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Dietary Supplementation with (−)-Epigallocatechin-3-gallate Reduces Inflammatory Response in Adipose Tissue of Non-obese Type 2 Diabetic Goto-Kakizaki (GK) Rats

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ABSTRACT: (−)-Epigallocatechin gallate (EGCG), a major catechin in green tea, is an antioxidant associated with the reduction of oxidative stress in vitro. However, the mechanisms underlying the effects of EGCG on adipose tissue-related metabolic disturbances in vivo are not understood. This study examined whether dietary supplementation of EGCG reduces the oxidative stress-associated inflammatory response in the mesenteric adipose tissue of non-obese type 2 diabetic Goto-Kakizaki (GK) rats. GK rats were fed a normal diet or diet containing 0.1, 0.2, or 0.5% EGCG (w/w) for 25 weeks. The mRNA levels of IL-1β were significantly reduced in GK rats given 0.1% EGCG (0.059 ± 0.008; means ± SEM in arbitrary unit) compared with those in GK rats given a control diet (0.135 ± 0.011), but not in those given 0.2% EGCG (0.123 ± 0.012) or 0.5% EGCG (0.112 ± 0.019). The mRNA and protein level of other genes for inflammatory responses such as IL-18, TNF-α, MCP-1, CD11s, CD18, and resistin were also significantly reduced in rats given 0.1% EGCG, but not in those given ≥0.2% EGCG. This suggests that supplementation with EGCG at relatively low concentrations (0.1%) in GK rats reduces expression of genes and proteins involved in inflammation in adipose tissue.

KEYWORDS: *EGCG, adipose tissue, chemokine, inflammatory cytokine, oxidative stress*

INTRODUCTION

Recent studies have shown that intermittent rises in blood glucose cause an acute immune activation with elevated serum concentrations of inflammatory cytokines and tumor necrosis factor (TNF)-α in subjects with impaired glucose tolerance, which suggests a link between postprandial hyperglycemia and the development of type 2 diabetes mellitus (T2DM) and related vascular complications.[1] It is well-known that obesity is a major factor associated with insulin resistance and onset of T2DM, and weight reduction through lifestyle intervention effectively reduces the incidence of T2DM in persons with elevated fasting glucose and impaired glucose tolerance.[2] An increased macrophage infiltration in the adipose tissue of obese subjects is believed to be the predominant factor affecting insulin resistance and the onset of T2DM.[3] However, diabetic patients are not always overweight, especially in Asia, where the prevalence of obesity is much lower than in Western countries. Thus, in addition to obesity, other factors such as immune response should be considered for the risk of onset of T2DM and development of its complications.[4]

Recently, we have demonstrated that hyperglycemia induces elevated mRNA levels of inflammatory cytokines such as interleukin (IL)-1β, TNF-α, and integrins such as CD11b, CD11c, and CD18 in peripheral leukocytes.[5−7] These inflammatory cytokines and integrins are associated with the risk of development of T2DM and related complications including cardiovascular disease (CVD). Previous studies have demonstrated that chronic hyperglycemia induces oxidative stress and the production of inflammatory cytokines.[8] Thus, reduction of oxidative stress and associated inflammation is important for preventing diabetic complications including CVD.

Experimental and epidemiological studies have reported that (−)-epigallocatechin gallate (EGCG), one of the major catechins in green tea and an antioxidant product, can reduce the risk of chronic diseases such as CVD[9] and obesity.[10,11] Moreover, the consumption of green tea extracts or EGCG was shown to help blood glucose control in obese and type 2 diabetic humans[11,12] and rodents.[12] Moreover, EGCG or green tea extract exerts potent obesity preventive effects in mouse and rat models of diet-induced obesity.[13,14] These effects are at least in part mediated by a direct impact of EGCG on the expression of several lipogenic genes in the adipose tissue.[14] In a previous study, Shimada et al. found that a diet containing 0.2% EGCG led to increased plasma adiponectin levels even in non-obese diabetic Goto-Kakizaki (GK) rats, a model of type 2 diabetes rats with insulin secretory dysfunction.[15] This suggested that EGCG ameliorates insulin resistance in the adipose tissue of non-obese diabetic animals as well as in obese ones. However, it was unclear whether EGCG ameliorates the inflammatory response in adipose tissue in the diabetic condition.

