Effect of Superoxide Dismutase plus Catalase on Ca^{2+} Transport in Ischemic and Reperfused Skeletal Muscle[1,2]

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Cytotoxic oxygen metabolites may contribute to skeletal muscle damage associated with ischemia and reperfusion. This study utilized a rat hindlimb ischemia model to investigate the effect of pretreatment with oxygen free radical scavengers superoxide dismutase (SOD) and catalase (CAT) on skeletal muscle Ca^{2+} uptake by sarcoplasmic reticulum (SR) in limbs subjected to periods of ischemia and reperfusion. SOD and CAT were conjugated to polyethylene glycol to prolong their half lives. Anesthetized rats (ca. 350 g) received an iv injection of either conjugated SOD (2 mg/kg) plus CAT (3.5 mg/kg) ($n = 6$, Treated Group) or 0.9 saline (4 ml/kg) ($n = 6$, Control Group) 5 min before unilateral hindlimb tourniquet ischemia of 3 hr duration. After 19 hr of reperfusion, muscle from each lower leg was excised and homogenized. Skeletal muscle SR was isolated by differential centrifugation. ATP-dependent Ca^{2+} uptake by the SR was then measured with dual wavelength spectrophotometry and used as an index of muscle function. Pretreatment with SOD and CAT maintained higher rates of Ca^{2+} uptake by SR of skeletal muscle from postischemic reperfused limbs (Treated Group 2.29 ± 0.21 vs Control Group, 1.61 ± 0.06 μmole Ca^{2+}/mg protein/min). These results implicate cytotoxic oxygen metabolites in the pathogenesis of ischemic reperfusion skeletal muscle injury. © 1987 Academic Press, Inc.

INTRODUCTION

Limb ischemia occurs during many forms of vascular insufficiency, including chronic atherosclerotic occlusive disease, arterial embolism, vascular trauma, and acute arterial thrombosis. In addition to disease and trauma-induced vascular impairment, limb ischemia is often produced electively in orthopedic procedures which utilize a tourniquet to provide a bloodless operative field. Surgical correction of these vascular disorders by techniques directed at increasing blood flow has necessitated a more thorough understanding of the pathogenesis of ischemia.

Ischemic damage is multifactorial, and includes inadequate delivery of oxygen and substrate, depletion of high energy phosphates, and accumulation of toxic end products of ischemic metabolism. After correction of the ischemic event, metabolic factors tend to normalize but, paradoxically, cell damage continues [25, 29, 30]. This persistent decline in cell function is indicative of ongoing muscle destruction during reperfusion. Evidence of postischemic degeneration of sleketal muscle in humans has been reported [1, 7].

Ischemia with reperfusion may produce tissue edema [20], compartment syndromes, fibrotic contractures [31], and contractile dysfunction [12]. The etiology of contractile dysfunction can be partly explained at the level of the sarcoplasmic reticulum (SR), the microsomal system required for both release of calcium (Ca^{2+}) to initiate contraction, and Ca^{2+} sequestration to cause relaxation. The velocity of calcium sequestration by skeletal muscle SR serves as a sensitive measure of intracellular membrane function and integrity.

In vitro studies [2] have shown that ischemia significantly depresses the activity of muscle SR to transport Ca^{2+}.

Recently, cytotoxic oxygen metabolites (oxygen free radicals) have been identified as significant mediators of reperfusion damage. Reoxygenation of ischemic tissue results in abundant oxygen radical production and subsequent cell damage. The participation of oxygen radicals in ischemic tissue injury has been suggested by studies involving the heart [5, 11, 14, 26–28], central nervous system [6, 8], gastrointestinal tract [23, 24], kidney [13, 32], liver [18], and skin [15, 17].

This study was designed to assess the involvement of oxygen free radicals in the damage of skeletal muscle due to ischemia and reperfusion by using the free radical scavengers superoxide dismutase (SOD) and catalase (CAT) as biologic probes. The rate of Ca^{2+} uptake by isolated SR vesicles was used as an index of ischemic damage and protection by drug intervention.

MATERIALS AND METHODS

Ischemic model. Healthy male Sprague–Dawley rats weighing approximately 350 g were anesthetized with an intraperitoneal injection of 2 ml of 5% chloral hydrate. Additional chloral hydrate was administered as needed throughout the experiment. The right jugular vein was cannulated with a polyethylene catheter, which was positioned in the superior vena cava for use as a drug and saline injection site.

