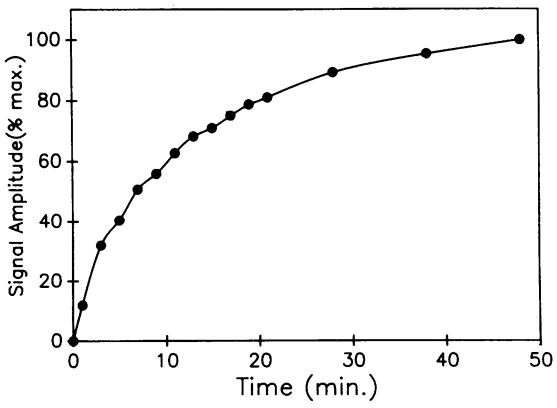

FIG. 2. Time course of the appearance of the DMPO-OH signal in a preparation of 1.6×10^7 endothelial cells in 1 ml exposed to 45-min 37°C ischemia followed by reoxygenation at time 0. Serial 2-min EPR spectra were recorded as described in Fig. 1.

increased cellular free radical generation upon reoxygenation.

To further demonstrate that the observed free radical generation was not due to an osmotic perturbation from the 100 mM DMPO, similar experiments were performed with DMPO concentrations of only 50 mM. In these experiments, prominent DMPO-OH signals were again observed; however, the signals were decreased by ≈40% as would be expected due to decreased efficiency of OH· trapping. Experiments performed assessing cell viability by trypan blue exclusion demonstrated that >98% of the cells excluded trypan blue after incubation with either 50 or 100 mM DMPO.

Experiments were performed in which the number of endothelial cells was varied to demonstrate that the observed radical generation was due to cellular production. In the absence of endothelial cells, no signal was observed. On reducing the number of cells in the anaerobic incubation solution, the observed radical signals were similarly reduced. A reduction by a factor of 2 in the number of cells to 8×10^6 cells per ml resulted in a reduction by a factor of 2–3 in observed radical concentration.

To determine whether the observed DMPO-OH signal was derived from $O_2^{-\cdot}$ or H_2O_2, endothelial cells were incubated in the presence of SOD or catalase (5000 units/ml). These experiments were performed with 8×10^6 cells in 1 ml of PBS under anaerobic conditions at 37°C for 45 min, followed by reoxygenation with a DMPO concentration of 50 mM. In the absence of SOD or catalase, a prominent DMPO-OH signal was observed with a radical concentration of 0.3 μM (Fig. 3, curve A). In the presence of SOD or catalase, no radical signals were observed (curves B and C). The dose dependence of free radical scavenging by SOD and catalase was studied. SOD concentrations of 500 units/ml were sufficient for complete radical scavenging and concentrations of only 100 units/ml still scavenged 90% of the radical signal. With catalase, it was observed that concentrations >500 units/ml were required for complete radical scavenging. To determine whether the radical scavenging observed with SOD and catalase was due to their specific enzyme activities, experiments were performed exposing endothelial cells to anoxia and reoxygenation in the presence of denatured SOD or catalase. With either denatured SOD or catalase concentrations matching those of the studies performed with the active enzymes (500–5000 units/ml), no decrease in radical generation was observed. To assess the role of iron in the generation of the observed signal, similar experiments were performed in the presence of 1.0 mM deferoxamine. Unlike SOD or catalase, which totally quenched radical generation, deferoxamine only reduced the observed signal by 10–20% (Fig. 3, curve D).

Experiments were performed in the presence of ethanol (1% volume) to further determine whether reoxygenated endothelial cells generate OH·. Observation of ethoxy radical formation would be expected in the presence of the OH· since OH· will extract a hydrogen atom from ethanol, generating the ethoxy radical. Similar reoxygenated preparations of endothelial cells gave rise to a spectrum consisting of two components. In addition to the 1:2:2:1 quartet DMPO-OH signal, a 1:1:1:1:1:1 sextet signal was observed (Fig. 4). Simulation of this spectrum demonstrated that the hyperfine couplings of this component signal were a_N = 15.8 G and a_H = 22.8 G, indicative of the trapped ethoxy radical (12).