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Table 1. Composition of Experimental Diets

ingredient	control diet g/kg	EGCG diet 0.1% g/kg	0.2% g/kg	0.5% g/kg	kcal/kg	energy %
casein	200	200	200	200	800	16.5
sucrose	250	250	250	250	1000	20.6
α-cornstarch	199.5	199.5	199.5	199.5	798	16.5
corn oil	50	50	50	50	450	9.3
lard	200	200	200	200	1800	37.1
AIN-93 vitamin mix[a]	35	35	35	35		
AIN-93 mineral mix[a]	10	10	10	10		
choline bitartrate	2.5	2.5	2.5	2.5		
L-cystine	3	3	3	3		
cellulose	50	49	48	45		
EGCG[b]	0	1	2	5		
total	1000	1000	1000	1000	4848	100

[a]AIN-93 vitamin mix and AIN-93G mineral mix (*J. Nutr.* **1993**, *123*, 1939−1951) were prepared by Oriental Co., Ltd. (Tokyo, Japan). [b]Sunphenon EGCg (Taiyo Kagaku, Mie, Japan) is extracted from green tea leaves and contains >90% EGCG, <5% other catechins, and <0.1% caffeine.

In this study, we examined whether supplementation with EGCG in the diet affects the expression of inflammation-related genes in the adipose tissue of GK rats.

MATERIALS AND METHODS

Chemicals. The EGCG preparation, Sunphenon EGCg (Taiyo Kagaku, Mie, Japan), is made by extracting tea leaves with hot water and subsequently purified to consist of >90% EGCG, <5% other catechins, and <0.1% caffeine. Theaflavin is not detected in the EGCG preparation. All other chemicals are of reagent grade and purchased from Wako Pure Chemical Industries, Inc. (Osaka, Japan), unless otherwise stated.

Animals. Male 4-week-old GK rats and age-matched male Wistar rats were purchased from Japan SLC Inc. (Hamamatsu, Japan). All animals were housed at 22 ± 3 °C under 12 h light−12 h dark cycles and allowed free access to a standard laboratory chow (CE-2; CLEA, Inc., Tokyo, Japan). At 9 weeks of age, GK rats were assigned to one of four groups (eight animals per group) with similar fasting serum glucose levels (309 ± 3 mg/dL, mean ± SEM) and body weights (387 ± 8 g), and they were fed either a control diet or diets containing 0.1, 0.2, or 0.5% (w/w) EGCG preparation for 25 weeks (Table 1). An oral glucose tolerance test (OGTT) was performed at 24 weeks after feeding the test diets. Rats were fasted overnight (8:00 p.m.−10:00 a.m.) and administered a single dose of glucose. The 20% glucose solution was administered from 10:00−10:30 a.m. using a gastric tube at a volume of 1 mL/kg (2 g/kg body weight). Blood samples for glucose and insulin measurements were obtained from the tail vein 0.25, 0.5, 1, 2, and 3 h after glucose loading. Wistar rats were fed the control diet for 25 weeks. Rats were killed at 25 weeks after feeding the test diets without fasting by decapitation, and whole blood was collected for glycated hemoglobin A1c (HbA1c) determination. Mesenteric adipose tissue was dissected, immediately frozen in liquid nitrogen, and stored at −80 °C until use. The experimental procedures used in the present study conformed to the guidelines of the Animal Usage Committee of the University of Shizuoka.

RNA Extraction. Total RNA was extracted from frozen mesenteric adipose tissue (70−100 mg) using RNeasy (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Total RNA concentration was quantified by a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). The total RNA samples were stored at −80 °C for subsequent quantitative RT-PCR analyses.

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA samples (300 ng) were converted to cDNA by reverse transcription using SuperScript III RT (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. To quantitatively estimate the mRNA levels of selected genes, PCR amplification was performed on a Light-Cycler instrument (Roche Molecular Biochemicals, Tokyo, Japan) using SYBER Green Master I (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's protocol. The PCR primer sequences are listed in Table 2. The cycle threshold (CT) values for each gene and hypoxanthine guanine phosphoribosyl transferase (HPRT) detected by real-time RT-PCR were converted to signal intensities by the delta−delta method.[16] The delta−delta method calculates the difference of one CT value as a 2-fold difference between each signal for the gene and the signal for a gene for normalization (HPRT). The formula is $[2^{(\text{CT of HPRT}-\text{CT of test gene})}]$.

