Oxidative Damage of Kidney, Heart, Lung, and Spleen Measured by Oxidized Heme Proteins in Tissue Slices

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Oxidative damage to rat kidney, heart, lung, and spleen was measured by the formation of oxidized heme proteins (OHP) in tissue slices. Kidney, heart, lung, and spleen slices were incubated in oxygenated KRP–glucose buffer at 37 °C with and without the presence of prooxidants. The absorbance spectra (500–640 nm) of heme proteins in the fresh and oxidized tissue slices were analyzed, and the concentrations of OHP, such as methemoglobin, ferrylhemoglobin, hemichrome, oxidized mitochondrial, and microsomal cytochromes were determined with a heme protein spectra analysis program. Bromotrichloromethane ($CBrCl_3$), *tert*-butyl hydroperoxide (TBHP), ferrous sulfate ($FeSO_4$), and cupric chloride ($CuCl_2$) accelerated the oxidative reactions, and the amount of OHP was dependent on the incubation time as well as the type and concentration of prooxidants.

INTRODUCTION

Oxidative damage of heme proteins involves a redox reaction of the heme group with a one- or two-electron transfer and the denaturation of the globin structure (Winterbourn, 1985, 1990). For instance, oxyhemoglobin is oxidized to methemoglobin due to the oxidation of heme ferrous ion to ferric ion with either OH^{1-} or H_2O in the sixth coordination site. When the sixth coordination site is occupied by either the distal histidine or an external ligand, methemoglobin is further converted to hemichrome, the main constituent of Heinz bodies (Winterbourn, 1985, 1990; Chiu et al., 1982; Wagner et al., 1988). Oxidized heme proteins (OHP) can be measured by spectrophotometry and may serve as a good index of oxidative stress in animal tissues (Winterbourn, 1990; van den Berg et al., 1991, 1992; Kollias et al., 1992). Recently, oxidative damage of heme proteins in liver slices has been studied by measurement of OHP using a computer-aided heme protein spectra analysis program (HPSAP) (Chen et al., 1993a,b; Andersen et al., 1993).

Halogenated hydrocarbons, hydroperoxides, and transition metals are well-known initiators of oxidative damage in animal tissues (Ames et al., 1982; Sies, 1985; Halliwell and Gutteridge, 1990) and have been used as prooxidants in many in vitro and in vivo studies.

Kidney, heart, lung, and spleen are constantly subjected to oxidative assaults (Sano and Tappel, 1990), and their oxidative damage has been linked to certain disease states. For instance, it is suggested that the injury caused by reactive oxygen species is involved in pulmonary microcirculatory damage (Yoshikawa, 1991). Studies of oxidative injury of animal organs in a tissue slice system thus may provide valuable information regarding the pathogenesis of various diseases. In this study tissue slices prepared from rat kidney, heart, lung, and spleen were incubated with and without the presence of $CBrCl_3$, TBHP, $FeSO_4$, and $CuCl_2$ at 37 °C. The absorbance spectra obtained between 500 and 640 nm were analyzed with HPSAP, and the concentrations of oxidized heme products were then determined. The effects of incubation time and concentration of prooxidant were studied.

MATERIALS AND METHODS

Chemicals. Chemicals used as prooxidants were bromotrichloromethane (Eastman Kodak Co., Rochester, NY), *tert*-butyl hydroperoxide (Polysciences, Inc., Warrington, PA), ferrous sulfate (Fisher Scientific, Fair Lawn, NJ), and cupric chloride (Merck & Co. Inc., Rahway, NJ). Dimethyl sulfoxide was obtained from Sigma Chemical Co. (St. Louis, MO).

Animals and Tissue Slices. Male Sprague-Dawley rats (220–250 g) were obtained from Bantin and Kingman (Fremont, CA). Rats were fed with a standard diet and distilled water before the experiments. The rats were decapitated, and organs of the rats were immediately dissected and immersed in ice-cold Krebs–Ringer phosphate (KRP) buffer (pH 7.4). Kidney, heart, and spleen were cut into 0.5-cm^3 cubes by a sharp surgical knife and sliced into about 0.5 mm thick pieces (80–100 mg) by a Stadie–Riggs tissue slicer (Thomas Scientific, Philadelphia, PA) according to a method described by Gavino et al. (1984). A sharp surgical scissor was used to prepare lung tissue slices.

