Heme Protein Oxidation and Lipid Peroxidation of Tissue Homogenates Induced by Halogenated Hydrocarbon, Hydroperoxide, and Transition Metals

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Damage of liver, heart, kidney, and spleen caused by spontaneous oxidative reaction or oxidative reactions induced by halogenated hydrocarbon, hydroperoxide, or transition metals was studied in a tissue homogenate system. Both thiobarbituric acid reactive substances (TBARS) and oxidized heme proteins (OHP) were measured in oxidatively damaged tissue homogenates. Bromotrichloromethane ($CBrCl_3$), *tert*-butyl hydroperoxide (TBHP), ferrous iron (Fe^{2+}), and cupric ion (Cu^{2+}) are potent prooxidants. In general, the production of OHP increased more rapidly than the production of TBARS, especially with short incubation periods and low concentrations of prooxidants. The correlation between heme protein oxidation and lipid peroxidation and the merits of using OHP to measure oxidative damage of tissues are discussed.

INTRODUCTION

Cellular components, such as polyunsaturated lipids, proteins, and DNA molecules, are continuously challenged with prooxidants that are generated either exogenously or endogenously (Pryor and Godber, 1991). The consequences of such oxidative assault have been linked to various pathological states such as atherosclerosis, cardiovascular disease, degenerative disease, and cancers (Halliwell and Gutteridge, 1985; Sies, 1985a,b; Johnson et al., 1986; Cross et al., 1987; Simic et al., 1988; Spatz and Bloom, 1992).

Numerous chemicals can initiate oxidative reactions in biological systems. For instance, oxidative damage processes in animal tissues can be promoted by halogenated hydrocarbons found in contaminated drinking water, foods, and polluted air (Recknagel et al., 1977; Reynolds and Molsen, 1980; *Chemical & Engineering News*, 1984; Hidalgo et al., 1990), hydroperoxides (Cadenas et al., 1983; Sies, 1985a,b; Sano et al., 1992), and transition metals (Thomas and Aust, 1988; Halliwell and Gutteridge, 1990; Schaich, 1992; Minotti and Aust, 1992).

A variety of methods have been employed to determine oxidative damages in biological systems (Pryor and Godber, 1991). Measurement of thiobarbituric acid reactive substances (TBARS), which targets the secondary products from lipid peroxidation, is probably the most widely used method to assess oxidative damage in tissues. Flourescent products, enzyme inactivation, release of amino acids from proteolysis, and oxidatively damaged DNA molecules have also been suggested as indexes of oxidative stress (Tappel, 1977; Davies, 1988; Pacifici et al., 1990; Shigenaga et al., 1990; Dillard et al., 1991; Sano et al., 1992).

Oxidized heme proteins (OHP), such as methemoglobin and hemichrome, can be measured by spectrophotometry (Winterbourn, 1990). Recently, measurement of OHP was used to determine oxidative damage in animal tissues (Chen et al., 1993a,b; Andersen et al., 1993).

It is desirable to employ multiple methods to determine oxidative damage to tissues due to the complexity of biological systems and the limitations of each individual analytical method (Tappel, 1991). In this study, it was our purpose to advance knowledge of lipid peroxidation and concurrent oxidative damage to proteins in animal tissues by measuring both TBARS and OHP. Tissue homogenates prepared from liver, kidney, heart, and spleen were incubated with and without the presence of bromotrichloromethane ($CBrCl_3$), *tert*-butyl hydroperoxide (TBHP), ferrous iron (Fe^{2+}), or cupric ion (Cu^{2+}) at 37 °C. Measurements of TBARS and OHP were conducted, and effects of incubation time and concentration of prooxidant on oxidative damage of tissue homogenates were studied. Correlations between lipid peroxidation and heme protein oxidation are reported.

