In Vivo Effect of Diosmin on Carrageenan and CCl_4-Induced Lipid Peroxidation in Rat Liver Microsomes

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ABSTRACT: The aim of this study was to compare the protective effect of a flavonoid, the 3',5,7-trihydroxy-4'-methoxyflavone 7-rutinoside or diosmin, on liver microsomal lipid peroxidation induced in rats by either carbon tetrachloride or carrageenan. Thirty rats were divided into five groups. Group 1 received no chemical product and was considered as control. Groups 2 and 3 received either an intraperitoneal injection of carrageenan or carbon tetrachloride 48 or 24 hours before killing, respectively. Groups 4 and 5 were treated first with an intraperitoneal injection of diosmin and then by carrageenan (group 4) or carbon tetrachloride (group 5) 48 or 24 hours before killing, respectively. The lipoperoxidant effect of carrageenan and carbon tetrachloride was demonstrated by both significant decreases in polyunsaturated fatty acids, principally 20:4 ($n - 6$) ($p < 0.05$) and of vitamin A ($p < 0.05$) in groups 2 and 3. With diosmin treatment, only thiobarbituric acid reactive substances significantly decreased in group 4, whereas vitamin A level increased. These results could suggest that the effect of diosmin differs with the choice of chemical product used; it seems a better antioxidant against products inducing inflammation. © 1996 John Wiley & Sons, Inc.

KEY WORDS: **Carbon Tetrachloride, Carrageenan, Diosmin, Lipid Peroxidation, Liver.**

INTRODUCTION

Lipid peroxidation (LPO) has been identified as a deleterious process involved in many pathological disorders such as liver injury (1). It was initiated by an excessive production of reactive oxygen species (ROS) that reacted with membrane lipids. The oxidative degradation of polyunsaturated fatty acids (PUFA) leading to damage of biological membranes is one of the major effects of free radical reactions (2). In physiological conditions, LPO occurs, but many enzymatic systems or antioxidant nutrients protect the biological membranes. LPO can be detected by measuring the breakdown products of lipid peroxides such as malondialdehyde (MDA) by the simple thiobarbituric acid (TBA) test, as thiobarbituric acid reactive substances (TBARS), which correlates well with other methods for measuring LPO (5).

Carbon tetrachloride (CCl_4) is a hepatotoxic agent that is known to stimulate LPO in rat liver microsomes and that produces also disturbances to other tissues (6). It has been effectively demonstrated (7) that most LPO in the liver of CCl_4-intoxicated rats occurs in the endoplasmic reticulum where most cytochrome P450 is located. The toxicity of CCl_4 is depending upon its metabolic activation to trichloromethyl free radical $CCl_3^{\cdot}$ that can function as a peroxidation chain initiator. $CCl_3^{\cdot}$ combinates with O_2 to yield the more reactive trichloromethylperoxy radical $CCl_3O_2^{\cdot}$ (8) that covalently binds to cellular components or sparks a LPO process (9). Some researchers indicated that the damage produced (10) will depend on the ratio between trichloromethyl and trichloromethylperoxy radicals. However, a direct CCl_4 action detected by enzyme leakage has been proposed by Berger et al. (11).

Carrageenan, a long-chain–sulfated polysaccharide, is known to induce inflammation and a lipoperoxidant effect in rats (12). An increased release of many factors like histamin, serotonin, prostaglandins, and leukotrienes has also been observed in the inflammatory tissues (13).

Diosmin, the 3',5,7-trihydroxy-4'-methoxyflavone 7-rutinoside, is one of the flavonoid family that shows antioxidant or free radical scavenger properties. It may reduce in rats the paw oedema induced by doxorubicin (14) and the inflammatory granuloma, affecting the synthesis of prostaglandins and thromboxane (15). This flavonoid protects isolated human lipoproteins from in vitro peroxidation (16) and is extensively metabolized by the liver (17).

In a previous study, we have demonstrated that treatment of rats with diosmin before an injection of carrageenan or CCl_4 decreased in serum the acute inflammatory reaction and the lipoperoxidant effect (12).

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The present study was designed to investigate in vivo the direct influence of diosmin on rat liver microsomes after carrageenan or CCl_4 intoxication and to find out whether diosmin treatment may compensate for the damage formed in the presence of a high level of peroxides.

