The Protective Effects of Tetrahydrocurcumin on Oxidative Stress in Cholesterol-fed Rabbits

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Tetrahydrocurcumin (THC) is an antioxidant substance which is derived from curcumin by hydrogenation. Curcumin is the main component of turmeric and is responsible for the yellow color of curried foods. First, LDL derived from a normal human volunteer was incubated in the presence of an antioxidant with 10 μM CuSO₄ at 37°C for 2 hours. All antioxidants tested (THC, curcumin, probucol, and α-tocopherol) dose-dependently (1-10 μM) inhibited the oxidative modification of LDL. Probucol was the strongest, followed by THC, α-tocopherol, and curcumin. Next, in order to evaluate the antioxidative activity of THC in vivo, we fed rabbits diets containing 1% cholesterol with or without 0.5% THC and examined their effects on oxidative stress and atherosclerosis. Animals were divided into two groups: the control group rabbits (n = 12) were fed a normal chow diet and the experimental group (n = 12) was fed a diet containing 0.5% THC for one week. Then, 1% cholesterol was added to the diets and the animals were allowed to feed further for either 6 (n = 4 for each group) or 12 weeks (n = 8 for each group). Although serum cholesterol levels rapidly increased after starting the high cholesterol diet, no difference was observed between the control and THC groups. TBARS formation in the absence of added copper ion was inhibited in the LDL separated from THC-treated animals compared with that from control animals. THC treatment tended to inhibit the area covered with atherosclerotic lesions compared with the control, although this was not significant (28.8 ± 17.5% vs. 40.0 ± 23.7%, p = 0.2). Formation of N′-(hexanoyl) lysine, 4-hydroxyxenoneal and dihydroxynine in liver and kidney also had a tendency to be inhibited by THC treatment. Although free THC was not detected in serum and liver, THC was detected in samples treated with β-glucuronidase and sulfatase, suggesting that THC is present as a conjugate with glucuronic acid or sulfate. In conclusion, the present results suggest that curcuminoids, particularly THC, which are contained in turmeric, may be useful as a functional food factor. J Atheroscler Thromb, 2002; 9: 243-250.

Key words: Curcuminoid, Antioxidant, Atherosclerosis, Conjugate

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oxidatively modified by incubation with endothelial cells, smooth muscle cells, or monocyte/macrophages in the presence of transition metals. This biological modification is mediated by a free radical-induced peroxidation of LDL that can be mimicked by transition metals such as copper or iron in the absence of cells. The potential atherogenic properties of oxidatively modified LDL include:

(a) chemotactic activity, which facilitates the recruitment of blood monocytes; (b) inhibition of the migration of macrophages from the arterial wall back to the plasma; (c) enhanced uptake by macrophages via scavenger receptors, resulting in foam cell formation; and (d) cytotoxicity to endothelial cells, which may facilitate the entry of LDL and monocytes in the early stages of disease and cause endothelial denudation at a later stage.

Many studies, including ours (4-7), have been performed in order to examine the antiatherogenic effects of antioxidative agents, using various models. Although many different types of natural antioxidants have been isolated and identified, the major natural antioxidants are plant phenols. We recently found a novel type of antioxidant that contains both a phenolic moiety and a β-diketone moiety in the same structure: tetrahydrocurcumin (THC), which is derived from curcumin by hydrogenation (8, 9). Curcumin is the main component of turmeric and is responsible for the yellow color of curried foods.

In this study, in order to determine the effect of THC on atherosclerosis, we fed rabbits diets containing cholesterol with or without THC. We also evaluated the effect of THC on the oxidation of isolated LDL.

