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Antioxidant Status Following Acute Ischemic Limb Injury: A Rabbit Model

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Although ischemic injury to skeletal muscle is a matter of great clinical importance, relatively little is known about the mechanisms which determine systemic responses. One purpose of this study is to elucidate the systemic antioxidant status following an episode of acute ischemic limb injury and subsequent reperfusion.

Twelve New Zealand white rabbits were used in this study. After the animals were anesthetized, an ischemic insult was created in the right hind limb for twelve hours, followed by four hours of reperfusion. Several series of blood samples were obtained. At the end of the experiment, the animals were killed and necropsies undertaken in order to evaluate the antioxidant status of various visceral organs.

The results link ischemia and reperfusion injury to a significant decline in antioxidative activity in various tissues. The weakening in antioxidant status after ischemic limb injury was most pronounced in the heart tissue, followed in descending order by the spleen, skeletal muscle, lung, liver, and kidney tissue. The levels of specific antioxidants and reactive oxygen species in various organs changed significantly, and the changes were tissue specific. Endogenous radical scavenging systems were not entirely overwhelmed in most of the tissues studied. But higher levels of malondialdehyde (MDA) found in cardiac tissue suggest that the production of oxygen free radicals is accelerated by an ischemic injury. Based on the study, we believe that the cardiac tissue is particularly susceptible to the effects of ischemia and reperfusion injury. Damage to cardiac tissue is probably the major cause of mortality following acute ischemic injury in a limb.

Keywords: Ischemia, reperfusion, antioxidant status, lipid peroxidation

INTRODUCTION

Oxygen free radicals are believed to play a key role in the cascade of biochemical changes which can lead to cellular damage, organ failure and death following acute ischemia.[1–8] In clinical medicine, ischemia/reperfusion injury arising from successful microvascular anatomoses is sometimes followed by the breakdown of cell membranes, rupture of intracellular organelles and extravasation of fluid into interstitial spaces, leading to edema, microcirculatory collapse and even the delayed failure of the transplanted

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part.[9] Cytotoxic oxygen species include superoxide ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$), as well as various free radical derivatives such as lipid peroxide. There is considerable evidence that H_2O_2 and other peroxides are capable of inflicting the most widespread oxidative damage owing to their high diffusibility and reactivity.[10]

Hydroperoxide-initiated chemiluminescence has been used to detect decreases in endogenous antioxidants in the liver homogenate of ethanol-treated rats.[11] Antioxidant parameters can also be used to assess the antioxidant properties of a plasma sample. The antioxidant defense mechanism in tissue can be used to assess the role of oxygen free radicals in tissue injury during ischemia and reperfusion. Free radicals exert cytotoxic effects on membrane phospholipids and produce lipid peroxides which increase membrane fluidity and permeability, leading to the loss of cell integrity.[12] Malondialdehyde (MDA), a product of lipid peroxidation, can serve as an indicator of free radical generation and provide a means of assessing the cytotoxicity of reactive oxygen species. The concomitant measurement of antioxidant levels and free radical activity in tissues and plasma during an ischemia/reperfusion insult can provide evidence of a systemic change in antioxidant status. In this study, we investigated the antioxidant parameter in plasma and examined free radical and antioxidant activity in the tissue of rabbits subjected to ischemic insult and reperfusion of an isolated hind limb. Specific tests measured the levels of MDA, H_2O_2, and four antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GSH-R) and glutathione peroxidase (GSH-Px).

MATERIALS AND METHODS

Animal Preparation

Twelve New Zealand white rabbits weighing 2.5–3.0 kg were used in this study. The animals were fed Purina Laboratory Chow *ad libitum* and housed in a temperature, humidity, and light-controlled environment. Surgical procedures and experimental protocols were approved and supervised by the Animal Research Committee of the Medical College at National Taiwan University. The rabbits were premedicated with 0.5 mg atropine and then anesthetized with ketamine (25 mg/kg, sc., Sintong, Taiwan, ROC) and Combelen® [N-(3′-dimethyl-aminopropyl)-3-propionylphenothiazine] (5 mg/kg, sc., Bayer AG, Leverkusen, Germany). The operative procedure was quite similar to that reported by Colburn *et al.*,[13] with some modifications. Each animal was positioned supine on an operating board, and a lower abdominal mid-line incision was made. Ischemia of the right hind limb was induced by placing two microvascular clamps (S&T Marketing AG, Neuhausen Am Rheinfall, Switzerland) directly on the right common iliac and external iliac arteries. Collateral circulation from the contralateral hind limb was occluded by microvascular clips (S&T Marketing AG, Neuhausen Am Rheinfall, Switzerland) placed at the iliolumbar; common and internal iliac and common, deep and lateral circumflex femoral arteries on the ischemic leg. Additional clips were applied to the internal iliac and deep femoral arteries of the nonischemic leg. Occlusion of the blood flow to the lower legs was monitored by confirming the absence of pulsation in the femoral artery and the lack of active bleeding after toes were amputated in the pilot study. The left hind limb of each animal served as an internal control and was sham operated but not clamped. The right hind limbs of the experimental rabbits experienced ischemia for 12 h. At the end of the ischemic period, blood flow was restored to the limb by releasing the clamps and observing the pulsation of the external iliac artery. The rabbits were kept under anesthesia (as described) until the end of the experiment. In an earlier study at this institute, 70% of the experimental rabbits died within an eight-hour period following reperfusion.[14] In this experiment, the rabbits were killed four hours after reperfusion began. Six control animals were

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treated the same way, except for the ischemic injury.