MATERIALS AND METHODS

Animals and Treatments

Thirty weanling male Wistar rats weighing 50–60 g were housed individually in a room with a temperature of 20°C. They were fed on commercial food pellets AO3 (UAR, Epinay sur Orge, France). Food and water were provided ad libitum. Rats were assigned to the basal diet for 12 weeks. Then they were weighed (400–420 g) and divided into five groups. Group 1 received no treatment before killing and was considered as control. Group 2 received an intraperitoneal injection of 2.5 mL of 2% suspension of carrageenan in 0.15 M NaCl (corresponding to 50 mg carrageenan) per kg body weight, 48 hours before killing. Group 3 received an intraperitoneal injection of 1.25 mL of a 1/4 (V/V) mixture of CCl_4 and vaseline oil (corresponding to 0.31 mL CCl_4) per kg body weight, 24 hours before killing. Groups 4 and 5 were pretreated with an intraperitoneal injection of 1.66 mL of a 90/1 (W/V) suspension of diosmin in 0.15 M NaCl (corresponding to 150 mg diosmin) per kg body weight and per week during the last 8 weeks, which preceded injections of carrageenan (group 4) or CCl_4 (group 5). Food consumption and body weights were measured weekly.

Tissue Preparation

After an overnight fast of 12 hours, rats were killed by decapitation. The livers were quickly excised; rinsed in saline; and then dried, weighed, and stored at −80°C until analyzed. Microsomal suspension was prepared as described previously (18). In brief, the homogenized rat liver (1 g wet weight/3 mL of 10 mM Tris-HCl, 150 mM KCl, 0.1 mM EDTA buffer, pH 7.4) was centrifuged initially at 9,000 × g for 15 minutes at 4°C, and the supernatant was recentrifuged at 105,000 × g for 1 hour at 4°C. The resulting pellet was suspended in 10 mM Tris-HCl, 150 mM KCl buffer, pH 7.4, so as to obtain a concentration of 5–10 mg protein/mL and was used immediately.

Analytical Procedure

Vitamins A and E and retinylpalmitate were quantitated by high-performance liquid chromatography (HPLC) using a method previously described (19). The results were expressed as pmol/mg of liver microsomal protein.

LPO was evaluated as TBARS, as described by Recknagel et al. (20). Tetraethoxypropane was used as standard. TBARS were spectrophotometrically determined at 532 nm, and the LPO level was expressed as nmol of MDA equivalents/mg of liver microsomal protein. Protein was estimated by the method of Lowry *et al.* (21) using bovine serum albumin as standard.

Fatty acid composition was determined from 2 mL of a lipid extract after transformation in isopropylic esters (22). Peak identification was made by comparison with reference fatty acids (Sigma, St. Louis, MO). Quantification of each fatty acid was expressed as the percentage of the total extract.

Statistical Analysis

Data are presented as means ± SD. Statistical analyses were done using a statistical software package (Stat-View). For comparison between paired data, the Mann Whitney test was chosen because of the sample size. A p value < 0.05 was considered significant.

RESULTS

At the end of the experimental period, no significant differences in body and liver weights were observed among the five groups.

As compared to control group 1, microsomal vitamin A (Table 1) was significantly decreased after carrageenan or CCl_4 treatment in groups 2 and 3; with diosmin, the decrease was still significant in groups 4 and 5. Retinylpalmitate was only significantly decreased in CCl_4 groups 3 and 5, as compared with group 1. A slight increase was observed between groups 2 and 4 and between groups 3 and 5. Unlike vitamin A, vitamin E levels were similar in all studied groups.

Levels of microsomal TBARS (Table 2) were significantly increased in groups 2 through 4 compared with control group 1, indicating the occurrence of LPO. However, the TBARS amount was significantly decreased in group 4 as compared with group 2, while no significant difference was observed between groups 3 and 5. Comparative spectra of TBARS formed during peroxidation (Figure 1) showed a peak at 532 nm in all cases and a slight absorption at 460 nm in CCl_4 groups 3 and 5.

Total PUFA percentages (Table 2) decreased significantly in groups 2 through 4. Fatty acid composition of experimental groups (Table 3) was determined after

TABLE 1. Vitamin A and E Concentrations[a] in Liver Microsomes

	Groups				
Measurement	*1 Control[b]*	*2 Carrageenan*	*3 CCl_4*	*4 Diosmin Plus Carrageenan*	*5 Diosmin Plus CCl_4*
Vitamin A	366 ± 77[c]	183 ± 33*	100 ± 16*	207 ± 45*	103 ± 32*
Retinyl palmitate[d]	245 ± 58	160 ± 36	129 ± 19*	197 ± 40	142 ± 17*
Vitamin E	199 ± 51	165 ± 34	146 ± 28	168 ± 27	136 ± 19

[a]Vitamins A and E are expressed as pmol/mg microsomal protein.
[b]Control group received no treatment before killing.
[c]Values are means ± SD for six animals per group. Significance level for each group compared with control group 1 was studied by the Mann Whitney test (*$p < 0.05$).
[d]Retinylpalmitate is expressed as pmol/mg microsomal protein.

