Effects of Oxidized Frying Oil on Proteins Related to α-Tocopherol Metabolism in Rat Liver

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Summary An oxidized frying oil (OFO) diet has been reported to induce an increase in lipid peroxidation and a reduction in vitamin E status in animal tissues. This study was performed to investigate how vitamin E metabolism is influenced by OFO. Male Wistar rats were divided into three groups, a control group (CO) and two OFO-fed groups (OF and OFE). The diet of the OFE group was supplemented with an extra 50 mg/kg of α-tocopherol acetate and thus contained twice as much vitamin E as that of the OF group. After six weeks on these diets, liver α-tocopherol levels in the OF group were the significantly lowest among the three groups. Excretion of the α-tocopherol metabolite, α-carboxyethyl hydroxychroman (α-CEHC) in the urine was significantly lower in the OF group than in the other two groups. There were no significant differences in protein levels of α-tocopherol transfer protein (α-TTP) and multidrug resistance protein among the three groups. Protein levels of cytochrome P450 monooxygenase (CYP) 3A, CYP4A, and catalase were markedly increased in both groups on the OFO diet. This suggests that an OFO diet may interfere with medicine metabolism and needs further investigation.

Key Words: oxidized frying oil, α-tocopherol, α-carboxyethyl hydroxychroman, cytochrome P450, rats

Introduction

Fried foods are popular and provide a high fat source in the diet. Levels of lipid peroxide and toxic polymer [1] and polar compounds [2] increase with time during the frying process due to oil oxidation. The effects of oxidized frying oil (OFO) on animals has been widely studied. The induction of total cytochrome P450 monooxygenase (CYP) activity [3] by OFO suggests that OFO contains substances that need to be metabolized by CYPs in the liver. CYP1A1 [4] and CYP4A1 [5] protein levels and CYP4A1 and CYP2E1 mRNA levels [6] are also increased when rats are fed thermally oxidized dietary fat.

In rats fed an OFO-diet, increased oxidative stress is an important issue, since higher lipid peroxidation and lower vitamin E status have been reported [7–9]. Vitamin E is an important lipid soluble-antioxidant in oil and biomolecules. Loss of vitamin E during heating and the reduced absorption of dietary vitamin E are factors causing a decreased vitamin E status in rats fed an OFO diet [9]. A faster catabolism/turn-over of vitamin E in OFO diet-fed rats was suggested by Liu and Huang [10], who found higher excretion of radiolabeled vitamin E in the urine and feces after 14C-tocopherol injection, but the mechanism is still uncertain.

This study was performed to investigate how vitamin E metabolism is influenced by OFO. First, levels of the metabolite product of α-tocopherol in the urine, α-
carboxyethyl hydroxychroman (α-CEHC) [11], might be increased if α-tocopherol catabolism in liver were enhanced. α-CEHC is reported to be produced by cytochrome P450 3A (CYP3A), the xenobiotic metabolizing enzyme [12, 13]. Second, since α-tocopherol can be excreted in the feces with bile [14], the higher levels of radiolabeled α-tocopherol in the feces could come from increased bile excretion. Multidrug resistance (MDR) protein, or P-glycoprotein, in the liver is involved in the biliary excretion of various drugs and xenobiotics [15, 16]. Third, plasma α-tocopherol levels might be decreased by lower secretion of α-tocopherol from the liver by downregulation of the expression of α-tocopherol transfer protein (α-TTP). Levels of proteins related to vitamin E metabolism (CYP3A, MDR1, and α-TTP) and antioxidant enzymes in the rat liver were therefore measured in this study.

**Materials and Methods**

**Materials**

The α-CEHC standard was generously donated by Dr. C.J. Huang (Laboratory of Nutritional Biochemistry, Institute of Microbiology and Biochemistry, National Taiwan University, Taipei, Taiwan). Primary antibodies against rat α-TTP were prepared as described previously [17] except they were raised in a rabbit instead of Balb/c mice. Antibodies obtained as the following: rabbit anti-rat CYP3A1 polyclonal antibody (Chemicon, Billerica, MA); goat anti-rat CYP4A (Daichi pure chemicals, Tokyo, Japan); mouse monoclonal to β-actin, mouse monoclonal (C219) to P Glycoprotein, MDR1, rabbit polyclonal to superoxide dismutase (SOD), rabbit polyclonal to glutathione peroxidase (GPxs) and rabbit polyclonal catalase (Abcam, Cambridge, UK). The secondary antibodies used were: mouse IgG antibody (Abcam, Cambridge, UK); Horseradish peroxidase-conjugated (HRP)-goat anti-rabbit IgG antibodies (Zymed Co., South San Francisco, CA); anti-goat HRP (KPL, Gaithersburg, MD).