Six treated animals received iv injections of 2 mg/kg SOD and 3.5 mg/kg CAT, each drug coupled covalently to polyethylene glycol[3]. A control group of six rats received an equivalent volume of 0.9% saline (4 ml/kg). The timing of drug injection and subsequent tourniquet application was selected empirically, but should allow adequate time for circulation and distribution of the drugs throughout the peripheral vasculature. Five minutes after the drug or saline injection, a tourniquet (sash cord) was positioned above the right knee at midthigh and tightened to a tension that completely occluded hindlimb arterial flow, as determined by fluorescein injection in a pilot study.

Tourniquet occlusion was maintained for 3 hr, during which time the rat remained anesthetized. After removing the tourniquet the injured leg was massaged for 2 min in order to more adequately restore blood flow. The venous catheter was removed and the cervical incision closed using nylon suture. The leg muscle was then allowed 19 hr of reperfusion (Fig. 1).

Coupling of polyethylene glycol to SOD and CAT. Superoxide dismutase and catalase were coupled to polyethylene glycol (average molecular weight—5000) using the method of Beauchamp *et al.* [3]. Coupling increases the enzyme half-life from 3.5 min to over 9 hr, while preserving 95% of original enzyme activity.

Isolation of sarcoplasmic reticulum. After 19 hr of reperfusion each animal was killed by anesthetic overdose. Sarcoplasmic reticulum vesicles were isolated and purified according to a variation of the method used by Okabe *et al.* [22] (Fig. 2). All steps were performed at 4°C. Skeletal muscle from below the knee
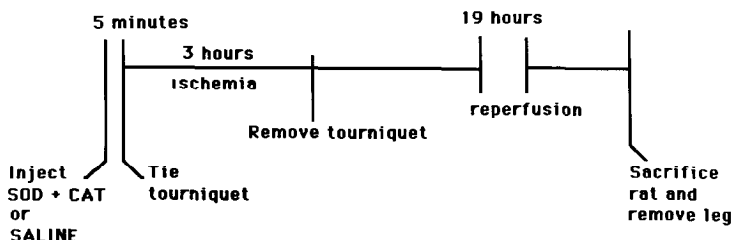

FIG. 1. Sequence of experimental procedures used to study rat hindlimb ischemia and reperfusion. Abbreviations used: SOD, superoxide dismutase; CAT, catalase.

rat hindlimb skeletal muscle
minced, ground and homogenized in
16 ml 10 mM imidazole buffer

centrifuge 10,000 G x 20 min.

save supernatant (S1)

resuspend pellet in
10 mM imidazole buffer
centrifuge 10,000 G x 20 min

combine supernatant (S2)   discard pellet
with S1

strain S1 + S2 through gauze
centrifuge 12,000 G x 15 min.

discard pellet

strain supernatant (S3) through gauze
centrifuge 31,000 G x 90 min

resuspend pellet in 30 ml KCl solution*   discard
centrifuge 105,000 G x 60 min.   supernatant

