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7-Hydroperoxycholesterol as a Marker of Oxidative Stress in Rat Kidney Induced by Paraquat

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The *in vivo* paraquat-induced oxidative stress in rat tissue was studied by analyzing cholesterol-derived hydroperoxide as an index of lipid peroxidation. Paraquat (10 mg/kg) was administered i.p. to rats. Rats were sacrificed and lung, liver, and kidney were collected 2, 24 h, and 5 d after paraquat injection. Lipids were extracted and analyzed by HPLC with post-column chemiluminescence. We found that two cholesterol-derived hydroperoxides, 7α-hydroperoxycholest-5-en-3β-ol (7α-OOH) and 7β-hydroperoxycholest-5-en-3β-ol (7β-OOH) were present in lungs of control animals (0.06 and 0.06 nmol/g, respectively), in livers (6.5 and 15.8 nmol/g, respectively) and in kidneys (3.7 and 8.9 nmol/g, respectively). In liver paraquat increased lipid peroxidation approximately by 60% over the levels of control animals only at 2 h after paraquat treatment. In kidney, augmented lipid peroxidation, 7α-OOH and 7β-OOH (by 70% and 147%, respectively) above levels was found at 2 h after paraquat treatment. Interestingly, these increase remained in kidney of rats 5 d after a single dose of paraquat. In contrast, cholesterol-derived hydroperoxides were not affected in lung of paraquat dosed rats. This is the first report on 7α-OOH and 7β-OOH accumulations in rat liver and kidney, and it seems to reflect greater oxidative stress in the pathology of kidney of rats treated with acute paraquat at low dose.

Keywords: Paraquat, 7-hydroperoxycholesterol, lipid peroxidation, rat, kidney

INTRODUCTION

Paraquat (1,1′-dimethyl-4,4′-bipyridinium) is a commonly used herbicide which has caused many deaths either accidently or after its deliberate ingestion. It is well known that paraquat undergoes a redox-cycling reaction which can lead to the production of reactive oxygen species such as the superoxide anion, hydrogen peroxide, and the hydroxyl radical.[1–3] Some investigators have suggested that paraquat produces its toxic effects by inducing membrane lipid peroxidation mediated by reactive oxygen species.[4,5]

We have developed a method for separating, detecting, and quantifying cholesterol-derived hydroperoxides, based on extraction, purification by solid-phase extraction cartridge, high-performance liquid chromatography with

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chemluminescent detection (HPLC-CL). Furthermore, we have succeeded in avoiding artifact formation during extraction and purification procedure.[6] We found two cholesterol-derived hydroperoxides, 7α-hydroperoxycholest-5-en-3β-ol (7α-OOH) and 7β-hydroperoxycholest-5-en-3β-ol (7β-OOH) in alcoholic fatty liver in evidently elevated amounts and showed augmented lipid peroxidation by chronic alcohol intake.[7]

The aim of this study is to evaluate lipid peroxidation in rats treated with paraquat at a very small dose. We measured the cholesterol-derived hydroperoxide in lung, liver, and kidney of rats to examine in which tissue and when paraquat cause peroxidation of lipids.

MATERIALS AND METHODS

Materials

Paraquat dichloride salt was purchased from Sigma (St. Louis, MO). 3,5-Di-tert-butyl-4-hydroxytoluene, luminol (3-aminophthaloylhydrazine) and cytochrome *c* (from horse heart, type VI) were purchased from Wako Pure Chemical Co. (Osaka, Japan). Cholesterol hydroperoxides, 5α-hydroperoxycholest-6-en-3β-ol (5α-OOH), 7α-OOH and 7β-OOH were synthesised as described previously.[8,9] β-Sitosterol-5α-hydroperoxide (as internal standard) was prepared by irradiating a solution of β-sitosterol and hematoporphyrin in pyridine.[8] Hydrogen chloride (5%, w/v) in methanol was obtained from Nacalai Tesque, Inc. (Kyoto, Japan).

Animals

Six-week-old female Wistar rats, weighing 100–120 g, were used. The rats received a single intraperitoneal injection of 10 mg/kg of paraquat dissolved in 1.0 ml saline. Control animals received 1.0 ml of saline solution only. The rats were sacrificed under deep anesthesia with Nembutal (pentobarbital sodium, Abbott Lab., Abbott Park, IL, USA) at 2, 24 h and 5 d after administration. The liver, kidney, and lung tissues were collected on ice. These experiments were approved by the Animal Research Committee of Kawasaki medical school and conducted according to the "Guide for the Care and Use of Laboratory Animals" of our School.