**Oxidized frying oil**

OFO was prepared by frying dough sheets in non-stripped soybean oil at 205 ± 5°C for four 6-h periods, as described previously [18]. The vitamin E-stripped fresh soybean oil, fresh soybean oil, and OFO were analyzed for acid value, TBA value, carbonyl values, and UV absorbance at 233 nm [3]. The vitamin E content of the test oils was analyzed by HPLC as described below for plasma and liver homogenate.

**Animals and diets**

Male Wistar rats (3-weeks-old), purchased from BioLASCO Co. (Taipei, Taiwan), were housed individually in stainless-steel wire cages in a room maintained at 23 ± 2°C with a controlled 12-h light/dark cycle (lights on at 7 am) and with free access to water and food. The feeding period is 6 weeks, body weights and food intake were recorded weekly. The compositions of the test diets (groups OF and OFE) and control diet (group CO) are given in Table 1. Vitamin E in oil is largely lost during the deep-frying process [9], so vitamin E-stripped soybean oil was used as the fat source of the control diet in this study. The vitamin E-stripped fresh soybean oil used for group CO was prepared from fresh soybean oil by treatment with active carbon, as described by Liu and Huang [9] to remove vitamin E. The diet of the OFE group was supplemented with 50 mg/kg of

### Table 1. Composition of the test diets.

| Ingredient                                      | Group     |
|-------------------------------------------------|-----------|
| Corn starch (g/kg)                              | CO 572    |
| Casein (g/kg)                                   | OF 200    |
| Oxidized frying oil (g/kg)                      | OFE 200   |
| Vitamin E-stripped fresh soybean oil (g/kg)     | CO 150    |
| Cellulose (g/kg)                                | OF 30     |
| Mineral mixture (g/kg)                          | OFE 30    |
| Vitamin mixture (g/kg)                          | CO 10     |
| DL-Methionine (g/kg)                            | OF 3      |
| Choline bitartrate (g/kg)                       | OFE 3     |
| All-rac-α-tocopheryl acetate (mg/kg)            | CO 50     |
| Final vitamin E content (mg/kg)                 | OF 50     |
|                                                  | OFE 100   |

1Oxidized frying oil was prepared by frying dough sheets in soybean oil (President Co., Tainan, Taiwan) at 205 ± 5°C for 24 h.
2Vitamin E-stripped fresh soybean oil was prepared using active carbon.
3Vitamin mixture contained all-rac-α-tocopheryl acetate 5000 IU/kg.
α-tocopherol acetate to give a diet with 2-fold higher levels of vitamin E than that of the OF group.

**Tissue sampling and preparation**

On the last day of the feeding period, each rat was transferred to a metabolic cage and urine collected for the last 24 h. The urine was kept ice-cold during collection, then ascorbic acid was added (100 mg/mL urine) and the sample stored at −20°C under nitrogen until used for the α-CEHC assay. Food was then withheld overnight and the rats killed by carbon dioxide asphyxiation in the morning. Blood was collected from the abdominal vena cava and centrifuged at 1,000 × g for 10 min at room temperature and the plasma stored at −80°C. A small piece of liver was homogenized and cytosol and microsome fractions prepared as described previously [19] and stored at −70°C until analysis.

**Biochemical analysis**

Concentrations of α-tocopherol in the plasma and liver homogenate were analyzed by HPLC as described previously [20]. Thiobarbituric acid-reactive substances (TBARS) levels in the liver homogenate were measured using a fluorescence method [21]. α-CEHC levels in the urine were determined using HPLC with an electrochemical detector as described previously [19]. Creatinine levels in the urine were measured by colorimetric methods and triglyceride levels in the plasma and liver were measured by enzymatic methods using commercial kits (Randox Lab, Northland, UK). Liver triglycerides were extracted using a 2:1 v/v mixture of chloroform/methanol, and a 10 uL aliquot dried down for assay.

**Western blot analysis**

Aliquots of the liver cytosolic fraction (for detection of α-TTP, SOD, GPxs, and catalase) or the microsomal fraction (CYP3A, CYP4A and MDR protein detection) containing 10 μg of protein were separated by SDS-polyacrylamide electrophoresis and transferred to a PVDF membrane. The details of the Western blot process have been described previously [19].

