

Dietary Epicatechin Promotes Survival of Obese Diabetic Mice and *Drosophila melanogaster*¹⁻³

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Abstract

The lifespan of diabetic patients is 7–8 y shorter than that of the general population because of hyperglycemia-induced vascular complications and damage to other organs such as the liver and skeletal muscle. Here, we investigated the effects of epicatechin, one of the major flavonoids in cocoa, on health-promoting effects in obese diabetic (*db/db*) mice (0.25% in drinking water for 15 wk) and *Drosophila melanogaster* (0.01–8 mmol/L in diet). Dietary intake of epicatechin promoted survival in the diabetic mice (50% mortality in diabetic control group vs. 8.4% in epicatechin group after 15 wk of treatment), whereas blood pressure, blood glucose, food intake, and body weight gain were not significantly altered. Pathological analysis showed that epicatechin administration reduced the degeneration of aortic vessels and blunted fat deposition and hydropic degeneration in the liver caused by diabetes. Epicatechin treatment caused changes in diabetic mice that are associated with a healthier and longer lifespan, including improved skeletal muscle stress output, reduced systematic inflammation markers and serum LDL cholesterol, increased hepatic antioxidant glutathione concentration and total superoxide dismutase activity, decreased circulating insulin-like growth factor-1 (from 303 ± 21 mg/L in the diabetic control group to 189 ± 21 mg/L in the epicatechin-treated group), and improved AMP-activated protein kinase- α activity in the liver and skeletal muscle. Consistently, epicatechin (0.1–8 mmol/L) also promoted survival and increased mean lifespan of *Drosophila*. Therefore, epicatechin may be a novel food-derived, antiaging compound. *J. Nutr.* 141: 1095–1100, 2011.

Introduction

It is estimated that at least 23.6 million, or ~8%, of Americans presently suffer from diabetes, and 57 million people are prediabetic in the US (1). The life expectancy of diabetic individuals is reduced by 7.5–8.2 y compared with that of nondiabetics (2), which is primarily due to diabetes-caused complications. Previous studies indicate that hyperglycemia, dyslipidemia, and hyperinsulinemia cause vascular complications and subsequent damage to multiple organs, including the liver, skeletal muscle, and the nervous system (3,4). Therefore, ameliorating these complications can promote health and survival of diabetic patients.

Recent epidemiological studies indicate that people living on San Blas island, who are known to consume a large quantity of flavanol-rich cocoa beverage daily, have a considerably lower incidence of ischemic heart disease, stroke, and diabetes, and a remarkably longer lifespan compared with those who live on the mainland of Panama (5). Interestingly, these differences disappeared when people from San Blas island migrated to Panama City, where the quantity of cocoa consumption was considerably reduced (6). Consistently, recent human studies demonstrated that dietary intake of cocoa or chocolate can improve blood vessel function, insulin sensitivity, blood pressure, and inflammation (7). These data suggest that cocoa may exert health-promoting effects, although the specific cocoa components primarily responsible for these actions are not known.

It was recently reported that consumption of a flavanol-rich cocoa beverage increased flow-mediated vasodilation in humans that was associated with an increased plasma level of NO, the major vasodilator in the circulation (8). In line with this finding, another study found that intake of cocoa flavanols improved flow-mediated dilatation in type 2 diabetic patients (9), suggesting that the beneficial effects of cocoa on human vascular function may be at least partially attributable to flavanols. Although the various beneficial effects of cocoa were primarily investigated using healthy humans or animals (5), there are very few studies, to our knowledge, that have examined if cocoa flavanols have

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³ Supplemental Figure 1 and Table 1 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.

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health-promoting effects in diabetic individuals. In the present study, we investigated whether and how dietary consumption of epicatechin improves health and promotes survival of obese diabetic mice.

