

Inhibition of hepatic glucose 6-phosphatase system by the green tea flavanol epigallocatechin gallate

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Abstract Effect of 5–100 μ M epigallocatechin gallate (EGCG) on hepatic glucose 6-phosphatase (G6Pase) system was investigated. EGCG inhibited G6Pase in intact but not in permeabilized rat liver microsomes, suggesting the interference with the transport. However, EGCG did not hinder microsomal glucose 6-phosphate (G6P) uptake. Instead, it increased the accumulation of radioactivity after the addition of [¹⁴C]G6P, presumably due to a slower release of [¹⁴C]glucose, the product of luminal hydrolysis. Indeed, EGCG was found to inhibit microsomal glucose efflux. Since G6Pase activity is depressed by glucose in a concentration-dependent manner, we concluded that EGCG inhibits G6Pase through an elevated luminal glucose level. © 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Endoplasmic reticulum; Transport; Glucose; Green tea; Catechin; Epigallocatechin gallate

1. Introduction

The various beneficial health effects of green tea consumption have received significant scientific attention recently [1]. Extensive investigations revealed that most of these effects can be attributed to polyphenolic compounds, which have long been known to possess widespread biological functions [2]. Green tea is especially rich in polyphenols; they represent 30% of the leaf dry matter [3]. Major polyphenols in the fresh tea leaves are flavanol (catechin) monomers, among which epigallocatechin, epicatechin, and their gallic esters are particularly abundant [4].

The best studied effects of epigallocatechin gallate (EGCG) are those related to the prevention of cancer and cardiovascular disease. In addition, several studies demonstrated its anti-

obesity and anti-diabetic effects [5]. EGCG decreases body weight and fat mass, influences adipocyte differentiation and proliferation, hormone levels and intermediary metabolism [6]. It was also reported to reduce blood glucose level by decreasing hepatic glucose production [7–9] through down-regulation of phosphoenolpyruvate carboxykinase and glucose 6-phosphatase (G6Pase) at transcriptional level [9–11].

G6Pase catalyzes the final step of hepatic glucose production, i.e. the hydrolysis of glucose 6-phosphate (G6P) derived either from glycogen breakdown or from gluconeogenesis. The active site of G6Pase is localized in the lumen of the endoplasmic reticulum (ER); therefore, transporter proteins are needed to allow the entry of G6P into, as well as the exit of glucose and phosphate from this compartment [12]. Glucose transport across the ER membrane was shown to be a high-capacity bidirectional facilitated uniport [13], though the glucose transporter protein of the ER has not been identified. Similarly to several other luminal enzymes, G6Pase has higher activity and lower specificity if the ER membrane is permeabilized. This phenomenon is referred to as latency and indicates that the transmembrane fluxes – presumably both the G6P uptake and the glucose release – are rate-limiting for the overall process [12].

G6Pase as well as hexose 6-phosphate dehydrogenase (H6PDH), the other G6P consuming enzyme in the ER lumen are provided with substrate by the G6P translocase (G6PT) that catalyzes the selective transport of G6P across the ER membrane [14]. Chlorogenic acid (CHA) and its derivatives (e.g. S3483) are potent inhibitors of G6PT [15]. Accordingly, they increase the latency of G6Pase and H6PDH by reducing their activity in intact microsomes or in the intact ER in situ.

CHA and curcumin were shown to induce glioma cell death through the inhibition or suppressed expression of G6PT, respectively [16,17]. The interesting observation that this effect can be mimicked by EGCG inspired the hypothesis that CHA and EGCG can be linked to a common intracellular target, namely G6PT [17]. It also implies that EGCG might inhibit the hepatic G6Pase system directly, in addition to the long-term transcriptional effect.

In the present work, we investigated the potential effect of EGCG and other green tea catechins on G6Pase and related transport activities in rat liver microsomes. Our aim was to reveal whether a direct inhibition of the hepatic G6Pase system can contribute to the metabolic effects of tea flavanols. Our results show that EGCG reduces the activity of G6Pase through the inhibition of glucose efflux from the ER lumen.