Materials and methods

Animals and diets

The protocols for animal experiments were approved by the Laboratory Animal Care Advisory Committee of Nagoya University. Male New Zealand White (NZW) rabbits (2.2-2.7 kg, Kitayama, Japan) (n = 24) were housed individually at 24 ± 1°C with a 12-hour light:dark cycle. They were allowed free access to water and a commercial rabbit diet for 7 days to enable them to adapt to the new environment. Then, rabbits were assigned to two groups: the control group rabbits (n = 12) were fed a normal chow diet and the experimental group (n = 12) was fed a diet containing 0.5% THC for one week. Then, 1% cholesterol was added to the diets and the animals were allowed to feed for either 6 (n = 4 for each group) or 12 weeks (n = 8 for each group). THC was kindly provided by Nippon Fine Chemicals (Shizuoka, Japan). All of the diet ingredients were products of Clea Japan (Tokyo, Japan). The food intake of each rabbit was restricted to 100 g/day. No food was supplied for 18 hours before blood collection. Blood samples were collected from the ear vein after 1, 7, 10, and 13 weeks of the experiment, and serum and plasma were isolated from blood by centrifuge. After 7 and 13 weeks, rabbits were killed by administration of a bolus injection of pentobarbital; the liver, kidneys, and aorta were removed and washed three times with cold physiological saline solution. Serum and tissues were immediately stored at –80°C.

LDL preparation

LDL (d=1.019-1.063) was isolated from the plasma stepwise at 150,000 x g for 20 hours at 16°C using a rotor (Hitachi, Japan), followed by extensive dialysis against phosphate-buffered saline (PBS) containing 0.01% EDTA for 48 hours at 4°C. To remove EDTA, LDL was dialyzed against a 2,000-fold volume of PBS without EDTA for 48 hours at 4°C. LDL was sterilized by passage through a 0.22 µm filter, stored at 4°C, and used within one week. Protein concentration was determined using bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL)

Oxidation of LDL

Isolated LDL was diluted with PBS to a final concentration of 100 µg protein/ml. The oxidation was observed with or without the addition of a freshly prepared 10 µM (final concentration) CuSO₄ solution. Samples were incubated for 2 hours at 37°C in a thermostatically controlled waterbath. The reaction was stopped by the addition of 1 mM EDTA and 10 µM butylated hydroxytoluene (BHT) (final concentration). The lipid peroxidation of LDL was determined by measuring the formation of 2-thiobarbituric acid-reactive substances (TBARS). Briefly, each reaction mixture contained a 0.1 ml sample, 0.2 ml of 0.28 mM SDS (Wako, Osaka, Japan), 1.5 ml of 3.3 M acetic acid solution (pH 5.0), and 1.5 ml of 56 mM aqueous solution of TBA (Merck, Darmstadt, Germany), brought up to 4.0 ml with distilled water. The samples were boiled at 95°C for 60 minutes, cooled on ice, extracted with 4.0 ml of n-

Abbreviations:

BHT: butylated hydroxytoluene
EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
HEL: N’-(hexanoyl) lysine
HDL: high-density lipoprotein
HDL-C: HDL-cholesterol
4-HNE: 4-hydroxynonenal
LDL: low-density lipoprotein
LDL-C: LDL-cholesterol
MDA: malondialdehyde
PBS: phosphate-buffered saline
PL: phospholipid
SDS: sodium dodecyl sulfate
TBARS: 2-thiobarbituric acid-reactive substances
TC: total cholesterol
TG: triglycerides
THC: tetrahydrocurcumin
TPBS: PBS containing 0.05% Tween 20
butanol/pyridine (15:1, v/v), and centrifuged at 800 × g for 10 minutes. The absorbance of the upper layer at 532 nm was measured, and levels of TBARS were calculated as malondialdehyde (MDA) equivalents, using freshly diluted malondialdehyde-bis-(dimethylacetal), i.e., 1,1,3,3-tetraethoxypropane (Aldrich Chemical, WI) as the standard. MDA was prepared by hydrolysis of 1,1,3,3-tetraethoxypropane with 1N HCl.