Western Blot Analysis. Total proteins were extracted from the adipose tissue with RIPA buffer (1% NP-40, 0.1% SDS, 0.1% sodium deoxycholate, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, protease inhibitor tablet (Roche Molecular Biochemicals)/10 mL, phosphatase inhibitors (1 mM $NaMoO_4$, 50 mM NaF, 1 mM Na_3VO_4)). Lysates were centrifuged at 10000*g* for 20 min at room temperature. The soluble supernatants were normalized for their total protein concentrations using the Lowry method.[17] Aliquots (10 μg per lane) were subjected to 12 or 15% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). The proteins developed by SDS-PAGE were electrotransferred to Immobilon transfer membranes (PVDF) (Millipore, Bedford, MA, USA) at 80 V for 120 min in Tris/glycine/methanol transfer buffer. The membranes were blocked with 3% nonfat milk (Megmilk Snow Brand, Tokyo, Japan) in phosphate-buffered saline containing 0.1% Tween 20 (PBS-Tween) for 1 h at room temperature. Membranes were then incubated with primary monoclonal antibodies against α-tubulin (Sigma, St. Louis, MO, USA), MCP-1 (Millipore, Tokyo, Japan), IL-18 (Medical and Biological Laboratories, Nagoya, Japan), IL-1β (Santa Cruz, Heidelberg, Germany), CD11c (Abcam, Tokyo, Japan), CD18 (Santa Cruz), or resistin (Millipore, Tokyo, Japan) for 16 h at 4 °C. After five washings with PBS-Tween, the membranes were incubated with anti-rabbit or anti-mouse IgG conjugated with biotin in 3% nonfat milk in PBS-Tween. The membranes were washed with PBS-Tween five times and then incubated with horseradish peroxidase conjugated with avidin for 16 h at 4 °C. After five washings with PBS-Tween, the membranes were exposed to chemiluminescence ECL Pro (Perkin-Elmer, Boston, MA, USA). Protein bands were quantified by Multi Gauge version 3.0 Densitograph software (Fuji Film, Tokyo, Japan) and normalized to the α-tubulin signal as an internal control.

Other Assays. Blood was collected from the tail tips of rats after 8 h of fasting before and 23 weeks after starting the experimental diets. Serum glucose concentration was measured by a commercial kit (Glucose C II-test Wako, Wako Pure Chemical Industries). HbA1c in the whole blood was measured by HPLC using a TSKgel Boronate-5PW column system. Insulin concentration was measured by REBISU insulin rat ELISA kits (Shibayagi Co., Ltd., Gunma, Japan).

Table 2. Sequences of Oligonucleotide Primers Used for RT-PCR

gene	sequence
TNF-α	5′-AGCATGATCCGAGATGTGGAA-3′ 5′-AATGAGAAGAGGCTGAGGCACA-3′
IL-1β	5′-AAAATGCCTCGTGCTGTCTGA-3′ 5′-CAGGGATTTTGTCGTTGCTTG-3′
IL-6	5′-CAGCGATGATGCACTGTCAGAA-3′ 5′-TCCAGGTAGAAACGGAACTCCA-3′
IL-12p35	5′-TGAGGACGGCCAGAGAAAAAT-3′ 5′-AGGCACAGGGTCATCATCAAA-3′
IL-12p40	5′-CCTGCAGGTGAAACCTTTGAA-3′ 5′-CAGATATTCGCCCCTTTGCAT-3′
IL-18	5′-CAGACCACTTTGGCAGACTTCA-3′ 5′-ACACAGGCGGGTTTCTTTTGT-3′
MCP-1	5′-CAGAAACCAGCCAACTCTCA-3′ 5′-GTGGGGCATTAACTGCATCT-3′
CD11a	5′-TCAGTTCCCAGTCAAGTCCAGC-3′ 5′-AGCTCCTACAACCACATCTGC-3′
CD11b	5′-ACTCTGATGCCTCCCTTGG-3′ 5′-CCTGGACACGTTGTTCTCAC-3′
CD11c	5′-AAGCCCAAGTGTTCCTTCG-3′ 5′-AATGACGTGTCGGCTGCT-3′
CD18	5′-GGGCTGCTGGATAACCTACAT-3′ 5′-AATGACGTGTCGGCTGCT-3′
PAI-1	5′-TGCTGGTGAATGCCCTCTACT-3 5′-CGGTCATTCCCAGGT TCTCTA-3′
resistin	5′-ATCAAGACTTCAGCTCCCTACTG-3′ 5′-GTGACGGTTGTGCCTTCTGTCA-3′
HPRT	5′-AGTCAACGGGGGACATAAAAG-3′ 5′-CTTCAACAATCAAGACGTTCTTTTC-3′

Statistical Analyses. Results were expressed as means ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance and Tukey's multiple-range tests or Student's *t* tests. Differences were considered significant at $p < 0.05$. All statistical analyses were performed using Excel Statistics software version 2007 for Windows.