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Abbreviations: EGCG, epigallocatechin gallate; G6Pase, glucose 6-phosphatase; G6P, glucose 6-phosphate; ER, endoplasmic reticulum; G6PT, glucose 6-phosphate translocase; H6PDH, hexose 6-phosphate dehydrogenase; CHA, chlorogenic acid; GCG, gallic acid; EGCG, epigallocatechin gallate; PG, propyl gallate; MOPS, 4-morpholinepropanesulfonic acid; 11 β -HSDH1, 11 β -hydroxysteroid dehydrogenase type 1; ME, metyrapone

2. Materials and methods

2.1. Materials

G6P, glucose, EGCG, gallic acid, gallo catechin gallate (GCG), epicatechin gallate (ECG), propyl gallate (PG), alamethicin, 4-morpholinepropane-sulfonic acid (MOPS) were purchased from Sigma Chemical Co. [^{14}C]G6P and [^{14}C]glucose were obtained from MP Biomedicals. S3483 was kindly supplied by Aventis Pharma. All other reagents were of analytical grade.

2.2. Preparation of rat liver microsomal vesicles

Microsomes were prepared from livers of overnight fasted male Wistar rats (180–230 g), using fractional centrifugation [18]. The ER vesicles were washed and re-suspended in MOPS–KCl buffer (100 mM KCl, 20 mM NaCl, 3 mM MgCl_2 , 20 mM MOPS; pH 7.2) then immediately frozen in liquid nitrogen and kept in liquid nitrogen until use (within 6 months). The protein concentration in microsomal samples was determined using the method of Lowry et al. [19] with bovine serum albumin as a standard.

Purity of the microsomes was assessed by a marker-enzyme analysis as described earlier [20]. The integrity of the microsomal membranes was ascertained by using the mannose-6-phosphatase assay [21], which showed a latency greater than 95%.

2.3. G6Pase activity measurements and calculation of the latency

The microsomes (1 mg protein/ml) were incubated in MOPS–KCl buffer containing 5 mM G6P for 3 min at 37 °C to determine the rate of G6P hydrolysis. Vanadate (1 mM), S3483 (50 μM), PG (100 μM) or the investigated tea flavanols (at the indicated concentrations) were added to the microsomes 2–3 min before starting the reaction with G6P. Parallel incubations were set up containing intact microsomes and microsomes permeabilized with the pore-forming antibiotic alamethicin (0.1 mg/mg microsomal protein) [22]. The initial and final concentrations of inorganic phosphate (P_i) were measured in the samples using the sensitive assay of Chen et al. [23] with slight modification. The reagent mixture, which was prepared daily, contained 1 part of 10% ascorbic acid, 2 parts of 10% SDS and 6 parts of 0.42% ammonium molybdate $\cdot 4\text{H}_2\text{O}$ in 1 N H_2SO_4 . One milliliter of reagent was added to 100 μl sample and after 20 min at 46 °C the optical density was measured at 820 nm wavelength against a blank containing 1 ml reagent and 100 μl MOPS–KCl buffer. G6Pase activity was calculated as the amount of inorganic phosphate produced in 1 min by 1 mg microsomal protein. To determine the latency quantitatively, the difference between the activities measured in intact and permeabilized microsomes was expressed as the percentage of the latter [12].

2.4. Rapid filtration transport assay

G6P influx and glucose efflux were studied at 20 °C, using a rapid filtration method with radiolabeled tracers. In both cases, the microsome-associated radioactivity was measured after rapid filtering of the samples (100 μl volume) through cellulose acetate/nitrate filter membranes (MF-Millipore; 25 mm diameter; 0.22 μm pore size). The filters were immediately washed with 2.5 ml of ice-cold MOPS–KCl buffer and counted for radioactivity in a liquid scintillation counter. The filtration and washing procedure took about 10 s. In order to distinguish the intravesicular and membrane-bound radioactivity, parallel measurements were carried out in intact and alamethicin-permeabilized [24] microsomes. Alamethicin-treatment did not alter the recovery of microsomal proteins on the filters, indicating that the vesicular structure of microsomes was maintained. The alamethicin-releasable portion of radioactivity was regarded as intravesicular.

For G6P uptake measurements, the microsomes (2 mg protein/ml) were incubated in MOPS–KCl buffer containing 1 mM G6P and 600 000 cpm [^{14}C]G6P in a final volume of 140 μl and the samples were filtered after 30 s incubation. S3483 (50 μM), EGCG (50 or 100 μM) and vanadate (1 mM) were administered 2–3 min before the addition of G6P and the radioactive tracer. For glucose release measurements, the microsomes (4 mg protein/ml) were loaded for 1 h in 140 μl MOPS–KCl buffer containing 5 mM glucose and 300 000 cpm [^{14}C]glucose. EGCG (0, 50 or 100 μM) was added in a small volume of MOPS–KCl buffer to the samples 2–3 min before filtering. Glucose release occurred when the vesicles were washed on the filters, so the wash buffer contained EGCG at the same concentration as the corresponding sample.