**Enzyme-linked immunosorbent assay (ELISA)**

Microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 µl of sample (10 µg protein/ml, diluted with PBS). The coating solution was discarded, and the wells were washed three times with PBS containing 0.05% Tween 20 (TPBS), followed by distilled water. The wells were blocked with 200 µl of 4% skim milk for 1 hour at 37°C with shaking. After being washed three times with TPBS and once with distilled water, each well was incubated with 100 µl of monoclonal antibody against Nα-(hexanoyl) lysine (HEL) (10), 4-hydroxynonenal (4-HNE) (11), or citriline (12) for 2 hours at 37°C with shaking. After being washed with TPBS and distilled water, the wells were incubated for 1 hour at 37°C with 100 µl of peroxidase-labeled goat anti-mouse IgG (American Qualex, La Mirada, CA) diluted 1:5,000 in TPBS. After another wash, 100 µl of o-phenylenediamine solution (5 mg of o-phenylenediamine and 10 µl of 30% H2O2 in 10 ml of 0.1M citrate phosphate buffer, pH 5.5) was added to each well. The plates were sealed with aluminum foil and incubated for 15 minutes at room temperature. Adding 50 µl of 2N sulfuric acid terminated the reaction. Absorbance at 492 nm was read using a micro-ELISA plate reader (Spectra Max 250, microplate spectrophotometer, Molecular Devices, USA).

**THC and α-tocopherol determination**

Tissue samples were homogenized for 15 seconds in 2 volumes of PBS using a Brinkman homogenizer equipped with a PT20ST probe generator (Hitachi, Tokyo, Japan). To each sample (200 µl) was added 500 U/g of β-glucuronidase and 40 U/g of sulfatase (both from Sigma Chemical, St. Louis, MO) in 10mM PBS (pH 5.0) with 2% vitamin C and 0.01% EDTA and then the mixture was incubated at 37°C for 45 minutes. Following this, each sample was added 1.5 ml of ethyl acetate, and the samples were then centrifuged at 3,500 rpm for 15 minutes. The upper layer was transferred to a microfuge tube, evaporated in vacuo under nitrogen, then resuspended in 50 µl of methanol. HPLC was performed by injection of a 20 µl sample onto a Develosil ODS-Column (4.6 i.d. × 250 nm, Nomura Chemical, Aichi, Japan) at room temperature, using 1.0 ml/min of acetonitril/H2O (1:1, v/v) with 0.1% trifluoroacetic acid as a mobile phase, and monitored at 280 nm.

The concentrations of α-tocopherol in the serum and liver were analyzed using HPLC as described previously (4).

**Other biochemical analyses**

Serum triglyceride (TG), total cholesterol (TC), phospholipid (PL) and HDL-cholesterol (HDL-C) concentrations were determined enzymatically using commercial kits (Nippon Shouji, Osaka, Japan). To determine TC and TG levels, the liver was weighed and homogenized, and total lipid was isolated by means of extraction with chloroform/methanol (2:1, v/v). TC and TG concentrations were then determined enzymatically using the commercial kits. Protein concentrations were determined using a biocinchonic acid protein assay kit (Pierce, Rockford, IL), employing bovine serum albumin as the standard. The extent of lipid peroxidation in serum and liver was determined as TBARS as described (13, 14).

**Extent of aortic atherosclerosis**

At the end of the 7- and 13-week study period, rabbits were killed by administration of a bolus injection of sodium pentobarbital. Immediately thereafter, aortas were removed from the arch to the descending thoracic aorta. The aortas were cleaned of excess adventitial tissues, rinsed with PBS and opened longitudinally and photographed. The photographs were copied onto graph paper with magnification and the atherosclerotic lesions were delineated. The areas of the lesions were calculated, and the percentage of the lesion areas was determined. After the photographing, the aortas were fixed with formalin for histological analysis. The weights of the excised heart, kidneys, and liver were also measured.

**Statistical analysis**

Results are presented as the mean ± SD. The data were tested by ANOVA, followed by Fisher's test to identify significant differences. All analyses were performed using Stat View software version 5.0J (Abacus Concepts, Berkeley, CA). A level of p < 0.05 was considered significant.

