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The Effect of Crude Drugs on Experimental Hypercholesteremia: Mode of Action of (−)-Epigallocatechin Gallate in Tea Leaves

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The mechanism of the anti-hypercholesteremic effect of (−)-epigallocatechin gallate (EGCG), a component of green tea, was explored in rats from the viewpoint of cholesterol metabolism.

1) The *in vitro* incorporation of ^{14}C-acetate into cholesterol was not affected by the presence of EGCG in liver slices from normal rats or by oral pretreatment of the animals with EGCG in liver slices from normal and Triton-induced hypercholesteremic rats.

2) The kinetics of serum levels of ^{14}C-cholesterol given orally and of ^{3}H-cholesterol given intravenously revealed that EGCG, when orally administered, suppressed the absorption of ^{14}C-cholesterol from the digestive tract and had an enhancing effect on elimination of serum ^{3}H-cholesterol at higher doses.

3) *In situ* uptake in the intestine of ^{14}C-cholesterol given in the lumen was suppressed by the presence of EGCG.

In conclusion, these results indicated that the anti-hypercholesteremic effect of EGCG in rats is mainly due to suppression of the absorption of exogenous cholesterol from the digestive tract, and partly due to the enhancement of the elimination of endogenous cholesterol.

Keywords——tea leaf; (−)-epigallocatechin gallate; hypercholesteremia; cholesterol absorption; cholesterol excretion

Introduction

(−)-Epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG) obtained from green tea inhibit the synthesis of lipid peroxides[1] and interfere with the Ames mutagenicity test.[2] It has been reported[3] that ECG and EGCG were effective against high fat emulsion-induced and high cholesterol diet-induced hypercholesteremia. In addition, in Triton-treated hypercholesteremia, EGCG inhibited the increase of serum cholesterol level. In order to clarify the mechanism of action of EGCG on hypercholesteremia, the effect of EGCG on cholesterol biosynthesis, absorption and excretion was examined.

Materials and Methods

ECG and EGCG were isolated and purified by the reported method.[3] Drugs used were Clofibrate (Aldrich Chemical Co.) and Nicomol (Kyorin Pharmaceutical Co.). Compactin was obtained from the Central Laboratories of Kuraray Co., Ltd.

The incubation medium used in these experiments had the following composition: NaCl 120.1 mM, KCl 4.8 mM, $MgSO_4$ 1.3 mM, $CaCl_2$ 2.5 mM, KH_2PO_4 1.3 mM, $NaHCO_3$ 25.2 mM, glucose 11.6 mM (pH 7.4). The medium was aerated with a 95% O_2—5% CO_2 gas mixture.

Triton-Induced Hypercholesteremia——Male Wistar rats weighing approximately 200 g (Kitayama Labs Co.) were injected intravenously with Triton WR-1339 (Ruger Chemical Co.) at 400 mg/kg. Eight hours thereafter, blood

was sampled from the abdominal aorta under ether anesthesia and the levels of serum cholesterol and neutral lipids were determined. Serum cholesterol was measured by the COD-*p*-chlorophenol colorimetric method (Cholesterol CII Test Wako, Wako Pure Chemical) and serum neutral lipids were measured by the GPO-*p*-chlorophenol colorimetric method (Triglyceride G-Test Wako, Wako Pure Chemical). Immediately after the collection of the blood, the liver was excised in order to examine cholesterol biosynthesis by using radioisotopes, as described later. The test drug was administered orally at 0 and 4 h after the Triton injection.

Cholesterol Biosynthesis in Liver Slices from Triton-Induced Hypercholesteremic Rats[4]——Triton-treated rats, described in section 1), were sacrificed and the livers were rapidly removed under ether anesthesia. The livers were rinsed in an incubation medium at 4 °C and blotted with filter paper. Slices with a thickness of 0.5—1 mm were manually prepared from the left lobe of the liver. Slices weighing 40—60 mg were first incubated for 15—20 min in 0.9 ml of the incubation medium in stoppered test tubes (12 × 100 mm). To this incubation system was added 100 μl of [1-^{14}C]acetic acid solution (20 mM; 0.25—0.5 mCi/mmol) (Amersham), and incubation was continued for a further 90 min at 37 °C. The reaction was stopped by adding 1 ml of 15% KOH in alcohol and the whole mixture was saponified for 2 h at 75—80 °C. The non-saponified fraction was extracted with petroleum ether and the solvent was evaporated by off by means of a stream of nitrogen gas. The residue was dissolved in 0.5 ml of 0.1% cholesterol solution in acetone as a carrier plus 1 ml of 0.5% digitonin solution and the mixture was left overnight prior to centrifugation. The resultant pellets were rinsed with 1.5 ml of acetone and their radioactivity was measured in a liquid scintillation counter to estimate the amount of radioactive acetate incorporated into digitonin-precipitable cholesterols.[5]