2.5. Measurement of metyrapone-dependent accumulation of radioactivity upon [^{14}C]G6P addition

Inward G6P transport was also detected using the method described by Gerin and Van Schaftingen [25] with minor modifications. Microsomes (8 mg protein/ml final concentration) were incubated in MOPS–KCl buffer containing 1 mM metyrapone, 10 μM G6P, and 300 000 cpm [^{14}C]G6P in a final volume of 50 μl . S3483 (50 μM) and EGCG (50 or 100 μM) were administered 2–3 min before the addition of G6P and the radioactive tracer. Metyrapone was omitted from the samples where indicated. After 6 min incubation at 20 °C, the samples were mixed with 500 μl of ice-cold MOPS–KCl buffer, and rapidly filtered through cellulose acetate/nitrate filter membranes (MF-Millipore; 25 mm diameter; 0.22 μm pore size), which were immediately washed with 2.5 ml of ice-cold MOPS–KCl buffer. Finally, the filter-retained radioactivity was determined with a liquid scintillation counter.

2.6. Statistical analysis

Results are expressed as means \pm S.E.M or means \pm S.D. Results were compared using the Student's two-tailed *t*-test: $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Inhibition of G6Pase activity by EGCG

The hypothesis that EGCG can inhibit the hepatic G6Pase system directly was first tested by enzyme activity measurements in intact and permeabilized liver microsomes. The maximal G6Pase activity of liver microsomes can only be detected when the membrane barrier is eliminated. This can be achieved without destroying the vesicular structure by permeabilizing the microsomal membrane with a pore-forming antibiotic, alamethicin [22,24]. In our experiments, the activity measured in intact microsomes was only about 65% of the one observed after alamethicin-treatment, i.e. the latency was around 35% (Table 1). Inhibitors of G6P transport (e.g. S3483 [15]) only depress G6Pase activity in intact microsomes, thus increase the latency (Table 1). In contrast, inhibitors of G6Pase enzyme (e.g. vanadate [26]) reduce the activity both in intact and permeabilized microsomes; and usually decrease the latency because the transport is no longer rate-limiting when the enzyme itself works slower (Table 1). The metabolic effects of green tea flavanols – the insulin-like effects on hepatic glucose production in particular – were studied *in vitro* in the concentration range of 10–100 μM and they were found to be most pronounced at 100 μM level [9,11]. Therefore, the selected tea flavanols were studied in similar conditions in our experiments. EGCG and its isomer GCG were found to increase the latency of G6Pase, because their inhibitory effect was greater in intact than in permeabilized microsomes. ECG acted similarly but less efficiently, while PG, a non-catechin gallate was apparently ineffective (Table 1). The comparison of these agents suggests that the core catechin structure is essential for the effect, which is enhanced by the gallo moiety and also by the gallate group, though to a lesser extent.

The observed inhibitory effect of the main tea catechin, EGCG was further studied by determining its concentration-dependence. As it is also shown in Table 1, the higher concentrations of EGCG moderately affected G6Pase activity in the permeabilized microsomes (Fig. 1), which must be due to a weak direct interaction with the enzyme protein; nevertheless this effect was statistically not significant ($P = 0.0894$ for 100 μM EGCG vs. control). In contrast, the increasing concentration of EGCG caused an increasing inhibition of G6Pase activity in the intact microsomes (Fig. 1). The dissociation of

Table 1
Inhibition of G6Pase activity in intact and permeabilized microsomes

Inhibitor	G6Pase activity in intact microsomes (nmol/min/mg protein)	G6Pase activity in permeabilized microsomes (nmol/min/mg protein)	G6Pase latency (%)
None	80.2 ± 4.3	123.6 ± 8.1	35.1
100 μM GCG	42.3 ± 3.8**	100.6 ± 13.4*	57.9
100 μM ECG	60.0 ± 6.7**	107.0 ± 11.0	43.9
100 μM EGCG	47.5 ± 4.2**	111.0 ± 9.5	57.2
100 μM PG	74.8 ± 7.5	119.3 ± 10.4	37.3
50 μM S3483	6.0 ± 1.1**	118.5 ± 7.3	94.9
1 mM vanadate	1.7 ± 0.5**	1.9 ± 0.8**	7.3

Enzyme activity was measured at 5 mM G6P concentration in intact and alamethicin-permeabilized liver microsomes (1 mg protein/ml). The putative inhibitors were administered 2–3 min prior to the substrate. Latency represents the difference between G6Pase activities measured in intact and permeabilized microsomes expressed as the percentage of the latter. Data are shown as means ± S.D., $n = 4$, * $P < 0.05$, ** $P < 0.01$ vs. control values (None).