MATERIALS AND METHODS

Chemicals. Chemicals used were $CBrCl_3$ (Eastman Kodak Co., Rochester, NY), TBHP (Polysciences, Inc., Warrington, PA), ferrous sulfate ($FeSO_4$) (Fisher Scientific, Fair Lawn, NJ), and cupric chloride ($CuCl_2$) (Merck & Co. Inc., Rahway, NJ). Dimethyl sulfoxide, malonaldehyde (MDA), and 2-thiobarbituric acid (TBA) were obtained from Sigma Chemical Co. (St. Louis, MO). Trichloroacetic acid (TCA) was obtained from Fisher Scientific.

Preparation of Tissue Homogenates. Male Sprague-Dawley rats were obtained from Bantin and Kingman (Fremont, CA) and fed with a standard diet and distilled water before the experiments. The rats were decapitated, and liver, heart, kidney, and spleen were immediately dissected and immersed in ice-cold Krebs-Ringer phosphate (KRP) buffer (pH 7.4). Organs were blotted with filter papers and stored at -22 °C. Frozen organs were cut into 0.5-cm^3 cubes by a sharp surgical knife, and homogenates were prepared by homogenizing 1 g of tissue with 9 mL of oxygenated KRP buffer containing glucose (10 mmol, pH 7.4). A motor-driven tissue homogenizer was used.

Oxidation of Heme Proteins in Tissue Homogenates. Tissue homogenates (1.5 mL) were transferred to a 10-mL glass serum bottle and covered with a layer of parafilm. The homogenates were incubated in a gyrotory water bath shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ) at 37 °C with continuous shaking (180 cycles/min). Prooxidants were dissolved either in dimethyl sulfoxide ($CBrCl_3$ and TBHP) or in distilled water ($FeSO_4$ and $CuCl_2$) and added to the glass serum bottles immediately before the incubation.

Spectrophotometric Measurement of Heme Proteins from Tissue Homogenates. After incubation, the absorbance spectra of tissue homogenates were obtained with a Beckman DU-50 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). Tissue homogenates (0.6 mL) were transferred to a microcuvette with a light path of 10 mm and mixed with 0.6 mL

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of glycerol. Four layers of parafilm representing turbidity were used as background to subtract some of the absorbance caused by turbidity inherent in tissue homogenates. The sample was scanned from 500 to 640 nm, and absorbance vs wavelength at 5-nm intervals was automatically recorded by a scan program in the spectrophotometer.

Analysis of Absorbance Spectra of Heme Proteins of Tissue Homogenates with HPSAP. HPSAP is a spreadsheet program written with Lotus 123 (Lotus Development Corp., 55 Cambridge Parkway, Cambridge, MA) that contains the micromolar absorbance from 500 to 640 nm of the visible spectra of individual heme proteins. The analysis is based on the knowledge that the absorbance spectrum of a mixture of heme proteins is the sum of the spectra of the individual heme proteins including any contribution from turbidity of the biological samples. Quantitation is achieved by matching the calculated spectrum with the experimental spectrum through successive approximations. The determination of the amounts of heme proteins by the HPSAP can be highly accurate because the absorbance of heme proteins closely obeys Beer's law. Any interaction between individual heme proteins, such as redox reactions, will result in the production of other measurable heme proteins. The development and application of HPSAP have been described (Chen et al., 1993a,b; Andersen et al., 1993).

To determine the concentrations of individual heme proteins from a mixture, the experimental visible absorbance vs wavelength at 5-nm intervals from 500 to 640 nm was entered into HPSAP. To match the experimental spectrum, a calculated spectrum was established. Micromolar values for expected individual heme proteins were entered into concentration cells of HPSAP. Values for contributions due to turbidity at 500 to 640 nm were also entered into HPSAP. The turbidity contributions at other wavelengths can be calculated with HPSAP by constructing a slanted baseline between the turbidity values entered for 500 and 640 nm. Contributions of the individual heme proteins were calculated using Beer's law and the micromolar absorptivities for those specific heme compounds. All of the contributions were summed for each wavelength, and the results were displayed on the spreadsheet as calculated absorbance, along with the difference between the experimental and calculated absorbance and the percent error at each wavelength. Both experimental and calculated spectra were viewed on the CRT screen. To achieve the best superposition of calculated and experimental spectrum, micromolar values of individual heme proteins and values for turbidity contributions at 500 and 640 nm were adjusted to minimize the differences between the two spectra. Some restrictions were established for the approximation. For instance, the ratio hemoglobin:mitochondria:microsomes in fresh liver tissue was estimated as 7:2:1 on a weight basis on the basis of the literature (Long, 1961; Lehninger, 1964; De Robertis et al., 1965). Since the amount of hemoglobin in nonperfused tissues was not a constant during the experiment, the ratio may vary from time to time. The ratio used was assumed to remain constant during the oxidation; that is, the total percentage of both reduced and oxidized hemoglobin, mitochondrial cytochromes, and microsomal cytochromes was constant. Besides the superposition of experimental and calculated spectra, the average calculation error, defined as the average difference between experimental and calculated spectra, was used to support the investigator's judgment of the best fit. When the best matching was achieved, the concentrations of individual heme proteins in the homogenates were determined.