Oxidative Reactions in Tissue Slices. Approximately 90 mg of tissue slices in a 10-mL serum bottle containing 5 mL of oxygenated KRP–glucose buffer (concentration of glucose was 10 mM) was incubated in a gyrotory water bath shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ) at 37 °C with continuous shaking (180 cycles/min). $CBrCl_3$ and TBHP were dissolved in dimethyl sulfoxide. $FeSO_4$ and $CuCl_2$ were dissolved in distilled water. Prooxidants were added into serum bottles immediately before incubation.

Spectrophotometric Measurements of Tissue Slices. After incubation of tissue slices, the absorbance spectrum of each sample was obtained with a Beckman DU-50 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). A 50-mg sample was blotted with a filter paper and then transferred to a spectrophotometer cell of 5.5 mm i.d. and a light path of 2.0 mm. In the airtight cell the heme proteins in the tissue slices came to a redox equilibrium determined by the physiological conditions of the tissues. Four layers of parafilm representing turbidity were used as a background to blank the spectrophotometer and to subtract some of the absorbance of turbidity caused by the tissues. Spectra can be corrected for scattered light loss by using a reference material with similar scattering properties to blank the spectrophotometer (Erickson, 1973). We have found parafilm to be a useful reference material for tissues over the wavelength range 500–640 nm. The cell was sealed by a microscope cover glass and mounted on the center of the window of the spectrophotometer near the photoreceptor to reduce the light scattering caused by the tissue. The sample was scanned from 500 to 640 nm, and the 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 Slices with HPSAP. HPSAP is a spreadsheet calculation program written with Lotus 1-2-3 (Lotus Development Corp., Cambridge, MA) that contains visible spectra of individual heme proteins from 500 to 640 nm. The program is based on the

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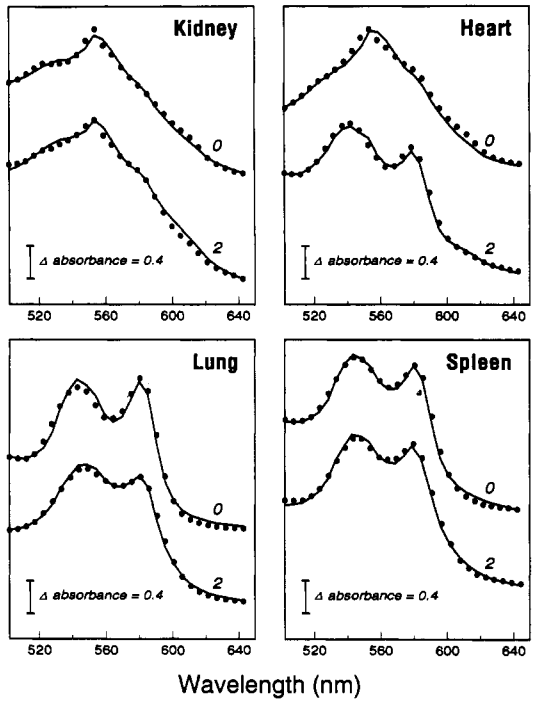

Figure 1. Visible absorbance spectra of heme proteins in kidney (A), heart (B), lung (C), and spleen (D) tissue slices incubated at 37 °C for 0 and 2 h: (···) experimental spectrum; (—) calculated spectrum.

knowledge that the absorbasnce spectrum of a mixture of heme proteins is the combination of the spectra of individual heme proteins including any absorption from turbidity of the biological samples. Quantitation of individual heme proteins from the mixture is achieved by matching the calculated spectrum with the experimental spectrum through successive approximations. The analysis of HPSAP can be highly accurate since the absorbance of heme proteins obeys Beer's law and any interaction between individual heme proteins, such as redox reactions, will result in other measurable heme proteins. The details of the development, application, and operation of HPSAP in tissue slice model system are described in our previous studies (Chen et al., 1993a,b; Andersen et al., 1993).