resuspend pellet in 1-4 ml   discard supernatant
sucrose solution *

determine protein concentration of SR by Lowry method

Dilute to 700 ug/ml

*KCl solution: 1.0 M KCl, 10 mM imidazole pH 7.0   all steps
*Sucrose solution: 30% sucrose, 20 mM tris/HCl pH 7.0   performed
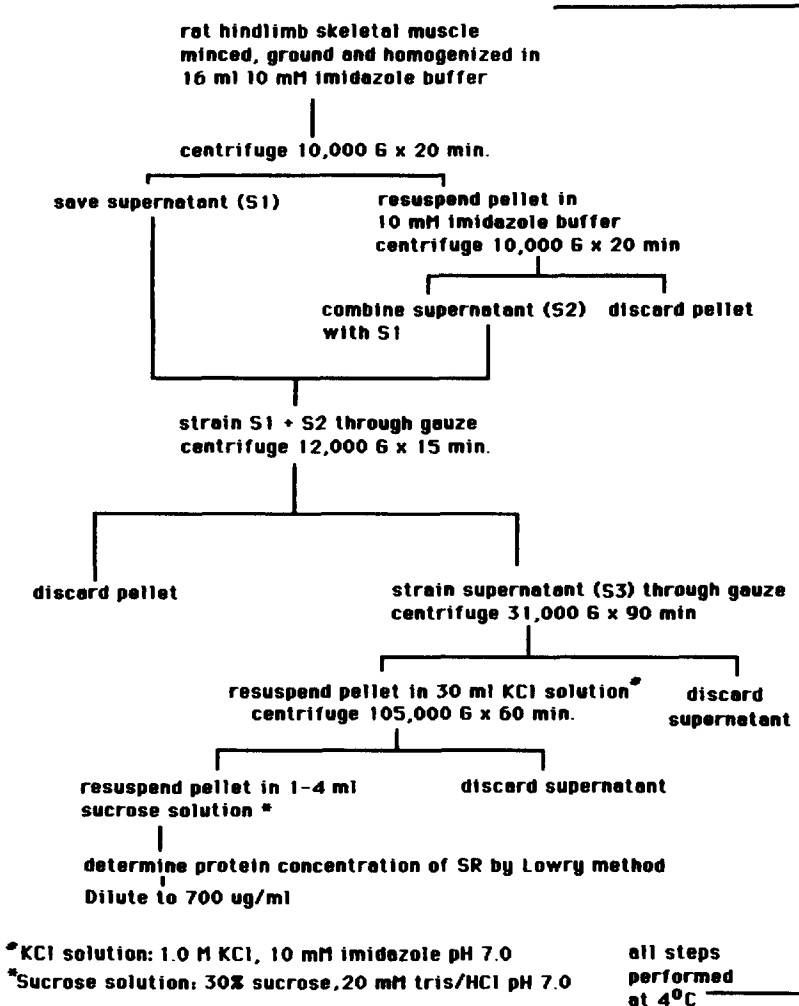   at 4°C

FIG. 2. Flow chart for isolation of skeletal muscle microsomes (SR). The final pellet was suspended in 30% Tris-buffered sucrose.

was removed from both lower limbs and separately processed. Each muscle grouped was dissected free of fat and connective tissue and trimmed until a final weight of 4 ± 0.1 g was reached. The muscle was minced with scissors for 60 sec in 16 ml of 10 mM imidazole buffer (pH 7.0). The mince was homogenized using six 5-sec bursts from a Tekmar Tissuemizer. A power-driven Potter–Elvehjem homogenizer was used for further disruption of the muscle cell membranes. The homogenate was centrifuged at 10,000g for 20 min and the resulting supernatant (S1) was saved. The pellet was resuspended in 16 ml imidazole buffer and centrifuged again at 10,000g for 20 min. The sediment, containing predominantly connective tissue, myofibrils, and mitochondria, was discarded. The supernatant obtained (S2) was combined with S1 and filtered through four layers of gauze. The combined S1 and S2 fractions were then centrifuged at 12,000g for 15 min to remove additional mitochondria. The resulting pellet was discarded and the supernatant (S3) was strained through eight layers of gauze. The S3 fraction, containing soluble protein and microsomal vesicles, was centrifuged at 31,000g for 90 min. The supernatant was discarded. The microsomal pellet was re-

suspended in 30 ml 1.0 M KCl, (in 10 mM imidazole buffer, pH 7.0), using a power-driven Potter–Elvehjem homogenizer in order to solubilize non-microsomal proteins (i.e., actomyosin). The suspension was centrifuged at 105,000g for 60 min and the supernatant discarded. The sediment, composed predominantly of sarcoplasmic reticulum vesicles (SR), was then suspended in 1–4 ml of 30% sucrose in 20 mM Tris–HCl (pH 7.0). A portion of each sample was frozen at $-20°C$, while the remainder was kept at 4°C for immediate analysis of Ca^{2+} uptake velocity. Protein concentrations were determined by the Lowry method [16] and the SR solutions were subsequently diluted to a concentration of 700 μg/ml immediately before assaying Ca^{2+} transport.