Sample Preparation

For all rabbits, whole blood samples were obtained with heparinized plastic syringes immediately before surgery as a control blood sample. Series of blood samples were obtained immediately after 12 h of ischemia, upon reperfusion after the vascular clamps were released, and again at the end of the four-hour post-reperfusion period. The heparinized blood was immediately wrapped with aluminum foil to avoid exposure to light and placed under refrigeration until testing for chemiluminescence was carried out, generally within 2 h. After the rabbits were killed, necropsies of the heart, lung, liver, kidney, spleen and muscle tissue were excised rapidly and immediately frozen in liquid nitrogen. The tissue was kept in liquid nitrogen until homogenates were prepared.

The preparation of tissue homogenates for chemiluminescence testing was a modification of the procedure used by Naito *et al.*[15] The samples were trimmed of fatty tissue, placed in a near-freezing (0–4°) container, minced finely with scissors and suspended in 10 volumes (v/w) of Tris-sucrose buffer (0.25 M sucrose in 20 mM Tris HCl buffer containing 1 mM EDTA at pH 7.4). Homogenization was performed with a Polytron homogenizer (PT-10, Brinkman Instruments, Rexdale, Ontario, Canada) at a setting of 5.5 for two 30 s periods at 0–4°C.

The tissue homogenates were stored frozen at −80°C for enzyme assays or were centrifuged at $400 \times g$ in Beckman Model J2-21 refrigerated centrifuge (4°C) for 30 min. The supernatant was immediately wrapped with aluminum foil and kept under refrigeration until the testing for chemiluminescence, which was usually performed within 2 h.

Determination of Chemiluminescence

The measurement of luminol-amplified tert-butylhydroperoxide-initiated chemiluminescence was similar to that described by Prasad *et al.*,[16] with some modifications. Briefly, 0.2 ml of luminol in a PBS buffer (concentration: 5 mg/L, pH 7.4) was added to 0.4 ml of sample (heparinized blood or homogenate supernatant) in a stainless cell (5 cm in diameter). The mixture was then incubated at 37°C for 10 min. Chemiluminescence was measured in the absolutely dark chamber of a Chemiluminescence Analyzing System (Tohoku Electronic Industrial Co., Sendai, Japan). This system contains a photon detector (Model CLD-110), chemiluminescence counter (Model CLC-10), water circulator (Model CH-20), and a 32-bit IBM personal computer system. The cooler circulator was connected to the CLD-110 photon detector to maintain the temperature at 5°C. The CLD-110 is extremely sensitive, capable of detecting radiant energy as weak as 10^{-15} W, according to the manufacturer's documentation. Photon emission from the whole blood was measured at 10 s intervals at 37°C and atmospheric conditions. At 100 s, 0.1 ml of tert-butylhydroperoxide (t-BHP, Sigma Co., USA) in PBS (pH 7.4) was injected into the cell. The chemiluminescence in the sample was measured continuously for a total period of 1000 s. The total amount of chemiluminescence was calculated by integrating the area under the curve and subtracting it from the background level, which was equivalent to the dark average. The assay was performed in duplicate for each sample and the results expressed as chemiluminescence counts/10 s for blood chemiluminescence and chemiluminescence counts/10 s/mg protein for tissue chemiluminescence. A mean (SE: Standard Error) chemiluminescence level for all samples was also calculated.

Enzyme Measurement

Glutathione (GSH)

The presence of GSH in biological samples was determined by the glutathione-S-transferase assay described by Brigelius *et al.*[17] Briefly, the reaction between chlorodinitrobenzene and GSH

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in a 0.1 M potassium phosphate buffer, pH 7.0, catalyzed by glutathione-S-transferase, is followed at 340 nM. This method is specific for GSH measurement due to the specificity of glutathione-S-transferase for glutathione. Cysteine, homocysteine, or other biological thiols do not serve as sulfur donors.