Methods and Materials

Experimental animals

Obese diabetic mice. Five-week-old male, obese, diabetic (BKS.Cg-m +/-Lepr db/J, *db/db*) mice and their lean littermates [C57BLKS/J, used as normal control (Con)] were purchased from Jackson Laboratory. Mice were housed in an environmentally controlled ($23 \pm 2^\circ\text{C}$; 12-h-light/dark cycle) animal facility and were provided free access to a rodent diet (AIN 93G diet; Dyet) (10). Diabetic mice were randomly divided into 2 groups ($n = 12$) and given either 0% [diabetic control (db)] or 0.25% epicatechin (db+EC) in drinking water for 15 wk. This dose of epicatechin [~ 150 mg/kg body weight (BW)]⁸ equivalent] was calculated based on previous experiments using cocoa products in humans (100–400 mg/kg BW) and the mean concentration of flavanols in cocoa (11). To ensure its stability, stock epicatechin was stored at -80°C and its water solution kept away from light. Fresh epicatechin solution was made and provided to mice every other day. BW and food and water intake were monitored weekly. The general clinical condition and mortality of the mice were monitored daily. The criteria for euthanasia of mice were independently assessed by a veterinarian according to Association for Assessment and Accreditation of Laboratory Animal Care guidelines. Mice with a BW $< 30\%$ of original BW were euthanized by inhalation of CO_2 and censored. Before and at the end of the experiment, blood pressure was measured using a computerized noninvasive blood pressure system (Kent Scientific) as previously described (12) and blood glucose levels in tail vein blood samples were determined by using a glucometer (Roche). At 20 wk of age, the remaining mice were euthanized using CO_2 and their blood and tissues were collected for biochemical and physiological analyses. The animal protocol was approved by the Institutional Animal Care and Use Committee at Virginia Tech.

Drosophila melanogaster. Wild-type *Drosophila* strain Canton-S was purchased from Bloomington *Drosophila* Stock Center at Indiana University. Fruit flies were incubated in the refrigerator incubator at 25°C with a 12-h-light/dark cycle, a standard condition for this type of research. Newly eclosed male adults were divided into 5 groups (30 flies/group) and provided a standard diet (2% agar, 10% yeast, 10% sucrose; Carolina Biological Supply) containing 0, 0.01, 0.1, 1, or 8 mmol/L epicatechin (Sigma-Aldrich), which are equivalent to a range of daily intake of $41.76 \mu\text{g}$ – 41.76 mg/kg BW. We used this dose range, because it overlaps that humans consumed through the intake of cocoa products or flavanol supplements [14 – 42 mg flavanols/(kg BW·d)] (13). Flies were transferred to fresh food vials every 3 d and the mortality was scored after each transfer.

Measurements of serum biological markers and hepatic antioxidants

Serum total cholesterol, HDL-cholesterol, and TG in feed-deprived mice were measured using a PTS CardioChek Blood Analysis Meter (Maria Stein) according to the manufacturer's instructions. LDL-cholesterol was calculated using the formula from the manual of the analysis meter: $\text{LDL-cholesterol} = \text{total cholesterol} - \text{HDL-cholesterol} - \text{TG}/5$. Serum C-reactive protein (CRP) was measured using a highly sensitive 2-site ELISA kit (Immunology Consultants Laboratory). Cytokines and chemokines from serum, including IL-5, IL-1 β , and TNF α , were tested by using a Luminex mouse cytokine array assay (Capital Biosciences) as

previously described (14). The level of glutathione (GSH) and total activity of superoxide dismutase (SOD) in the liver were measured as we previously described (15). The serum insulin-like growth factor-1 (IGF-1) concentration was measured using an ELISA kit (R&D Systems).