Dual wavelength spectrophotometric measurement of Ca^{2+} uptake. Calcium accumulation by isolated SR, in the presence of ATP and oxalate, was measured using the dual wavelength procedure of Ohnishi and Ebashi [21] (SLM-Aminco DW2-C spectrophotometer) and the chelometric dye tetramethylmurexide (TMX). Absorbance differences between the wavelength pair 507 nm and 554 nm were used as the index of Ca^{2+} uptake. Absorbance measurements were linearly related to added Ca^{2+} between zero and 75 μM (Fig. 3).

The reaction medium was maintained at 37°C and stirred constantly in the cuvette. The reagents in the uptake reaction mixture were added according to a timed sequence. The cuvette was initially filled with 1.8 ml of incubation medium (composed of 175 mM KCl, 31.7 mM Imidazole, 16.7 mM K-oxalate, 8.3 mM $MgCl_2$), 700 μl of H_2O, 150 μl of TMX dye, and 200 μl SR membrane (700 μg/ml). After 60 sec allowance for mixing and temperature equilibration, 150 μl of 0.1 M ATP was added. The uptake reaction was initiated 90 sec later by the addition of 7.5 μl of 30 mM $CaCl_2$. The final composition in the cuvette was 105 mM KCl, 19 mM imidazole buffer
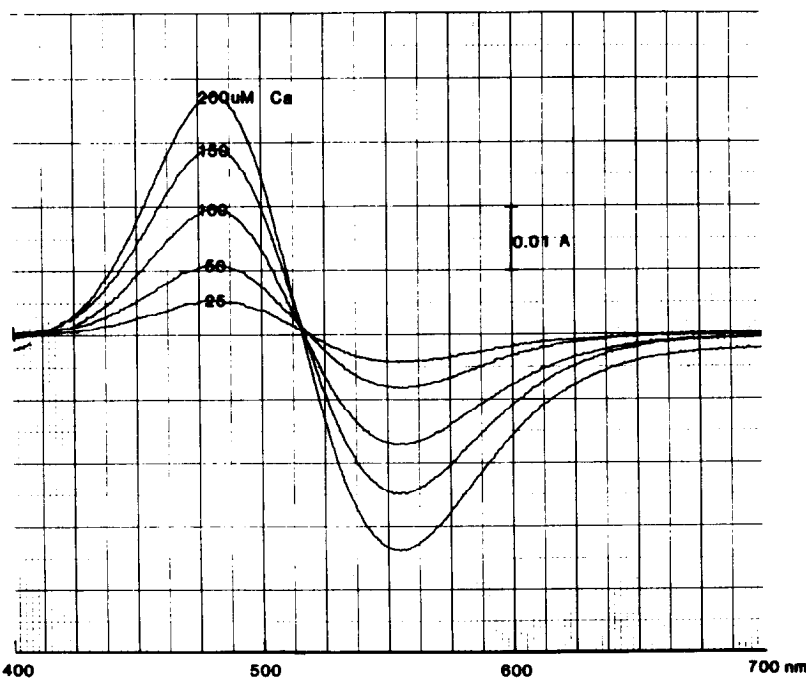

FIG. 3. Dual wavelength scan demonstrating calcium-TMX complex absorbance maxia and isosbestic point. Reaction mixture: 105 mM KCl, 19 mM Imidazole (pH 6.8), 5 mM $MgCl_2$, 10 mM K-oxalate, 5 mM ATP, 50 μM TMX, and discrete additions of $CaCl_2$ up to a maximum final concentration of 250 mM. The temperature was 37°C.

(pH 6.8), 5 mM MgCl$_2$, 10 mM K-oxalate, 46.6 μg/ml SR membrane protein, 5 mM Tris-ATP, 50 μM TMX and 75 μM CaCl$_2$, in a final volume of 3 ml. The reaction was allowed to proceed for 3 min. The rate of Ca^{2+} uptake (Fig. 4) was measured as the slope of the absorbance change over time, after a 3 sec allowance for mixing. Calcium uptake velocities are expressed as micromoles Ca^{2+} transported per milligram SR protein per minute of reaction time.

Statistics. Data comparison between enzyme treated rats and control rats was performed using Student's t test. Statistical comparison of nonischemic and ischemic limbs within the same treatment group was made using paired t tests. Values are expressed as means ± one standard error of the mean. Significance was defined as $P < 0.05$.