RESULTS

Absorbance spectra obtained from both fresh and partially oxidized nonperfused kidney, heart, lung, and spleen slices are presented in Figure 1. There are evident differences between the spectra obtained from fresh tissues and from tissues incubated for 2 h at 37 °C. The difference in the absorbance spectra is obviously caused by the formation of OHP. With the application of HPSAP analysis, concentrations of individual heme proteins and the total amount of OHP can be quantitatively determined (Table I). In fresh kidney and heart slices, the dominating heme proteins are oxyhemoglobin, hemoglobin, reduced mitochondrial cytochromes, and a small amount of reduced microsomal cytochromes. In fresh lung and spleen slices, on the other hand, oxyhemoglobin and hemoglobin appeared to be the major heme pigments. Heme proteins can be readily converted to their oxidized states even without the presence of prooxidants. For instance, after 2 h of incubation, ferrylhemoglobin, methemoglobin, and hemichromes as well as oxidized mitochondrial and microsomal cytochromes were formed. The concentrations of total OHP in kidney, heart, lung, and spleen slices were 40%, 20%, 28%, and 30%, respectively, after 2 h of incubation at 37 °C.

Figure 2 presents the effect of incubation time on the spontaneous oxidation of heme proteins and the heme

Table I. Analysis of Spectra Obtained from Spontaneous Oxidation of Kidney, Heart, Lung, and Spleen Slices Incubated at 37 °C with HPSAP

	micromolar, %			
heme proteins	kidney	heart	lung	spleen
Incubation Time = 0 h				
oxyhemoglobin	12	11	78	66
hemoglobin	56	64	22	28
reduced mitochondrial cytochromes	28	21	0	3
reduced microsomal cytochromes	4	4	0	3
total oxidized heme proteins	**0**	**0**	**0**	**0**
average calculation error, %	0.2	3	0.1	0.1
Incubation Time = 2 h				
oxyhemoglobin	12	62	37	42
hemoglobin	25	0	35	24
methemoglobin	0	0	3	5
ferryl hemoglobin	27	0	15	20
hemichrome	4	17	10	3
reduced mitochondrial cytochromes	20	14	0	2
oxidized mitochondrial cytochromes	8	3	0	1
reduced microsomal cytochromes	3	4	0	2
oxidized microsomal cytochromes	1	0	0	1
total oxidized heme proteins	**40**	**20**	**28**	**30**
average calculation error, %	0.7	0.6	0.1	0.3

protein oxidation induced by $CBrCl_3$. Lung tissues were more susceptible to oxidation than other tissues during the early stages of the spontaneous oxidation (0.5 and 1 h). For instance, after 0.5 h of incubation at 37 °C, lung tissue contained 20% OHP and heart tissue had 10% OHP. As incubation time increased to 1.5 h, concentrations of OHP rose steadily in all tissue slices. Beyond 1.5 h, concentrations of OHP did not increase except for that in kidney slices.

Animal tissues were sensitive to $CBrCl_3$, especially the heart tissues (Figure 2B). For example, after 0.5 h of incubation at 37 °C, there were only 10% OHP in the heart tissues (Figure 2A). With the same incubation time, however, 0.4 mM $CBrCl_3$ induced 30% OHP in heart tissues. As the incubation time increased from 0.5 to 2 h, the concentration of OHP in heart tissues rose linearly. After 2 h of incubation, more than 60% of the heme proteins in heart tissues were oxidized. Kidney appeared to be most resistant to exposure to $CBrCl_3$. For instance, there were approximately 15% OHP in kidney tissues after 0.5 h of spontaneous oxidation (Figure 2A). During the same reaction time, $CBrCl_3$ increased the OHP to only 20%.

Figure 3 presents the effect of TBHP, Fe^{2+}, or Cu^{2+} on the oxidation of heme proteins during 2 h of incubation at 37 °C. Heart tissues were exceptionally susceptible to TBHP (Figure 3A). For instance, as the concentration of TBHP increased from 0.05 to 0.15 mM, the amount of OHP in heart tissues increased by more than 200%. In fact, with the presence of 0.15 mM TBHP, more than 95% of the heme proteins in heart tissues were oxidized. With the same concentration of TBHP, however, there were less than 50% of OHP in the other tissues.