Pathological analysis

Fresh aorta and livers were fixed in 10% phosphate buffered neutral formalin, embedded in paraffin, cut at thicknesses of $5 \mu\text{m}$, and then stained with hematoxylin and eosin for histological examination of atherosclerotic and hepatic lesions. Three sections from each mouse were examined. The pathological alterations in the aorta were graded based on the presence of vacuoles, subendothelial deposits, loss of elastic fibers, and plaques (0 = normal, 1 = smooth muscle vacuoles, 2 = smooth muscle vacuoles and subendothelial deposits, 3 = vacuoles, subendothelial deposits, loss of elastic fibers, and 4 = vacuoles, subendothelial deposits, loss of elastic fibers, plaques). The liver lesions were scored according to the levels of vacuolar change (hydropic degeneration or lipidosis) in hepatocytes (1 = 0–10%, 2 = 10–30%, 3 = 30–50%, and 4 = $>50\%$ of hepatocytes affected).

Skeletal muscle function assays

Extensor digitorum longus (EDL) muscles were surgically excised from dead mice and secured via a 4–0 suture to a dual-mode servomotor (Aurora Scientific) to determine contractile function as we described (16). Stress output (g/mm^2) was calculated as the force output (g) for a given stimulation normalized to the estimated cross-sectional area (mm^2) of the muscle. The cross-sectional area of each muscle was determined using the following equation: $\text{muscle cross-sectional area} = \text{muscle mass in g}/(1.056 \text{ g}/\text{cm}^3 \times \text{muscle length in cm})$.

Immunoblot analysis

Equal amounts of proteins from the liver or skeletal muscle lysates were subjected to immunoblot analysis as previously described (12). Membranes were probed with an antibody against phospho-AMP-activated protein kinase- α (AMPK α) (Cell Signaling Technology). The protein bands were digitally imaged for densitometric quantitation with a software program Genetools (Synoptics Limited). The expression of phospho-AMPK α was normalized to the total AMPK α level from the same sample.

Statistical analysis

Survival curves were plotted using the Kaplan-Meier method including all available mice at each time point (17), and the Logrank test was applied to compare the survival distributions of control and epicatechin-treated groups. We used a mixed-model ANOVA with repeated measures for comparison of stress-frequency (factors: genotype and stimulation frequency) and fatigue (factors: genotype and time) profiles of skeletal muscle, and Tukey's test was used for post hoc analyses. The results from pathological analysis of the liver and aorta were analyzed using Kruskal-Wallis test and significant differences between treatment groups were further analyzed using the Mann-Whitney U test. All other data were analyzed with 1-way ANOVA and significant differences between treatment groups were further analyzed using t test. $P < 0.05$ was considered significant.

Results

Dietary supplementation of epicatechin promotes survival of diabetic mice. At 20 wk of age, 50% of the mice in the db group had died, whereas the mortality rate was only 8.3% in the db+EC group ($P = 0.02$) (Fig. 1). Although the sample size ($n = 12/\text{group}$) was relatively small for a typical survival study, we think that the observed effect of epicatechin on the survival of diabetic mice is a real effect of this compound, because such a large difference between the 2 groups was unlikely due to random variation. In addition, the statistical power from this sample size and obtained data was 0.88, which exceeds the minimal statistical power of 0.80 required for the adequacy of

⁸ Abbreviations used: AMPK, AMP-activated protein kinase; BW, body weight; Con, normal control; CRP, C-reactive protein; db, diabetic control; db+EC, diabetic mice treated with epicatechin; EC, epicatechin; EDL, extensor digitorum longus; GSH, glutathione; IGF-1, insulin-like growth factor-1; SOD, superoxide dismutase.

