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THE BIOLOGICAL REDUCTIVE CAPACITY OF TISSUES IS DECREASED FOLLOWING EXPOSURE TO OXIDATIVE STRESS: A CYCLIC VOLTAMMETRY STUDY OF IRRADIATED RATS

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The reductive capacity of rat tissue homogenates and body fluids was determined by cyclic voltammetric measurements. The reductive capacity of rat lung, liver and kidney homogenates was significantly reduced four days after total body γ-ray irradiation with 5.5 Gy as compared to controls. In parallel, reduced ability of the irradiated organ homogenates to scavenge hydroxyl radicals and to destroy hydrogen peroxide was recorded. However, no difference in their superoxide dismutase activity was found. The possible use of cyclic voltammetry as a method for qualitative evaluation of the ability of biological tissues to cope with oxidative stress is discussed.

KEY WORDS: Reducing agents, cyclic voltammetry, antioxidant, irradiation, oxidative stress, measurement.

INTRODUCTION

Characterization of antioxidant activity in biological samples may be of special importance for understanding many pathological processes in which oxidative stress is involved. The broad definition of antioxidants includes various compounds which enhance the tissues capacity to cope with different types of oxidative stress.[1] Halliwell[2] suggested that normally, such compounds must be present at low concentrations compared to those of an oxidizable substrate in order to serve as an antioxidant. In mammalian systems a wide variety of antioxidants have been characterized.[3] They include protective enzymes such as catalase, superoxide dismutase, glutathione peroxidase and others, small molecules which are synthesized in the body and other antioxidant molecules derived from the diet.[4] These antioxidants may function through different mechanisms, which include blocking free-radical formation, removing oxidants from biological targets, reacting with reactive species (chemical scavengers), thus sparing the biological targets, transforming a reactive species into a nonreactive one, stabilizing membranes and acting indirectly by removing mediators that could catalyze free-radical damage.[1] In recent years it has been suggested that many pathological states are a result of insufficient defence against reactive oxygen metabolites. Evaluation of antioxidant activity of biological fluids and tissues is,

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therefore, of great importance.[5] Most of the common procedures used to evaluate the ability of an organ, tissue or biological fluid to cope with oxidative stress provide only a partial view of the antioxidant capacity of the sample under investigation.[6,7] These various methods include the quantitation of specific levels of antioxidants such as: uric acid,[8] bilirubine,[9] carnosine and its derivatives,[1] ascorbic acid,[10] and tocopherol,[11] or the detection of their metabolites following oxidative stress. Other methods deal with the general capacity of an organ, tissue, cell or biological liquid to interfere with reactive oxygen species such as hydroxyl radicals, superoxide radicals and hydrogen peroxide. The data obtained from these various methods contributes to the evaluation of the total antioxidant capacity. Melino *et al.*[12] suggested a method for evaluating the antíoxidant capacity of cells based on the rate of oxidation of 2′,7′-dichlorofluorescin diacetate. However, this method measures the antioxidant ability only against hydrogen peroxide.

By definition, every reducing agent in the cell may act as an antioxidant *via* its ability to donate an electron to the oxidizing agent and by doing so, blocking the oxidative process.[13] Rossum[14] suggested that the measurement of the reducing potential of biological tissues, may indicate its contribution to the oxy-radical tissue damage.

In the present study we suggest the use of the cyclic voltammeter instrument for evaluating the ability of tissue homogenate or biological fluid to cope with oxidative stress induced by γ-irradiation. The damage induced to biological targets by ionizing radiation is mediated, in part, by oxygen free radicals. It is suggested that biological reductive capacity (BRC) is well correlates to some of the antioxidant quality of the tissues under study.

MATERIALS AND METHODS

Unless otherwise specified all the chemical reagents used in this study were purchased from Sigma Chemical Co., St. Louis, Mo, USA.

Animals

Sabra Rats, 3 months old, weighing about 250 g were used throughout the experiments. They were handled according to the regulations regarding the use of experimental animals and were fed ad libidum.

