

Antioxidant Enzymes and Lipid Peroxidation in Cold Ischemic Lung Preservation

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Abstract

Objectives: Our purpose was to investigate antioxidant enzymes and lipid peroxidation in time course ischemic lung preservation in rats.

Materials and Methods: Thirty-six Wistar rats were divided into 6 groups of 6 rats each. After having been anesthetized, the rats were intubated and connected to a rodent ventilator. Lung-heart blocks were excised. In the control group, the lungs were immediately stored at -80°C after removal. The lungs from the other groups were preserved in 40 milliliters of low potassium dextran solution at 4°C for 6, 12, 24, 48, and 72 hours, respectively. Antioxidant enzyme activity and malondialdehyde levels were then measured.

Results: Superoxide dismutase activity significantly increased at the 12th hour and remained higher up to the 72nd hour ($P < .001$). Glutathione peroxidase activity was higher than that in the control group from the 6th to the 24th hour but was significant only at the 12th hour ($P < .001$) and decreased below the level in the control group after the 48th hour. Catalase activity was significantly higher than that in the control group in all preservation periods ($P < .001$). The nitric oxide level slowly increased and reached a significantly higher level than that in the control group at the 24th and 72nd hours ($P = .028$) and then decreased to the level found in the control group. The malondialdehyde level slightly increased from the 6th to the 24th hour, but that increase, when compared with the level in the

control group, had no statistical significance ($P = .110$).

Conclusions: In ischemic lung preservation, oxidative stress begins during the early phase of preservation and continues for up to 72 hours. Although oxidative stress continues for a significant period, an antioxidant mechanism adequately prevents its harmful effects on the lung. Thus no significant lipid peroxidation occurred in long-term ischemic lung preservation in the murine model studied.

Key words: Transplant, Lung preservation, Oxidative stress, Antioxidant enzyme, Lipid peroxidation, Rat

Introduction

Oxidative stress in the lung differs from that in other organs because of the anatomic characteristics of the lung. Ischemia, which is the interruption of blood flow, does not cause anoxia in the lung because adequate oxygen is supplied from residual air in the alveoli. However, it has been reported that ischemia can cause lipid peroxidation and oxidative stress in the lung (1). Those conditions are explained by the mechanotransduction hypothesis (2), according to which endothelial cells are highly sensitive to physical forces resulting from blood flow variations and can transform those mechanical forces into electrical and biochemical signals. The absence of the mechanical component of flow during lung ischemia stimulates the membrane depolarization of endothelial cells via the activation of nicotinamide adenine dinucleotide phosphate oxidase, nuclear factor-kappa beta, and calcium/calmodulin-dependent nitric oxide synthase.

The endothelium is one of the main sources of reactive oxygen species generation. Other cells (eg, macrophages and/or margined neutrophils, which

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have a high level of nicotinamide adenine dinucleotide phosphate oxidase activity) also contribute to the lung oxidant burden that occurs during ischemic storage (1). The purpose of this study was to investigate antioxidant enzyme activity and lipid peroxidation in lung tissue in time course cold ischemic preservation.

Materials and Methods

This study was approved by the ethics committee of Gaziosmanpasa University. All animal subjects received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* (3).

Thirty-six male Wistar rats (weight range, 280-320 g) were divided into 6 groups of 6 rats each. After having been anesthetized with intraperitoneal thiopentone 30 mg/kg, rats were intubated via tracheostomy and were connected to a rodent ventilator (Harvard instrument, MA, USA). All rats were maintained via ventilation with room air (positive end-expiratory pressure, 4 cm H₂O; frequency, 60 bpm). Heparin 50 IU was administered through the penile vein. Five minutes later, a laparosternotomy was performed. The inferior vena cava and the right and left atrial appendages were cut, and the lung was flushed through the pulmonary artery with 20 mL of cold Perfadex solution at a pressure of 20 cm H₂O. The trachea was tied, the lungs were inflated, and the lung-heart block was removed. In the control group, the lungs were stored immediately after removal at -80°C. The lungs from the other groups were preserved in 40 mL of Perfadex solution and were stored at 4°C for 6, 12, 24, 48, and 72 hours, respectively, and then stored at -80°C.