Xanthine oxidase has been hypothesized to be an important source of free radical generation in postischemic tissues; however, this hypothesis remains quite controversial (13, 14). To investigate whether xanthine oxidase is an important

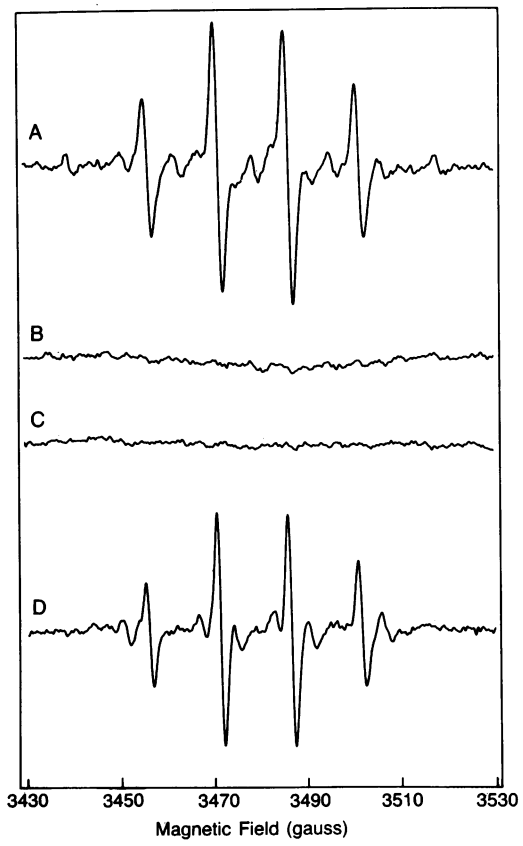

FIG. 3. EPR spectra of preparations of endothelial cells (8×10^6 cells in 1 ml) subjected to 45-min 37°C anoxia and then reoxygenation in the presence or absence of radical scavenging agents. The DMPO concentration was 50 mM. Spectra: A, no added enzyme; B, SOD at 5000 units/ml; C, catalase at 5000 units/ml; D, in the presence of 1 mM deferoxamine. Spectra were recorded as described in Fig. 1.

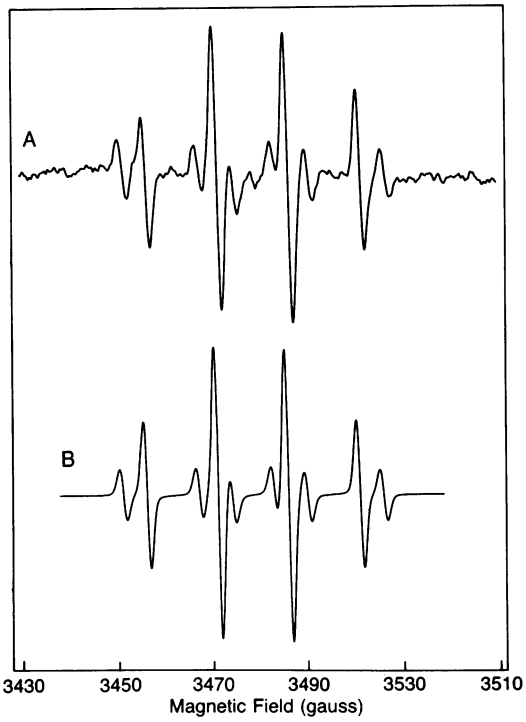

FIG. 4. EPR spectra of reoxygenated endothelial cells subjected to anoxia and reoxygenation in the presence of 1% ethanol. Spectra: A, experimental spectrum recorded as described in Fig. 1; B, computer simulation of experimental spectrum consisting of two components: a 1:2:2:1 quartet with $a_N = a_H$ = 14.9 G, linewidth 1.5 G, weight 0.7; and a 1:1:1:1:1:1 sextet a_N = 15.8 G and a_H = 22.8 G, linewidth 1.6 G, weight 0.3.

source of endothelial free radical generation, the effects of xanthine oxidase inhibitors were studied. Experiments were performed in which preparations of 8×10^6 endothelial cells were incubated in 1 ml of PBS under anaerobic conditions at 37°C for 45 min in the presence or absence of 4 mM allopurinol or 4 mM oxypurinol followed by reoxygenation, with a DMPO concentration of 50 mM. In the presence of allopurinol, a 60% decrease in the DMPO-OH signal was observed compared to that observed in the absence of the drug from a concentration of 0.3 μM to 0.12 μM; however, an alkyl signal was observed with a concentration 0.08 μM (Fig. 5, spectrum B; Table 1). The observed alkyl signal could be due to the formation of a drug radical generated by the scavenging of OH· by the drug. It has previously been suggested that allopurinol may prevent free radical injury by scavenging OH· rather than by specifically blocking $O_2^{-\cdot}$ generation by xanthine oxidase (15). The observed spectrum suggests that allopurinol is only partially effective as a blocker of OH· generation and partially effective as a OH· scavenger. Experiments performed with the more potent xanthine oxidase inhibitor oxypurinol showed a marked 80–90% reduction in the DMPO-OH signal (Fig. 5, spectrum C). This marked reduction in OH· generation suggests that the enzyme xanthine oxidase is an important source of endothelial free radical generation.