Total body irradiation of rats

Total body irradiation of rats was performed using a ^{60}Co γ-source. The rats were restricted to the center of the γ-source in which the dose distribution did not divert by more than 5%. A dose of 5.5 Gy was delivered at a dose rate of 22 Gy/min. Four days later, the irradiated animals were sacrificed. The lung, kidney and liver were removed, weighed, homogenized, and analyzed for protein content. The reductive capacity of these tissue homogenates was recorded. The fluid samples, cerebral spinal fluid (CSF) and whole blood, were freshly collected and analyzed for their protein content and their cyclic voltammetry (CV) potentials. When the samples were frozen in $-20°C$ for a month and reanalyzed, no significant changes in their CV potentials was recorded.

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Skin, brain, the intestinal epithelium, kidney, liver and lung were also examined. The natural reducing ability of these tissues was recorded. The samples homogenates were diluted 1:1 vol:vol with 0.01 M phosphate buffer (pH 7.2) and measured for both their protein content and CV potentials. Each experiment was carried out with at least 3 repetitions.

Cyclic voltammetry measurements

The cyclic voltammetry measurements (CV) were carried out using a BAS (West Lafayette, IN) cyclic voltammetry apparatus model CV-1B modified for a 250-μl cell volume. All cyclic voltammograms were performed between +0 and +2.0 volt. The measurements were carried out at 37°C in 0.01 M phosphate buffer (pH 7.2). A three electrode system was used throughout the study. The working electrode used was a glassy carbon disk (BAS MF-2012, Bioanalytical Systems), 3.2 mm in diameter. The counter electrode used was a platinum wire and the reference electrode was a Ag/AgCl (BAS). The working electrode was polished before each measurement with a polishing kit (BAS PK-1, Bioanalytical Systems, Inc.).

Hydroxyl radical scavenging ability

Tissue homogenates and fluids were tested for their ability to scavenge hydroxyl radicals. The analysis was performed using a modification of the method described by Halliwell *et al.*[15] The hydroxyl radicals were induced by adding the combination of ascorbic acid (0.1 mM) and copper ions (20 μM), or hydrogen peroxide (10 mM) and EDTA (100 μM) chelated ferrous ions (1 mM). The reaction mixture (2 ml) contained sodium salicilate (25 mg) and 100 μl of the tested homogenate in phosphate buffer (50 mM, pH 7.4). The reaction was stopped by the addition of 80 μl of concentrated HCl, 125 μl of 10% TCA, 250 μl of 10% sodium tungstate and 250 μl of 5% $NaNO_2$. The reaction mixture was centrifuged at 18,000 g for 2 minutes. The hydroxylation product 2,3-dihydroxybenzoic acid was monitored using a Kontron model 930 spectrophotometer. The absorption at 378 nm inversely correlates with the degree of protection affected by the homogenates against hydroxyl radicals. In separate control experiments, homogenates were incubated with salicylate, without the addition of ascorbic acid. This was done in order to measure the levels of non-enzymatic and enzymatic hydroxylations by endogenous agents. The results obtained were subtracted from these obtained in the experiments described above.

In order to exclude the possibility that the homogenates remove hydrogen peroxide, the samples were heat inactivated (100°C, 5 min) and were subsequently tested for their hydroxyl radical scavenging ability. No difference in the results between the heat inactivated and native homogenates were recorded.

Superoxide dismutase activity in the homogenates

In order to measure the changes in the level of the protective enzymes following exposure to the ionizing irradiation, we have monitored the activity of catalase and superoxide dismutase in the tissues homogenates. Superoxide dismutase activity was measured as described by McCord and Fridovich.[16] In brief the reaction mixture contained cytochrome c (3 mM), 33 μl tissue homogenate, xanthine (1 mM) in phosphate buffer (50 mM, pH 7.8) and diethylenetriaminepentaacetic acid (detapac)

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(0.1 mM) at a total volume of 1 ml. The reaction was carried out at 37°C and was initiated by adding the enzyme xanthine oxidase. The absorption of ferro cytochrome c was followed at 550 nm.