**Statistical analysis**

The data are expressed as the mean ± SD. The significance of differences between two groups or among the three groups were analyzed separately by Student’s t test or one-way ANOVA and Duncan’s multiple range test. The general linear model of the SAS package (SAS institute, Cary, NC) was used, and differences were considered significant at p<0.05.

**Results**

**Quality and vitamin E content of the test oils**

The acid value, TBA value, carbonyl value, and UV233 were all increased in the OFO compared to fresh soybean oil (Table 2). α-Tocopherol and γ-tocopherol levels in OFO were decreased by 62% and 76%, respectively, indicating vitamin E degradation during the frying period. The vitamin E-stripped soybean oil showed the same quality as fresh soybean oil. Levels of α-tocopherol and γ-tocopherol in the vitamin E-stripped oil were reduced by 80% and 32%, respectively. These results show that the active carbon treatment procedure removed most of the vitamin E, but did not lead to oil oxidation.

**Animal growth and food intake**

Food intake, feed efficiency, final body weight, and body weight gain are shown in Table 3. There were no significant differences in food intake among the three groups. Feed efficiency and body weight gain in rats fed the OF and OFE diets were significantly lower than in those fed the CO diet. Liver weight and liver relative body weight in the OF and OFE groups were significantly higher than in the CO group (Table 3), results often observed in OFO-fed rats [8, 22, 23].
Vitamin E status and lipids

As shown in Table 4, plasma α-tocopherol levels and the molar ratio of α-tocopherol to triglyceride in the plasma were significantly lower in groups OF and OFE than in group CO. Two-fold higher vitamin E levels in the diet did not result in significantly increased plasma α-tocopherol levels (OFE group compared to the OF group). The liver α-tocopherol concentration and whole liver α-tocopherol levels in the OF group were the lowest among the three groups, followed by those in the OFE group. This shows that vitamin E supplementation of the diet can increase α-tocopherol levels in the livers of rats fed an OFO diet. Liver TBARS levels were not reduced significantly by 2-fold vitamin E supplementation (Table 4). The liver triglyceride levels were significantly decreased in the OF and OFE groups (about 21% and 25% of those in the CO group, respectively). The molar ratio of α-tocopherol to triglyceride in the liver was significantly higher in the OFE group than in the OF and CO groups. α-CEHC excretion in the urine was significantly decreased in the OF and OFE groups, with no significant difference between the CO and OFE groups (Table 4). The urine α-CEHC/liver α-tocopherol ratio was highest in the OFE group and lowest in the CO group.

Western blot of proteins related to vitamin E metabolism

There was no difference in protein levels of cytosolic α-TTP (Fig. 1A) and microsome MDR protein (Fig. 1C) among the three groups. Microsomal levels of CYP protein levels were significantly decreased in the OF and OFE groups (about 21% and 25% of those in the CO group, respectively). The molar ratio of α-tocopherol to triglyceride in the liver was significantly higher in the OFE group than in the OF and CO groups. α-CEHC excretion in the urine was significantly decreased in the OF group, with no significant difference between the CO and OFE groups (Table 4). The urine α-CEHC/liver α-tocopherol ratio was highest in the OFE group and lowest in the CO group.

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were showed in Fig. 1B. Levels of both CYP4A and CYP3A were markedly increased in both groups fed the OFO diet.

**Protein levels of antioxidant enzymes**

Protein levels of the antioxidant enzymes glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase in cytosol were also analyzed by Western blotting (Fig. 2). Catalase levels were significantly increased by about 2-fold in both the OF and OFE groups compared to the CO group. There was no significant difference in GPx and SOD protein levels among the three groups.

**Discussion**

Vitamin E in oil is largely lost during the deep-frying process [9], so vitamin E-stripped soybean oil was used as
the fat source of the control diet in this study to ensure α-tocopherol levels in the oil were closely comparable in each group. Given the lower absorption of vitamin E (about 56%) in OFO-fed rats [9], the amount of vitamin E absorbed into the body could be different between the OF and CO groups, so the OFE group was also included in this study. Since this diet contained twice as much vitamin E as the OFO diet, the amount of vitamin E absorbed in the OFE group should be similar to that in the CO group.

Liver TG levels in rats fed an OFO diet are decreased [24, 25] and this has been shown to be due to the activation of PPAR-α and its target genes, acyl coenzyme A oxidase and sterol regulatory element-binding protein-1, which are involved in fatty acid oxidation, and also to a reduction in lipogenic enzymes levels [26, 27]. The increased expression of CYP4A1, which is involved in fatty acid oxidations [27], would also contribute to the enhanced fatty acid oxidation in OFO-fed rats seen in the present study.