■ RESULTS

Effects of EGCG on Body Weight and Adipose Tissue Weight. GK rats were fed a control diet or diet containing 0.1, 0.2, or 0.5% EGCG for 25 weeks. The body weight and average food intake during the experimental period did not differ among the GK rat groups, whereas food intake of GK rats fed the control diet or the diets supplemented with EGCG was significantly greater than that of Wistar control rats. The mesenteric adipose tissue weight of GK control rats was significantly lower ($P < 0.05$) than that of Wistar control rats, and the mesenteric adipose tissue weight did not differ among the GK rat groups (Table 3).

Effects of EGCG on Blood Glucose Parameters. HbA1c concentrations were higher in GK control rats than in Wistar control rats. Fasting serum glucose concentration was significantly higher ($P < 0.05$) in GK control rats than in Wistar control rats. However, the serum glucose concentration in GK rats fed a diet containing 0.1% EGCG was lower than that in GK control rats, and it was not significantly different from that in Wistar control rats. In GK rats fed diets containing 0.2 or 0.5% EGCG, serum glucose concentrations were similar to those in GK control rats and significantly higher than in Wistar control rats. HbA1c levels in the blood were significantly higher in all GK rat groups than in Wistar control rats, and they did not differ among the GK rat groups. Glucose concentrations at 0, 60, and 120 min after oral glucose loading in the OGTT were higher in GK rats than in Wistar control rats. Insulin concentrations at 0, 30, and 60 min after oral glucose loading in the OGTT were lower in GK rats than in Wistar control rats. However, glucose and insulin concentrations were not different among GK rats (Table 3).

Effects of EGCG on mRNA Levels of Inflammation Genes in the Adipose Tissue of GK Rats. In GK control rats, the mRNA levels of IL-1β, TNF-α, IL-6, and MCP-1 were significantly higher than in Wistar control rats. Supplementation of the diet with 0.1% EGCG led to significantly lower mRNA levels of IL-1β, TNF-α, IL-6, and MCP-1 (by 68, 71, 78, and 57%, respectively) in the mesenteric adipose tissue, compared with control GK rats. In GK rats fed diets containing 0.2 or 0.5% EGCG, mRNA levels of IL-1β, TNF-α, IL-6, and MCP-1 were similar to those in control GK rats. However, the MCP-1 mRNA levels in GK rats fed a diet containing 0.2% EGCG were significantly lower than those in GK control rats (Figure 1).

In GK control rats, the mRNA levels of IL-12p35, IL-12p40, and IL-18 were higher than those in Wistar control rats. Supplementation of the diet with 0.1% EGCG led to significantly reduced mRNA levels of IL-12p40 (by 74%) and IL-18 (by 49%). In GK rats fed diets containing 0.2 or 0.5% EGCG, mRNA levels of IL-12p35, IL-12p40, and IL-18 did not differ from those in control GK rats. The mRNA levels of CD11a, CD11b, and CD11c were significantly higher in GK control rats than in Wistar control rats. Similarly, mRNA levels of CD18 in GK control rats tended to be higher than those in Wistar control rats. Supplementation of the diet with 0.1% EGCG led to significantly lower mRNA levels of CD11a, CD11b, and CD11c (by 49, 46, and 57%, respectively) in mesenteric adipose tissue, compared with control GK rats. In GK rats fed diets containing 0.2 or 0.5% EGCG, mRNA levels of CD11a, CD11b, and CD11c were similar to those in control GK rats. Additionally, mRNA levels of resistin and PAI-1 were significantly higher in GK control rats than in Wistar control rats. Supplementation of diets with 0.1% EGCG led to significantly lower resistin and PAI-1 mRNA levels (by 62 and 74%, respectively) in mesenteric adipose tissue of GK rats (Figure 1).

Effects of EGCG on Inflammatory Protein Levels in the Adipose Tissue of GK Rats. In GK control rats, the protein levels of the active form of IL-1β, the active form of IL-18, MCP-1, CD11c, CD18, and resistin in mesenteric adipose tissue were significantly higher than in Wistar control rats. Supplementation of the diet with 0.1% EGCG led to significantly reduced protein levels of the active form of IL-

Table 3. Effects of EGCG on Body Weight, Food Intake, Adipose Tissue Weight, and Blood Glucose Parameters of Rats Fed a Control Diet and Those Fed Diets Containing 0.1, 0.2, or 0.5% EGCG for 25 Weeks[a]

