Effect of Polyphenols on 3-Hydroxy-3-methylglutaryl-Coenzyme A Lyase Activity in Human Hepatoma HepG2 Cell Extracts

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When carbohydrate metabolism is impaired, fatty acid metabolism is activated. Excess acetyl-CoA (CoA) is generated from fatty acids by β-oxidation and is used for the formation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and subsequently for acetoacetate. High levels of secreted ketone bodies (acetoacetate and 3β-hydroxybutyrate) lower the pH of blood and urine, resulting in ketoadidosis. HMG-CoA lyase in hepatic cells is a rate-limiting enzyme catalyzing the cleavage of HMG-CoA to acetoacetate, and thus inhibition of this enzyme results in reduced acetoacetate production, in other words, impaired ketoadidosis. Inhibition of HMG-CoA lyase activity possibly prevents ketoadidosis and should be the therapeutic target. Polyphenols are common and abundant dietary constituents with beneficial effects on human health. We examined the inhibitory effects of dietary polyphenols on HMG-CoA lyase activity in cellular extracts of human hepatoma HepG2 cells. Of the nine representative dietary polyphenols tested, (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG), and gallic acid (GA) effectively inhibited HMG-CoA lyase activity. Lineweaver–Burk analysis revealed that EGC and EGCG are likely to be mixed-type noncompetitive inhibitors. Pyrogallol with the gallyl structure also inhibited HMG-CoA lyase activity, suggesting that the gallyl moiety of polyphenols is important for the inhibition of HMG-CoA lyase activity.

**Key words** 3-hydroxy-3-methylglutaryl-CoA lyase; ketoadidosis; polyphenol; inhibition; (−)-epigallocatechin gallate

Under normal conditions, glucose serves as the primary energy source in most human tissues. It is converted via glycolysis to pyruvate and then to acetyl-coenzyme A (CoA), which feeds into the tricarboxylic acid cycle to produce energy. During fasting and starvation on the other hand, ketone bodies are readily utilized as an energy source in the brain and muscular tissues. However, in patients with diabetes or soft drink ketosis—which is called “PET bottle syndrome”—which is caused by consuming excessive amounts of soft drinks in summer and/or after exercise, ketone bodies are overproduced due to impaired glucose metabolism.1–3 Long-term impairment of glucose metabolism induces ketoadidosis, which itself can induce a coma.4

Fatty acid metabolism is activated when carbohydrate metabolism is impaired. Excess acetyl-CoA is generated from fatty acids by β-oxidation and is used for the formation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and subsequently acetoacetate. Some of the acetoacetate is converted to 3β-hydroxybutyrate through a reaction catalyzed by 3β-hydroxybutyrate dehydrogenase. In addition, acetoacetate is spontaneously converted into acetone by decarboxylation, albeit in small quantities. Acetoacetate, 3β-hydroxybutyrate, and acetone are ketone bodies, which in high levels lower the pH of blood and urine, resulting in ketoadidosis. Polyphenols are common and abundant dietary constituents with beneficial effects on human health. Recent research strongly supports the notion of polyphenols contributing to the prevention of serious diseases such as cardiovascular disease, cancer, neurodegenerative disease, and diabetes mellitus.5–6 We recently reported that apigenin, a flavonoid found in several herbs including parsley, thyme, and peppermint, inhibits steroidogenic enzymes of cytochrome P450 (CYP)17 and CYP21, as well as 3β-hydroxysteroid dehydrogenase.7 HMG-CoA lyase in hepatic cells is a rate-limiting enzyme catalyzing the cleavage of HMG-CoA to acetoacetate, and thus inhibition of this enzyme results in reduced acetoacetate production, in other words, impaired ketoadidosis. Based on the notion that inhibition of HMG-CoA lyase possibly prevents ketoadidosis and the identification of therapeutic targets, we examined the inhibitory effects of polyphenols on HMG-CoA lyase activity in cellular extracts of human hepatoma HepG2 cells.

**MATERIALS AND METHODS**

**Materials** Acetoacetic acid lithium salt (purity ≥99%), di-3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA, purity ≥90%), 3-(2-hydroxyphenyl)propionic acid (purity ≥99%, internal standard), magnesium chloride (molecular biology grade), glyccylglycine (molecular biology grade), polyoxyethylene octylphenyl ether (Triton X-100, molecular biology grade), and dithiothreitol (molecular biology grade, purity ≥99%) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Glycine (molecular biology grade), potassium dihydrogenphosphate, disodium hydrogen-phosphate, citric acid monohydrate, trisodium citrate dihydrate, methanol (HPLC grade), and hydrochloric acid were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and were special grade chemicals unless stated otherwise. Zirconia beads (di-
diameter, 0.5 mm) for cell disruption were from Tomi Seiko (Tokyo, Japan). The diazo reagent p-nitrobenzenediazonium fluoroborate (purity ≥98%) was from Tokyo Chemical Industry (Tokyo, Japan). Bond elut-C18 cartridges (50 mg, 1 mL) were from Agilent Technologies (Santa Clars, CA, U.S.A.). Coomassie Plus the Better Bradford Assay Kit for protein determination was from TaKaRa Bio (Shiga, Japan).