To determine whether endothelial free radical generation causes cell injury and cell death, cell viability was assessed in parallel with the above EPR measurements. Prior to transfer to the EPR flat cell, small aliquots of cells were removed for measurement of trypan blue exclusion. As shown in Table 1, cells not subjected to anoxia exclude trypan blue while cells subjected to anoxia and reoxygenation take up the dye. The time course of this reoxygenation endothelial cell death was studied by preparing slides of cells stained at different times after reoxygenation. Cell counts performed in the first 2 min after reoxygenation showed that only 25% of the cells took up trypan blue. After 4 min 50% of the cells took up the dye, after 10 min 90% of the cells took up the dye, and after 20 min >95% of the cells took up the dye. Cells not subjected to anoxia continued to exclude the dye with >95% of the cells excluding dye even after 30 min. These studies suggest that the ongoing generation of free radicals in reoxygenated endothelium induces gradually increasing cellular damage as radical production continues. In the presence of either SOD or catalase (5000 units/ml), this cell damage was almost totally prevented in accordance with the abolishment of free radical generation (Table 1). Denatured SOD or denatured catalase, however, did not decrease the observed radical concentrations or prevent cell damage. The radical concentrations and the uptake of trypan blue were identical to preparations with no added enzyme. Allopurinol did decrease the proportion of the cells that took up the dye, but it was considerably less effective than SOD or catalase (Table 1). Oxypurinol, however, was considerably more effective than allopurinol. Thus, cell death appeared to correlate closely with measured radical concentrations, suggesting that the observed free radical generation was sufficient to cause cell injury and cell death.

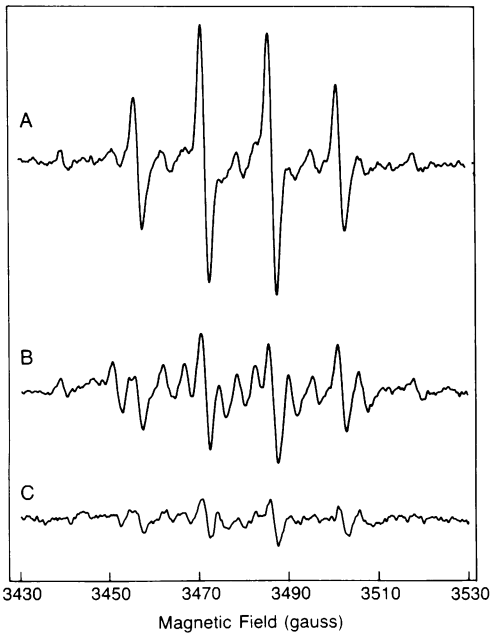

FIG. 5. EPR spectra of reoxygenated endothelial cells in the presence and absence of xanthine oxidase inhibitors. Spectra: A, no added inhibitor; B, in the presence of 4 mM allopurinol; C, in the presence of 4 mM oxypurinol. Spectra were recorded as described in Fig. 1 with 8×10^6 cells in 1 ml.

Table 1. Correlation of radical generation and cell injury

	Radical concentration		Cell viability, % cells excluding trypan blue
	DMPO-OH, μM	DMPO-R, μM	
Control Cells	0	0	99
Reoxygenated	0.3	0.03	10
Reoxygenated + SOD	0	0	96
Reoxygenated + catalase	0	0	92
Reoxygenated + allopurinol	0.12	0.08	70
Reoxygenated + oxypurinol	0.03	0.0	82

Cells were counted 10 min after reoxygenation.

DISCUSSION

Ischemic cell damage is the major source of morbidity and mortality in the United States today. Therefore, a variety of techniques have been developed to revascularize ischemic tissues. Unfortunately, when ischemic tissues are reperfused oxidative injury is often observed (1). Oxidative reperfusion injury is thought to be a central mechanism of cell damage affecting all organs and tissues; however, the mechanism(s) of this cell damage is not known. It has been demonstrated in the isolated crystalloid perfused heart that oxygen free radicals are generated on postischemic reperfusion (2–7). This radical generation in a non-blood-perfused organ suggests that there must be a solid tissue cell type responsible for radical generation. While EPR studies have demonstrated that activated leukocytes are potent radical generators, no solid tissue cell type has ever been similarly shown to endogenously generate these radicals (16). Spin-trapping EPR studies of exogenous radical generation in endothelial cells have been performed suggesting the feasibility of measuring radicals in these cells (17).