Superoxide Dismutase (SOD)

SOD activity in the homogenate and supernatant was measured by the method of Sun *et al.*[18] The following reagents were mixed for a 40-tube assay: 40 µl of 0.3 mmol/L xanthine; 20 µl of 0.6 mmol/L diethylene-triaminepenta-acetic acid (DETAPAC); 20 µl of 150 µmol/L nitro blue tetrazolium (NBT); 12 µl of 400 mmol/L sodium carbonate (Na_2CO_3) and 6 µl of bovine serum albumin (1 g/L). The reagents were mixed thoroughly and 2.45 µl of the mixture was placed in each of 40 tubes, followed by 0.5 µl of water in the blank and reagent blank tubes, 0.5 µl of standard SOD in different concentrations (30–300 ng/ml) in the standard tubes, and 0.5 µl of test samples (supernatant of tissue homogenate, approximately 10–250 µg protein) in the sample tubes. Fifty microliters of xanthine oxidase (20 U/ml) were added to all 40 tubes at intervals of 20 s. After the tubes were incubated in a 25° water for 20 min, the reaction was terminated by the addition of 1 ml of 0.8 mmol/L cupric chloride. The production of formazan was measured spectrophotometrically by reading the absorbance at 560 nm. The percent inhibition is calculated as follows, where A is absorbance:

$$\%\ \text{inhibition} = (A_{\text{blank}} - A_{\text{sample}}) \times 100\%/A_{\text{blank}}.$$

The experiment was conducted using test samples with a range of protein concentrations. The data was used to plot a graph with protein concentration as the X axis and % inhibition as the Y axis. One unit of SOD activity is defined as the amount of protein that inhibits reduction of the added NBT by 50%. SOD activity was calculated by comparison with a standard curve.

Catalase

Catalase activity was measured essentially according to the method of Aebi.[19] One hundred microliters of tissue supernatant (200–500 µg protein) was added to 1.9 ml of 50 mM phosphate buffer (pH 7.0) in cuvette and a reaction started with the addition of 1.0 ml freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was determined from the absorbance changes at 240 nm in a spectrophotometer. The activity of the catalase measured was calculated by comparison with a standard curve.

Glutathione Peroxidase (GSH-Px)

GSH-Px activity was measured by the method of Paglia and Valentine as modified by Lawrence and Burk.[20,21] GSH-Px activity of the supernatant was assayed in a 3 ml cuvette containing 2.0 ml of 75 mM phosphate buffer (pH 7.0); glutathione, 50 µl of 60 mM; glutathione reductase, 0.1 ml of 30 U/ml; disodium salt of ethylene diaminetetracetic acid (Na_2EDTA), 0.1 ml of 15 mM; reduced nicotinamide adenine dinucleotide phosphate (NADPH), 0.1 ml of 3 mM; various aliquots of supernatant (200–500 µg protein in 200 µl) and H_2O (0.3 ml). The reaction was started by addition of 0.1 ml of 7.5 mM H_2O_2, and conversion of NADPH to $NADP^+$ was monitored continuously on a spectrophotometer at 340 nm for 3–4 min. The final volume of the reaction mixture in the cuvette was 3.0 ml. The activity of GSH-Px was expressed as nmoles of NADPH oxidized to $NADP^+$ per minute per mg of protein using an extinction coefficient for NADPH at 340 nm of 6220 $M^{-1}\,cm^{-1}$.

Glutathione Reductase (GR)

For the assay of glutathione reductase (GR) activity, the appropriate amount of tissue supernatant was added to 1.0 ml of a reaction mixture containing 0.1 M potassium phosphate buffer, pH 7.4, 1.0 mM EDTA, 0.16 mM NADPH and 1.0 mM GSSG.[22] Blanks were prepared without GSSG. NADPH disappearance was followed

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spectrophotometrically at 340 nm. Results are expressed as mU (nmole NADPH oxidized/min/mg protein) (NADPH extinction coefficient: $6220\,M^{-1}\,cm^{-1}$).

Malondialdehyde (Thiobarbituric Acid-Reactive Substances)

The assay method for malondialdehyde (MDA) was similar to that of Yagi,[23] Prasad and Kalra.[24] Three hundred mg of tissue was added to 10 volumes (3 ml) of Hank's balanced salt solution (HBSS), which has the following composition (pH 7.4): $CaCl_2$, 0.14 g; KCl, 0.4 g; KH_2PO_4, 0.06 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; NaCl, 8.0 g; $NaHCO_3$, 0.35 g; $Na_2HPO_4 \cdot 7H_2O$, 0.09 g; distilled water, 1 L. The samples were homogenized with a Polytron homogenizer (PT-10, Brinkman Instruments, Rexdale, Ontario, Canada) at setting of 5 for two periods of 10 s at 0–4°C, and then centrifuged for 3 min at 3000 rpm. Homogenate (0.2 ml) was added to 2 ml of normal saline in a centrifuge tube and shaken gently. After centrifugation at 3000 rpm for 10 min, 0.5 ml of the supernatant was transferred to another centrifuge tube containing 4.0 ml of 0.083 N sulfuric acid. Half a milliliter of 10% phosphotungstic acid was added and the tube was mixed. After standing at room temperature for 5 min, the mixture was centrifuged at 3000 rpm for 10 min, the sediment was suspended in 2 ml of 0.083 N sulphuric acid and 0.3 ml of 10% phosphotungstic acid, and the mixture centrifuged at 3000 rpm for 10 min. The sediment was suspended in 4 ml of distilled water. One milliliter of TBA reagent (mixture of equal volumes of 0.67% thiobarbituric acid aqueous solution and glacial acetic acid) was added. Then, the reaction mixture was heated for 60 min at 95°C. After cooling, 5.0 ml of n-butanol was added, the mixture was centrifuged at 3000 rpm for 15 min and the n-butanol layer was used for fluorometric measurement at 553 nm with 515 nm excitation. Tetraethoxypropane was used as a standard and the results were expressed as nmol of malondialdehyde equivalents.