TABLE 2. Lipid Peroxide (TBARS) Values[a] and Polyunsaturated Fatty Acids (PUFA) Percentages[b] in Liver Microsomes

	Groups				
Measurement	*1 Control*	*2 Carrageenan*	*3 CCl_4*	*4 Diosmin Plus Carrageenan*	*5 Diosmin Plus CCl_4*
TBARS	108 ± 50[c]	670 ± 131*	190 ± 57*	284 ± 56*	170 ± 44
PUFA	50 ± 1	41 ± 1*	45 ± 1*	47 ± 1*	49 ± 1

[a]TBARS values are expressed as nmol/mg microsomal protein.
[b]PUFA are expressed as area % of total fatty acids.
[c]Values are means ±SD for six animals per group. Significance level for each group compared with control group 1 was studied by the Mann Whitney test (*$p < 0.05$).

an overnight fast of 12 hours. The most abundant microsomal PUFA of control rats were arachidonic acid, 20:4 ($n - 6$), 21.73 ± 2.06%; linoleic acid, 18:2 ($n - 6$), 16.53 ± 1.36%; and docosahexaenoic acid, 22:6 ($n - 3$), 7.84 ± 0.71%. In group 2, we observed a significant decrease in 20:4 ($n - 6$) and in 22:6 ($n - 3$) mainly involved in LPO. In group 3, only 20:4 ($n - 6$) significantly decreased. This last PUFA was also depressed in groups 4 and 5. However, diosmin treatment led to an increase in PUFA percentages, higher in group 4 than in group 5, compared with groups 2 and 3, respectively.

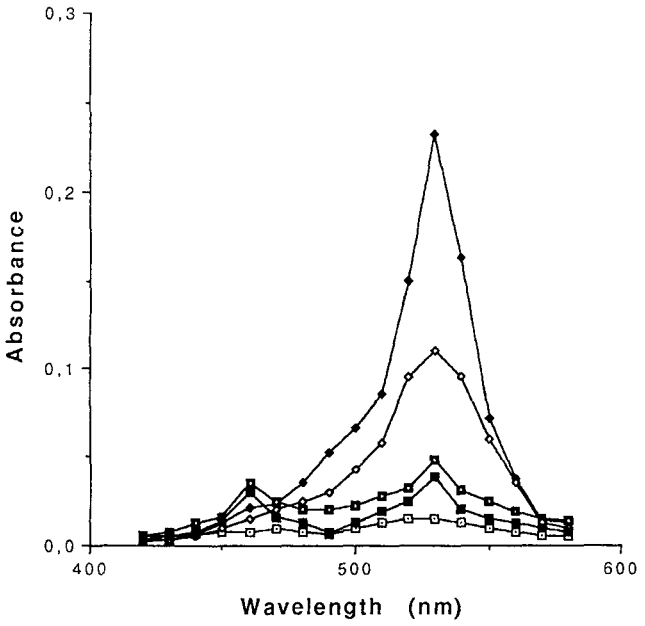

FIGURE 1. Dependence of absorption on wavelength. The absorption was measured in rat liver microsomal suspension after TBA reaction. Reaction conditions are described in Materials and Methods. Symbols used are as follows: -□- control (1); -◆-, carrageenan (2); -■-, CCl_4 (3); -◇-, diosmin plus carrageenan (4); -■-, diosmin plus CCl_4 (5).

DISCUSSION

This study investigated the in vivo effect of a flavonoid, the diosmin, on the peroxidative process induced by chemical products, such as carrageenan and CCl_4 in rat liver microsomes.

At the end of the experimental period, body and liver weights of the different groups did not differ one from another, indicating that the nutritional requirements of rats were not altered by the different treatments. However, in serum, the inflammatory effect of carrageenan was demonstrated by elevated levels of $\alpha 1$, $\alpha 2$, and β globulins and the hepatotoxicity of CCl_4 proved by the increase of $\alpha 1$ globulin (data not shown).

Microsomal vitamin A levels significantly decreased, more in group 3 than in group 2 versus group 1. Such a decrease in hepatic vitamin A storage was

TABLE 3. Fatty Acid Composition[a] of Experimental Groups

	Groups				
	1	*2*	*3*	*4*	*5*
Fatty Acid	*Control*	*Carrageenan*	*CCl_4*	*Diosmin Plus Carrageenan*	*Diosmin Plus CCl_4*
16:0	21 ± 2[b]	24 ± 2	23 ± 2	22 ± 2	22 ± 2
16:1 (n − 7)	<2	<2	<2	<2	<2
18:0	20 ± 2	26 ± 2	23 ± 2	23 ± 2	20 ± 1
18:1 (n − 9)	7 ± 1	7 ± 1	8 ± 1	7 ± 1	8 ± 1
18:2 (n − 6)	17 ± 1	16 ± 1	17 ± 2	19 ± 2	19 ± 2
20:4 (n − 6)	22 ± 2	16 ± 1*	16 ± 1*	17 ± 2*	18 ± 2*
20:5 (n − 3)	<2	<2	<2	<2	<2
22:5 (n − 3)	<2	<2	<2	<2	<2
22:6 (n − 3)	8 ± 1	6 ± 1*	8 ± 1	7 ± 1	8 ± 1