Cholesterol Biosynthesis in Liver Slices from Normal Rats——Male Wistar rats weighing 250—300 g were decapitated and exsanguinated between 10 : 00 p.m.—11 : 00 p.m., and the liver was excised. Slices was prepared in the same manner as described above. Preincubation was performed in the medium containing the test drugs. ^{14}C-Acetate was added and incubation was further continued as previously described. Treatment of the tissue thereafter was also the same as described in section 2).[5]

Analysis of Liver Slices from EGCG-Treated Rats——Male Wistar rats weighing approximately 200 g were given the test drug orally once a day for 5 d. The control group was given 5% acacia gum solution instead of the test drug. Each rat was killed by decapitation and exsanguination between 10 : 00 p.m.—11 : 00 p.m., 20 h after the final administration, and the liver was excised. The tissue was then treated described in section 2).[5]

Isotope-Ratio Method——Male Wistar rats weighing approximately 200 g were orally administered with the test drug plus ^{14}C-cholesterol, and injected immediately thereafter with ^{3}H-cholesterol intravenously. The solution containing ^{14}C-cholesterol for oral administration consisted of 3 μCi of [4-^{14}C]cholesterol (57.5 mCi/mmol) (New England Nuclear) and cold cholesterol (6 mg), which were dissolved in 156 mg of triolein (Wako Pure Chemical), and 1 ml of water containing 7.5 mg of cholic acid (Wako Pure Chemical). ^{3}H-Cholesterol solution for i.v. use consisted of 3 μCi of [1,2-^{3}H]cholesterol (60.0 mCi/mmol) (New England Nuclear) in 25 μl of 95% ethanol and 475 μl of 0.9% NaCl. After administration of these solutions, animals were further fasted for 6 h and then allowed food and water. Blood (0.2 ml) was sampled from the tail artery 12, 48, 72, and 96 h after administration of the labeled compounds. Serum was added to scintillator fluid (Scintisol EX-H (Dojindo Laboratories)) and radioactivity was measured by the use of a liquid scintillation counter.[6]

Cholesterol Uptake in Rat Intestine *in Situ*——Male Wistar rats weighing approximately 250 g were fasted for 48 h prior to experimentation. Anesthesia was induced by administration of pentobarbital (Abbott Laboratories) intraperitoneally. The abdominal wall was opened in a room maintained at 37 °C. Jejunal segment of approximately (0.30 g wet weight) were ligated with intact blood supply. The first of these segments was located just distal to the ligament of Treitz. Before the segment was completely closed, 0.4 ml of a micellar solution was injected through one end. The segment was resected 20 min after the injection.[7] Micellar solution consisted of 1 mM monoolein (Wako Pure Chemical), 5 mM taurodeoxycholic acid (Wako Pure Chemical) and 0.09 μCi of [4-^{14}C]cholesterol (57.5 mCi/mmol) in 0.15 M sodium phosphate solution previously mixed by ultrasonic vibration and brought to 1 ml with EGCG solution.

Jejunal slices of approximately 2 cm (0.20 g wet weight) were carefully resected, opened along the anti-mesenteric border and immediately transferred to 2.0 ml of the micellar solution.

At the end of the experiment, the mucosal surface of the segment was exposed to ice-cold 2 mM sodium cholic acid/physiological saline. The segment was then washed 3 times in 10 ml of the same solution (10 s in each) with gentle agitation. Tissues were blotted with filter paper, weighed and solubilized in 1 ml of Soluene 350 (Packard). The solubilized sample was added to 10 ml of scintillator and the radioactivity was measured with a liquid scintillation counter.

Effect of EGCG on the Serum and the Liver ^{3}H-Cholesterol Levels after an Intravenous Administration of ^{3}H-Cholesterol——EGCG (250 or 500 mg/kg) was orally administered to male Wistar rats, weighing approximately 200 g, twice at approximately 9 : 00 a.m. and 9 : 00 p.m. They were injected intravenously with the solution containing 3.0 μCi of [1,2-^{3}H]cholesterol (60.0 mCi/mmol) in 25 μl of 95% ethanol dissolved in 0.5 ml of physiological saline. Blood was then collected from the tail artery 24, 48, 72, and 96 h after administration of the labeled compounds. The serum was obtained and the radioactivity was measured.