**Results**

**Effect of THC on LDL oxidation in vitro**

The in vivo effects of antioxidants on copper-induced oxidation of LDL were examined. Normal human LDL was incubated in the presence of the antioxidants with 10 µM CuSO4 at 37°C for 2 hours. The positive control without added antioxidants was defined as 100% lipid peroxidation. The extent of LDL lipid peroxidation induced by 10 µM CuSO4 was assessed by monitoring the formation of TBARS. All antioxidants tested dose-dependently (1-10 µM) inhibited the oxidative modification of LDL (Fig. 1). Probiotic was the strongest, followed by THC, α-tocopherol, and curcumin.
Effect on body, liver and kidney weight
There were no differences in body weight between the control and THC-treated groups during the study period (Table 1). Food intake and liver and kidney weights also did not differ between the two groups (data not shown). Before the experiment began, serum TC, TG, and HDL-C concentrations did not differ between the groups. Although serum TC levels rapidly increased after the initiation of the high cholesterol diet, no difference was observed between the control and THC groups. LDL-C and LDL-TG levels also did not differ between the groups (data not shown).

Effect of THC administration on LDL oxidation ex vivo
LDL (100 μg protein/ml) was incubated for 2 hours at 37°C with or without CuSO₄ (final concentration: 10 μM). The reaction was halted by the addition of 1 mM EDTA and 10 μM butylated hydroxytoluene. The oxidation was monitored as the formation of TBARS at 532 nm and was expressed as MDA. The formation in the absence of added copper ion was inhibited in the LDL separated from THC-treated animals compared to those from control animals (Fig. 2). However, there was no difference in the formation of TBARS with CuSO₄ between the control and THC-treated animals.

Effect of THC administration on serum and tissue levels of TBARS
We examined the effects of THC administration on serum and tissue levels of TBARS (Fig. 3). At 13 weeks, there was a significant difference in the amount of TBARS in kidney between the two groups (p < 0.05). In the liver, there was a tendency for the formation of TBARS to be inhibited in the THC-treated groups at 13 weeks (p = 0.085). There was no difference in serum TBARS between the two groups during the study period.

Effect of THC on HEL- and 4-HNE-modification of proteins
We measured an early peroxidation product, HEL, and a late aldehyde product, 4-HNE, in serum and tissues (Table 2). The formation of HEL in liver was significantly inhibited in the THC-treated group compared to the control group at 13 weeks (p < 0.05). There was a tendency...
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A) Liver

![Graph showing TBARS levels in liver, control vs. THC treated, with error bars for weeks 7 and 13.]

TBARS (μmol MDA/mg protein)

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for the formation of HEL in kidney to be inhibited in the THC-treated group. There was also a tendency for the formation of 4-HNE in liver and kidney to be inhibited in the THC-treated group after 13 weeks.

Effect of THC administration on dihydroxy formation in liver and kidneys

We also measured dihydroxy, which is a fluorescent dimer of tyrosine and is formed by reactive oxygen species (12), in organs (Table 2). There was a tendency for dihydroxy formation to be inhibited in liver at 13 weeks and in kidney at 7 and 13 weeks.

Effect of THC administration on the extent of atherosclerosis

At the end of the 13-week study period, the rabbits (n = 8 for each group) were killed and their aortas were removed to quantify the extent of atherosclerotic lesion formation (Fig. 4). THC tended to result in a somewhat smaller area covered with atherosclerotic lesions compared with the control, although the difference was not significant (28.8 ± 17.5% vs. 40.0 ± 23.7%, p = 0.2).

Concentrations of THC and α-tocopherol in serum and liver

At the end of the 13-week treatment period, THC was not detected in the serum or liver of untreated animals.

![Graph showing the percentage of intimal surface area covered with atherosclerotic lesions, with markers for Control and THC groups.]

