When carbohydrate metabolism is impaired, fatty acid metabolism is activated. Excess acetyl-coenzyme A (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 3-hydroxybutyrate) lower the pH of blood and urine, resulting in ketoacidosis. 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 ketoacidosis. Inhibition of HMG-CoA lyase activity possibly prevents ketoacidosis 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; ketoacidosis; 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 converted from fatty acids are readily utilized as an energy source in the brain and muscular tissues. However, in patients with diabetes or soft drink ketosis—so-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 ketoacidosis, 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 ketoacidosis. 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 ketoacidosis. Based on the notion that inhibition of HMG-CoA lyase possibly prevents ketoacidosis 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%), 3β-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA, purity ≥99%), 3-(2-hydroxyphenyl)propionic acid (purity ≥99%, internal standard), magnesium chloride (molecular biology grade), glycylglycine (molecular biology grade), polyethylene octylphenyl ether (Triton X-100, molecular biology grade), glycylglycine (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 (diameter, 0.5 mm) for cell disruption were from Tomy 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 Clara, 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.). (−)-Catechin (purity ≥98%), (−)-epicatechin gallate (ECG; 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.8) HepG2 human hepatoma cells (6×10⁶ 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 glycyglycine, 20 mM MgCl₂, 0.2% Triton X-100, and 20 mM dithiothreitol (pH 9.25). To these aliquots were added 5 µL DMSO (as a control) or nine representative dietary polyphenols (daidzein, hesperetin, naringenin, catechin, ECG, EGC, 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 method9) 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 precolumn derivatization, developed by us.10) 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 citrate buffer solution (pH 3.5) and 50 µL of 10 mM 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 5 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-

(a) daidzein
(b) hesperetin
(c) naringenin
(d) (+)-catechin
(e) (−)-epicatechin
(f) (−)-epicatechin gallate
(g) (−)-epigallocatechin
(h) (−)-epigallocatechin gallate
(i) gallic acid
(j) pyrogallol

Fig. 1. Structures of Polyphenols and Related Compounds
the Michaelis–Menten kinetic parameters, that is, \( V_{\text{max}} \) and \( K_m \), whereas GA did not affect the enzyme activity in a concentration-dependent manner (Fig. 3).

Types of Inhibition by Polyphenols Using the Lineweaver–Burk Plot

Following the findings that EGC, EGCG, GA, and Pg inhibit HMG-CoA lyase activity, we determined the types of inhibition using the Lineweaver–Burk plot. EGC, EGCG, 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, \( V_{\text{max}}, K_m, K_i, \) and \( IC_{50} \) (the concentration of inhibitor that inhibits 50\% of enzyme activity) (Table 1). We found that \( V_{\text{max}} \) and \( K_m \) of EGC, EGCG, and Pg were decreased compared with the controls, suggesting that the polyphenols were mixed-type noncompetitive inhibitors (Table 1). Although \( V_{\text{max}} \) 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 a gallyl structure was smaller than that of EGC with a gallyl 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 EGC and ECG. 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.\textsuperscript{12,13} 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\textsuperscript{40} and that the galloyl moiety of catechins has a binding affinity toward human serum albumin.\textsuperscript{41} 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.\textsuperscript{42} HMG-CoA lyase activity requires a divalent cation, such as Mg\textsuperscript{2+} or Mn\textsuperscript{2+}.\textsuperscript{43} 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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