Extraction

For the analysis of lipid peroxidation products, total lipid was extracted as follows: 5 ml of ice-cold chloroform/methanol (2 : 1, v/v), containing 0.005% (v/v) butylated hydroxytoluene as antioxidant and 500 pmol β-sitosterol 5α-hydroperoxide as the internal standard, was added to approximately 0.2 g of liver and kidney and 0.4 g of lung, and the mixture homogenised under ice-cold conditions. The homogenate was mixed with 5 ml of chloroform/methanol (2 : 1, v/v) and 1 ml of distilled water, vortexed vigorously for 1 min and centrifuged at 800*g* for 20 min. The chloroform layer was aspirated, concentrated in a rotary evaporator and dried under a nitrogen stream. A cholesterol-rich fraction was subsequently isolated from the total lipid by solid phase extraction; a silica column (Sep-Pak, Waters Co. Milford, MA) of 3 ml capacity packed with aminopropyl-derivatized silica ($-NH_2$) was initially conditioned by washing with 5 ml of acetone and 10 ml of n-hexane. The total lipid sample, dissolved in a small amount of chloroform, was applied to the column, which then was flushed with a mixture of 2 ml chloroform : 1 ml iso-propanol, giving an eluate that mainly consisted of cholesterol. This was concentrated in a rotary evaporator and dried under a nitrogen stream. The cholesterol-rich fraction was dissolved in 500 μl methanol, and a 10 μl portion injected on to the HPLC column (see below).

LC-MS

A Hitachi L-7000 series liquid chromatography system fitted with Spherisorb ODS-2-5

(250 × 4.6 mm i.d.) and a model M-1200AP LC-MS system which incorporated an atmospheric chemical ionization system (Hitachi, Tokyo, Japan) were used to identify 7-hydroperoxycholesterol (7-OOH). The mobile phase, methanol containing 0.1 M ammonium acetate, was delivered at the flow rate of 0.7 ml/min.

HPLC-CL Analysis

Cholesterol hydroperoxides were determined by reverse phase HPLC-CL, comprising of two LC-10AD vp pumps (Shimadzu, Kyoto, Japan), a CLD-10A chemiluminescence detector (Shimadzu), and a Chromatopac C-R4A integrator (Shimadzu). A TSK gel Octyl-80Ts column (Tosoh, Tokyo, Japan) was used (150 × 4.6 mm internal diameter). As the mobile phase of methanol/water/acetonitrile (89:9:2, v/v/v) was delivered by one pump (at the flow-rate of 0.7 ml/min), the chemiluminescent reagent was delivered by the second pump (at the flow-rate of 0.5 ml/min). The reagent consisted of cytochrome *c* and luminol (10 and 2 μg/ml, respectively) in alkaline borate buffer (0.4 g boric acid and 1.06 g sodium carbonate in 1000 ml; pH 10) as described previously.[10] After the column eluant passed through a UV detector (set at 210 nm), it was mixed with the luminescent reagent in the post-column mixing joint of the chemiluminescence detector. Standard curves were prepared from the analyses of 1, 2, 4, 10, and 20 ng of 5α-OOH and 7β-OOH and from 0.5, 1, 2, 5, and 10 ng of 7α-OOH using 2.5 ng of the internal standard. Regression lines of the ratios of hydroperoxides to the internal standard versus the standard concentrations (pmol) were linear ($r^2 > 0.998$).

Statistical Analysis

All data are expressed as mean ± SD of 6 observations in each group. Differences between groups were assessed with Student's unpaired *t*-test.

RESULTS

In Control Tissues

Typical HPLC-CL chromatograms for cholesterol hydroperoxide are shown in Figure 1, which illustrates the chromatographic separation of 5α-OOH, 7α-OOH, 7β-OOH standards together with the internal standard using a TSKgel Octyl-80Ts column with methanol–water–acetonitrile as the mobile phase. The retention times of standard 7β-OOH, 7α-OOH, 5α-OOH, and the internal standard, respectively, on the chromatograms were 6.9, 7.4, 8.0 and 9.7 min, respectively, and they were clearly separable. Figure 1 also shows HPLC-CL chromatograms for cholesterol hydroperoxides in kidney samples from the control and paraquat groups. Peaks 1 and 2 in the kidney samples, with retention times of 6.9 and 7.4 min, respectively, corresponded with those of the respective standards for 7β-OOH and 7α-OOH. Lipid extracts from kidney contained 7α-OOH and 7β-OOH, but no 5α-OOH was detected in