	Wistar	GK			
	control	EGCG 0%	EGCG 0.1%	EGCG 0.2%	EGCG 0.5%
body wt (g)					
initial	260 ± 10	298 ± 4	280 ± 5	290 ± 7	287 ± 9
final	449 ± 9	449 ± 6	458 ± 14	437 ± 13	442 ± 11
weight gain (g/25 weeks)	198 ± 8a	162 ± 12ab	188 ± 11ab	155 ± 13b	160 ± 8ab
av food intake (g/day)	14.0 ± 0.8a	17.2 ± 0.4b	16.6 ± 0.4b	16.3 ± 0.3b	17.2 ± 0.3b
mesenteric adipose tissue (g/kg BW)	28.3 ± 0.2a	17.7 ± 0.2b	17.5 ± 0.2b	17.8 ± 0.1b	16.1 ± 0.2b
glucose (mg/dL) (8 h fasted)[b]	206 ± 12a	356 ± 37b	296 ± 19b	328 ± 33b	344 ± 24b
glycated hemoglobin A1c (HbA1c: %)[c]	4.7 ± 0.1a	6.7 ± 0.3b	6.2 ± 0.3b	6.4 ± 0.3b	6.7 ± 0.3b
oral glucose tolerance test (OGTT)[d]					
fasting glucose (14 h fasted) (mg/dL)	110 ± 3.3a	213 ± 6.7b	207 ± 6.6b	210 ± 11.8b	218 ± 8.0b
60-min glucose after OGTT (mg/dL)	198 ± 11a	546 ± 12b	528 ± 21b	554 ± 23b	556 ± 18b
120-min glucose after OGTT (mg/dL)	173 ± 4.2a	425 ± 17b	432 ± 27b	447 ± 24b	450 ± 21b
fasting insulin (ng/dL)	1.1 ± 0.3	0.9 ± 0.2	1.0 ± 0.2	0.9 ± 0.1	0.5 ± 0.1
30-min insulin after OGTT (mg/dL)	2.5 ± 0.3a	1.3 ± 0.2b	1.3 ± 0.1b	1.4 ± 0.2b	0.8 ± 0.2b
60-min insulin after OGTT (mg/dL)	2.3 ± 0.2a	1.3 ± 0.3b	1.4 ± 0.2b	1.1 ± 0.2b	0.8 ± 0.1b

[a]At the end of the experiment, body weight, adipose tissue weight, and HbA1c were measured without fasting, and serum glucose levels were measured after 8 h of fasting. Values are means ± SEM for eight rats per group. Values not sharing a common letter (a, b) in the same row are significantly different from one another at $P < 0.05$ (Tukey–Kramer multiple-comparison test). [b]Fasting glucose concentration at 8 h was measured at 23 weeks after feeding the test diets. [c]HbA1c (%): National Glycohemoglobin Standardization Program (NGSP). [d]OGTT were performed at 24 weeks after feeding the test diets.

1*β*, the active form of IL-18, MCP-1, CD11c, and resistin in mesenteric adipose tissue (by 33, 69, 92, 84, and 44%, respectively). CD18 protein levels were not altered. In GK rats fed a diet containing 0.5% EGCG, protein levels of the active form of IL-1*β*, the active form of IL-18, MCP-1, CD11c, and resistin were similar to those in control GK rats, except for CD18 protein levels (Figure 2).

DISCUSSION

Recent studies have suggested that inflamed adipose tissue in obese animals is associated with the development of metabolic abnormalities such as insulin resistance.[18] The development of these metabolic abnormalities is positively associated with secretion of cytokines from visceral adipose tissue.[19] These results indicate that the inflammation of adipose tissue is related to the development of these metabolic abnormalities. However, it is still unknown whether expression of inflammation genes such as cytokines is reduced by EGCG supplementation in diabetic animals.

In this study, we found that intake of dietary EGCG at a relatively low concentration (0.1%) suppresses the enhanced inflammatory cytokine production induced by hyperglycemia in non-obese animals with insulin secretory dysfunction. However, these results were not found or were less significant at higher concentrations (0.2 and 0.5%). These findings suggest that dietary EGCG opposes the development of hyperglycemia-induced inflammatory responses in adipose tissue even in non-obese animals. GK rats had lower adipose tissue weight compared with normal Wistar rats. Supplementation of the diet with EGCG in GK rats did not affect the adipose tissue weight. Nevertheless, the mRNA levels of IL-1*β*, TNF-*α*, and IL-6 were reduced in the mesenteric adipose tissue of GK rats fed the diet containing 0.1% EGCG. IL-1*β* and TNF-*α* are cytokines produced mainly by inflammatory cells, for example, monocytes, macrophages, and neutrophils. Cytokines play a very important role in the pathophysiology of atherosclerosis.[20] The expression of IL-1*β* in adipose tissue is indicative of acute inflammation.[21] In addition, TNF-*α* and IL-6 are known to be expressed from adipose tissue itself. Overproduction of TNF-*α* from visceral adipose tissue, which usually occurs in adipose tissue with excessive fat, is induced by infiltrating macrophages and is closely related to insulin resistance.[22] Thus, the results of this study suggest that acute inflammation is present in the adipose tissue of GK rats, presumably because of hyperglycemia, and that the acute inflammation is alleviated by EGCG supplementation.