Measurement of TBARS. Measurements of TBARS of oxidized tissue homogenates followed the method described by Zalkin and Tappel (1960) with some modifications. One gram of frozen tissue was cut into small pieces and mixed with 9 mL of 0.9% NaCl and homogenized for 2 min using a motor-driven tissue homogenizer. Two milliliters of homogenates was transferred to a 10-mL glass serum bottle containing 3 mL of oxygenated 0.9% NaCl. Prooxidant was added to the serum bottle immediately before the reaction began. The homogenates were incubated in the water bath shaker at 37 °C with continuous shaking (180 cycles/min). After incubation, the bottles were cooled with dry ice. Homogenates (2.5 mL) were transferred to a 10-mL centrifuge tube containing 2.5 mL of 10% TCA. The homogenates were centrifuged at 3000 rpm for 2 min at 4 °C.

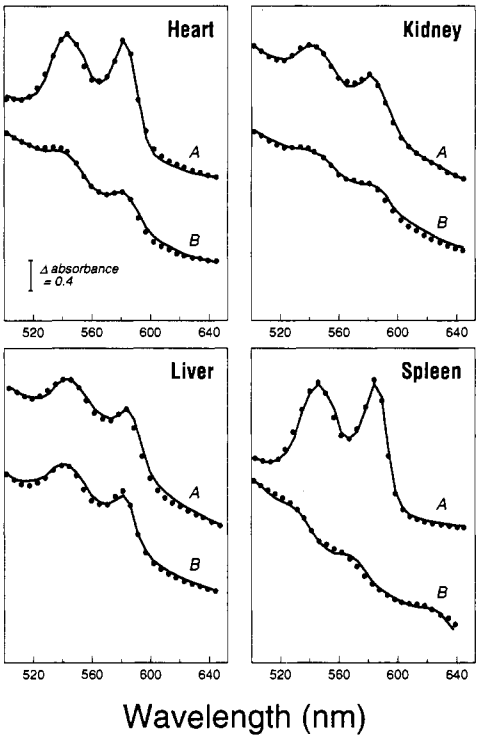

Figure 1. Visible spectra of liver, kidney, heart, and spleen homogenates: A, fresh tissue homogenates; B, oxidized tissue homogenates; experimental spectra (···); calculated spectra (—). The oxidized homogenates were incubated at 37 °C for 1 h with the presence of prooxidants. The corresponding prooxidants and the concentrations are presented in Table I.

Table I. Production of Oxidized Heme Proteins (OHP) Measured with HPSAP[a] in Homogenates of Liver, Heart, Kidney, and Spleen

tissue	prooxidant	concn of prooxidant, mM	OHP, M %
liver	$CBrCl_3$	0.40	52
heart	$CuCl_2$	0.08	77
kidney	$FeSO_4$	0.08	57
spleen	TBHP	0.40	91

[a] Tissue homogenates were incubated at 37 °C for 1 h.

After centrifugation, 4 mL of supernatant was reacted with 1.25 mL of 0.75% TBA (freshly prepared) at 100 °C for 10 min. The resulting pink color was measured at 530 nm. TBARS were expressed as MDA equivalents per gram of tissue.