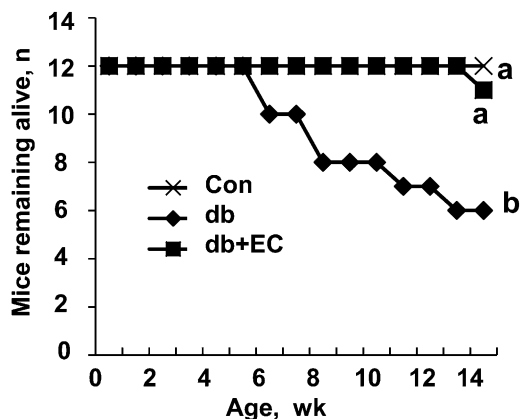


FIGURE 1 Lifespan in normal Con, db, and db+EC mice treated for 15 wk. $n = 12$. Curves without a common letter differ, $P < 0.01$.

sample sizes at $\alpha = 0.05$. The BW of db+EC mice tended to be lower (9.4%) than that of db mice ($P = 0.10$) (Table 1). Notably, epicatechin did not alter food intake, blood glucose levels, or blood pressure, except that db+EC mice drank less water than db mice ($P = 0.01$) (Table 1). These results suggest that the effect of epicatechin on lifespan is not secondary to changes in these variables.

Epicatechin ameliorates pathological alterations in the aorta and liver. Ingestion of epicatechin for 15 wk prevented the development of aortic lesions caused by diabetes ($P < 0.01$) (Supplemental Fig. 1; Table 1), which was evaluated according to the levels of vacuoles, subendothelial deposits, loss of elastic fibers, and plaques in the aorta. In the liver, histopathological examination indicated severe fat deposition and hydropic degeneration in hepatocytes of db mice, which was prevented in the db+EC group ($P < 0.05$) (Supplemental Fig. 1; Table 1). These protective effects in the aorta and liver may contribute to the increased survival rate of diabetic mice by dietary intake of epicatechin.

Epicatechin improves age-related biomarkers in diabetic mice. Consistent with observations of the pathological alterations in vital organs and remarkably shortened lifespan of mice in the db group, circulating levels of CRP and IL-1 β were significantly elevated in the db group compared with those in the Con group (Table 2). However, dietary intake of epicatechin significantly reduced these inflammatory markers, indicating that epicatechin may suppress chronic inflammation caused by diabetes. In addition, the GSH concentration and total SOD activity in the livers of the db+EC group were significantly greater, whereas these antioxidants were not altered in the db group

compared with the Con group (Table 2). Furthermore, serum total and LDL-cholesterol levels were significantly greater in the db group than those in the Con group, but these increases were prevented in the db+EC group, which did not differ from the Con group (Table 2). Moreover, serum IGF-1 levels were significantly lower in the db+EC group than in the db and Con groups (Table 2). Low serum IGF-1 levels have been shown to play an important role in lifespan regulation and aging in several animal models (18).

Epicatechin improves skeletal muscle function in diabetic mice. Absolute EDL stress output at all stimulation frequencies tested was significantly improved in the db+EC group compared with the db and Con groups (Fig. 2A). Consistently, EDL from the db+EC mice generated more stress than that from db and Con mice throughout a fatigue protocol (Fig. 2B). These data suggest that epicatechin improved skeletal muscle function.

Epicatechin improves AMPK α activity in the liver and skeletal muscle of diabetic mice. AMPK α activity was reduced in the liver of diabetic mice. However, epicatechin restored AMPK α activity to the level comparable to that in Con mice, whereas protein expression of AMPK α was not altered (Fig. 3A). In addition, the levels of AMPK α phosphorylation in skeletal muscle from the db+EC group were significantly higher than those from the db group (Fig. 3B). This result suggests that epicatechin may be a novel lifespan extension molecule through regulation of AMPK activity.

Epicatechin increase lifespan of Drosophila. To confirm whether epicatechin is a novel lifespan extension agent, we treated *Drosophila* with various doses of epicatechin throughout their lifespan. We found that mean lifespan was greater in the groups receiving 0.1–8 mmol/L epicatechin than in those receiving no or less epicatechin (Fig. 4A). Consistently, epicatechin (0.1 mmol/L) significantly promoted survival of the fruit flies (Fig. 4B), further indicating that epicatechin is a food-derived, antiaging compound.