In this study, we have shown that the mRNA levels of MCP-1 and macrophage activation markers, CD11s, are reduced in GK rats fed a diet containing EGCG at a concentration of 0.1%. However, mRNA levels were reduced less or not at all at concentrations of 0.2 and 0.5% EGCG (Figure 2). MCP-1 is a key mediator of monocyte trafficking and, therefore, plays a major role in chronic inflammation in adipose tissue. One change in adipose tissue during inflammation is an increase in the percentage of macrophages within the tissue. Macrophages are a primary source of inflammatory cytokines secreted by adipose tissue. The primary adipokine responsible for this infiltration is MCP-1. As the level of macrophages increases in adipose tissue, the level of inflammatory cytokines secreted by the tissue increases. Our results suggest that the levels of both MCP-1 and CD11c increase as adipose tissue is exposed to hyperglycemia-induced oxidative stress, and these changes are directly associated with insulin resistance and development of T2DM.

In this study, we have demonstrated that resistin and PAI-1 mRNA levels were significantly reduced in the mesenteric adipose tissue of rats fed a diet supplemented with EGCG at a concentration of 0.1%. However, mRNA levels were reduced less or not at all at concentrations of 0.2 and 0.5% EGCG. Previous studies suggested that resistin links insulin resistance and adipogenesis, inflammation, and CVD.[23] PAI-1 has been shown to be a regulator of the endogenous fibrinolytic systems,[24] and it is a potent risk factor for CVD in humans.[25] The results of this study suggest that in inflamed adipose tissue,