To determine the liver ^{3}H-cholesterol level, the liver was excised at 96 h after ^{3}H-cholesterol administration, rinsed in physiological saline at 4 °C and blotted with filter paper. Tissues weighing 100 to 200 mg from the left lobe of the liver were saponified with 15% KOH in alcohol for 1 h at 75—80 °C. The non-saponified fraction was extracted with petroleum ether and the solvent was evaporated under a stream of nitrogen gas. The residue was dissolved in scintillator and the radioactivity was determined in a liquid scintillation counter.

Statistical Analysis——Statistical analysis was performed by Dunnett's method.[8] Values were expressed or plotted as the mean ± S.E.

Results

Effect of EGCG on Cholesterol and Neutral Lipid Levels in Triton-Induced Hypercholesteremic Rats

Table I shows that in the control group, the level of serum cholesterol increased approximately 3.5 times and neutral lipids increased approximately 43 times as compared to the normal group. The EGCG-treated group (250 and 500 mg/kg) showed a significant inhibition of the serum cholesterol increase.

Effect of EGCG on Cholesterol Synthesis in Triton-Induced Hypercholesteremic Rats

Table II shows that in the control group, cholesterol synthesis increased to approximately 5.5 times that of the normal group. Clofibrate,[9] which is an inhibitor of cholesterol synthesis, significantly antagonized the increase of the cholesterol synthesis. EGCG, however, had no significant effect on it.

Effect of EGCG on Cholesterol Synthesis in Normal Rats

Table III indicates that EGCG did not have any significant effect on cholesterol

Table I. Effect of EGCG on the Serum Lipids in Triton-Treated Rats

Compounds	Dose (mg/kg, *p.o.*)	Total cholesterol (mg/dl)	Triglyceride (mg/dl)
Normal	—	90.8 ± 3.6[a]	66.4 ± 6.1[a]
Control	—	308.9 ± 7.1	3092.4 ± 180.3
EGCG	250	271.8 ± 9.4[a]	2831.2 ± 94.7
	500	264.3 ± 11.8[a]	3011.7 ± 167.7
Clofibrate	300	264.4 ± 12.5[a]	2310.2 ± 125.5[a]

Each compound was administered orally at 0 and 4 h after injection of Triton. Serum lipids were measured at 8 h after injection of Triton. Each value represetns the mean ± S.E. of seven male rats. Significantly different from the control at *a*) $p < 0.01$.

Table II. Effect of EGCG on Incorporation of ^{14}C-Acetate into the Digitonin-Precipitable Sterols in Liver Slices of Triton-Treated Rats

Compound	Dose (mg/kg, *p.o.*)	Incorporation of ^{14}C-acetate (nmol/g(tissue)·h)	Inhibition[c] (%)
Normal	—	40.9 ± 7.8[b]	—
Control	—	230.7 ± 15.1	—
EGCG	250	192.9 ± 18.1	19.9
	500	197.5 ± 30.5	17.5
Clofibrate	300	147.4 ± 23.8[a]	43.9

Each compound was administered orally at 0 and 4 h after injection of Triton. Each value represents the mean ± S.E. of seven male rats. Significantly different from the control at *a*) $p < 0.05$, *b*) $p < 0.01$. *c*) Inhibition = ((control − normal) − compound)/(control − normal).

TABLE III. Effects of EGCG and Compactin on Incorporation of ^{14}C-Acetate into the Digitonin-Precipitable Sterols in Rat Liver Slices (*in Vitro*)

Compounds	Concentration (M)	Incorporation of ^{14}C-acetate (nmol/g(tissue)·h)	Inhibition (%)
EGCG	0	36.9± 5.5	—
	10^{-6}	44.0± 5.6	−19.2
	3×10^{-6}	39.1± 6.6	−6.0
	10^{-5}	42.7± 8.0	−15.7
	3×10^{-5}	36.6± 9.1	0.8
	10^{-4}	36.1± 5.8	2.2
Compactin	0	42.8± 5.3	—
	3×10^{-8}	46.8±11.4	−9.3
	10^{-7}	22.2± 2.7[a]	48.1
	3×10^{-7}	25.7± 1.1	40.0
	10^{-6}	16.1± 1.8[b]	62.4
	3×10^{-6}	5.8± 0.2[b]	86.4

Each value represents the mean±S.E. of six experiments. *a*) $p<0.05$. *b*) $p<0.01$.