RESULTS

Figure 1 shows the spectral changes of heme proteins in liver, heart, kidney, and spleen homogenates during oxidative reactions. Strong absorptions at 540 and 580 nm indicate that oxyhemoglobin is the predominating heme protein in a fresh tissue homogenate. As oxidation was initiated by $CBrCl_3$, TBHP, Fe^{2+}, or Cu^{2+}, absorptions at 540 and 580 nm were decreased due to oxidative conversion of oxyhemoglobin. On the other hand, absorptions at 500, 570, and 630 nm were increased, indicating the formation of methemoglobin, ferrylhemoglobin, and hemichrome in the oxidized homogenate. The concentration of total OHP was determined with HPSAP and is shown in Table I.

Figure 2 presents oxidative damage of liver homogenates measured by both OHP and TBARS during oxidation with or without adding TBHP. Heme proteins were more vulnerable to oxidative damage than polyunsaturated lipids during spontaneous oxidation. For instance, at 30 min of incubation, 30% of heme proteins were oxidatively

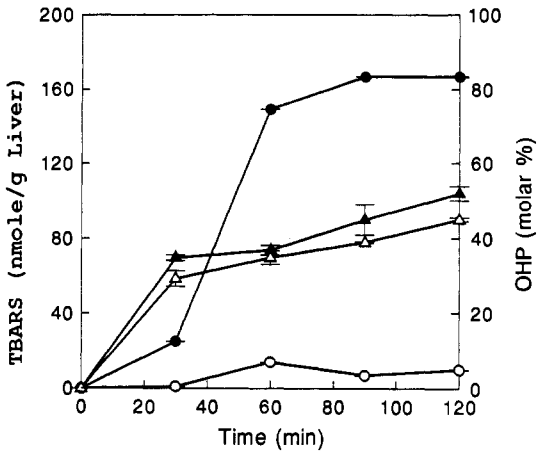

Figure 2. Oxidative damage of liver homogenates caused by spontaneous oxidation or oxidation induced by 0.4 mM TBHP: OHP of spontaneous oxidation (△); TBARS of spontaneous oxidation (○); OHP of oxidation induced by TBHP (▲); TBARS of oxidation induced by TBHP (●). Incubation was conducted at 37 °C. The correlations between TBARS and OHP in spontaneous oxidation and oxidation induced by TBHP were both $r = 0.7$ ($P < 0.001$). The values are expressed as mean ± SD for three measurements.

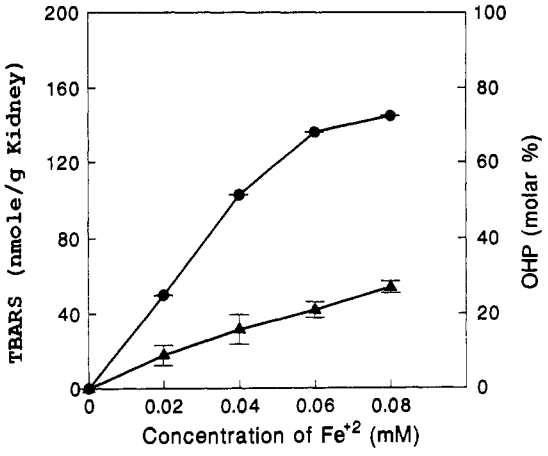

Figure 3. Oxidative damage of kidney homogenates induced by Fe^{2+}: OHP (▲) and TBARS (●). Homogenates were incubated at 37 °C for 1 h. The correlation between TBARs and OHP was $r = 0.94$ ($P < 0.001$). The values have background subtracted and are expressed as mean ± SD for three measurements.

converted, while very little TBARS could be measured. At 2 h, approximately 45% of heme proteins were oxidized. In contrast, there was only a small increase in TBARS. Thus, OHP appeared to be a more sensitive measure of oxidative damage than TBARS under moderate oxidative conditions. Although adding TBHP promoted both heme protein oxidation and lipid peroxidation, the effect of TBHP on oxidative damage of polyunsaturated lipid was much greater than that on heme protein oxidation. For example, after 2 h of incubation, approximately 55% of heme proteins were oxidized, which was a 25% increase from the spontaneous oxidation. On the other hand, the concentration of TBARS increased from 10 to 170 nmol/g of tissue.