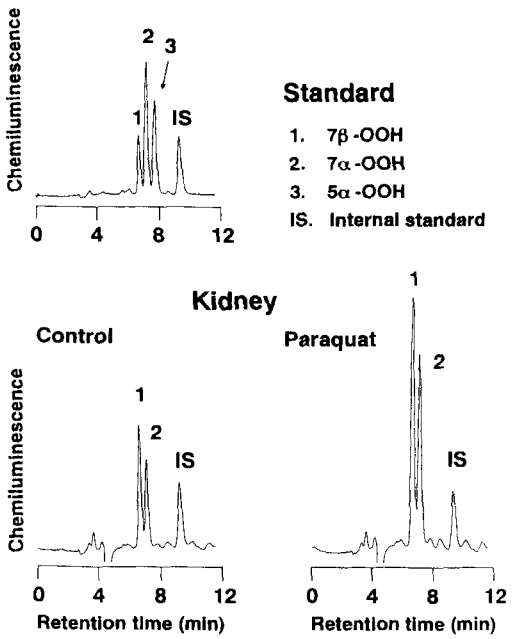

FIGURE 1 Representative HPLC-CL chromatograms for standard and kidney samples of control and paraquat groups.

these samples. The presence of 5α-OOH would have suggested artificial formation during processing of the tissue.[6]

The mean concentrations of 7α-OOH and 7β-OOH in control rat samples are shown in Tables I (liver), II (kidney) and III (lung). In the liver of the control group, 7β-OOH (15.8 nmol/g) was significantly higher than 7α-OOH (6.5 nmol/g). Similarly, in kidney of the control group, 7β-OOH (8.9 nmol/g) was significantly higher than 7α-OOH (3.7 nmol/g). In contrast, in the lung of the control group, 7β-OOH (0.06 nmol/g) was similar to 7α-OOH (0.06 nmol/g). Compared to kidney, significantly higher values of 7α-OOH and 7β-OOH were found in liver (6.5 and 15.8 nmol/g, respectively). Among the three tissues, the smallest concentrations of 7α-OOH and 7β-OOH were found in lung (0.06 and 0.06 nmol/g, respectively). Higher levels of 7β-OOH compared to 7α-OOH may reflect the fact that the latter is easily epimerized to 7β-OOH.[6] The smallest levels of 7α-OOH and 7β-OOH in lung compared to the liver and kidney may reflect the higher antioxidant capacity of lung.

TABLE I Changes of lipids in rat liver following acute paraquat administration (10 mg/kg i.p.)

	Control	2 h	24 h	5 d
7α-OOH	6.5 ± 1.5	10.2 ± 3.3*	8.1 ± 2.8	7.8 ± 1.8
Ratio		1.56	1.24	1.20
7β-OOH	15.8 ± 1.5	25.6 ± 9.2**	18.9 ± 6.0	16.5 ± 5.0
Ratio		1.62	1.20	1.04

Values (nmol/g tissue) are mean ± SD ($n = 6$). *$P < 0.05$, **$P < 0.01$ vs value before loading. The ratio of lipid concentration at each time to corresponding control value.

TABLE II Changes of lipids in rat kidney following acute paraquat administration (10 mg/kg i.p.)

	Control	2 h	24 h	5 d
7α-OOH	3.7 ± 1.5	6.3 ± 2.4**	8.3 ± 2.0*	7.3 ± 3.2*
Ratio		1.70	2.24	1.97
7β-OOH	8.9 ± 1.5	22.0 ± 2.4**	23.0 ± 2.0**	23.0 ± 1.2**
Ratio		2.47	2.58	2.58

Values (nmol/g tissue) are mean ± SD ($n = 6$). *$P < 0.05$, **$P < 0.01$ vs value before loading. The ratio of lipid concentration at each time to corresponding control value.

TABLE III Changes of lipids in rat lung following acute paraquat administration (10 mg/kg i.p.)