Discussion

Individuals with diabetes live 7.5–8.2 y less than their nondiabetic equivalents (2), and diabetes-triggered complications, particularly cardiovascular and liver diseases (1), are the primary causes of death in diabetic patients. In the present study, we found that epicatechin treatment promoted survival of obese diabetic mice. Notably, epicatechin treatment had no effect on hyperglycemia, food intake, and BW gain of diabetic mice, suggesting that a lifespan extension effect of epicatechin is not a secondary action

TABLE 1 Physiological variables of mice at baseline and 15 wk of epicatechin treatment¹

	Baseline			15 wk		
	Con	db	db+EC	Con	db	db+EC
BW, g	19.0 \pm 0.4 ^b	31.5 \pm 0.6 ^a	31.1 \pm 0.6 ^a	24.2 \pm 0.3 ^{a*}	28.4 \pm 3.4 ^{a*}	25.8 \pm 1.8 ^{a*}
Food intake, g/d	2.4 \pm 0.5 ^b	4.9 \pm 0.8 ^a	5.2 \pm 0.7 ^a	2.3 \pm 0.6 ^b	5.2 \pm 1.0 ^a	5.3 \pm 0.9 ^a
Water intake, g/d	5.6 \pm 1.2 ^b	18.2 \pm 2.7 ^a	16.7 \pm 2.3 ^a	7.8 \pm 1.3 ^c	44.4 \pm 1.4 ^{a*}	35.5 \pm 1.4 ^{b*}
Blood glucose, mmol/L	181 \pm 18.2 ^b	312 \pm 28.4 ^a	376 \pm 30.4 ^a	179 \pm 5.3 ^b	512 \pm 24.2 ^{a*}	569 \pm 16.8 ^{a*}
Systolic blood pressure, mm Hg	159 \pm 4.8	154 \pm 5.0	162 \pm 5.4	169 \pm 8.5	148 \pm 8.0	146 \pm 3.5
Mice, n	12	12	12	12	6	11

¹ Values are means \pm SEM. Means at a time with superscripts without a common letter differ, $P < 0.05$. *Different from baseline, $P < 0.05$.

TABLE 2 Serum lipids, inflammatory markers, IGF-1, and hepatic antioxidants levels in Con, db, and db+EC mice treated for 15 wk¹

Group	n	LDL		Total	CRP-1	IL-1 β	GSH	SOD
		cholesterol	cholesterol	cholesterol				
		mg/L	mmol/L	mmol/L	mg/L	mg/L	nmol/mg protein	Units/mg protein
Con	12	307 \pm 17.0 ^a	1.5 \pm 0.2 ^a	4.4 \pm 0.2 ^a	28.9 \pm 2.9 ^a	61 \pm 11.0 ^a	28.2 \pm 0.8 ^b	15.7 \pm 2.3 ^a
db	6	303 \pm 21.0 ^a	2.6 \pm 0.4 ^b	7.0 \pm 0.5 ^b	41.0 \pm 6.1 ^b	815 \pm 23.0 ^b	25.9 \pm 0.7 ^a	14.2 \pm 4.5 ^a
db+EC	11	189 \pm 21.0 ^b	1.4 \pm 0.3 ^a	5.3 \pm 0.5 ^a	31.1 \pm 1.7 ^a	351 \pm 17.0 ^c	28.5 \pm 0.4 ^b	25.8 \pm 3.2 ^b

¹ Values are means \pm SEM. Means in a column without a common letter differ, $P < 0.05$.

whereby it modulates these variables in diabetic mice. However, epicatechin attenuated diabetes-induced aortic and hepatic pathogenesis and improved various biomarkers that are associated with a longer and healthy lifespan, including improved skeletal muscle function, reduced systematic inflammation and blood cholesterol, increased hepatic antioxidant status, decreased circulating IGF-I, and improved AMPK α activity in the liver and skeletal muscle. These findings suggest that epicatechin may be a food-derived, antiaging agent that directly exerts protective effects on the tissues such as the liver, skeletal muscle, and vasculature.