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Statistical Analysis

Differences in the results were evaluated using a repeated measurements analysis of variance statistical method. The *post hoc* test was Bonferroni's *t*-test. A student's *t*-test was used to compare the two sets of variables resulting from the biochemical evaluation of the control and experimental tissue samples. Differences were considered statistically significant when P was less than 0.05.

RESULTS

Chemiluminescence Indicates Lower Antioxidant Activity

Luminol-amplified chemiluminescence (CL) testing is a highly sensitive method of measuring the production of oxygen free radicals induced by tert-butylhydroperoxide (t-BHP). The results of luminol-amplified t-BHP-initiated CL testing on the blood samples taken during the course of this experiment are summarized in Table I. Ischemia is associated with a significant increase in the t-BHP-initiated chemiluminescence of blood. The luminol-amplified t-BHP-initiated CL of the blood rose sharply after 12 h of ischemia, and then decreased gradually during the 4 h of

TABLE I Effect of luminol-amplified tert-butyl hydroperoxide t-BHP-initiated whole blood chemiluminescence (CL) following acute ischemic limb injury. The results are expressed as a mean (SE: standard error) of 12 experiments

	Luminol-amplified t-BHP-initiated CL (counts/10 s)
Control	4358.4 (SE: 1760.8)
12 h ischemia	17284.2** (SE: 390.7)
Reperfusion	15517.3** (SE: 499.3)
4 h post-reperfusion	12182.5** (SE: 422.4)
P value	< 0.001

Note
*Significant difference existed between blood sample and control with $P < 0.05$.
**Significant difference existed between blood sample and control with $P < 0.001$.

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TABLE II Effect of luminol-amplified tert-butyl hydroperoxide-initiated tissue chemiluminescence following acute ischemic limb injury. The results are expressed as a mean (SE: standard error)

Chemiluminescence (1000 counts/s/mg protein)	Control ($n=6$)	Experiment ($n=12$)
Heart	178.4 (SE: 13.1)	908.4 (SE: 274.0)
Spleen	32.9 (SE: 2.9)	460.8 (SE: 95.5)
Rt leg	33.5 (SE: 24.9)	374 (SE: 81.2)
Lung	187.6 (SE: 12.9)	521.1 (SE: 89.6)
Lt leg	32.0 (SE: 25.5)	292.6 (SE: 59.8)
Liver	22.5 (SE: 2.5)	185.3 (SE: 16.5)
Kidney	39.2 (SE: 3.8)	151.6 (SE: 66.4)

reperfusion ($P < 0.000$). In the control blood samples obtained before surgery, t-BHP-initiated CL was measured at 4358.4 counts/10 s (with a standard error of 1760.8). In the blood drawn after 12 h of ischemia, just after reperfusion, and four hours after reperfusion, it measured 17284.2 (SE: 390.7) counts/10 s, 15517.3 (SE: 499.3) counts/s, and 12182.5 (SE: 422.4) counts/s, respectively. The results indicate that the ischemic injury diminished antioxidative activity in the blood, while reperfusion of the ischemic tissue tended to bring the values back towards the control level.

Ischemia appears to weaken antioxidative activity in tissue as well as in plasma. The results of t-BHP-initiated CL testing on visceral organs following ischemia/reperfusion injury are summarized in Table II. t-BHP-initiated chemiluminescence of the visceral organ tissue was much higher among the rabbits that had experienced ischemic limb injury. In heart tissue, for example, the control group had a mean chemiluminescence level of 178.4 (SE: 13.1) $\times 10^3$ counts/10 s/mg protein, while the experimental group averaged 980.4 (SE: 274.0) $\times 10^3$ counts/10 s/mg protein. Similar, though smaller, increases in tissue chemiluminescence were observed in the