	Control	2 h	24 h	5 d
7α-OOH	0.06 ± 0.04	0.08 ± 0.04	0.08 ± 0.07	0.05 ± 0.01
Ratio		1.33	1.33	0.83
7β-OOH	0.06 ± 0.02	0.05 ± 0.02	0.12 ± 0.10	0.06 ± 0.02
Ratio		0.83	2.0	1.0

Values (nmol/g tissue) are mean ± SD ($n = 6$). The ratio of lipid concentration at each time to corresponding control value.

LC-MS

Analyses of lipid extracts from kidney of paraquat dosed rat by LC-MS gave four peaks in the total ion chromatograms.

The peak from rat kidney, appearing at an Retention time (Rt) of 7.0 min, was subjected to mass spectrometry. The mass spectra of standard 7-OOH and 7-hydroxycholesterol (7-OH), and the peak from kidney are shown in Figure 2. Standard

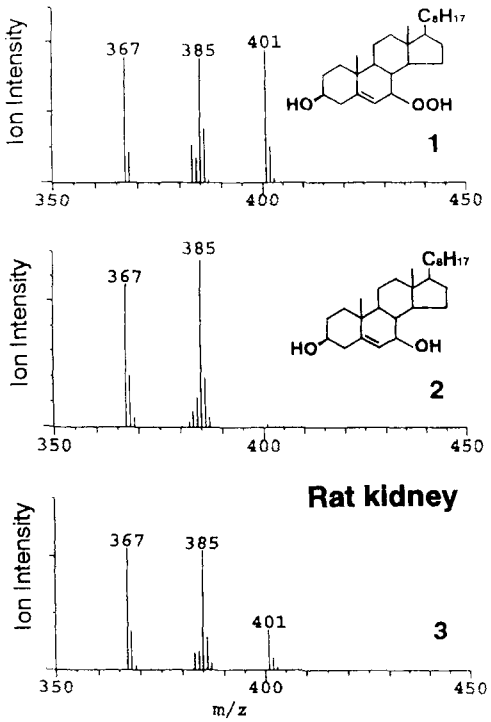

FIGURE 2 Mass spectra of standard 7-OOH, 7-OH, and the peak from rat kidney sample obtained by LC-MS with an atmospheric pressure chemical ionization interface.

7-OOH had an ion $[M+H-H_2O]^+$ at m/z 401 and fragment ions at m/z 385 and 367, whereas standard 7-OH had an ion $[M+H-H_2O]^+$ at m/z 385 and a fragment ion at m/z 367 but not at m/z 401. The Rts of standard 7-OOH, 7-OH, and the peak were all about 7.0 min, almost identical. Accordingly, the peak which had a larger ion at m/z 385 than at m/z 401 was assumed to be a mixture of 7-OOH and 7-OH.

Effects of Paraquat

Rats showed no clinical signs of paraquat poisoning. The concentrations of 7α-OOH and 7β-OOH in liver, kidney and lung tissues from paraquat groups are shown in Tables I (liver), II (kidney), and III (lung). The ratios of concentrations between paraquat and control group are also shown. There were significant increases of 7α-OOH (+56%) and 7β-OOH (+62%) in liver at 2 h after administration of paraquat at a dose of 10 mg/kg i.p. The increases returned at 24 h to the control values. Significant elevations of 7α-OOH (+70%) and 7β-OOH (+147%) were observed in kidney 2 h following acute paraquat. Marked elevations in 7α-OOH (+124%) and 7β-OOH (+158%) continued at 24 h. Surprisingly, these increases still remained on 5 d after a single 10 mg/kg i.p. of paraquat. In contrast, significant increases of 7α-OOH and 7β-OOH in lung were not seen 2, 24 h and 5 d following acute paraquat. Accordingly, quite low dose of paraquat affected evidently the membrane lipid from kidney as well as from liver, whereas it did not affect the lipid from lung.

DISCUSSION

Paraquat is used as a model for oxidative stress in both *in vivo* and *in vitro* studies.[11] The ensuing pulmonary damage induced by paraquat is due to enhanced membrane lipid peroxidation.[11,12] Recently, the concentrations of malondialdehyde (MDA),[5,13,14] MDA + 4-hydroxyalkenals (4HDA)[15,16] and diene conjugates[17] have been used as indices of the general peroxidative damage by paraquat. However, very little work has been undertaken to characterise the molecular species of the oxidised lipids formed during paraquat exposure. Below, we discuss our data in relation to observations made on liver, lung and kidney.