RESULTS

Calcium Uptake Velocity

The calcium transport mechanism of skeletal muscle SR was assessed as an indirect indicator of cell alterations following limb ischemia and reperfusion, and to evaluate the efficacy of superoxide dismutase and catalase to prevent these changes.

Calcium uptake velocity by SR isolated from ischemic limbs was significantly lower than that from the contralateral nonischemic limbs for both untreated (ischemic, 1.61 ± 0.06 vs nonischemic, 3.08 ± 0.22 μmole/mg/min; $P < 0.001$) and SOD plus CAT treated rats (ischemic 2.29 ± 0.21 vs nonischemic 3.12 ± 0.16; $P < 0.05$; Fig. 5).

Calcium uptake velocity by SR from ischemic muscle of rats pretreated with SOD and CAT was significantly higher than the corresponding values measured in SR isolated from ischemic limbs of saline-treated controls (SOD + CAT-treated, 2.29 ± 0.21 vs saline treated, 1.61 ± 0.06, $P < 0.01$).

Nonischemic limb muscle from both animal groups showed no significant difference in the ability of isolated SR to transport Ca^{2+}.

SR Protein Yield

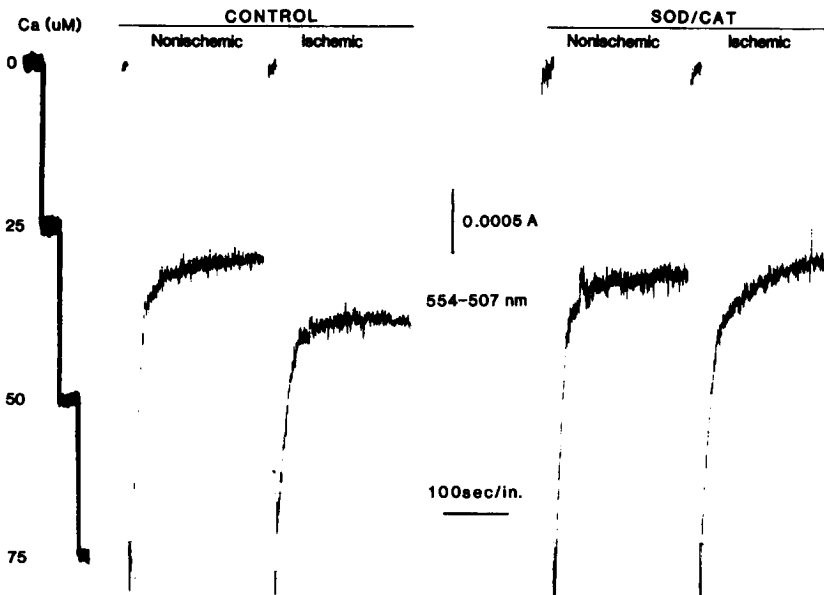In control animals a greater quantity of SR protein was recovered from the nonischemic

FIG. 4. Representative Ca^{2+} uptake curves are shown for control and SOD + CAT-treated rat skeletal muscle SR. The rate of disappearance of Ca^{2+} from solution was recorded and the slope of the uptake curve determined. Uptake was initiated by the addition of CaCl$_2$.
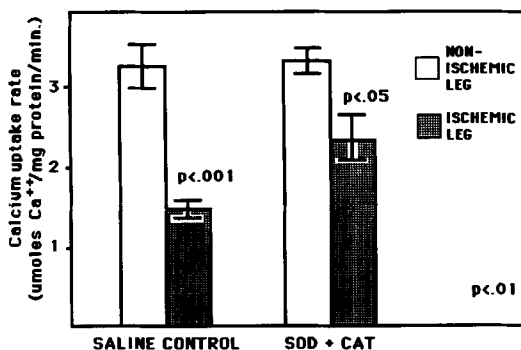
FIG. 5. SR isolated from ischemic limbs from rats pre-treated with SOD and CAT show significantly higher rates of Ca^{2+} uptake ($P < 0.01$, nonpaired t test). The experimental protocol had no effect on Ca^{2+} uptake velocities of SR isolated from nonischemic limbs.

limbs than from the ischemic limbs (nonischemic limbs, 0.97 ± 0.17 vs ischemic limbs, 0.58 ± 0.09 mg SR protein/g muscle wet wt; $P < 0.04$) (Fig. 6). There was no difference in SR yield between the ischemic and nonischemic limbs of the treated animals. Additionally, less SR membrane protein was obtained from the ischemic legs of the controls as compared to the ischemic legs of the SOD/CAT treated rats (controls, 0.58 ± 0.09 vs SOD/CAT treated, 1.00 ± 0.22 mg SR protein/g muscle wet weight, $P < 0.01$).