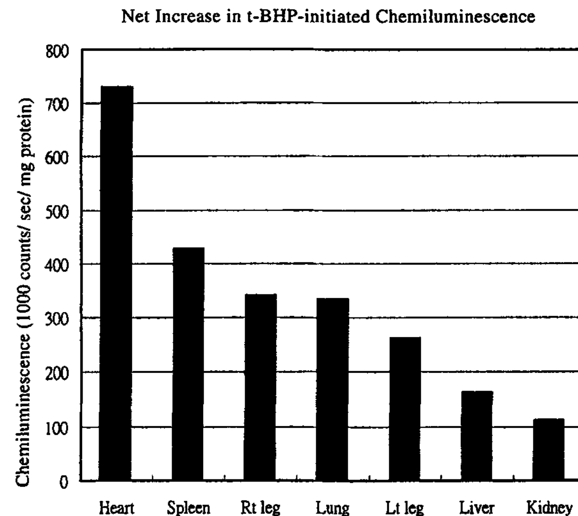

FIGURE 1 Loss of antioxidant activity in the visceral organs of rabbits after 12 h ischemia/reperfusion injury of the hind limb. The changes in t-BHP-initiated chemiluminescence following ischemic limb injury were most obvious in the heart, followed by the spleen, the skeletal muscle, lungs, liver and kidney.

spleen, skeletal muscle, lung, liver and kidney samples (Table II). In all of those cases, the differences in chemiluminescence levels following ischemic limb injury were statistically significant. ($P < 0.00001$). The difference was most pronounced in the heart tissue, followed by the spleen, skeletal muscle, lungs, liver, and kidney tissue (Figure 1).

The levels of superoxide dismutase (SOD), catalase, hydrogen peroxide (H_2O_2), malondialdehyde (MDA), glutathione (GSH), glutathione reductase (GR) and glutathione peroxidase (GSH-Px) in various organs are listed in Table III. The levels of some of these substances changed significantly following ischemic limb injury, although the changes were usually tissue specific rather than systemic.

Superoxide Dismutase, Catalase and Hydrogen Peroxide

Ischemic limb injury did not appear to trigger significant changes in the level of SOD present in various visceral organs. Catalase activity, in

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TABLE III Changes of superoxide dismutase (SOD), catalase, and hydrogen peroxide (H_2O_2) content in various organs after 12 h ischemia/reperfusion insult of hind limb. The results are expressed as the mean (SE: standard error) of 12 experiments

	SOD (U/mg protein)		Catalase (U/mg protein)		H_2O_2 (μM/mg protein)	
	Control	Experiment	Control	Experiment	Control	Experiment
Heart	7.22	7.74	187.71	205.77	926.75	937.84
	(SE: 1.42)	(SE: 1.61)	(SE: 18.96)	(SE: 17.70)	(SE: 115.70)	(SE: 178.86)
Spleen	4.29	5.67	187.05	207.99	1341.04*	1025.21*
	(SE: 0.83)	(SE: 1.06)	(SE: 17.08)	(SE: 20.10)	(SE: 141.29)	(SE: 90.26)
Rt leg	2.12	2.13	426.09**	236.49**	207.28	258.46
	(SE: 0.53)	(SE: 0.37)	(SE: 40.40)	(SE: 35.29)	(SE: 47.47)	(SE: 92.61)
Lung	4.02	4.99	213.41**	273.01**	1115.17	1049.48
	(SE: 1.28)	(SE: 0.98)	(SE: 6.09)	(SE: 15.02)	(SE: 119.32)	(SE: 182.89)
Lt leg	2.17	1.58	424.76	353.09	211.24	299.25
	(SE: 0.55)	(SE: 0.44)	(SE: 39.98)	(SE: 32.68)	(SE: 35.72)	(SE: 71.05)
Liver	12.63	16.66	285.19	333.49	901.59*	1407.07*
	(SE: 2.29)	(SE: 2.84)	(SE: 25.72)	(SE: 25.28)	(SE: 129.09)	(SE: 229.15)
Kidney	11.40	14.69	164.67	202.34	449.70	312.14
	(SE: 2.57)	(SE: 2.29)	(SE: 14.93)	(SE: 25.80)	(SE: 87.92)	(SE: 76.06)

Note
* Significant difference existed between blood sample and control with $P < 0.05$.
** Significant difference existed between blood sample and control with $P < 0.005$.
*** Significant difference existed between blood sample and control with $P < 0.0005$.

TABLE IV Changes in malondialdehyde (MDA) content in various organs 12 h after ischemia/reperfusion insult introduced at hind limb. The results are expressed as the mean (SE: standard error) of 12 experiments

	MDA (nmole/mg protein)	
	Control	Experiment
Heart	4.96*	10.87*
	(SE: 0.46)	(SE: 2.54)
Spleen	4.93	4.49
	(SE: 0.42)	(SE: 0.24)
Rt leg	1.36*	1.01*
	(SE: 0.16)	(SE: 0.06)
Lung	3.33	2.92
	(SE: 0.29)	(SE: 0.14)
Lt leg	0.76	0.64
	(SE: 0.08)	(SE: 0.06)
Liver	1.83	1.66
	(SE: 0.15)	(SE: 0.16)
Kidney	2.20**	1.51**
	(SE: 0.22)	(SE: 0.15)

Note
* Significant difference existed between blood sample and control with $P < 0.05$.
** Significant difference existed between blood sample and control with $P < 0.005$.
*** Significant difference existed between blood sample and control with $P < 0.0005$.

contrast, decreased markedly in the lung tissue and the muscles of the right hind limb, where the ischemic insult was located. The catalase status of the contralateral control muscle (left hind limb) and other visceral tissues did not appear to be greatly affected by the ischemic injury and reperfusion. Hydrogen peroxide levels soared in the liver while falling significantly in the spleen tissue homogenates, as shown in Table III. Its fluctuations in the other tissues were relatively minor.