Polyphenols hesperetin (purity ≥98%) and (−)-epicatechin

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(EC, purity ≥98%) were from Funakoshi (Tokyo, Japan). Daidzein (purity ≥98%) was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). (+)-Catechin (purity ≥98%), (-)-epicatechin gallate (ECG; purity ≥98%), and (-)-epigallocatechin gallate (EGCG; purity ≥98%) were from LKT Laboratories (St. Paul, MN, U.S.A.). (-)-Epicatechin (purity ≥98%), (-)-epigallocatechin gallate (EGCG; purity ≥98%), and pyrogallol (Pg, purity ≥98%) were from Wako Pure Chemical Industries, Ltd. Naringenin was from Sigma-Aldrich. (-)-Epigallocatechin (EGC; purity ≥98%), gallic acid (GA, purity ≥99%), and pyrogallol (Pg, purity ≥99%) were from Wako Pure Chemical Industries, Ltd.

For cell culture, Dulbecco’s modified Eagle’s medium and penicillin–streptomycin were purchased from Sigma-Aldrich. Fetal bovine serum and trypsin–ethylenediaminetetraacetic acid (EDTA) solution were from Life Technology (Tokyo, Japan), phosphate buffered saline was from Nissui Pharmaceutical (Tokyo, Japan), and dimethyl sulfoxide (DMSO, sterile-filtered) was from Nakalai Tesque (Kyoto, Japan).

**Instruments** The following were used for HPLC (all from Shimadzu unless stated otherwise): a Capcell Pak C8 Column (4.6×150 mm, particle size: 5 µm) and a guard column Capcell C8 SG 120 (4.0×10 mm, particle size: 5 µm) (both from Shiseido); a HPLC system controller (SCL-10 AVP); an autoinjector (SIL-10 AVP); a column oven (CTO-10A); a pump (LC-10ATVP); a degasser (DGU-14A); and an UV detector (SPD-10AVP). For protein determination, a Model 680 Microplate Reader (Bio-Rad) was used for measuring absorbance at 590 nm. A Microtec 1524R (Astec) was used for centrifugation. For cell culture, a CO2 incubator (MCO-5AC, Sanyo, Osaka, Japan) was used.

**HMG-CoA Lyase Activity Measurement** HMG-CoA lyase activity was measured by using a previously described method. HepG2 human hepatoma cells (6×10^6 cells) suspended in 100 µL of 0.02 m phosphate buffer (pH 7.5) in a 1.5 mL tube were mixed with 0.16 g of zirconia beads for 5 min, and the resulting homogenate was centrifuged at 20000×g for 5 min. Then, 25 µL aliquots of the supernatant were mixed with 120 µL of a buffer solution containing 50 mM glycylglycine, 50 mM glycylglycine, 20 mM MgCl2, 0.2% Triton X-100, and 20 mM diithiothreitol (pH 9.25). To these aliquots were added 5 µL DMSO (as a control) or nine representative dietary polyphenols (daidzein, hesperetin, naringenin, catechin, EC, ECG, EGCG, and GA) and Pg of the related compounds (Fig. 1) at final concentrations of 0, 25, 50, or 100 µM. After incubation for 5 min at 37°C, 25 µL aliquots of 10 mM HMG-CoA solution were added and the mixture was further incubated for 30 min at 37°C. The enzymatic reaction was stopped by adding 50 µL of 0.4 M citrate buffer (pH 3.5). The supernatant was collected to determine the level of acetoacetate formed by HMG-CoA lyase-catalyzed reaction.

Protein levels of cell homogenate samples were determined by the Bradford method using bovine serum albumin (BSA) solutions as standards. Cell homogenate samples or BSA solutions (10 µL/well) and Coomassie Brilliant Blue G250 (CBB-G250, 300 µL/well) were mixed on a 96-well microplate. After incubating for 10 min at room temperature, absorbance at 595 nm was measured.

**Determination of Acetoacetate Formed from the HMG-CoA Lyase-catalyzed Reaction** The levels of acetoacetate formed from the HMG-CoA lyase-catalyzed reaction were determined by the HPLC method with pre-column derivatization, developed by us. Briefly, 50 µL of 0.2 m 3-(2-hydroxyphenyl) propionic acid as an internal standard was mixed with 100 µL of a 0.4 m phosphate buffer solution (pH 3.5) and 50 µL of 100 µM p-nitrobenzenediazonium fluoroborate (diazo reagent solution) containing 0.2% Triton X-100 and incubated for 5 min at 37°C. Then, 25 µL aliquots of an acetoacetate standard solution (or cell extract sample) were added to the mixture. The derivatization of acetoacetate was performed for 2 min at 37°C. The mixture was then applied onto a solid phase cartridge (50 mg, 1 mL) preconditioned with 1 mL of methanol and 1 mL of water. After removing excess diazo reagent and matrix with 3 mL of water, the derivatives retained on the cartridge were eluted with 300 µL of metha-

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**Fig. 1. Structures of Polyphenols and Related Compounds**
nol, and 20 µL aliquots of the eluate were introduced into the HPLC system. HPLC was performed under the following conditions: mobile phase, methanol–HCl (1 m; 55:45); flow rate, 1.0 mL/min; column temperature, room temperature. HPLC eluates were monitored by UV absorbance at 380 nm to determine derivatives.