DISCUSSION

Substances which metabolize or "scavenge" reduced forms of oxygen have been shown to partially prevent cell injury due to ischemia and reperfusion in many organ systems. Studies of cardiac ischemic injury have demonstrated that SOD [5, 11, 14, 26–28], CAT [5, 14, 26, 27], mannitol [14, 28], and allopurinol [11, 22], afford protection against myocardial necrosis as evidenced by improved contractile and physiologic functions. Similarly, SOD and allopurinol pretreatment reduced the amount of mucosal degeneration in ischemic intestinal disorders [23, 24]. Scavengers were also efficacious in limiting tissue injury in ischemia of the kidney [13, 32], liver [18], and island skin flaps [15, 17]. The role of cytotoxic oxygen species is less clear with respect to skeletal

muscle injury. The purpose of this study was to assess the involvement of reduced oxygen species in skeletal muscle injury associated with ischemia and reperfusion.

Oxygen free radical scavengers (vitamin E, ascorbate, glutathione, and mannitol) chemically react with reduced oxygen metabolites. Enzymes such as SOD, CAT, and peroxidases, which are normal constituents of mammalian cells, do not react directly with oxygen radicals but instead catalyze their conversion to other oxygen metabolites [10] (Fig. 7). The production of cytotoxic oxygen metabolites is also affected by the xanthine oxidase inhibitor, allopurinol, and the iron chelator, deferoxamine. The ability of oxygen radical scavengers to partially eliminate the destructive manifestations of hypoxia or anoxia is good, albeit indirect evidence of the participation of cytotoxic oxygen radicals in the pathogenesis of cell damage due to hypoxia or ischemia.

This study demonstrated that pretreatment with the enzymes superoxide dismutase and catalase, conjugated to polyethylene glycol, reduced the degree of depression of ATP-dependent Ca^{2+} uptake by SR isolated from ischemic and reperfused rat hindlimb skeletal muscle. Preservation of this important biochemical process may specifically reduce postischemic rigor that is characteristic of ischemic muscle injury. In addition, relative maintenance of intracellular Ca homeostatis
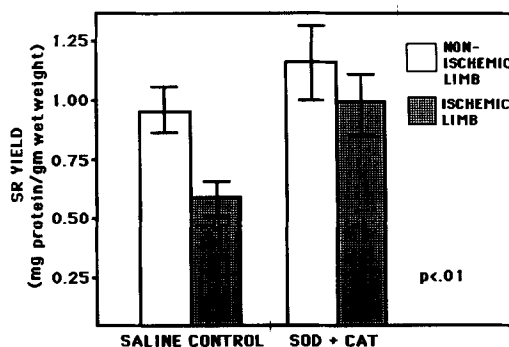

FIG. 6. Rats pretreated with SOD and CAT demonstrated significantly greater recovery of SR membrane protein from ischemic limbs compared to untreated controls ($P < 0.01$, nonpaired t test). Differences between nonischemic limbs were not significantly different.

Xanthine
Dehydrogenase

Cell Ca Influx → SR Ca Overload, Rigor

PURINES, O_2

Xanthine Oxidase ✕ Allopurinol

Mitochondria → O_2^- $\xrightarrow{SOD}$ H_2O_2 $\xrightarrow{CAT}$ $H_2O + O_2$

Fe^{3+} Fe^{2+} Deferoxamine ✕
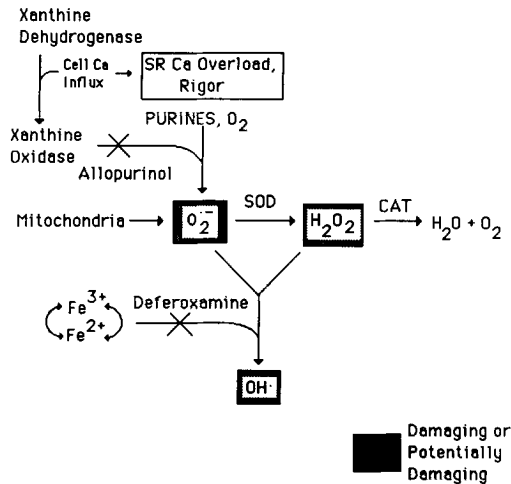
OH^-

Damaging or Potentially Damaging

FIG. 7. Schematic diagram indicating the pathways of production and catabolism of the major oxygen metabolites, as well as points of potential pharmacologic intervention.