Figures 3 and 4 present oxidative damage of kidney and heart homogenate caused by Fe^{2+} or Cu^{2+}. OHP increased linearly as concentration of transition metals increased. In the oxidative reaction initiated by Fe^{2+}, TBARS increased rapidly when the concentration of Fe^{2+} increased from 0.02 to 0.06 mM. In the reaction induced by Cu^{2+}, TBARS did not show significant increase until the concentration of Cu^{2+} reached 0.04 mM.

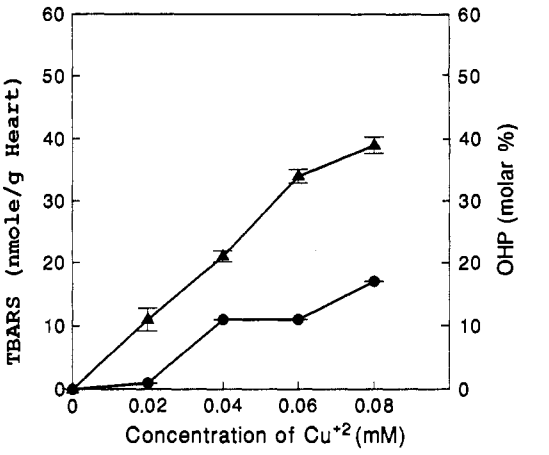

Figure 4. Oxidative damage of heart homogenates induced by Cu^{2+}: OHP (▲) and TBARs (●). Homogenates were incubated at 37 °C for 1 h. The correlation between TBARS and OHP was $r = 0.98$ ($P < 0.001$). The values have background subtracted and are expressed as mean ± SD for three measurements.

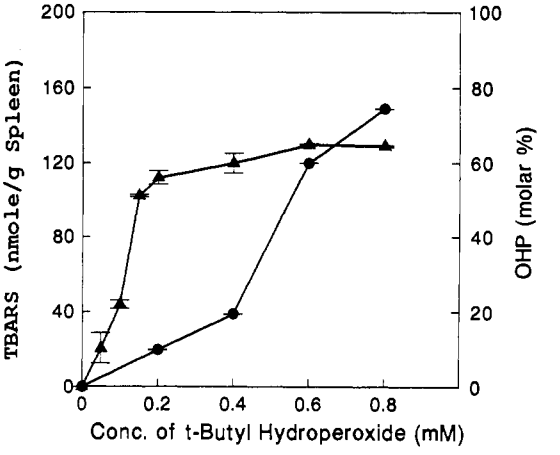

Figure 5. Oxidative damage of spleen homogenates induced by TBHP: OHP (▲) and TBARS (●). Homogenates were incubated at 37 °C for 1 h. The correlation between TBARS and OHP was $r = 0.98$ ($P < 0.001$). The values have background subtracted and are expressed as mean ± SD for three measurements.

Figure 5 shows the oxidative damage of spleen homogenates induced by TBHP. Heme proteins were much more susceptible to TBHP than polyunsaturated lipids. For instance, the concentration of OHP increased exponentially as TBHP increased from 0.05 to 0.2 mM. At 0.2 mM TBHP, approximately 50% of heme proteins were oxidized. At the same range of concentration of TBHP, however, only small amounts of TBARS can be measured. An increase in TBARS was observed when the concentrations of TBHP were beyond 0.4 mM.

Figure 6 presents the effect of $CBrCl_3$ on oxidative damage of liver homogenates. The amount of OHP rose steadily as the concentration of the $CBrCl_3$ increased. When 0.8 mM $CBrCl_3$ was added, more than 20% of the heme proteins were oxidized. There was no great change in TBARS until the concentration of the prooxidant increased to 0.4 mM, and beyond 0.4 mM the concentration of TBARS remained the same.