Malondialdehyde (MDA)

Malondialdehyde is a product of lipid peroxidation, so its presence in the tissue homogenates could serve as an indicator of free radical formation and oxidation activity in the visceral organs. As shown in Table IV, the greatest rise in MDA levels occurred in the cardiac tissue, suggesting that the ischemia/reperfusion insult boosted the activity of free radicals and accelerated lipid peroxidation in the heart ($P < 0.05$). In the kidney tissue homogenate, malondialdehyde content actually declined after the ischemia/reperfusion insult. Other tissue homogenates did not reveal any significant change in the MDA levels.

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TABLE V Changes in glutathione (GSH), glutathione reductase (GR), and glutathione peroxidase (GSH-Px) levels in various organs after 12 h ischemia/reperfusion insult of hind limb. The results are expressed as a mean (SE: standard error) of 12 experiments

	GSH (nmole/mg protein)		GR (mU/mg protein)		GSH-PX (nU/mg protein)	
	Control	Experiment	Control	Experiment	Control	Experiment
Heart	26.09** (SE: 2.10)	47.02** (SE: 6.07)	51.38 (SE: 4.37)	55.27 (SE: 2.52)	80.38* (SE: 18.51)	29.07* (SE: 4.22)
Spleen	23.46*** (SE: 2.87)	51.76*** (SE: 2.86)	42.36 (SE: 4.54)	42.15 (SE: 3.84)	63.41 (SE: 3.01)	57.11 (SE: 5.30)
Rt leg	7.56 (SE: 1.85)	6.45 (SE: 1.98)	5.21 (SE: 0.37)	7.23 (SE: 0.61)	34.85 (SE: 10.17)	19.29 (SE: 3.48)
Lung	17.01** (SE: 2.59)	31.89** (SE: 2.21)	40.89 (SE: 3.05)	45.38 (SE: 3.50)	64.95 (SE: 10.86)	72.80 (SE: 4.30)
Lt leg	5.31 (SE: 1.62)	5.91 (SE: 1.22)	7.50* (SE: 0.71)	12.12* (SE: 1.47)	31.03 (SE: 3.83)	21.86 (SE: 2.69)
Liver	63.71 (SE: 4.77)	73.36 (SE: 9.77)	74.06 (SE: 6.62)	69.23 (SE: 5.10)	490.01 (SE: 61.17)	379.42 (SE: 15.99)
Kidney	28.60 (SE: 3.09)	37.79 (SE: 3.38)	150.80 (SE: 9.96)	217.70 (SE: 22.48)	194.86 (SE: 17.93)	156.91 (SE: 14.18)

Note
* Significant difference existed between blood sample and control with $P < 0.05$.
** Significant difference existed between blood sample and control with $P < 0.005$.
*** Significant difference existed between blood sample and control with $P < 0.0005$.

Glutathione, Glutathione Peroxidase and Glutathione Reductase

The ischemia/reperfusion insults appeared to trigger a significant increase in GSH content in the heart, spleen and lung tissue homogenates (Table V). Glutathione peroxidase and glutathione reductase (GSH-Px and GR) are the two most important antioxidant enzymes in the GSH-GSSG cycle. During the period of ischemia, GSH-Px activity increased in the cardiac homogenate, whereas GR levels climbed most sharply in the kidney and skeletal muscle of the control limb (Table V).

DISCUSSION

Tissue ischemia is a major problem in many clinical situations. The result of hypoxia in the heart and brain is often immediate and catastrophic, but skeletal muscle is considered fairly resistant to long periods of ischemia. In spite of its clinical importance, relatively little is known about systemic responses to acute ischemic injury of skeletal muscle tissue. Experimental work has focused largely on myocardial ischemia, and peripheral ischemia has been examined less frequently.[25] While cardiac ischemia during the peri-operative period is indeed the primary cause of mortality in post-operative surgical patients,[26] the acute interruption of arterial blood flow to the extremities may also be associated with significant morbidity and mortality.[27] Rhabdomyolysis, compartment syndrome, and even circulatory shock can result from skeletal muscle ischemia.[27,28]