RESULTS AND DISCUSSION

Effect of Polyphenols on HMG-CoA Lyase Activity It has been reported that certain polyphenols inhibit digestive enzymes such as lipase, α-amylase, α-glucosidase, and steroidogenic enzymes. Nine representative dietary polyphenols and Pg of the related compounds were added to the cell extract sample to examine their effects on HMG-CoA lyase activity. The activity of HMG-CoA lyase in the presence of the above compounds was compared with that in the absence of inhibitor (the control) using the Student’s t-test. Daidzein, hesperetin, naringenin, catechin, and EC caused only a negligible change in activity. On the other hand, EGC, EGCG, GA, and Pg significantly decreased the activity of HMG-CoA lyase (p<0.01) by 54±5%, 87±6%, 79±7%, and 84±14%, respectively. On the other hand, ECG decreased the activity of HMG-CoA lyase by 37±4% (p<0.01), which revealed a slightly weak inhibition compared to the other four compounds (Fig. 2). All of these compounds inhibited HMG-CoA lyase activity in a concentration-dependent manner (Fig. 3).

Types of Inhibition by Polyphenols Using the Lineweaver–Burk Plot Following the findings that ECG, EGCG, GA, and Pg inhibit HMG-CoA lyase activity, we determined the type of inhibition using the Lineweaver–Burk plot. ECG, EGCG, GA, and Pg affected x- and y-intercepts and the slope of the plotted line of the uninhibited enzyme, indicating that they were mixed-type noncompetitive inhibitors, while GA did not affect the x-intercept, indicating that it is a noncompetitive inhibitor (Fig. 4). We also calculated the Michaelis–Menten kinetic parameters, that is, V_{max}, K_{m}, K_i, and IC_{50} (the concentration of inhibitor that inhibits 50% of enzyme activity) (Table 1). We found that V_{max} and K_{m} of ECG, EGCG, GA, and Pg were decreased compared with the controls, suggesting that the polyphenols were mixed-type noncompetitive inhibitors (Table 1). Although V_{max} of GA was slightly decreased and K_{m} of GA was unchanged compared with the controls, the results suggested that GA was a noncompetitive inhibitor (Table 1). Furthermore, K_{i} of EGCG with both gallyl and galloyl structures and that of EGCG with a gallyl structure were smaller than K_{i} of ECG with a galloyl structure. Similarly, K_{i} of Pg with a gallyl structure was also smaller than that of GA with galloyl structure. It can therefore be presumed that a structure with a gallyl moiety contributes mainly to noncompetitive inhibition, while that with a galloyl moiety contributes weakly to inhibition. In addition, IC_{50} of EGCG was smaller than that of ECG and EGCG. As a result, the Michaelis–Menten kinetic parameters of EGCG were the lowest, suggesting that EGCG is the strongest HMG-CoA lyase inhibitor of the dietary polyphenols tested (Table 1).

EGCG is the most abundant catechin found in green tea leaves and the most extensively studied catechin regarding health benefits. EGCG possesses a gallyl moiety in the B-ring and a galloyl moiety at the 3-position in the C-ring. The biological and/or biochemical effects of catechins focus mainly on the galloyl moiety. In fact, it has been reported that the galloyl moiety is critical for the inhibition of fatty acid synthase and that the galloyl moiety of catechins has a binding affinity toward human serum albumin. On the other hand, the galloyl moiety is less noteworthy. Recently, however, it has been reported that the galloyl moiety is a prerequisite for catechins to induce intermolecular cross-linking of membrane proteins. HMG-CoA lyase activity requires a divalent cation, such as Mg^{2+} or Mn^{2+}. On the other hand, the gal-
loyl and gallyl moieties of EGCG formed a hexa-coordination complex with the Mg$^{2+}$ ion. Therefore, we speculate that these moieties contribute to the inhibition of HMG-CoA lyase activity. Here we reported for the first time the inhibitory effect of EGCG on HMG-CoA lyase activity and the contribution of the gallyl moiety in EGCG to enzymatic inhibition.

**CONCLUSION**

Of the nine representative dietary polyphenols tested, EGC, EGCG, and GA effectively inhibited HMG-CoA lyase activity. Lineweaver–Burk analysis revealed that EGC and EGCG are likely to be mixed-type noncompetitive inhibitors. Pg with the gallyl structure also inhibited HMG-CoA lyase activity, suggesting that the gallyl moiety of polyphenols is important for the inhibition of HMG-CoA lyase activity. Taken together, EGC, EGCG, and GA are useful for preventing ketoacidosis or as adjuncts in the treatment of ketoacidosis.

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