In this study, we have demonstrated that vascular endothelial cells subjected to an ischemic equivalent of anoxic incubation of dense suspensions in the absence of substrate followed by reoxygenation become potent generators of oxygen free radicals. Endogenous endothelial radical generation was measured by EPR spectroscopy using the spin-trap DMPO. Increased duration and temperature of anoxic incubation resulted in increased radical generation on reperfusion. In ischemic tissues, prolongation of the duration of ischemia and maintenance of higher temperatures during ischemia increase tissue injury. Thus, the measured increase in endothelial cell radical generation under these conditions

parallels the occurrence of *in vivo* tissue injury. The chemical mechanism of oxygen reduction proceeded in one-electron steps from O_2 to $O_2^{-\cdot}$ to H_2O_2 to OH· with either superoxide dismutase or catalase exhibiting complete quenching of OH· production. OH· generation was not due only to simple iron-redox Fenton chemistry of weakly bound Fe^{3+} chelates since deferoxamine did not similarly abolish free radical generation.

Several cellular enzymes have been previously hypothesized to be important sources of free radical generation. These enzymes include NADPH oxidase, cytochrome P450, cyclooxygenase, and xanthine oxidase (18). McCord (19) has demonstrated that the enzyme xanthine dehydrogenase is converted to xanthine oxidase in ischemic tissues and has hypothesized that xanthine oxidase may therefore be an important source of free radical generation in ischemic tissues. Significant controversy has remained regarding whether xanthine oxidase is actually responsible for postischemic free radical generation and whether the xanthine oxidase mechanism is an important cause of postischemic cell injury. Much of this controversy has resulted from differing results obtained in studies investigating the protective effects of the xanthine oxidase inhibitor allopurinol, and based on these differences it has been questioned whether there is a free radical component of reperfusion injury at all (13, 14). Endothelial cells have been previously shown to have high concentrations of xanthine dehydrogenase or xanthine oxidase (20). We observed that the potent xanthine oxidase inhibitor oxypurinol markedly decreased the observed concentrations of superoxide or hydroxyl free radicals in reoxygenated endothelial cells. An 80–90% decrease in the radical concentrations was observed. Inhibition may have been incomplete because of either incomplete blocking of xanthine oxidase or the presence of another additional mechanism of free radical generation in these cells. Of particular interest was the observation that allopurinol was less potent than oxypurinol at blocking free radical generation by xanthine oxidase and that, as previously proposed, allopurinol appeared to be partially effective as a OH· scavenger (15). Allopurinol would be expected to be less potent than oxypurinol in that it is metabolized by the enzyme to oxypurinol, serving as a substrate for O_2 reduction to $O_2^{-\cdot}$ (21). Thus, allopurinol can serve initially as a potential source of $O_2^{-\cdot}$ generation. These observations could explain the variable results previously reported in studies testing the efficacy of allopurinol in preventing oxidative reperfusion injury. With allopurinol pretreatment for 24 hr, much of the drug would be metabolized to oxypurinol, which would block free radical generation from xanthine oxidase (13). Acute treatment with allopurinol, however, would be expected to be less effective.

Variable concentrations of xanthine oxidase or xanthine dehydrogenase have been observed in different species and in different tissues within a given species (22). Variations in the expression of this enzyme may thus serve to modulate the severity of reperfusion injury in different species and in different organs. While xanthine oxidase appeared to be an important source of free radical generation in reoxygenated endothelial cells, it may not be the only important mechanism of oxidative injury. SOD or catalase was effective at totally scavenging free radical generation and preventing cell injury; however, millimolar concentrations of oxypurinol, which would be expected to totally block xanthine oxidase, were only 80–90% effective at radical scavenging and only 80% effective at preventing cell death. Therefore, it is possible that there are additional important mechanisms of radical generation in endothelial cells and in whole biological tissues.

Measurements of cell viability demonstrated that the observed free radical generation caused cell injury and death. The duration and magnitude of radical generation directly correlated with the occurrence of cell injury. SOD and catalase concentrations that abolished free radical generation also prevented cell injury, while identical concentrations of the inactivated enzymes had no effect on radical generation and did not prevent cell injury.

Thus, reoxygenated endothelial cells are potent generators of $O_2^{-\cdot}$ and OH· free radicals. The concentrations of generated radicals were shown to be sufficient to induce cell injury and cell death. The enzyme xanthine oxidase appears to be an important source of the observed radical generation. Since vascular endothelial cells are present in all organs, this mechanism of free radical generation may be a central cause of oxidative reperfusion injury in a large variety of organs and tissues.

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