Progressive rises of systemic inflammation, oxidative stress, and circulating lipid abnormalities are increasingly recognized to play an important role in the aging process and age-related disorders such as diabetes and vascular dysfunction (19). Indeed, CRP is an independent vascular inflammation maker (20), and inflammatory cytokine IL-1 β , which participates in fundamental

inflammatory processes, is a predictor of mortality associated with age-related chronic diseases (21). Thus, agents that can lower inflammation and oxidative stress and improve lipid metabolism potentially prevent or delay various age-associated degenerative diseases. Indeed, epicatechin intake decreased serum proinflammatory markers such as CRP and IL-1 β and reduced serum LDL cholesterol in *db/db* mice. Impairment of the antioxidant defense system, including reduced GSH content and SOD activity and subsequent elevation in oxidative stress, often occurs in obesity and diabetes (22,23). In line with previous studies showing that tea catechins can upregulate hepatic or GSH content, thereby protecting the liver from injury (24), we found that epicatechin increased hepatic the GSH level and total SOD activity in diabetic mice. A limitation of this study is that we did not separately measure mitochondrial and cytosolic SOD activity, given that mitochondrial dysfunction is increasingly recognized to play an important role in the aging process. However, changes in these antioxidants by epicatechin may not make a major contribution to its beneficial effects on health and survival of diabetic mice, because neither GSH nor SOD were significantly altered in *db/db* mice compared with those in the Con group. Although epicatechin reportedly can scavenge free radicals at pharmacological doses (25), it is unclear from the present study whether oxidative stress in diabetic mice was reduced by epicatechin treatment. Collectively, our data suggest that epicatechin may protect vasculature and the liver and thereby extend lifespan of diabetic mice at least in part through modulating inflammation and lipid metabolism.

Although epicatechin increased the survival of diabetic mice, it is more important to see whether health-related quality of life was improved by this treatment. One of the biomarkers for this assessment was to measure skeletal muscle strength, which is progressively reduced in the elderly and patients with diabetes (26). Interestingly, dietary intake of epicatechin significantly improved muscular strength and fatigability compared with diabetic control mice. Although the exact mechanism underlying this effect of epicatechin is not clear, epicatechin may improve skeletal muscle strength partially through suppression of inflammation, which was shown to impair muscle strength and physical performance in diabetic patients (27). There is also a possibility that these changes could be a result of alterations in Ca²⁺ handling (28), evidenced by treatment-induced changes in fatigability, twitch, and tetanus temporal properties.

AMPK, an energy-sensing molecule that is highly conserved from yeast to animals, is increasingly recognized as a master regulator of whole body energy homeostasis (29). Activated AMPK promotes fatty acid oxidation and regulates mitochondrial biogenesis (29). To do so, AMPK α directly regulates the activity of acetyl-CoA carboxylase and the PPAR γ coactivator-1 α (30), 2 of the most important metabolic regulators. In addition, activated AMPK directly phosphorylates and inactivates 3-hydroxy-3-methylglutaryl-CoA reductase in the liver, the rate-