Preparation of lung tissue homogenate

Lung tissues were stored at -80°C until analysis. After the lung tissues had been weighed, they were homogenized in 5 volumes of ice-cold tris-HCl buffer (50 mM, pH 7.4) containing 0.50 mL/L of Triton X-100. Homogenization was performed for 2 minutes at 13 000 rpm with the Ultra-Turrax T25 basic, (IKA, Staufen, Germany). All procedures were performed at 4°C. Homogenate, supernatant, and extracted samples were prepared, and the following analyses were performed on the samples with commercial chemicals supplied by Sigma (St. Louis, MO, USA).

Protein measurements were determined in the samples according to the method of Lowry and colleagues (4).

Biochemical analyses

Total copper-zinc superoxide dismutase (EC 1.15.1.1) activity was determined as described by Sun and colleagues (5). The principle of that method is based on the inhibition of nitroblue tetrazolium reduction by the xanthine-xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the supernatant after 1.0 mL of ethanol-chloroform mixture (5:3, v/v) had been added to the same volume of sample and centrifuged. One unit of superoxide dismutase was defined as the amount causing a 50% inhibition of the nitroblue tetrazolium reduction rate. The superoxide dismutase activity is expressed as Umg⁻¹ protein. Glutathione peroxidase activity was measured according to the method of Paglia and Valentine (6). The enzymatic reaction in the tube containing nicotinamide adenine dinucleotide phosphate, reduced glutathione, sodium azide, and glutathione reductase was initiated by the addition of H₂O₂, and the change in absorbance at 340 nm was monitored with a spectrophotometer. Activity is expressed in units per gram of protein. Catalase activity was determined according to the method of Aebi (7). The principle of that method is based on the determination of the rate constant *k* (s⁻¹) of the H₂O₂ decomposition at 240 nm. The results are expressed as kg⁻¹ protein. All samples were assayed in duplicate.

The tissue thiobarbituric acid-reactive substance level was determined by the thiobarbituric acid test reaction (8), in which malondialdehyde or malondialdehydelike substances and thiobarbituric acid react at 90°C to 100°C to produce a pink pigment with an absorption maximum at 532 nm. The reaction was performed at a pH of 2 to 3 at 90°C for 15 minutes. The sample was mixed with 2 volumes of cold 10% (w/v) trichloroacetic acid to precipitate the protein. The precipitate was pelleted by centrifugation, and an aliquot of the supernatant was combined with an equal volume of 0.67% (w/v) thiobarbituric acid in a boiling-water bath for 10 minutes. After the mixture had cooled, the absorbance was 532 nm. The results were expressed as nanomoles per gram of wet tissue, according to

Table 1. Statistical analyses of data from the comparison of groups. The nonparametric Kruskal-Wallis test was used for comparisons.

Study Periods	SOD [†]	GPX [†]	CAT [‡]	NO [§]	MDA [§]
Control group	46 ± 5	3.022 ± 0.634	0.055 ± 0.014	0.063 ± 0.003	1.292 ± 0.077
6 h	47 ± 5	3.321 ± 0.623	0.110 ± 0.006	0.065 ± 0.010	1.377 ± 0.167
12 h	63 ± 6	4.347 ± 0.980	0.140 ± 0.025	0.069 ± 0.006	1.427 ± 0.141
24 h	75 ± 8	3.088 ± 0.290	0.155 ± 0.016	0.070 ± 0.004	1.327 ± 0.183
48 h	65 ± 12	2.174 ± 0.565	0.167 ± 0.009	0.070 ± 0.005	1.190 ± 0.208
72 h	65 ± 11	1.882 ± 0.493	0.240 ± 0.026	0.060 ± 0.004	1.189 ± 0.148
P value	< .001*	< .001*	< .001*	.028*	.110

*Statistically significant.

Abbreviations: CAT, catalase; GPX, glutathione peroxidase; MDA, malondialdehyde; NO, nitric oxide; SOD, superoxide dismutase.[†] Unit per gram of protein.[‡] Kilograms per gram of protein.[§] Nanomoles per gram of wet tissue.

the standard graphic prepared from measurements with a standard solution (1,1,3,3-tetramethoxypropane).