Catalatic ability

The ability of the examined tissues to decompose hydrogen peroxide was tested as described elsewhere.[17] In brief tissue homogenates (100 μl) were added to hydrogen peroxide (0.4 mM) in phosphate buffer (50 mM, pH 7.4) and were incubated for 5 min. The reaction was terminated by the addition of 100 μl TCA 35%, 50 μM $Fe(NH_4)_2(SO_4)_2$ (80 mg/5 ml) and 100 μM of NaSCN. The color produced was recorded at 480 nm.

RESULTS

The cyclic voltammetry (CV) chromatograms obtained were found to be specific and characteristic for each of the biological fluids and tissue homogenates examined (Figure 1a–d). Panels a, b, c and d show CV recorded for normal rat CSF, whole blood, lungs and skin, respectively. Each of these chromatograms have at least one potential wave indicating the ability of the sample to act as a reducing agent. The data on the reductive capacity of various fluids and tissues are summarized in Table I.

Rats exposed to γ-irradiation were used. It was found that following total body irradiation of 5.5 Gy, the anodic peaks shown in the CV were shifted to higher potentials as presented in Figure 2. The potential for lung homogenate which was found to be 708 ± 40 mV prior to the exposure to irradiation, was elevated to 798 ± 9 mV following this stress. All the changes were significant $p < 0.005$ (by Mann-Whitney test). The potential recorded for liver homogenate was 690 ± 18 mV prior to the exposure and increase to 786 ± 19 mV, following irradiation ($p < 0.005$). The potentials recorded for kidney were 708 ± 47 mV and 796 ± 33 mV respectively ($p < 0.005$). The increase in the potentials recorded in all the tissues tested suggests that irradiation selectively eliminated reducing equivalents with lower potentials, but did not affect other compounds with higher reducing potentials in the samples tested.

The antioxidant activity of the tissues was measured by recording their ability to scavenge hydroxyl radicals, to dismute superoxide radicals and to decompose hydrogen peroxide, prior to and following γ-irradiation. The results indicate a significant loss of the capacity of the kidney, liver and lung homogenates to react with hydroxyl radicals (Figure 3), and a loss of their capacity to decompose hydrogen peroxide (Figure 4). However, no change in superoxide dismutase activity was observed (Figure 5).

DISCUSSION

This study presents a novel method for the evaluation of the contribution of reducing equivalents to the antioxidant activity of biological samples. The cyclic voltammograms of various low molecular weight antioxidant compounds such as uric acid, carnosine,[1] and bilirubine,[9] show an anodic peak at the region of positive potential. Therefore CV, which measures the reductive capacity of biological tissues