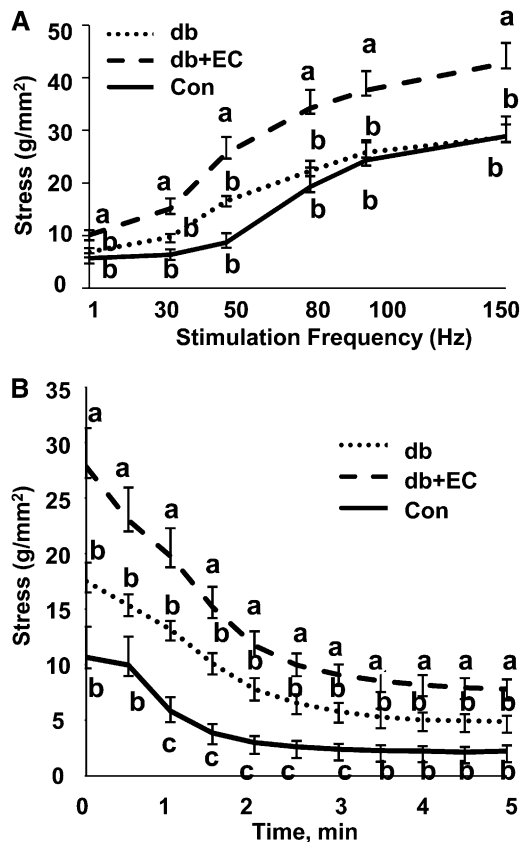


FIGURE 2 Stress output (A) and fatigability protocol (B) of EDL skeletal muscle in Con, db, and db+EC mice treated for 15 wk. Data are means \pm SE, $n = 12$ (Con), 6 (db), and 11 (db+EC). Means at each frequency (A) and time (B) without a common letter differ, $P < 0.05$.

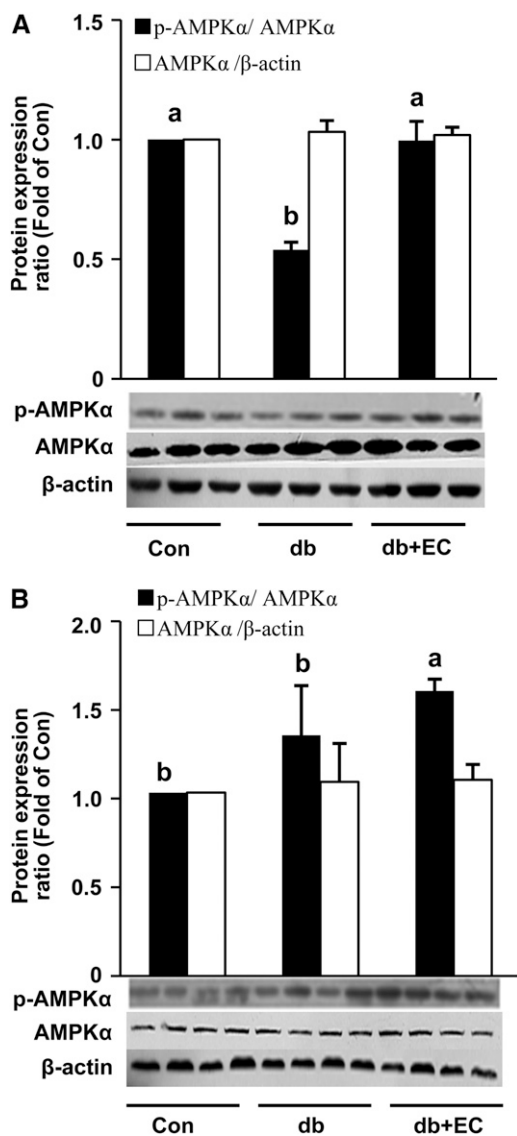


FIGURE 3 AMPK activity in the liver (A) and skeletal muscle (B) in normal Con, db, and db+EC mice treated for 15 wk. The level of p-AMPK α was normalized to that of total AMPK α , and total AMPK α expression was normalized to β -actin content in each sample. Values are means \pm SEM, $n = 12$ (Con), 6 (db), and 11 (db+EC). Columns without a common letter differ, $P < 0.05$.

limiting enzyme in cholesterol synthesis (31). Therefore, epicatechin might improve plasma and hepatic lipids through AMPK-mediated inhibition of their synthetic enzymes in the liver. Recently, several lines of evidence demonstrate that activation of AMPK increases lifespan and delays age-associated functional decline in various species (32). Conversely, reduction of AMPK activity leads to age-associated dysfunction of skeletal muscle (33), vessel (34), and the liver (35). In addition, emerging studies showed that AMPK also plays an essential role in the control of inflammation (36) and oxidative stress (37). Given these versatile roles of AMPK, it is conceivable that activation of the AMPK pathway by epicatechin may represent a central mechanism that mediates its various health-beneficial effects.