α-Tocopherol levels in the liver and plasma were decreased in these two OFO-fed groups. The α-tocopherol/triglyceride ratio in the plasma in the OF and OFE groups was reduced to 49% and 70% of that in the control group, respectively. Since α-tocopherol is secreted from the liver packaged with triglyceride in VLDL, one might speculate that secretion could be impaired in rats fed an OFO diet. α-TTP, a cytosolic protein in the liver with a high affinity for α-tocopherol, plays a role in regulating plasma vitamin E levels [28–31]. Its levels are decreased in rats fed a low protein diet, which also have a lower plasma α-tocopherol/triglyceride ratio [21]. When rats are exposed to hyperoxia for 48 h [32], α-TTP mRNA levels are reduced and lipid peroxidation increased. α-TTP mRNA and protein levels are also reduced in rat hepatoma [33] and galactosamine-induced liver injury [34]. These data reveal that α-TTP expression is downregulated by oxidative stress. However, in the present study, hepatic α-TTP protein levels in both groups of rats fed the OFO diet were unchanged, although lipid peroxidation increased. Unchanged hepatic α-TTP protein levels are also observed in rats exposed to tobacco smoke [35], despite a lower α-tocopherol concentration in lung and higher lipid peroxidation in the rat liver. The downregulation of α-TTP levels seen in galactosamine-induced liver injury [34] is an acute response (24–48 h), and, since the OFO diet was fed for a long time, α-TTP levels may have changed during the acute injury and restored subsequently. The decreased plasma α-tocopherol levels seen in the OFO-fed rats is therefore not due to downregulation of α-TTP, but probably due to the lower α-tocopherol levels in the liver.

Liu and Huang [10] demonstrated higher excretion of radiolabeled α-tocopherol via both the urinary and fecal routes in rats fed an OFO diet compared to control rats, and predicted that α-tocopherol catabolism would be increased in rats fed an OFO-diet. In the present study, α-CEHC, the major metabolite of α-tocopherol in the urine, was measured to examine the effect of OFO on α-tocopherol catabolism. α-CEHC excretion, presented as pmol of creatinine or per gram body weight, was lower in the OF group than the other two groups. This can be explained by the reduced absorption of α-tocopherol caused by the OFO diet. Assuming that the levels of α-tocopherol absorbed by the OFE group were comparable to those absorbed in the CO group, the observation that these two groups had similar levels of α-CEHC in the urine seems reasonable. The level of excretion of α-CEHC is reported to depend on α-tocopherol intake and plasma α-tocopherol levels [11]. Liver α-CEHC levels are correlated with serum levels of α-CEHC and liver α-tocopherol levels [36]. This suggests that urine α-CEHC levels increase when liver α-tocopherol increases. In other words, lower hepatic α-tocopherol levels would result in less α-CEHC in the urine. The ratio of urine α-CEHC (per gram body weight) to total liver α-tocopherol (per gram body weight) was therefore calculated and an interesting phenomenon was found, namely that the ratio in the OF and OFE groups was about 1.75- and 2.3-fold higher, respectively, than in the CO group. This implies that increased excretion of α-CEHC is seen in rats fed an OFO-diet and that the concentration of α-CEHC in the urine alone could not be used as a sensitive indicator of vitamin E catabolism in this study.

α-CEHC is thought to be produced by α-hydroxylation of α-tocopherol, followed by β-oxidation. CYP3A has α-hydroxylase activity and was first reported to be involved in vitamin E metabolism because of changes in levels of CEHC metabolites in HepG2 cells when a CYP inhibitor, ketoconazole (KCZ), or a CYP inducer, rifampicin, was added to the medium [12, 13]. In the present study, despite a significant induction of CYP3A protein in both OFO-treated groups, the excretion of α-CEHC in the urine was not significantly increased in the OFE group compared to the CO group. Our previous study also showed that α-CEHC levels are not increased by CYP3A induction, but are reduced [18]. α-Tocopherol metabolism is dependent on CYP3A activity, but CYP3A is not a direct and major contributor to α-CEHC metabolism. CYP3A in the liver is responsible for more than 50% of the metabolism of clinically used drugs [37]. The induction of CYP3A in rats fed an OFO diet shows that this diet contained CYP3A inducers, so there is a potential for an OFO diet to interfere with the effectiveness of clinically used drugs.