TABLE IV. Effect of EGCG on Incorporation of ^{14}C-Acetate into the Digitonin-Precipitable Sterols in Rat Liver Slices

Compound	Dose (mg/kg, *p.o.*)	Incorporation of ^{14}C-acetate (nmol/g(tissue)·h)	Inhibition (%)
Control	—	42.2±7.0	—
EGCG	300	31.5±5.7	25.4

Each value represents the mean±S.E. of seven male rats. Each compound was administered orally for 5 d.

biosynthesis even at the high concentration of 10^{-4} M. Compactin[10] (3×10^{-8} to 3×10^{-6} M), which selectively inhibits (hydroxymethylglutaryl)-coenzyme A (HMG-CoA) reductase and thereby cholesterol biosynthesis, concentration-dependently inhibited the cholesterol synthesis.

Effect of EGCG on Cholesterol Synthesis in EGCG-Treated Rats

Table IV shows that EGCG did not significantly inhibit the cholesterol biosynthesis.

Isotope-Ratio Method

Serum levels of ^{3}H- and ^{14}C-cholesterols are shown in Fig. 1. The serum level of ^{3}H-cholesterol in the EGCG-treated group was similar to that in the control group. However, in the EGCG-treated group, the serum level of ^{14}C-cholesterol was significantly lower at all time points examined in comparison with the control. In addition, between 12 and 24 h after the cholesterol administration, the serum ^{14}C-cholesterol level decreased in the control, whereas it increased in the EGCG-treated group.

Figure 2 shows the ratios of the serum ^{14}C-cholesterol to ^{3}H-cholesterol. In both the control and the EGCG-treated group, the ratio of the labeled cholesterols remained virtually constant (average 51.9% in the control, and 20% in the EGCG treated animals, respectively) at 24 h after administration of radioactive cholesterol.

Cholesterol Uptake in Rat Intestine *in Situ*

Table V shows that EGCG (1.0 to 8.0 mM) concentration-dependently inhibited the intestinal uptake of ^{14}C-cholesterol *in situ*.

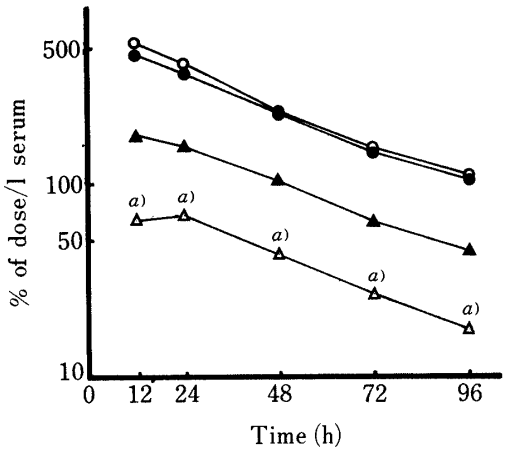

Fig. 1. Effect of EGCG on the Serum Levels of Orally Administered ^{14}C-Cholesterol and Intravenously Administered ^{3}H-Cholesterol in Rats

—●—, control (^{3}H-cholesterol); —○—, EGCG 150 mg/kg (^{3}H-cholesterol); —▲—, control (^{14}C-cholesterol); —△—, EGCG 150 mg/kg (^{14}C-cholesterol).

Each compound was administered orally at the same time as ^{14}C-cholesterol. Each value represents the mean of 5 male rats. Significantly different from the control at *a*) $p<0.01$.

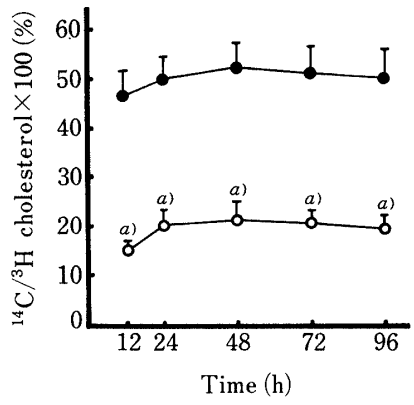

Fig. 2. Effect of EGCG on the ^{14}C/^{3}H Ratio in Serum

—●—, control; —○—, EGCG 150 mg/kg.

Each compound was administered orally at the same time as ^{14}C-cholesterol. Each value represents the mean with S.E. of 5 male rats. Significantly different from the control at *a*) $p<0.01$.