The development of microsurgical techniques for the revascularization of severed, replanted limbs and free transplantation of vascularized grafts will expose tissue to long periods of ischemia before successful revascularization can be accomplished.[28] The restoration of the blood supply after a period of ischemia permits the survival of cells not killed by the ischemia, but it may also result in further tissue injury.[29] It is therefore important to understand the mechanisms associated with ischemia–reperfusion

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injury and explore ways to ameliorate their effects. The pathogenic mechanism of tissue damage occurring after ischemia and reperfusion has been a subject of considerable interest in our institute. We have clarified that mortality arising from acute ischemic limb injury is mainly attributable to multiple organ failure, and tissue injury is maximal in areas with the greatest blood flow during reperfusion.[14] However, the biochemical mechanisms operating at the cellular and molecular levels after ischemic injury have not yet been elucidated. The purpose of this study is to delineate biochemical changes in various organs caused by acute limb injury and draw possible correlations to its clinical outcome.

After ischemic insult, blood chemiluminescence (CL) proved to be a valuable quantitative assay for measuring the effects of oxidative stress in the whole blood of rabbits.[30] Tert-butylhydroperoxide (t-BHP), a short chain analog of lipid hydroperoxide, can be metabolized by cytochrome P-450 into free radical intermediates. These can in turn initiate lipid peroxidation, affecting cell integrity and resulting in cell injury.[31] The product of tert-butylhydroperoxide (t-BHP) metabolized by cytochrome P-450 is similar to the product of oxidative stress in cells.[32] t-BHP-initiated CL can be amplified by the use of luminol, greatly increasing the sensitivity of organ chemiluminescence testing. Luminol-dependent CL provides a highly sensitive and continuous method for monitoring the production of oxygen free radicals by t-BHP through its interaction with cell membranes.[33–36] In this study, luminol-amplified, t-BHP-initiated chemiluminescence increased significantly after 12 h of ischemic insult to a hind limb, and decreased gradually during the 4 h reperfusion period (Table I). The results indicate that the effects of ischemia diminished antioxidative activity in the blood, while reperfusion of the ischemic muscle tended to bring the values back towards the control level.

Organ CL is a very sensitive technique and we have taken advantage of this sensitivity to detect tissue injury associated with ischemia and reperfusion. t-butylhydroperoxide has been used by various investigators to generate oxygen free radical and lipid peroxides,[37] and hydroperoxide-initiated CL has detected decreased levels of endogenous antioxidants in tissue homogenate.[11] In this study, the t-BHP-initiated chemiluminescence in all visceral organs increased significantly. The oxidative stress in these organs was thus elevated considerably by the ischemia/reperfusion injury. The levels of t-BHP-initiated tissue chemiluminescence were much higher in the experimental rabbits than in the control group (Table II). The differences were most pronounced in the heart, followed by the spleen, skeletal muscle of the right leg, lung, left leg, liver, and then the kidney (Figure 1). After 12 h of ischemia/reperfusion injury in the hind limb, t-BHP-initiated chemiluminescence had increased to 2.8 times the control level in lung tissue, 3.9 times in the kidney, 5.1 times in the heart, 8.2 times in the liver, 9.1 times in the left leg, 11.2 times in the right leg, and 14.0 times in the spleen. These large-scale increases in organ chemiluminescence could mean that these tissues had exerted a maximal effort to cope with the oxidative stress induced by the ischemia/reperfusion injury. The results could reflect a weakening in the antioxidant defenses of the visceral organs as a consequence of oxidative stress.

Other studies have reported decreases in the activity of muscular antioxidants (SOD, catalase and GSH-Px) during ischemia or hypoxia.[9,38] Our results did not show significant changes in SOD activity in any of the tissues examined, and GSH-Px activity in the skeletal muscles also seemed to be unaffected. This is consistent with the result of our previous study, which also found that an ischemia/reperfusion insult produced no significant pathological change in the skeletal muscle.[14] No explanation can be offered for this result at the present time. Catalase activity did decrease significantly in the muscular tissue of the experimental limb (Table III); while glutathione reductase activity increased significantly

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in muscular tissue of the contralateral control limb (Table V). The decrease in antioxidant defenses during the ischemia/reperfusion insult may result from decreased synthesis. Alternatively, an increase in the amount of oxygen free radicals, which had been reported during ischemia,[30] could exert oxidative stress leading to the exhaustion or inhibition of antioxidant defenses. The results suggest either a partial recovery of antioxidant activity (SOD, GSH-Px) or a complete recovery offset by the increased production of oxygen free radicals. During reperfusion the muscular tissue would use up or inhibit antioxidants (catalase) and the amount present in the tissue would decline.