could moderate other Ca-dependent processes such as activation of proteases that are thought to convert xanthine dehydrogenase to the oxidase. Xanthine oxidase is thought to reduce molecular oxygen and generate superoxide anion and hydrogen peroxide concomitant with catabolism of purines derived from catabolism of ATP during ischemia.

Although skeletal muscle damage was assessed indirectly in terms of an intracellular biochemical process, the large molecular weights of the enzyme additives, their conjugation to polyethylene glycol, and the probability that intracellular diffusion of this large complex was limited, suggests that protection may have been conferred at extracellular sites. It is nevertheless impossible to determine whether the superoxide or hydrogen peroxide that was degraded originated from within the skeletal muscle cell, the endothelium, or originated from circulating neutrophils.

Superoxide anion and hydrogen peroxide, which are degraded by SOD and CAT, respectively, appear to serve as direct or indirect cytotoxic mediators of ischemia/reperfusion damage in skeletal muscle. Similar hindlimb ischemia studies have demonstrated that a defect in the Ca^{2+} transport system is accom-

panied by the accumulation of lipid peroxidation products in the membrane of the sarcoplasmic reticulum [2]. Oxygen radicals can initiate chain reactions in the lipid bilayers of organelle membranes by removing hydrogen atoms from the polyunsaturated fatty acids, thus forming lipid free radicals that are also highly reactive and capable of propagating cell damage [9]. In addition to peroxides, lipid alcohols and aldehydes are formed and may disrupt membrane integrity and nonspecifically increase permeability. In addition to possible uncoupling of Ca^{2+} transport from ATP hydrolysis, peroxidation of microsomal membrane lipids in situ may render them more permeable to Ca^{2+}, such that accumulated Ca^{2+} cannot be retained. Additionally, free radicals can crosslink proteins and oxidise amino acids [9]. Alterations in the protein structure of Ca^{2+} dependent ATPase from skeletal muscle SR could depress enzyme activity following reperfusion damage. Thus, a defect in ATPase function, as well as permeability changes due to lipid peroxidation, could account for decreased Ca^{2+} uptake by postischemic SR as found in this study. Reduction of SR dysfunction by conjugated SOD plus CAT implicates oxygen radicals in the pathogenesis of damage.

The yield of microsomal protein isolated from the ischemic limbs of the control rats was markedly reduced. This change was prevented by pretreatment with SOD and CAT. Lipid peroxidation alters the lipophilicity of fatty acids creating a detergent effect, and disrupts protein–lipid interactions [33]. Reports of increased protein release from microsomal membranes during lipid peroxidation [4], allegedly due to this detergent phenomenon, may partially explain the reduction in the total SR protein yield from ischemic skeletal muscle. Dissolution of portions of the peroxidized membrane, and recovery of only a fraction of the total SR following differential centrifugation may account for additional protein loss. The diminished protein yield noted in ischemic and reperfused muscle (measured as milligrams protein per gram wet weight muscle) may also reflect cellular and interstitial

edema formation that would cause a relative decrease of protein content. Release of structual enzyme and components of the SR membrane during postischemic reperfusion could further compromise the ability of the SR to sequester Ca^{2+}.

The actual oxygen metabolites responsible for skeletal muscle ischemic damage, and the predominant source of production of these cytotoxic species are currently unknown. Future studies examining treatment with single enzymes, deferoxamine to chelate iron, or allopurinol to alter purine catabolism, should further elucidate the mechanism of skeletal muscle ischemic and reperfusion injury. Similarly, determination of the relative contributions of ischemia and reperfusion to overall damage, which would help predict whether these or related interventions would be efficacious when administered after the onset of ischemia, might be relevant to future clinical studies.

ACKNOWLEDGMENTS

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