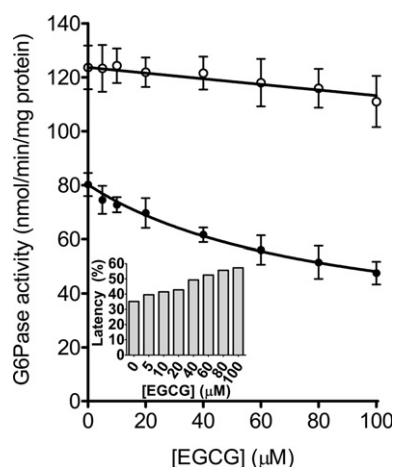


Fig. 1. Inhibition of G6Pase activity in intact and permeabilized microsomes. Enzyme activity was measured at 5 mM G6P concentration in intact (●) and alamethicin-permeabilized (○) liver microsomes (1 mg protein/ml). EGCG was administered at the indicated concentrations 2–3 min prior to the substrate. Latency of G6Pase activity as a function of EGCG concentration is shown in the inset. Data are shown as means ± S.E.M. from four experiments.

the activities measured in intact and permeabilized microsomes is also reflected by the gradually growing latency from 35% to nearly 60% (Fig. 1, inset). This observation strongly suggests that EGCG affects the G6Pase system through inhibiting one of the transport processes involved.

3.2. Effect of EGCG on the uptake of G6P into microsomes

Since the results of the enzyme activity measurements indicated that the target of EGCG is a transport associated to G6Pase activity, we investigated the effect of this flavanol on microsomal G6P influx using a direct transport assay. The accumulation of radioactivity in the microsomal vesicles was detected using rapid filtration after addition of G6P with radioactive tracer. Inhibition of G6Pase with vanadate reduced the apparent G6P uptake remarkably (Fig. 2), which demonstrates that a significant part of the luminal radioactivity can be attributed to glucose produced locally. It also indicates that a considerable amount of glucose is entrapped in the microsomal lumen during the sort time (30 s) of this incubation.

In fact, the measurement of pure G6P transport would require the inhibition of G6Pase. Therefore, the possible effect of EGCG on G6P uptake could be best assessed in the pres-

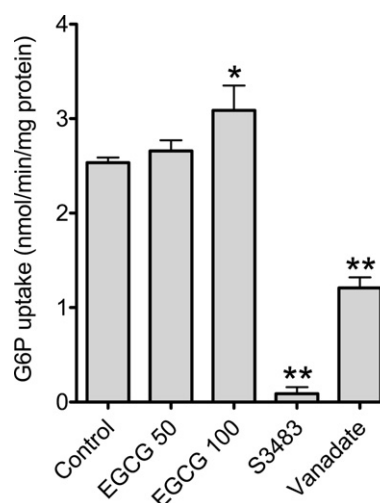


Fig. 2. Effect of EGCG on microsomal G6P uptake. Liver microsomes (2 mg protein/ml) were pretreated for 2–3 min with 50 μM EGCG, 100 μM EGCG, 50 μM S3483, 1 mM vanadate or none (control). Then G6P was added together with radiolabeled [14 C]G6P tracer at 1 mM final concentration, and after 30 s the amount of intraluminal G6P was determined in 100 μl samples using rapid filtration technique as described in Section 2. Data are shown as means + S.E.M., $n = 4$, * $P < 0.05$, ** $P < 0.01$ vs. control.

ence of vanadate but the two compounds are incompatible, i.e. vanadate oxidizes EGCG. Still, the experimental system (without vanadate) is suitable to detect the inhibition of G6P uptake, which is well proven by the nearly abolished accumulation of radioactivity in the presence of the known G6PT inhibitor, S3483 (Fig. 2). In contrast to S3483, EGCG was found to stimulate rather than inhibit the apparent G6P uptake (Fig. 2). It is theoretically possible, yet unlikely, that EGCG really enhances the influx of G6P. The stimulation of such a facilitated passive diffusion is seldom observed. The increased luminal accumulation of radioactivity in the presence of EGCG in our experiments is due presumably to a hindered glucose efflux.