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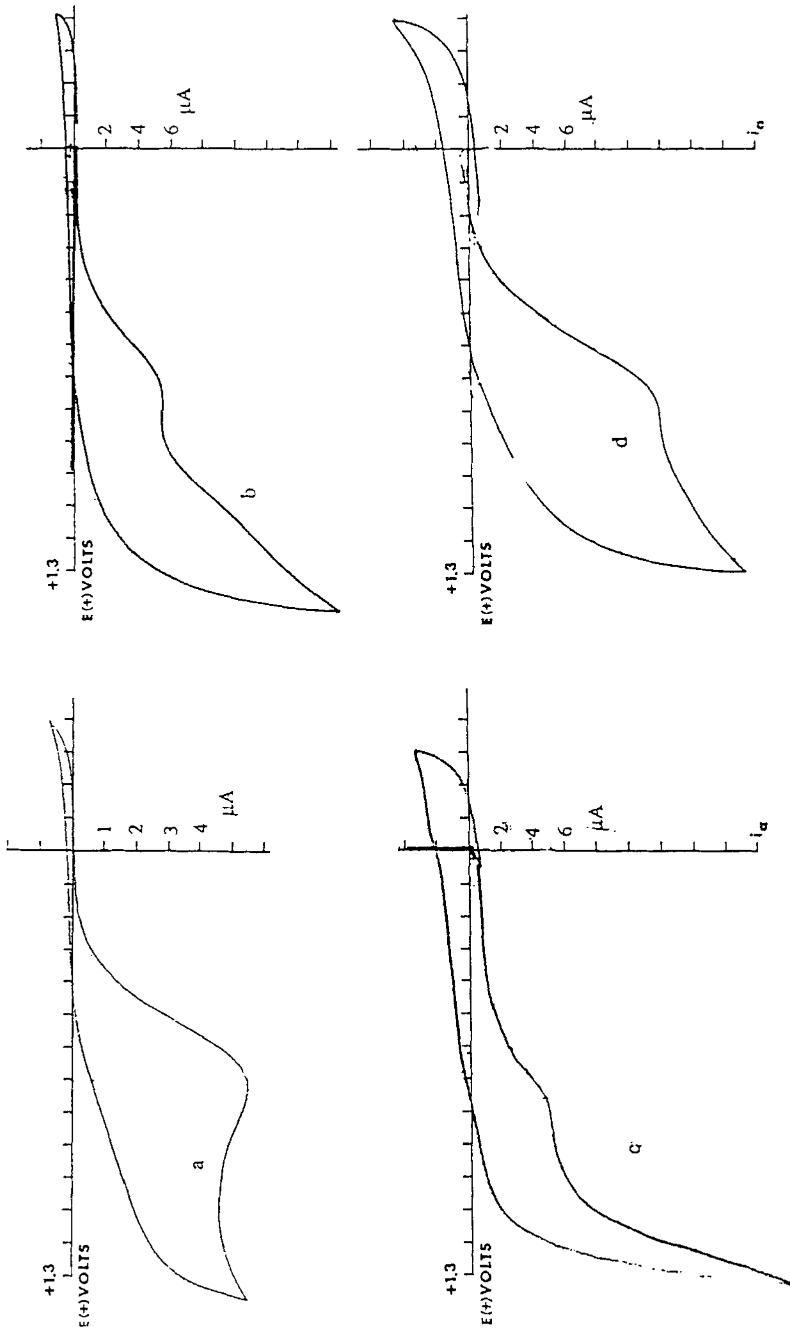

FIGURE 1 Cyclic voltammograms (CV) of tissue homogenates and biological fluids. The CV chromatograms were performed using the BAS cyclic voltammeter equipped with a glassy carbon working electrode as described in the Materials and Methods. a—Rat cerebral spinal fluid; b—rat whole blood; c—rat lungs homogenate; d—rat skin homogenate.

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TABLE I
The CV potentials ($E_{1/2}$) recorded for rats tissue homogenates and fluids from control

Source of biological sample	CV (mV)
CSF	490 ± 75
Whole blood	690 ± 30
Skin	640 ± 45
Intestinal epithelium	700 ± 30
Brain	580 ± 20
Lung	690 ± 18
Kidney	708 ± 40
Liver	682 ± 47

The samples were analyzed as described in Materials and Methods.

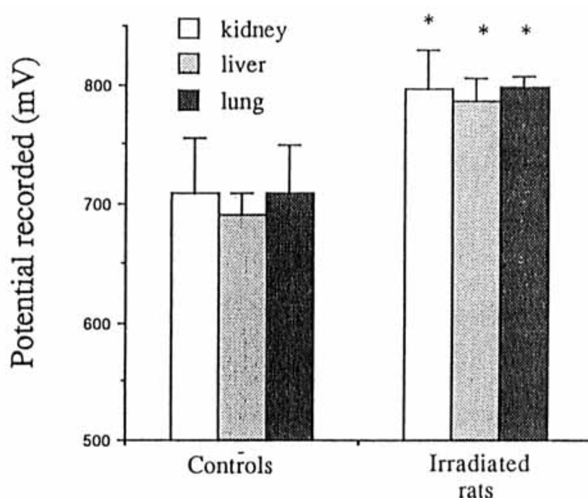

FIGURE 2 The potential recorded ($E_{1/2}$) by cyclic voltammetry of rat tissue homogenates. The animals were sacrificed four days following exposure to γ-radiation (5.5 Gy). The homogenates were prepared and analysis were performed as described in Materials and Methods. *Significant difference, ($p < 0.005$) by Mann-Whitney test.