Transition metals appeared to have more deleterious effects on kidney and heart tissues than on lung and spleen tissues (Figure 3B,C). For instance, after incubation with 0.2 mM Fe^{2+} or Cu^{2+} for 2 h at 37 °C, 60% of the heme proteins in kidney and heart tissue slices were oxidized and only 40% of the heme proteins in lung and spleen tissues were oxidized. Increasing the concentration of Fe^{2+} or Cu^{2+} beyond 0.05 mM did not markedly affect the oxidation of heme proteins.

DISCUSSION

Formation of OHP in tissue slices is highly correlated to other oxidative damage measures such as lipid perox-

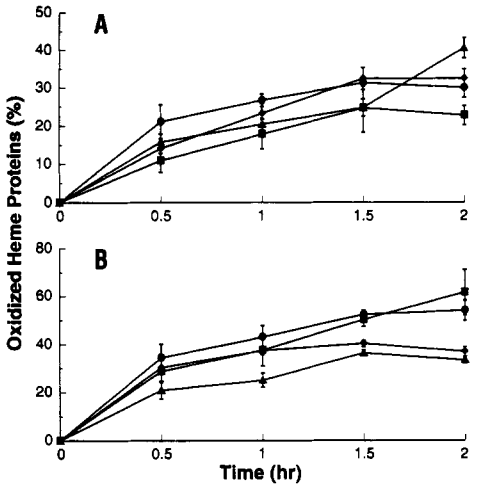

Figure 2. Effect of time on spontaneous oxidation of heme proteins (A) and on oxidation of heme proteins induced by $CBrCl_3$ (0.4 mM) (B): (▲) kidney; (■) heart; (●) lung; (◆) spleen. Tissue slices were incubated at 37 °C. The values are expressed as mean ± SD for three tissue slice measurements.

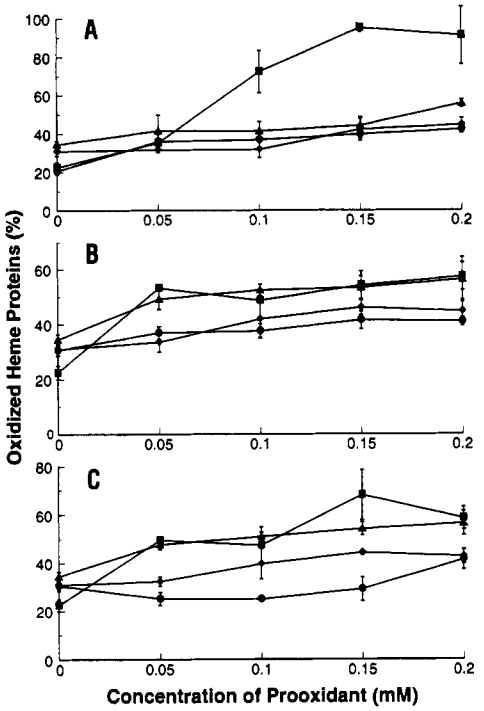

Figure 3. Effect of concentration of TBHP (A), Fe^{2+} (B), and Cu^{2+} (C) on oxidation of heme proteins: (▲) kidney; (■) heart; (●) lung; (♦) spleen. Tissue slices were incubated at 37 °C for 2 h. The values are expressed as mean ± SD for three tissue slice measurements.

idation and protein synthesis damage (Chen et al., 1993a). Heme protein oxidation initiated by TBHP (Chen et al., 1993a,b) is also parallel to the oxidative modification of cytochrome P-450 induced by cumene hydroperoxide (Weiss and Estabrook, 1986). Thus, the analysis of OHP in tissue slices with HPSAP appeared to be an appropriate approach to study oxidative damage in vivo.