Fig. 3. TBARS in liver, kidney and serum in rabbits fed a chow containing 1% cholesterol with or without 0.5% THC for 6 or 12 weeks. Data are expressed as the mean ± SD. n = 4 (at 7 weeks) or 8 (at 13 weeks). *p < 0.1, **p < 0.05 vs. control

Fig. 4. Percentage of intimal surface area covered with atherosclerotic lesions. Data are expressed as the mean ± SD. n = 8. *p = 0.2
suggested that the concentrations of free THC were negligible in liver and serum (Table 3). However, THC was detected in samples treated with β-glucuronidase and sulfatase, suggesting that it is present as a conjugate with glucuronic acid or sulfate. There was no difference in the concentration of α-tocopherol in serum and liver between the two groups.

Discussion

Curcumin was reported to inhibit the microsome-mediated mutagenicity of benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene (15). Huang et al. (16) subsequently reported that curcumin acts as a strong inhibitor of tumor promotion and that this effect can be explained by its antioxidant activity. Rao et al. (17) have reported that curcumin effectively inhibited colon cancer induced by azoxymethane. We used a food model system and the erythrocyte ghost system to compare the antioxidative activity of curcumin derivatives, and found THC to be the strongest (9). We also showed that the beta-diketone moiety exhibits antioxidative activity by cleavage of the C-C bond at the active methylene carbon between two carboxyls in the beta-diketone moiety. Because THC is one of the major metabolites of curcumin, it may also exhibit the same physiological and pharmacological properties as the active form of curcumin in vivo by means of the beta-diketone moiety as well as phenolic hydroxy groups. However, the antithrombogenic activity of THC has never been tested. In this study, to determine the effect of THC on atherosclerosis, we fed rabbits diets containing cholesterol with or without THC. We also evaluated the effect of oxidative stress ex vivo.

First, we examined the in vitro effect of THC on the copper-induced oxidation of LDL. All antioxidants tested dose-dependently inhibited the oxidative modification of LDL. Although probucol is the strongest, THC is almost as strong as α-tocopherol, and is stronger than curcumin. These in vitro results suggest that THC may be beneficial as an antioxidant in food.

Although Kim et al. (18) reported that curcumin decreased cholesterol in serum and LDL, administration of THC had no effect on hyperlipidemia in the present study. The formation of TBARS in the absence of added copper ion was inhibited in the LDL separated from THC-treated animals compared with that from control animals. However, there was no difference in the formation of TBARS with CuSO4 between the control and THC-treated animals. These results suggest that LDL oxidation induced by CuSO4 may be too strong to be inhibited by THC. How-

| Table 1. Changes of body weight, serum TC, HDL-C, and TG levels. |
|---------------------------------|----------------|----------------|----------------|--------|
|                                | Time (weeks) |
|--------------------------------|-------------|----------------|----------------|--------|
| Body weight (kg)               | 1            | 7              | 10             | 13     |
| Control                        | 2.28 ± 0.06  | 3.06 ± 0.06    | 3.13 ± 0.15    | 3.21 ± 0.20 |
| THC                            | 2.27 ± 0.06  | 3.01 ± 0.13    | 3.14 ± 0.10    | 3.32 ± 0.17 |
| TC (mg/dl)                     | 45 ± 10      | 1179 ± 459     | 975 ± 389      | 691 ± 329 |
| Control                        | 53 ± 16      | 1414 ± 588     | 894 ± 219      | 884 ± 401 |
| THC                            | 24 ± 5       | 23 ± 5         | 35 ± 5         | 28 ± 9  |
| HDL-C (mg/dl)                  | 23 ± 6       | 18 ± 4         | 34 ± 3         | 27 ± 6  |
| Control                        | 61 ± 16      | 140 ± 64       | 207 ± 135      | 163 ± 116 |
| THC                            | 69 ± 26      | 186 ± 79       | 195 ± 79       | 193 ± 105 |

Data are expressed as the mean ± SD. n = 12 (at 1 and 7 weeks) or 8 (at 10 and 13 weeks).