MDR protein, or P-glycoprotein, in the liver is involved in the biliary excretion of various drugs and xenobiotics [15, 16]. Mdr2 knockout mice have lower biliary α-tocopherol levels than wild-type mice [38]. These data indicate that the MDR transporters could be responsible for α-tocopherol excretion in the bile. Bjorneboe et al. [14]
demonstrated that about 14% of injected α-3H-tocopherol is recovered in the bile. This shows that bile is one of the excretion pathways for tocopherol, although the amount secreted in the bile expressed as a percentage of the α-tocopherol concentration in the liver is only about 0.3–0.7% [39]. Increased excretion of radiolabeled α-tocopherol in the feces is observed in OFO-fed rats [9]. The metabolites of α-tocopherol in the feces have not been identified due to a lack of suitable standards. We speculated that induction of MDR protein could be the factor that increases α-tocopherol excretion from the bile into the feces, so MDR protein was measured in this study, but, unexpectedly, its levels were unchanged by the OFO diet. Mustacich et al. [40] found that hepatic levels of MDR1 protein increase at the same time as liver and serum α-tocopherol concentrations decrease in rats receiving daily subcutaneously injections of α-tocopherol. It seems that MDR protein might be regulated by a high dose α-tocopherol injection, but not by the oxidative stress of OFO treatment. Many transporters are involved in bile acid excretion in the liver, e.g. MDR2, MDR, and the bile acid export pump [41]. These transporters are regulated by PXR [42], which also activates CYP3A, so an OFO diet could affect bile excretion, but which protein is involved is still unknown and needs further investigation.

The reaction steps of CYPs involve one-electron transfers, which can give rise to by-products, such as superoxide and hydrogen peroxide [43–45]. CYP3A-expressing microsomes are reported to have a higher rate of superoxide production than CYP1A1-expressing microsomes [46]. CYP4A plays a major role in oxidative stress induced in liver microsomes of Cyp2e1−/− mice with steatosis [47]. In a rat model of nonalcoholic steatohepatitis, an increase in TBARS and hepatic CYP4A1 expression is observed [48]. We therefore suggest that the increased oxidative stress caused by induction of CYP3A and CYP4A in both OFO-fed groups contributed to increased consumption of α-tocopherol, leading to a lower vitamin E status and higher lipid peroxidation in the rat liver.

GPx, SOD, and catalase are well-known cellular antioxidant enzymes and changes in enzyme activity show their adaptation to oxidative stress [49]. The regulation of hepatic antioxidant enzyme activity in OFO-feeding rats has been studied previously, but the results were not consistent. GPx and SOD activity is reported to be decreased, and catalase activity increased, in heated and fried oil-fed rats [50]. In rats fed 10% heated oil, GPx activity in the liver is reported to be increased [7]. In our study, we measured protein levels of antioxidant enzymes and found no change in GPx and SOD levels in rats fed the OFO diet. In contrast, catalase protein levels were significantly increased in the OFO-fed groups, which is agreement with the results for catalase mRNA in rats [6], catalase activity in mice [23] fed an OFO diet, and those mentioned above. Catalase is a peroxisome enzyme which cleaves hydrogen peroxide. OFO causes peroxisome proliferation by activating PPARα [5] and this could be the cause of the increased levels of catalase protein in OFO-fed rats.

In this study, 15% fat in diet (w/w) was used. Indeed, this is a high fat diet to rats since the oil percentage recommended by AIN-76 [51] and AIN-93G [52] is 5% and 7% fat in diet. The typical Western diet contains high thermally oxidized fats. In fast food restaurants and Chinese food-stall, fat is heated for up to 18 h daily. The heated fats are continually used for up to 1 wk before they are discarded. Assuming that the dry-weight of total food intake in one day a person is 500 g [53], 15% fat (w/w) in the diet is equal to 75 g fat. The total energy a day from fat is about 33.75%. If a person always choose fried food as their dietary fat source, it is possible to consume large amount of OFO in daily life.

In conclusion, dietary OFO had no effect on protein levels of MDR protein and α-TTP, despite a decrease in the plasma α-tocopherol/triglyceride ratio. CYP3A expression was induced by OFO, suggesting that an OFO diet might interfere with drug metabolism and this needs further investigation.

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Abbreviations

α-CEHC, α-carboxyethyl hydroxychroman; α-TTP, α-tocopherol transfer protein; CYP, cytochrome P-450; GPx, glutathione peroxidase; OFO, oxidized frying oil; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substance.

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