Lung and spleen tissues appeared to be more vulnerable to oxidative stress than kidney and heart during spontaneous oxidation (Figure 2A). As shown in Table I, the major heme proteins present in lung and spleen slices are oxyhemoglobin and hemoglobin. Hemoglobin readily undergoes oxidation due to the ferrous iron in the heme group and the presence of oxygen. The oxidation of hemoglobin produces O_2^- and H_2O_2 directly or indirectly (Winterbourn, 1990). Under suitable circumstances, both O_2^- and H_2O_2 are capable of initiating a series of reactions that generate reactive oxygen species and thus result in oxidative denaturation of hemoglobin (Winterbourn, 1985, 1990). The higher content of oxyhemoglobin and hemoglobin in lung and spleen tissues may be responsible for the more severe damage of the tissues.

In general, there is only a small difference in the oxidative damage among different tissues. In other words, oxidative damage is independent of the type of the tissues. This observation can be attributed to the following facts: (1) the contents of polyunsaturated fatty acids, proteins, and glutathione are similar in all tissues; (2) all tissues are exposed to the similar environment of oxygen and catalysts in vivo; (3) the deposits of antioxidants are similar in all tissues.

The chemical mechanisms of the hepatotoxicity caused by halogenated hydrocarbons have been extensively studied using CCl_4 as a model (Bulter, 1961; Noguchi et al., 1982a,b). The highly reactive oxygen species generated during the metabolism of halogenated hydrocarbons in liver, such as the 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, 1973; Recknagel et al., 1977; Brattin et al., 1985). Recent studies showed that $CBrCl_3$ also causes serious oxidative damage to membranous antioxidant enzymes and heme proteins in rat liver (Dillard et al., 1990; Chen et al., 1993a,b). In this study $CBrCl_3$ accelerates the oxidation of heme proteins in kidney, heart, lung, and spleen tissues (Figure 2B). The exact mechanism of $CBrCl_3$-induced oxidation of heme proteins in kidney, heart, lung, and spleen tissues is not clear at present. It is probable that the one-electron reduction of $CBrCl_3$ that initiates the generation of highly reactive oxygen species is the main contributor to the oxidative damage of the tissues (Recknagel and Glende, 1973; Recknagel et al., 1977).

When transition metals are present, organic hydroperoxides or lipid hydroperoxides are rapidly decomposed. The decomposition of hydroperoxides generates reactive oxygen species such as hydroxyl and peroxyl radicals (Halliwell and Gutteridge, 1990). The decomposition of TBHP promotes the oxidation of heme proteins in tissues, especially in heart tissues (Figure 3A). On the other hand, the deleterious effect of TBHP can be reduced by the cellular defense systems, particularly by the glutathione peroxidase system (Sies, 1985). Compared to heart tissues, kidney, lung, and spleen appear to be more resistant to TBHP. The diminished reactivity of TBHP may be attributed to the higher concentration of GSH peroxidase and the higher activity of the enzyme in kidney, lung, and spleen.

The oxidative damage of proteins catalyzed by metal ions is a site-specific process (Stadtman, 1990). The extent of oxidation of proteins, therefore, will depend on the availability and the number of metal-binding sites in the protein molecule. As shown in Figure 3B,C, even though the addition of Fe^{2+} and Cu^{2+} promotes the oxidation of heme proteins, increasing the concentration of metal ions above the threshold level did not substantially increase the OHP. In fact, only a small increase in OHP was observed when the concentration of Fe^{2+} and Cu^{2+} was raised from 0.05 to 0.2 mM. This observation suggests that the metal-binding sites of heme proteins are probably nearly saturated by the presence of 0.05 mM Fe^{2+} or Cu^{2+}. This finding, therefore, could be an indication that

oxidative damage of heme proteins catalyzed by metals is indeed a site-specific process.

In summary, spectrophotometric measurements of oxidatively damaged heme proteins and the determination of individual heme proteins with HPSAP have been applied to kidney, heart, lung, and spleen slices. HPSAP appears to be an appropriate method to quantify individual heme proteins in tissue slice systems. The extent of oxidative damage of tissues is dependent on the time of incubation and the type of prooxidants as well as the concentration of the prooxidants.

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