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or fluids, may indicate the ability to donate an electron(s). This ability governs the antioxidant activity of the entire tissue.

The reductive capacities of most tissues and body fluids tested decreased following exposure to oxidative stress (Table I, Figures 1, 2). The prolonged effect of irradiation, which persisted for four days after the exposure, was in parallel with a significant reduction in the ability of the tested sample to scavenge hydroxyl radicals (Figure 3), or to decompose hydrogen peroxide (Figure 4). Nevertheless, no significant change in the ability of the sample to dismute superoxide radicals was noticed (Figure 5). Since most of the hydroxyl radical scavengers are reducing agents, the correlation

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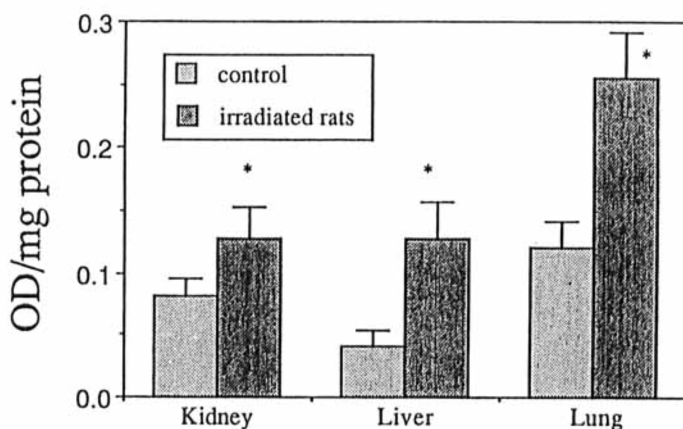

FIGURE 3 Scavenging capacity of hydroxyl radical in rat tissue homogenates prior to and four days following total body irradiation of 5.5 Gy. The tissue homogenates were prepared and analyses were performed as described in Materials and Methods. *Significant difference, ($p < 0.005$) by Mann-Whitney test.

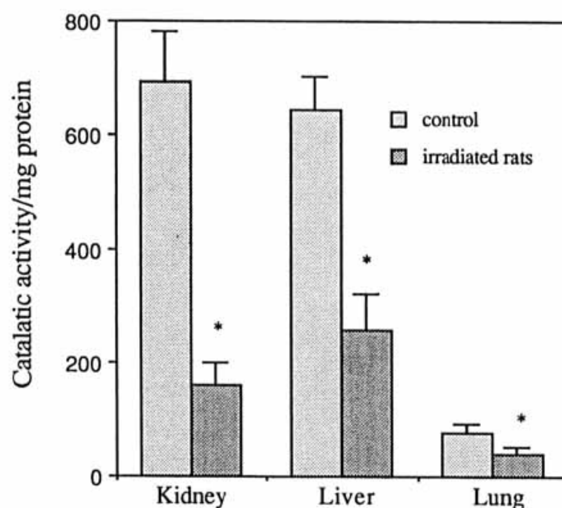

FIGURE 4 The capacity of rat tissue homogenates to decompose hydrogen peroxide prior to and four days following total body irradiation of 5.5 Gy. The cell homogenates were prepared and analyses were performed as described in Materials and Methods. *Significant difference, ($p < 0.005$) by Mann-Whitney test.

between the observed decrease in the reducing capacity of the exposed tissues and the demonstrated decrease in the hydroxyl radical scavenging capacity can be easily explained. The hydrogen peroxide removing system includes the enzymes, catalases and glutathione peroxidase. Catalase requires reducing equivalents for its activity. Glutathione peroxidase requires for its action the glutathione molecule (a known reducing agent) and other reducing agents [NAD(P)H] for its recycling mechanism.