To minimize the interference of glucose production without vanadate administration, G6P uptake was also studied in microsomes where the luminal metabolism of G6P was diverted from G6Pase [25]. This was achieved by activating H6PDH that binds G6P with high affinity and converts it to 6-phosphogluconate as long as luminal NADP⁺ is available. The luminal NADP⁺ generation can be fuelled by metyrapone

(ME), which oxidizes luminal NADPH in a reaction catalyzed by 11 β -hydroxysteroid dehydrogenase type 1 (11 β HSDH1) [12]. The presence of ME greatly enhances the luminal accumulation of radioactivity after the addition of G6P with radiolabeled tracer (Fig. 3, control vs. without ME). It indicates that the majority of luminal radioactivity in these conditions can be attributed to 6-phosphogluconate, which does not have efficient membrane transport to leave the microsomes as reported earlier [25]. In contrast to S3483, EGCG did not affect the accumulation of radioactivity in the microsomal lumen (Fig. 3), which supports that the target of this tea catechin is not the G6PT.

3.3. Inhibition of microsomal glucose release by EGCG

The above results strongly suggest that EGCG affects G6Pase system by inhibiting glucose efflux from the microsomal lumen. Although the glucose transporter of the ER has not been identified at molecular level, several observations suggest the existence of a protein-mediated transport [12]. The assumption that EGCG inhibits this yet unidentified transporter was tested in direct transport measurements. Preloaded microsomal vesicles retained significantly more glucose when washed with a buffer containing EGCG, as compared to the control (Fig. 4). The relatively high rate of glucose efflux does not allow the precise determination of the initial luminal glucose content with rapid filtration. However, it can be estimated to be around 18 nmol/mg protein assuming that the intravesicular water space (3.6 μ l/mg protein) of rat liver microsomes reported earlier [27] completely equilibrated with the extravesicular fluid containing 5 mM glucose during the 1 h long preincubation period. Therefore, it can be figured that the control microsomal vesicles and those treated with 100 μ M EGCG released 9.90 ± 0.86 and 3.37 ± 2.27 nmol/mg protein glucose, respectively, i.e. glucose efflux was nearly three-times slower in the presence EGCG. These results pro-

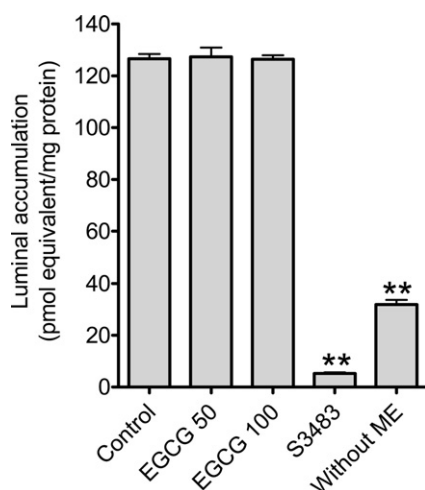


Fig. 3. EGCG fails to affect metyrapone-induced accumulation of radio-labeled G6P metabolites. Liver microsomes (8 mg protein/ml) were pre-incubated with or without 1 mM metyrapone for 10 min. The metyrapone-treated microsomes were then treated with 50 μ M EGCG, 100 μ M EGCG, 50 μ M S3483 or none (control). Intravesicular accumulation of radioactive metabolites was detected 6 min after addition of 10 μ M G6P with radio-labeled [14 C]G6P tracer. Data are shown as means + S.E.M., $n = 4$, * $P < 0.05$, ** $P < 0.01$ vs. control.

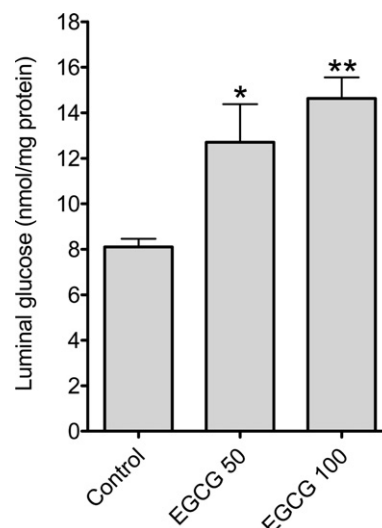


Fig. 4. Inhibition of glucose release from microsomal vesicles by EGCG. Liver microsomes (4 mg protein/ml) were loaded with 5 mM glucose and radio-labeled [14 C]glucose tracer by incubation for 1 h at room temperature. Then 50 μ M EGCG, 100 μ M EGCG or none (control) was administered and 100 μ l samples were filtered and washed to determine the amount of glucose retained in the vesicles as described in Section 2. Data are shown as means + S.E.M., $n = 6$, * $P < 0.05$, ** $P < 0.01$ vs. control.

vide further evidence for the existence of a microsomal glucose transporter, which has been disputed for a long time.