| Table 2. HEL, dihydroxy and 4-HNE in liver and kidney. |
|---------------------------------|----------------|----------------|--------|
|                                | Control THC |
| HEL                            | Liver 7 weeks | 0.268 ± 0.029 | 0.247 ± 0.030 | 0.44 |
|                                | 13 weeks      | 0.343 ± 0.060 | 0.274 ± 0.039 | 0.04** |
|                                | Kidney 7 weeks| 0.300 ± 0.082 | 0.254 ± 0.075 | 0.50 |
|                                | 13 weeks      | 0.333 ± 0.057 | 0.289 ± 0.045 | 0.19 |
| 4-HNE                          | Liver 7 weeks | 0.330 ± 0.108 | 0.302 ± 0.084 | 0.73 |
|                                | 13 weeks      | 0.438 ± 0.085 | 0.368 ± 0.036 | 0.09* |
|                                | Kidney 7 weeks| 0.310 ± 0.063 | 0.266 ± 0.096 | 0.12 |
|                                | 13 weeks      | 0.375 ± 0.041 | 0.300 ± 0.072 | 0.07* |
| Dihydroxy                      | Liver 7 weeks | 0.282 ± 0.060 | 0.245 ± 0.033 | 0.30 |
|                                | 13 weeks      | 0.332 ± 0.062 | 0.283 ± 0.028 | 0.11 |
|                                | Kidney 7 weeks| 0.302 ± 0.060 | 0.283 ± 0.020 | 0.12 |
|                                | 13 weeks      | 0.334 ± 0.062 | 0.284 ± 0.056 | 0.20 |

Data are expressed as the optical density at 490 nm and the mean ± SD.

n = 3 at 7 weeks, n = 6 at 13 weeks. *p < 0.1. **p < 0.05

| Table 3. Effects of THC feeding on the concentration of α-tocopherol and THC in serum and liver. |
|---------------------------------|----------------|----------------|--------|
|                                | Serum (µg/ml) | Liver (µg/g tissue) |
| group                          | α-Tocopherol THC | α-Tocopherol THC |
| Control group                  | 10.8 ± 3.8 ND | 2.5 ± 0.8 ND |
| THC group                      | 13.0 ± 4.2 4.8 ± 1.7 | 3.6 ± 1.7 21.7 ± 2.6 |

Data are expressed as the mean ± SD. n = 8. ND: not detected.
ever, oxidation without CuSO₄ ex vivo may be milder and more natural, suggesting the validity of THC in inhibiting LDL oxidation in vivo.

No difference in serum TBARS was seen between the two groups. At 13 weeks, a significant difference in the amount of TBARS in kidney was seen between the two groups. There was also a tendency for the increase of TBARS in the liver to be inhibited in the THC-treated group. HEL in liver was significantly inhibited in the THC-treated group compared to the control after 13 weeks, suggesting that an early peroxidation product, HEL (10), may be a useful marker of peroxidation in vivo. Other oxidation products, 4-HNE and dityrosine in liver, also had a tendency to be inhibited by the THC treatment. The levels of THC were higher in the liver than the serum, explaining why the HEL concentration is lower in liver, but not in serum, of the THC-group compared with the control. There was also a tendency for the formation of HEL and 4-HNE in kidney to be inhibited in the THC-treated group. In THC-treated animals, somewhat smaller areas of aorta were covered with atherosclerotic lesions compared with the control animals, although this difference was not significant.

These results suggest that the influence of severe hyperlipidemia induced in this study may be too strong to be overcome by THC, although some parameters had a tendency to be improved by the administration of THC to cholesterol-fed rabbits. The dose used in the present study was chosen according to the doses used in anti-cancer studies of THC (19, 20). It was presumed to be high enough for the anti-oxidative effects as shown by its anti-cancer effects. However, higher doses may inhibit atherosclerosis in this model.

Although THC was not detected in either serum or liver that had not undergone enzyme treatment, it was detected in the enzyme-treated samples, suggesting that THC is present as a conjugate with glucuronic acid or sulfate. However, it remains to be elucidated how the conjugated forms of THC exert anti-oxidative effects in vivo.

In conclusion, curcuminoids, particularly THC, which are contained in turmeric, may be useful as a functional food ingredient.

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