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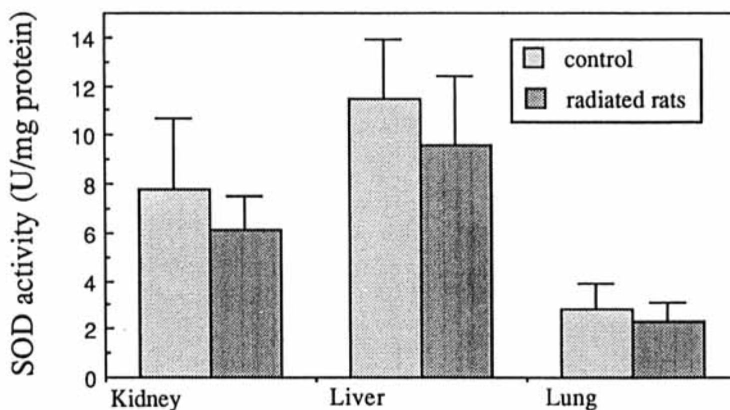

FIGURE 5 Superoxide dismutase activity of rat tissue homogenates prior to and four days following total body irradiation of 5.5 Gy. The cell homogenates were prepared and analyses were performed as described in Materials and Methods. The differences were found to be non significant.

Decreased levels of these compounds will result in a loss of the enzymes activity. Therefore, a decrease in the reducing capacity of the tissues following exposure to oxidative stress, will result in a decrease in the hydrogen peroxide removing ability, and will be reflected in the CV measurements. The results obtained support this explanation (Figures 2, 4). The observed conservation of the ability to dismute the superoxide radical, following irradiation, may result from the relatively high stability of the superoxide dismutase and the independence of its activity on reducing equivalents. In a recent work the redox potentials of blood samples obtained from geriatric patients were shown to be significantly higher than those of a group of age-matched controls.[14] The high potentials recorded in the geriatric patients, could result from the chronic exposure of these patients to a common oxidative stress. Cyclic voltammetry can be used for quantiation of reducing agents with a high degree of sensitivity (down to μM concentrations). The sensitivity of the method may be enhanced to the range of femtomolar, by the use of advanced CV in combination with HPLC equipped with an electrochemical detector. Another limitation of this technique is due to the fact that antioxidant activity *in vivo* is determined by the concentration of the antioxidant agents, site of their activity and the kind of oxidative stress induced. The use of cyclic voltammetry may not reflect these factors. However, the results obtained in this study indicate that there is a correlation between some of the antioxidant and the reducing capacity (see below). Moreover, Kissinger *et al.*[19] has already shown that ascorbic acid, a known antioxidant and reducing agent, can be detected *in vivo* in site specific locations using the CV instrument.

The measurements in the cyclic voltammetry give both qualitative and quantitative information. However, in this study we tested only the qualitative aspects. We monitored qualitatively the disappearance of the anodic peaks and changes in their position following exposure of the rats to oxidative stress. Knowledge of the contribution of each reducing agent present in the tissue to the reductive capacity measured, their concentration, their CV appearance and their reactivity towards reactive oxygen species may also lead to a quantitative determination of these compounds.

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One could question the relevance of reductive capacity as an indicator of antioxidant activity. Reducing equivalents may act as prooxidants rather than antioxidants.[20] In biological systems loosely bound transition metals can be reduced directly by such reducing equivalents. The reduced metal, in turn, can react with hydrogen peroxide to produce the highly toxic hydroxyl radicals.[20] Another possibility is that reducing agents donate their electron(s) directly to the tissues, and by doing so they induce damage. In a previous work[13] we showed that reducing equivalents, detected by CV, are involved in scavenging free radicals. We showed that exposure of biological fluids and tissues to peroxy radicals[18] or to hydroxyl radicals resulted in a significant decrease in the reducing capacity of these samples. We have also conducted some experiments in which rat skin homogenate were exposed to hydroxy radicals induced by hydrogen peroxide and chelated ferrous ions. The results obtained show a decrease in the reductive capacity of the skin homogenate (unpublished data).

The exact correlation between the antioxidant activity of biological tissues, fluids and organs and the reductive capacity needs further clarification. The selective contribution of each of the various reducing agents in the tissue or fluids to the total reductive capacity is currently under investigation.

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