3.4. G6Pase activity at various glucose concentrations

The inhibition of glucose efflux can lead to the reduction of G6Pase activity in intact microsomal vesicles if the build-up of glucose in the lumen acts as an obstacle to G6P hydrolysis – as reported earlier [28]. The phenomenon was confirmed in the conditions of our experiments. Glucose reduced the G6Pase activity both in intact and permeabilized liver microsomes in a concentration-dependent manner (Fig. 5); and the extent of inhibition was comparable with that achieved by EGCG in in-

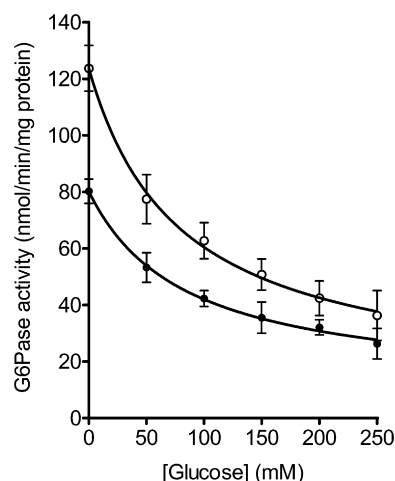


Fig. 5. Dependence of G6Pase activity on glucose concentration. Enzyme activity was measured at 5 mM G6P concentration in intact (●) and alamethicin-permeabilized (○) liver microsomes (1 mg protein/ml) in the presence of glucose at the indicated concentrations. Data are shown as means \pm S.E.M. from four experiments.

tact microsomes. It should be noticed that G6Pase activity is higher in the permeabilized microsomes at any glucose concentration, though glucose can equally take effect in the intact and permeabilized vesicles. The fact that G6Pase latency only decreased from 35% to 27% at high glucose concentration is in accordance with the assumption that G6P transport is rate-limiting for the overall process.

4. Concluding remarks

In summary, our results show that certain tea catechins can inhibit hepatic G6Pase system directly. The observed 40% inhibition likely contributes to their anti-diabetic, glucose-lowering effect by measurably reducing hepatic glucose production. EGCG was administered to a purified microsomal system in our experiments; therefore, the phenomenon is independent of the previously reported G6Pase inhibition, which was due to a lowered gene expression.

The EGCG concentrations found to be effective in this study are similar to those (10–100 μM) usually applied in cellular models to investigate specific effects of this catechin. Reduced glucose production and depressed expression of gluconeogenic enzymes were also found to be most evident at 100 μM EGCG in hepatoma cells [9,11]. It has been demonstrated in human pharmacokinetic studies that regular green tea consumption can only maintain as high as 1–4 μM level of EGCG in blood plasma [29,30]. However, the concentrations achieved in the cells and particularly in the hepatic ER have not been measured. The metabolic effects described *in vitro* at 10–100 μM EGCG concentrations [9,11] were in accordance with those observed *in vivo* after ip injections of pure green tea catechins [7] or after oral administration of green tea or EGCG [8,10]. These results justify the conditions applied *in vitro* and suggest the accumulation of tea flavanols in certain cells well above plasma concentrations.

The target of EGCG action seems to be the yet-unidentified glucose transporter component of the G6Pase system. In addition to its medical importance, this observation provides further experimental evidence for the protein-mediated glucose transport across the ER membrane. Neither the G6Pase enzyme nor G6PT was affected while glucose release was hindered and signs of luminal glucose accumulation were detected in the presence of EGCG. The glucose-sensitivity of G6Pase further supports the concept that luminal glucose accumulation may be responsible for the inhibitory effect of EGCG. Since the signaling pathways associated with the ER have not been reported to be activated or modulated by the luminal glucose concentration, there is no reason to assume that glucose accumulation in the ER lumen caused by EGCG could be mechanistically linked to changes in phosphoenolpyruvate carboxykinase expression and activity. The direct inhibition of the G6Pase system and the depressed expression of gluconeogenic enzymes can independently, yet synergistically reduce hepatic glucose production.

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