DISCUSSION

As demonstrated in this study, there are advantages in using multicomponent analysis, such as HPSAP, to quantitatively determine oxidation of heme proteins in tissue homogenate systems. One of the challenges in applying multicomponent analysis to biological systems

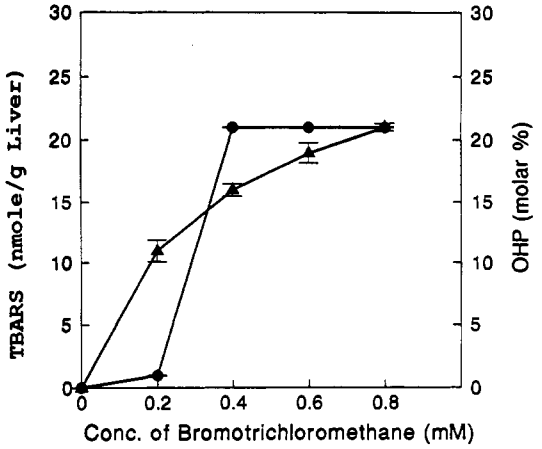

Figure 6. Oxidative damage of liver homogenates induced by $CBrCl_3$: OHP (▲) and TBARS (●). Homogenates were incubated at 37 °C for 1 h. The correlation between TBARS and OHP was $r = 0.98$ ($P < 0.001$). The values have background subtracted and are expressed as mean ± SD for three measurements.

is the turbidity. As shown in Figure 1, turbidity severely interferes with the measurements of absorption peaks of heme proteins. To avoid such interference, multicomponent analysis is normally conducted in a homogeneous system (Winterbourn, 1990). Establishing a homogeneous system for the analysis, however, could cause problems. First, the simple homogeneous system is hardly comparable to the complicated biological systems. Second, extraction or separation of individual heme proteins from tissues could be tedious. Third, artifacts could be introduced to the analysis due to the extraction or separation processes, and the reuslts could be altered or distorted. In this study, two measures have been taken to overcome the interference by turbidity. First, four layers of parafilm, which have light scattering properties similar to those of tissue homogenates, are used to blank the spectrophotometer. As a result, the absorbance of 2.5 of tissue homogenates can be reduced to an absorbance of approximately 1.5, wherein the DU-50 spectrophotometer is able to function properly. Second, the remaining absorbance of turbidity over 500–640 nm is measured and incorporated into HPSAP as a linear function of wavelength. The turbidity is subtracted during the spectra matching so that its effect on the analysis is significantly reduced. The determination of heme protein oxidation can be conducted directly in a homogenate system that is comparable to an in vivo situation. In addition, the analysis does not require any extraction or separation of individual heme proteins; thus, artifacts from sample preparation can be eliminated. Absorbance spectra of all known heme proteins that may exist during oxidation of tissues are incorporated into the program. Thus, with HPSAP individual heme proteins from a complicated composite spectra can be determined. Another important feature of the application of HPSAP is that this method directly targets oxidatively damaged proteins rather than the oxygen radical intermediates or secondary products from oxidative damage processes. Normally, it is quite difficult to achieve the quantitative determination of oxidative stress by measuring the intermediates or secondary products from oxidative reactions. The measurement of oxidized heme proteins, therefore, can serve as a more reliable and quantitative index of oxidative stress in biological systems.

Oxidative damage of heme proteins has been linked to various pathological processes. For example, oxidatively damaged hemoglobin, such as methemoglobin and hemichrome, is implicated in the formation of Heinz bodies, hemolysis of red blood cells, and sickle cell anemia (Chiu et al., 1982; Wagner et al., 1988). Heinz bodies have been used as an indicator of oxidation of hemoglobin. The measurement, however, is considered to be more qualitative than quantitative because of its limitations (Chiu et al., 1982). Measurement of OHP, on the other hand, is a more quantitative measure. Thus, it is potentially a quantitative index of the development of certain diseases.

Since OHP have relatively high absorptivities (Winterbourn, 1990), heme protein oxidation can be a sensitive measure of oxidative stress. Heme protein oxidation appeared to be more sensitive than TBARS under mild oxidative conditions (Figure 2). Even though a strong prooxidant, such as TBHP, $CBrCl_3$, Fe^{2+}, or Cu^{2+}, was added to the homogenates, the lag time for lipid peroxidation was still longer than that of heme protein oxidation (Figures 4–6). With its high sensitivity to oxidative stress, heme protein oxidation is especially applicable to the detection of the early stage of an oxygen radical chain reaction which normally has a low concentration of OHP (Chen et al., 1993a,b).