In the hepatic tissue, only H_2O_2 increased after the ischemia/reperfusion insult in the hind limb (Table III). This is the possible cause of the massive necrosis and nearly complete removal of hepatocytes at the peri-portal areas noted in our previous report.[14] In renal tissue, only glutathione reductase increased significantly during ischemia and reperfusion (Table V). Increased oxidative stress and rising GSSG production may have spurred an increase in the activity of glutathione reductase. This corresponded with characteristic histopathological changes noted in the renal tissue, including marked cellular swelling (hydropic degeneration) with the displaced dark nuclei of renal tubular cells adjacent to the glomerulus.[14]

In the spleen and lung tissue, the GSH titer increased significantly after the insult; and in the lung tissue, catalase activity was also elevated (Table V). This may reflect the infiltration of some inflammatory cells into the respiratory tract.[14] The ischemia/reperfusion injury also bolstered H_2O_2 production in spleen tissue (Table III). The data suggests that acute ischemia/reperfusion injury lead to systemic neutrophil activation which in turn triggers the production of toxic oxygen products. The release of these free radicals and their toxic metabolites results in the destabilization of cell membranes, peroxidation of enzyme and antienzymes, increases in capillary permeability, and changes in vascular reactivity. All of these modifications strongly resemble prominent characteristics of circulatory shock and distant organ injury, increases in capillary permeability, and changes in vascular reactivity.

As noted earlier in this study, no significant alteration of muscular GSH was observed in response to ischemia/reperfusion insult. The GSH titer was also essentially maintained, despite the imposed oxidative stress. However, caution should be exercised in drawing conclusions regarding hepatic GSH status under these experimental conditions, because the liver represents the largest pool of GSH in the body and the rapid efflux of GSH in response to physiological stimuli (such as hormonal signals) is known to occur.[39] Furthermore, *de novo* synthesis of GSH as well as inter-organ transport could be involved; thus confounding the GSH response. The response of the GSH system and the activation of antioxidant enzymes support the concept that ischemia/reperfusion injuries impose oxidative stress on skeletal muscle and other visceral organs.

Moreover, elevated GSH-Px activity was observed in the cardiac tissue, and as reported by Frank and Messaro, this is commonly associated with oxidative stress.[40] The heart is thus the main organ to come under oxidative stress, with decreased antioxidant activity as observed in this study (Figure 1). Our results also indicate that only cardiac tissue showed significant lipid peroxidation after the ischemia/reperfusion insult. The MDA titer in the heart increased significantly after the ischemia/reperfusion insult; while glutathione peroxidase levels decreased and the GSH titer rose in the same way (Table III). Prasad *et al.* recently reported an increase in cardiac MDA levels after ischemia and reperfusion.[16] In this study, the ischemia–reperfusion insult to a hind limb led to higher malondialdehyde content in cardiac tissue (Table IV), suggesting an increase in the production of oxygen free radicals. Highly reactive oxygen metabolites attack membrane phospholipids and act on unsaturated fatty acids to produce lipid peroxides, such as MDA.[12] Oxygen-derived free radicals

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have been implicated in various types of myocardial injury, especially in ischemia/reperfusion injury.[41,42] Different reactive oxygen species have been known to induce negative inotropic effects, contracture, cell damage, greater lipid peroxidation, and depleted high-energy phosphate levels in the myocardium.[43–45] The main targets of reactive oxygen species in myofibrils are the essential SH groups of creatine kinase (CK). Oxidation of these groups would inhibit the enzyme and bring about an alteration of the tissue's contractile properties as a consequence of a decreasing ATP-to-ADP ratio in the myofibrillar component.[46] This is consistent with the finding in our previous report that myocardial creatine kinase (CK-MB) levels were heightened after 12 h of exposure to an ischemia/reperfusion insult.[14] We also suggest that the main cause of mortality following ischemia/reperfusion injury of the hind limb is cardiac tissue injury, followed by a deterioration of the heart's ability to contract. In this study, the H_2O_2 titer in the cardiac tissue did not increase significantly. The particular type of free radical involved in the pathologic mechanism of multiple organ failure is still unknown. Khalid and Ashraf[46] provided evidence of OH– formation by isolated cells during cycles of cell damage and enzyme leakage. Other *in vivo* sources of free radicals and reactive oxygen species may also be involved, such as the very reactive hypochlorous acid that was shown to induce similar effects on skinned fibers at very low concentrations.[47] The possible role played by various sources of free radicals and reactive oxygen species in ischemia/reperfusion injury is now under investigation.

Ischemic limb injury remains a major problem in clinical medicine, and relatively little is known about its systemic effects. In this study, we demonstrated that ischemia in a limb can produce a significant decline in tissue antioxidative activity. The decline of antioxidative activity is most significant in the heart. Although normal levels of different antioxidants and reactive oxygen species persisted in various organs, certain tissue-specific changes in antioxidant status were also recorded. Most importantly, a rise in the malondialdehyde content of cardiac tissue suggests that the production of reactive oxygen species is accelerated following ischemia–reperfusion injury. From this study, we conclude that cardiac tissue is relatively more susceptible to ischemic/reperfusion injury than other types of organ tissue. The main cause of mortality after ischemia/reperfusion injury of the hind limb is probably cardiac tissue injury.

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