TABLE V. Effect of EGCG on ^{14}C-Cholesterol Uptake from Micellar Solutions by the Rat Small Intestine *in Situ*

Compound	Concentration (mM)	Cholesterol uptake (dpm/mg (tissue))	Inhibition (%)
Control	—	91.1±3.6	—
EGCG	1.0	86.9±3.7	4.6
	2.0	76.0±5.3	16.6
	4.0	67.2±4.9[a)]	26.2
	8.0	62.1±6.7[a)]	31.8

Each value represents the mean±S.E. of ten experiments. Significantly different from the control at *a*) $p<0.01$.

TABLE VI. Effect of EGCG on the Serum Levels of Intravenously Administered ^{3}H-Cholesterol in Rats

Compounds	Dose (mg/kg, *p.o.*)	Serum levels of ^{3}H-cholesterol (% of dose/l serum) 24 h	48 h	72 h	96 h
Control	—	750.6±35.9	418.2±22.2	246.4±17.0	180.8±11.3
EGCG	250	796.4±25.5	390.4±11.7	237.6± 8.0	183.0±19.1
	500	721.0±20.0	328.6±14.6[b)]	191.2±20.0	164.2±16.9
Nicomol	500	648.3±20.9	337.7±18.1[a)]	195.3±25.4	164.0±14.5

Each value represents the mean of 5 male rats. Significantly different from the control at *a*) $p<0.05$, *b*) $p<0.01$.

Effect of EGCG on the Serum and the Liver ^{3}H-Cholesterol Levels after Intravenous Administration of ^{3}H-Cholesterol

Table VI shows that EGCG decreased the serum ^{3}H-cholesterol at doses higher than

TABLE VII. Effect of EGCG on the Liver Level of Intravenously Administered ^{3}H-Cholesterol

Compounds	Dose (mg/kg, *p.o.*)	Liver level of ^{3}H-cholesterol (% of dose/g liver tissue)
Control	—	0.727±0.055
EGCG	250	0.639±0.018
	500	0.486±0.032[a]
Nicomol	500	0.600±0.057

The liver was removed 96 h after the injection of ^{3}H-cholesterol. Each value represents the mean±S.E. of 5 male rats. Significantly different from the control at *a*) $p<0.01$.

250 mg/kg. In addition, the effect of EGCG at 500 mg/kg was as potent as that of Nicomol,[11] which is known to facilitate excretion of cholesterol catabolites.

Table VII shows that EGCG decreased the ^{3}H-cholesterol level in liver as compared to the control, in a dose-dependent manner. EGCG at 500 mg/kg had a significant effect.

Discussion

It has been reported[12] that Clofibrate, used here as a reference drug, inhibits not only the activity of HMG-CoA reductase, thereby inhibiting cholesterol biosynthesis, but also the biosynthesis of acid and neutral lipids. The results in the present experiment also indicate that Clofibrate and EGCG significantly inhibit the increase in the level of cholesterol, so the inhibitory effect of EGCG on the biosynthesis of cholesterol from ^{14}C-acetate was examined in liver slices from Triton-induced hypercholesteremic rats[4] and normal rats. The results showed that EGCG does not inhibit cholesterol synthesis, indicating that the inhibitory effect of EGCG on the increase in serum cholesterol level is not related to the inhibition of cholesterol synthesis. Therefore, the effect of EGCG on absorption and excretion of cholesterol was further examined.

The effect of EGCG on the cholesterol absorption was examined by using isotope-ratio method. Since the serum ^{14}C-cholesterol level in the EGCG group is lower than that in the control group, it is possible that EGCG interferes with the absorption of cholesterol. In addition, in the experiment on cholesterol uptake in rat intestine *in situ*, EGCG also inhibited the absorption of cholesterol from the lumen. These results clearly indicate that EGCG inhibits the absorption of cholesterol. In this animal model, it has been reported that the mode of inhibitory action on absorption is not related to esterification of cholesterol in mucosal cells. Therefore, it can be concluded that EGCG inhibits the absorption step in absorptive cells. EGCG was also shown to facilitate the decrease of the serum level of ^{3}H-cholesterol administered intravenously at higher dose. These results suggest that EGCG facilitates the excretion of blood cholesterol.

We have previously reported[3] that ECG and EGCG, polyphenols found in tea leaves used as pungent drinks, are the main constituents effective against experimental hypercholesteremia.

As described above, EGCG inhibits the absorption of cholesterol and facilitates the excretion of serum cholesterol. EGCG inhibited the absorption at the level of the cholesterol uptake in the intestine, and it may facilitate the excretion of serum cholesterol when the cholesterol level increases beyond the normal level. EGCG does not inhibit the biosynthesis of cholesterol. These results support the concept that tannin components found in everyday tea may be beneficial, though the effect may be weak, for prevention and treatment of hypercholesteremia.

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