In liver, MDA + 4HDA levels have been shown to significantly increase 24 h following paraquat administration in doses of 50 mg/kg i.p.[16] and 70 mg/kg i.p.[15] However, hepatic MDA + 4HDA does not increase when paraquat is administered in a dose of 20 mg/kg i.p.[15] MDA concentrations in liver do not increase by dietary paraquat (26.6 mg/kg), though activities of liver glutathione peroxidase and catalase are increased.[13] In doses of 26–129 mg/kg s.c., paraquat increases hepatic metallothionein (MT) levels, which is associated with elevations in the levels of hepatic MT-1 and MT-2 mRNA.[18] In the present study, we report increased levels of 7α-OOH and 7β-OOH cholesterol hydroperoxides in liver only 2 h after paraquat administration, even with low doses (i.e., 10 mg/kg i.p.). Accordingly, it seems that some of the above mentioned analytes (i.e., MDA, for example) are relatively insensitive for identifying oxidative stress in liver tissue of paraquat-exposed rats.

In lung, MDA + 4HDA[15,16] and diene conjugate levels[17] are significantly elevated 24 h after paraquat administration at a dose of 20 mg/kg i.p. Two injections of 20 mg/kg i.p. paraquat have been shown to increase lung MDA at 5, 7, and 15 d.[14] An increase in lung GSH-Px has been observed after 5 d.[14] In our study, by contrast, levels of 7α-OOH and 7β-OOH at either 2, 24 h or 5 d after paraquat were not significantly affected in lung, because paraquat dosage (10 mg/kg) was too low.

In man, moderate to severe poisoning occurs following ingestion of paraquat at doses of 20–40 mg/kg body weight, with the concomitant development of renal failure and pulmonary fibrosis.[19] Paraquat, in doses greater than 40 mg/kg

p.o., causes acute multiple organ failure (i.e., cardiac, respiratory, hepatic, renal, adrenal, pancreatic, neurological).[19] Thus, membrane disorders due to paraquat-induced lipid peroxidation must occur not only in liver and lung, but in kidney as well. Nevertheless, levels of neither MDA nor diene conjugates in kidney have been reported as indices of lipid peroxidation.

We are the first to succeed in identifying 7-OOH directly from kidney of paraquat dosed rat using LC-MS. In addition, we report increased levels of 7α-OOH and 7β-OOH in kidney of rats at 2, 24 h and 5 d following 10 mg/kg paraquat. These data therefore describe for the first time (a) 7α-OOH and 7β-OOH in kidney as well as liver and (b) evidence for paraquat-induced free radical formation in rat kidney as reflected by increased levels of cholesterol hydroperoxides as specific markers of lipid peroxidation.

Studies of paraquat excretion, following its oral administration in rats, show that 64% of the herbicide is excreted in the faeces and urine over 14 days. The remainder is retained in organs such as the lung, liver, and kidney.[20] Our finding that the sustained increases of 7α-OOH and 7β-OOH were observed at 5 d following acute paraquat is consistent with above finding. A delayed increase of lung lipid peroxides, as described in a previous study,[14] may be indicative of increased lipid peroxidation in the macrophages after initial cell injury in the paraquat-treated lung. We found an accumulation of cholesterol hydroperoxide in kidney and liver even at 2 h following paraquat administration. Thus, an indication of paraquat-induced lipid peroxidation seems to be determined not only by the appropriate marker, but the dose and timing of the tissue sampling.

Bus *et al.*[21] reported that paraquat stimulates lipid peroxidation *in vitro* and suggested the involvement of superoxide anions and singlet oxygen. Hara *et al.*[5] showed that SOD and quenchers of singlet oxygen decrease the stimulation of lipid peroxidation by paraquat in brain and pulmonary microsomes, supporting previous studies.[21] These authors suggested that generation of superoxide anion and singlet oxygen, rather than hydrogen peroxide and the hydroxyl radical, may play the major role in microsomal lipid peroxidation in both brain and lung under the conditions where paraquat stimulates NADPH-dependent lipid peroxidation.[5,21] Peroxidation of cholesterol is known to be induced by such active oxygen species as singlet oxygen, producing 5α-OOH as the first step, which is consistent with their observations.

In conclusion, we are able to demonstrate for the first time evidence for increased lipid peroxidation in kidney following acute paraquat administration to rats. Furthermore, we are able to demonstrate the different degrees of vulnerability among lung, liver and kidney to the paraquat-stimulated peroxidations. We have confirmed that cholesterol-derived hydroperoxides are suitable and sensitive indices of oxidative stress.

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