Measuring nitric oxide in biological specimens is very difficult; therefore, tissue nitrite and nitrate levels were estimated as an index of nitric oxide production. Samples were initially deproteinized with Somogyi reagent. The total nitrite (nitrite plus nitrate) level was measured with a spectrophotometer at 545 nm after conversion of nitrate to nitrite by copperized cadmium granules. A standard curve was established with a set of serial dilutions (10^{-8} to 10^{-3} mol/L) of sodium nitrite. The point from the nitrite standard was used to perform linear regression. The resulting equation was then used to calculate the unknown sample concentrations. Results are expressed as nanomoles per gram of wet tissue (9).

SPSS software (Statistical Product and Service Solutions, pocket program version 15.0, SSPS Inc, Chicago, IL, USA) was used for statistical evaluation. Data are expressed as the mean ± SD. Intergroup comparisons were calculated with the nonparametric Kruskal-Wallis test, and post hoc comparisons were performed with the Mann-Whitney *U* test. A *P* value less than .05 was considered statistically significant.

Results

The superoxide dismutase level had significantly increased at the 12th hour and remained elevated up to the 72nd hour ($P < .001$). The glutathione peroxidase level was higher than that in the control group from the 6th to 24th hours but was significant only at the 12th hour ($P < .001$) and decreased after the 48th hour to a level below that in the control group. The catalase level was significantly higher than that in the control group in all preservation

periods ($P < .001$). The nitric oxide level slowly increased, reached a level significantly higher than that in the control group at the 24th and 72nd hours ($P = .028$), and then decreased to the level of the control group. The malondialdehyde level increased slightly from 6th to 24th hours, but that increase, when compared with the level in the control group, was not statistically significant ($P = .110$). The data from the statistical analyses are shown in Tables 1 and 2.

Table 2. *P* Values of multiple comparison in the time periods studied.

Study Periods	SOD	GPX	CAT	NO	MDA
Control vs 6 h	.818	.485	.002*	1.000	.310
Control vs 12 h	.004*	.041*	.002*	.065	.132
Control vs 24 h	.002*	.937	.002*	.026*	.394
Control vs 48 h	.009*	.026*	.002*	.041*	.589
Control vs 72 h	.009*	0.009*	.002*	.180	.240
6 h vs 12 h	.004*	.093	.065	.394	.699
6 h vs 24 h	.002*	.699	.002*	.310	.818
6 h vs 48 h	.015*	.015*	.002*	0.310	.132
6 h vs 72 h	.009*	.002*	.002*	.495	.093
12 h vs 24 h	.026*	.041*	.310	.699	.589
12 h vs 48 h	.818	.002*	.065	.699	.093
12 h vs 72 h	.589	.002*	.002*	.015	.015*
24 h vs 48 h	.132	.004*	.240	.937	.310
24 h vs 72 h	.240	.002*	.002*	.009*	.132
48 h vs 72 h	.937	.485	.002*	.009*	.937

*Statistically significant.

Abbreviations: CAT, catalase; GPX, glutathione peroxidase; MDA, malondialdehyde; NO, nitric oxide; SOD, superoxide dismutase.

Discussion

In normal metabolic process, reactive oxygen species generation and antioxidant defense mechanisms are in balance. Any disturbance in the prooxidant-antioxidant systems is considered oxidative stress, which can cause damage to lipids, proteins, carbohydrates, and nucleic acids. Consequently, cellular enzymes, structural proteins, simple and complex sugars, deoxyribonucleic acid, and ribonucleic acid are susceptible to oxidative damage (10).

Antioxidant defense systems are usually classified as indirect enzymatic antioxidant enzymes or small-molecular-weight molecules that scavenge free radicals and related reactants. The antioxidant enzymes act as a first line of defense against those toxic reactants by metabolizing them into innocuous byproducts (11). Superoxide dismutase, which causes superoxide breakdown and the subsequent production of hydrogen peroxide, has a central role regulating reactive oxygen species levels (10). Yang and Block (12) reported that vascular smooth muscle cells, pulmonary endothelial cells, and lung macrophages have been shown to generate superoxide under basal and stimulated conditions. Three isoforms of superoxide dismutase have been found in the lung: copper-zinc superoxide dismutase, manganese superoxide dismutase, and extracellular superoxide dismutase. Copper-zinc superoxide dismutase, which is particularly associated with pulmonary endothelial and vascular smooth muscle cells, is found in cytoplasm (13). In this study, we measured copper-zinc superoxide dismutase enzyme activity. In hypoxic conditions, superoxide dismutase activation initially decreases (14). After 48 hours of hypoxia, superoxide dismutase activity increases by increasing xanthine oxidase activity in the pulmonary artery endothelial cells (14). In our study, the enzyme activity increased after the 12th hour and remained elevated until the 72nd hour of lung preservation. Superoxide dismutase activity did not decrease during the early period of lung preservation as it did in hypoxic conditions. It is likely that ischemia immediately triggered superoxide dismutase activity through the mechanotransduction mechanism, but in our study, that increased activity reached a significant level at the 12th hour.