Virtually all cellular components are subjected to certain degrees of oxidative damage. It is desirable, therefore, to use complementary multiple methods to analyze oxidatively damaged tissues (Tappel, 1991). Multiple methods have been applied to the study of oxidative damage in animal tissues (Davies, 1988; Fraga et al., 1989; Dillard et al., 1991; Sano et al., 1992). Polyunsaturated lipids are mainly distributed in the lipid phase of the cell, such as the plasma membrane and membranes of other cellular organelles, while heme proteins are mainly located in the aqueous phase of the cell. Determination of both lipid peroxidation and heme protein oxidation, therefore, can be appropriate measures of oxidative damage in the whole cell or the entire tissue. Polyunsaturated lipids and heme proteins are both oxygen labile; thus, a close association between formation of TBARS and oxidized heme proteins is expected. As shown in this study, heme protein oxidation is highly correlated with lipid peroxidation during the oxidative reactions in liver, kidney, heart, and spleen homogenates, indicating that heme protein oxidation and lipid peroxidation concurrently occurred under oxidative stress.

Animal tissues are protected by cellular antioxidant defense systems against the attack of reactive oxygen species and free radicals (Burton et al., 1985; Halliwell, 1990). The antioxidant systems include small molecules such as vitamins E and C and enzymes of high molecular weight such as selenium glutathione peroxidase, catalase, and superoxide dismutase (Niki, 1990). Under normal physiological conditions, therefore, polyunsaturated lipids and heme proteins in tissue homogenate were protected effectively by the antioxidant defense systems. As a result, the spontaneous oxidation of heme proteins and polyunsaturated lipids was rather slow. Halogenated hydrocarbons, hydroperoxides, and transition metals are potent prooxidants (Ames et al., 1982; Sies, 1985a,b; Halliwell and Gutteridge, 1990). When $CBrCl_3$, TBHP, Fe^{2+}, or Cu^{2+} was introduced to the homogenates, the protection by antioxidants was overwhelmed, and the tissues were under oxidative stress. Highly reactive oxygen species generated from reactions initiated by $CBrCl_3$, TBHP, Fe^{2+}, or Cu^{2+} can seriously impair the antioxidant defense systems and cause oxidative damage of heme proteins and polyunsaturated lipids. Halogenated hydrocarbons are well-known hepatotoxins (Reynolds and Moslen, 1980). The chemical mechanisms of hepatotoxicity of halogenated hydrocarbons have been extensively studied using CCl_4

as a model (Butler, 1961; Noguchi et al., 1982a,b). The highly reactive oxygen species generated during the metabolism of halogenated hydrocarbons in liver, such as trichloromethyl and trichloromethylperoxyl radicals, react with various amino acids and unsaturated lipids rapidly, thus causing oxidative denaturation of proteins and lipid peroxidation (Recknagel and Glende, 1977). The mechanisms of the reaction of transition metals and hydroperoxides in cellular oxidative damage have been established (Stadtman, 1990; Halliwell and Gutteridge, 1990; Cadenas and Brigelius, 1983; Sies, 1985a,b). Transition metals play a key role in the production of reactive oxygen species during oxidative stress because the ions directly participate in the formation of hydroxyl radicals as an essential reactant and catalyst of Fenton chemistry (Halliwell and Gutteridge, 1990). Hydroperoxides, such as TBHP, can readily produce *tert*-butoxyl or *tert*-butylperoxyl radicals by radical decay when transition metals are present (Sies, 1985a,b).

In summary, we have demonstrated that oxidatively damaged tissue homogenates can be analyzed by measuring both OHP and TBARS. Under oxidative stress, heme protein oxidation and lipid peroxidation were concurrent and highly correlated. The experimental results suggested that the measurement of OHP was more sensitive than that of TBARS during spontaneous oxidative reaction.

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