It is well recognized that circulating IGF-1 levels are inversely associated with lifespan in mammals (38). Our results indicated that IGF-1 serum levels were reduced by epicatechin in diabetic

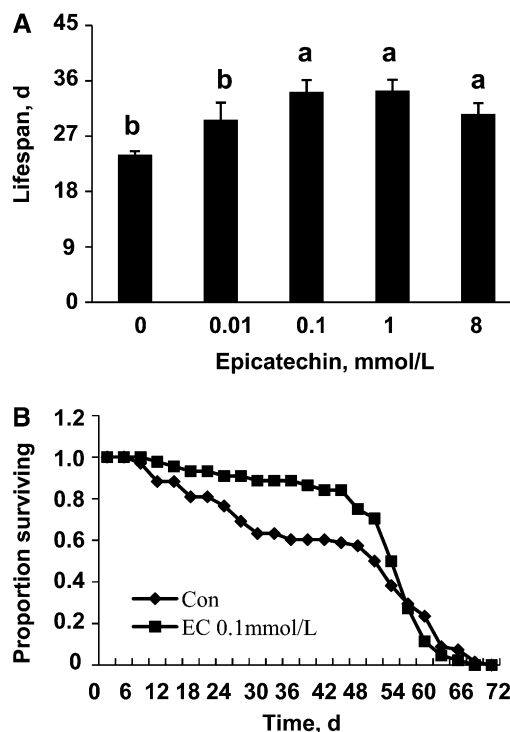


FIGURE 4 Mean lifespan (A) and up-shifts survival curve (B) of *Drosophila* treated with epicatechin. Values are means \pm SEM from 3 independent experiments, $n = 3$, with 40 fruit flies/treatment each. Columns without a common letter differ, $P < 0.05$.

mice without alteration in hepatic IGF-1 mRNA expression (data not shown). One possible interpretation is that epicatechin may affect the binding of IGF-1 and its carrier IGF binding proteins and therefore extend its half-life (39). Another possibility is that epicatechin reduces the IGF-1 level through activation of AMPK, which inhibits IGF-1 signaling and its protein synthesis through the phosphorylation of insulin receptor substrate-1 (40). Nevertheless, this result further suggests that epicatechin may be a food-derived, antiaging compound given the important role of IGF-1 in regulating the lifespan of organisms. Indeed, we confirmed this possibility by showing that epicatechin treatment significantly extended the mean lifespan of *Drosophila*, one of the widely used model organisms for longevity research. However, it is still unknown whether epicatechin extends lifespan through the same mechanisms in fruit flies and mice, although AMPK is highly conserved from yeast to all animal species (29).

Although our finding that epicatechin improved the lifespan of *db/db* mice is striking, it should be noted that there are limitations on the use of this animal model for longevity studies, because *db/db* mice have a much shorter lifespan than that of normal mice due to the development of obesity and hyperglycemia at a relatively young age, conditions that trigger various complications. To address this issue, a follow-up study using middle-aged normal mice is currently planned to confirm the antiaging effect of epicatechin.

In summary, the findings in this study demonstrate that epicatechin may be an antiaging compound, as evidenced by the improved *db/db* mouse survival and the favorable changes in a variety of age-related biomarkers. However, more preclinical studies are needed to further characterize the potential antiaging effects of this compound and to define the exact molecular mechanism(s) by which it may act.

Acknowledgments

D.L., H.S., and Z.F. designed research; H.S., Z.F., Z.J., P.V.A.B., T.L., M.P.M., K.A.V., and W.Z. conducted research; H.S., D.L., and R.W.G. analyzed data and wrote the paper; and D.L. and H.S. had primary responsibility for final content. All authors read and approved the final manuscript.

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