Glutathione peroxidase, which is also a tetramer and an 85 000-Dalton protein containing selenium, uses glutathione as a cosubstrate. Glutathione peroxidase is a cytosolic enzyme that also eliminates H_2O_2 but has a wider range of substrates (including lipid peroxides) than does catalase. The kinetics of glutathione peroxidase is very complex, but that enzyme is thought to have a greater affinity for H_2O_2 than catalase does. The primary function of glutathione peroxidase is that of detoxifying low levels of H_2O_2 in cells (15,16). Glutathione peroxidase activity may be selenium dependent or independent (17). In our study, glutathione peroxidase activity reached its maximum level at the 12th hour and then

gradually decreased. After the 48th hour, the enzyme level decreased to a value lower than that at baseline. We suggest that this decrease may have been caused by a decrease in the level of selenium and/or glutathione in the tissue because of the increased consumption of them. Although to our knowledge no data on selenium levels in preserved lung are available, an increased glutathione consumption that occurred in preserved lung tissue was reported by Pincemail and colleagues (18).

Catalase is a homotetrameric heme-containing enzyme that catalyzes the conversion of hydrogen peroxide into water and oxygen with one of the highest turnover rates known in enzymology (19). Catalase is a H_2O_2 -scavenging enzyme with optimal activity at high H_2O_2 concentrations. In the lung, catalase is found primarily in alveolar macrophages and the alveolar epithelium (20). That enzyme is relatively constitutive, and no major induction of catalase by cytokines or oxidants in the lung has, to our knowledge, been reported (21). In our study, the level of catalase progressively increased in all preservation periods. That continuous increase showed that the gradual increases in oxidative stress and H_2O_2 generation were continuing at the 72nd hour of ischemic lung preservation.

Nitric oxide, a stable free radical, is a potent regulator of vascular tone in systemic and pulmonary vessels and has an important role in cellular signaling and respiration. Nitric oxide is both an oxidant and an antioxidant (10). Endothelial cells, which contain endothelial nitric oxide synthase and inducible nitric oxide synthase isoforms, respond with an increase in cytosolic Ca^{2+} and the generation of reactive oxygen species when the flow is terminated but oxygenation is maintained. An increased level of intracellular Ca^{2+} activates endothelial nitric oxide synthase, which results in an increase in nitric oxide generation. It has been suggested that endothelial nitric oxide synthase causes endothelial nitric oxide generation. Endothelial nitric oxide synthase requires increased levels of Ca^{2+} , calmodulin binding, and protein kinase B/Akt phosphorylation to enable activation in oxygenated ischemia (22). In our study, nitric oxide generation increased slightly up to 48 hours and then decreased to a level lower than that at baseline. We suggest that this decrease may have resulted from the decrease in intracellular Ca^{2+} levels, although no data support that hypothesis.

Oxygen radicals react with polyunsaturated fatty acid residues in phospholipids, and malondialdehyde is one of the final products of that reaction in the cells. An increase in free radicals causes the overproduction of malondialdehyde, the level of which is a marker for oxidative stress. In our study, there was no statistically significant increase in the malondialdehyde level, although the activity of antioxidant enzymes increased. We suggest that in our study, antioxidant enzymes destroyed reactive oxygen species and that oxidative stress was not severe enough to create cell damage. However, more detailed data will be provided by further studies.

In conclusion, in ischemic lung preservation, oxidative stress begins at the early phase of the preservation and continues for up to 72 hours. Oxidative stress causes antioxidant enzyme activity to increase significantly, and some enzyme levels (superoxide dismutase, catalase) remain elevated at the 72nd hour of preservation. Although oxidative stress persists in the preserved lung, an antioxidant mechanism adequately prevents its harmful effects on cells. Thus, no significant lipid peroxidation occurs during long-term ischemic lung preservation.

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