[a]Fatty acids are expressed as area % of total fatty acids. Fatty acid composition was determined after on overnight fast of 12 h.
[b]Values are means ± SD for six animals per group. Significance level for each group compared with control group 1 was studied by the Mann Whitney test (*$p < 0.05$).

also observed after chronic ethanol consumption (23). It is known that the major site of CCl_4 activation within the liver is the endoplasmic reticulum where most cytochrome P450 is located (7). Since an increase in cytochrome P450 has been reported in rats treated with CCl_4 (23) or with phenobarbital (24), its contribution on the observed decrease in vitamin A level can be suggested. Oxidation of vitamin A and increased catabolism of retinoic acid (25) have also been postulated to explain the vitamin A decrease. The low vitamin A concentration observed with carrageenan can be due to its utilization in the synthesis of glycoproteins (26) and also to its consumption as antioxidant like ascorbate, SH groups, and urate (27). Diosmin treatment led to a slight increase in vitamin A level in groups 4 and 5 (13% and 3%, respectively) compared with groups 2 and 3. Thus the interaction between CCl_4 and vitamin A appeared drastic for the microsomal membrane and led to vitamin A degradation in the liver, as observed after ethanol administration (28). Similarly, retinylpalmitate, the major tissue storage form of liver vitamin A, decreased significantly after CCl_4 intoxication; with diosmin treatment, the decrease was less marked (group 5). In the carrageenan group, retinylpalmitate was not significantly depressed, indicating the lower effect of carrageenan on vitamin A metabolism. Although it is widely accepted that vitamin E acts as an antioxidant and protects biological membranes against free radical damage, microsomal vitamin E level remained quite unchanged in the carrageenan and CCl_4 groups. Miyazawa *et al.* (29) found a relatively large amount of vitamin E in the liver, 24 hours after the CCl_4 dose, as a consequence of antioxidative metabolic adaptation in the rat body. So regeneration of vitamin E (30) by a synergistic action in vivo between vitamins E and C can explain the similar vitamin E level observed especially in carrageenan groups 2 and 4.

Among the markers of LPO, TBARS were mainly increased in the carrageenan group since carrageenan induced an extensive inflammatory process. The CCl_4 group exhibited a lower but significant increase in TBARS production, as reported in in vitro (9, 31) as well as in vivo experiments (32, 33). An explanation of the slight increase observed in group 3 can be that lipid dienyl radicals generated after CCl_4 injection in part decompose to form alkanes that do not undergo significant oxidative degradation to produce MDA (34). The marked decrease in TBARS values between groups 2 and 4 shows the protective antioxidant role of diosmin against the carrageenan inflammatory process. As shown in Figure 1, although MDA is known to be the main aldehydic peroxidation product to have an absorbance maximum at 532 nm after TBA reaction (35), spectra of TBARS in groups 3 and 5 showed another peak at 460 nm, indicating the presence of interfering aldehydic compounds inducing in a slight overestimation of LPO, as observed by others in erythrocyte hemolysate (36). This phenomenon can explain that no significant difference in TBARS values was observed between groups 3 and 5.

Since TBARS are not the exact reflect of LPO (37), determination of PUFA that are the direct targets of ROS seems to be a better marker. A significant decrease in PUFA percentages takes place both in groups 2 and 3 versus control group 1. Peroxidative degradation of unsaturated fatty acids can greatly alter the function of cell membranes (38). The more unsaturated fatty acids are most susceptible to LPO. 20:4 (n − 6) and 22:6 (n − 3) are the major fatty acids to be destroyed during CCl_4-stimulated LPO in liver microsomes (8); they are significantly decreased in our experiments, especially in group 2. Only 20:4 (n − 6) decreased in group 3. With diosmin treatment, the PUFA level increased in groups 4 and 5, compared with groups 2 and 3, respectively.

So the decreased LPO observed in rats treated with diosmin compared with untreated rats may be attrib-

uted to the antioxidant effect of diosmin. However, the difference in the response of the liver microsomes to diosmin correlates well with their susceptibility to carrageenan or CCl_4-induced LPO.

Our results suggest that diosmin can be used to maintain sufficient protection against liver oxidative damage, especially in the carrageenan inflammatory process. Its antioxidant efficiency may be influenced by several factors, including the rate of radical production and the type of radical initiator. Further studies should be conducted to investigate the biochemical sequences preceding the pathological manifestations of carrageenan and CCl_4 in liver cell membranes.

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