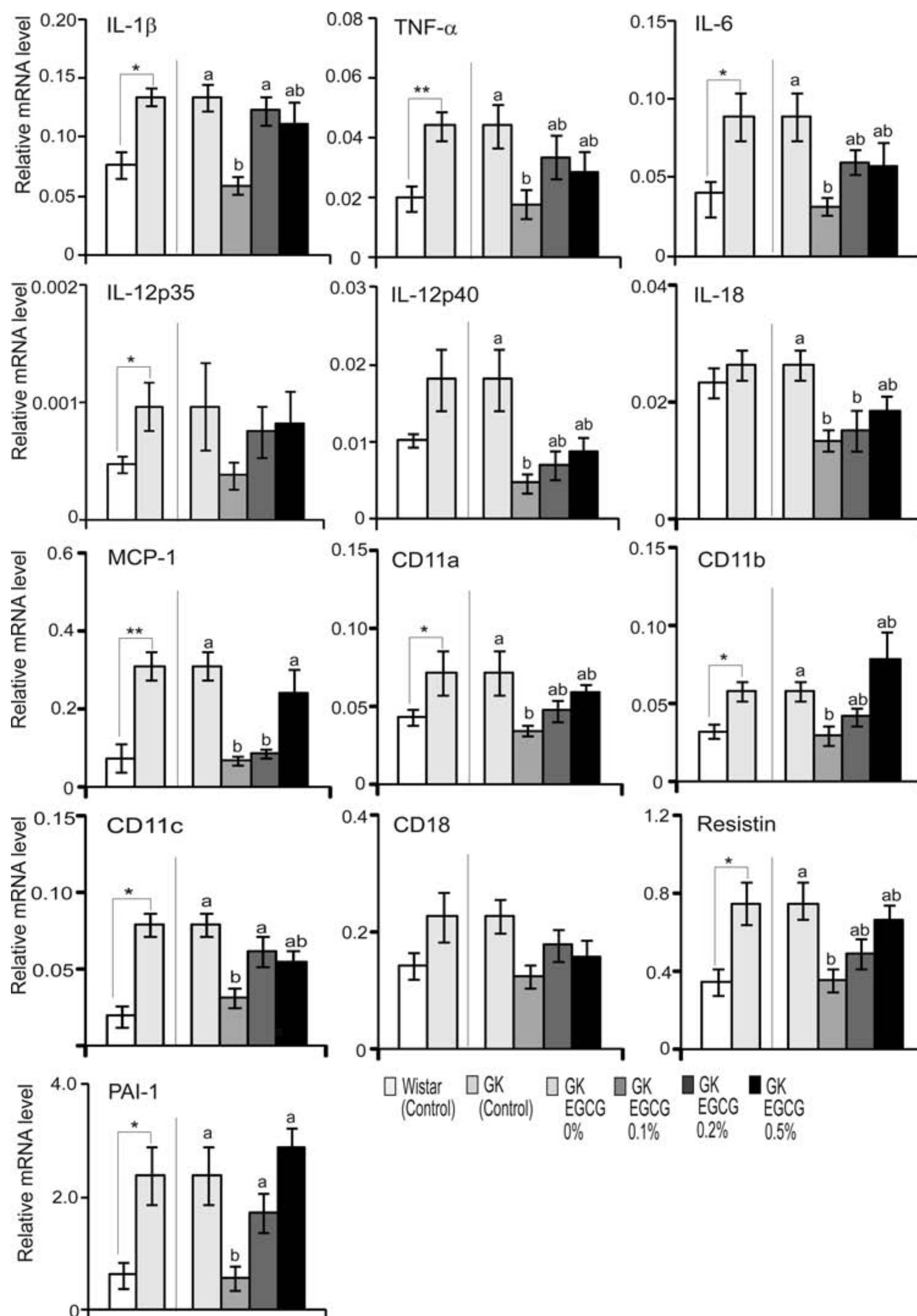

Figure 1. Quantitative RT-PCR analysis of inflammation genes in mesenteric adipose tissue of GK rats fed a control diet or diets containing EGCG (0.1, 0.2, 0.5%) for 25 weeks. The results for each sample were normalized for HPRT mRNA abundance and are expressed as arbitrary units, representing the mean values in mesenteric adipose tissue of GK rats. Values are means ± SEM for six to eight animals per group. Values not sharing a common letter (a, b) are significantly different from one another by Tukey's multiple-range test at $p < 0.05$. (*, **) Asterisks indicate a significant difference between Wistar control rats and GK control rats by Student's t test at $P < 0.05$ and $P < 0.01$, respectively. TNF-α, tumor necrosis factor α; MCP-1, monocyte chemoattractant peptide-1; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-12p35, interleukin-12 subunit p35; IL-12p40, interleukin-12 subunit p40; IL-18, interleukin-18; CD11a, integrin α-L (LFA-1α); CD11b, integrin α-M (MAC-1α); CD11c, integrin α-X (p150, CR4); CD18, integrin β-2 (CTB104); PAI-1, plasminogen activator inhibitor-1.

even if excessive fat is not accumulated, typical adipocytokines such as PAI-1 and resistin are largely expressed.

Among these genes, we found that the protein levels of typical inflammatory cytokines secreted from monocytes and macrophages (IL-1β and IL-18), a chemokine for inducing infiltration of these leukocytes into adipose tissue (MCP-1), proteins expressed in surface of the activated macrophages (CD11c and CD18), and an adipocytokine to induce insulin resistance (resistin) were reduced by supplementation with EGCG concentration at 0.1%, but less so or not at all at 0.2 and 0.5%, except of CD18. These results indicate that supplementation with EGCG at a concentration of 0.1%, but not 0.2 or 0.5%, can reduce protein and mRNA expression of inflammation gene cascades in adipose tissue in GK rats. It should be

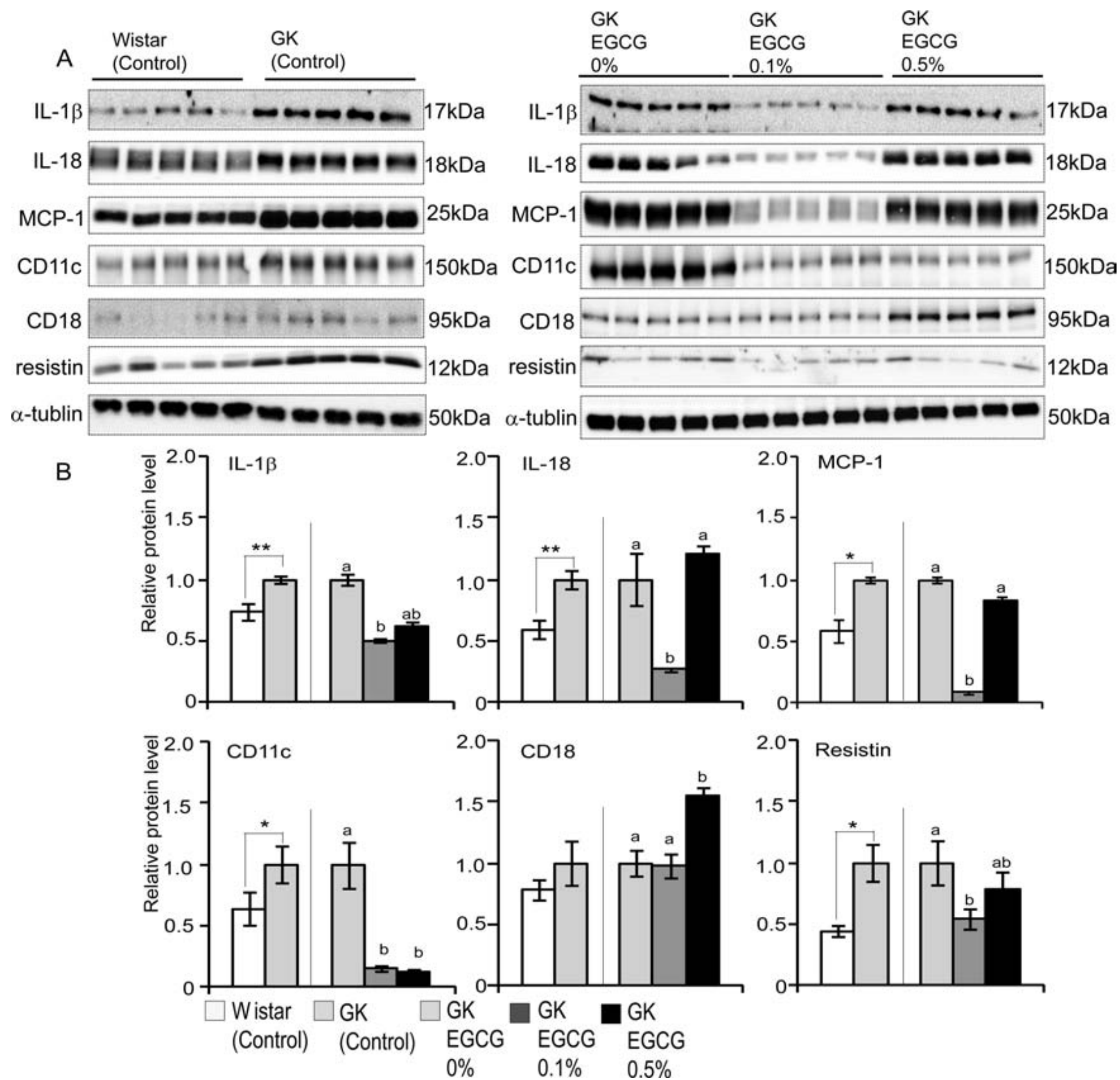

Figure 2. Western blot analysis of inflammation protein levels in mesenteric adipose tissue of GK rats fed a control diet or diets containing 0.1 or 0.5% EGCG. (A) Protein levels were analyzed by Western blot. (B) The results for each sample were normalized for α-tubulin protein abundance and are expressed in arbitrary units, representing the mean value in mesenteric adipose tissue of control GK rats as 1.0. Values are means ± SEM for five animals per group. Values not sharing a common letter (a, b) are significantly different from one another by Tukey's multiple-range test at $P < 0.05$. (∗, ∗∗) Asterisks indicate significant differences between Wistar control rats and GK control rats by Student's *t* test at $P < 0.05$ and $P < 0.01$, respectively. IL-1*β*, interleukin-1*β*; IL-18, interleukin-18; MCP-1, monocyte chemoattractant peptide-1; CD11c, integrin *α*-X (p150, CR4); CD18, integrin *β*-2 (CTB104); PAI-1, plasminogen activator inhibitor-1.

examined in further works whether protein levels of other genes such as TNF-*α*, IL-6, IL-12p40, CD11a, CD11b, and PAI-1 in adipose tissue are reduced by supplementation with EGCG at a concentration of 0.1%, but not at 0.2 or 0.5%. Supplementation with 0.5% EGCG induced changes in CD18 protein levels, but not the mRNA level. A previous study has shown that CD18 protein expression is maintained by one of the proteases, cathepsin B.[26] Thus, expression of CD18 protein in adipose tissue of GK rats can be induced by 0.5% EGCG supplementation by inhibiting protein degradation. Whether supplementation with 0.5% EGCG inhibits protein degradation of CD18 in adipose tissue of GK rats should be examined.

The molecular mechanism by which EGCG alters inflammation genes in adipose tissue is still unknown. Chronic hyperglycemia induces reactive oxygen species (ROS) production through enhancing glucose autoxidation, oxidative phosphorylation in mitochondria, and ROS producing enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, cytochrome p450, lipoxygenases, xanthine oxidase, and nitric oxide synthase.[27,28] ROS production induces inflammatory cytokine gene expression by activating a transcriptional factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-*κ*B).[29] Thus, supplementation with EGCG at a concentration of 0.1%, but not 0.2 or 0.5%, could reduce ROS. Moreover, EGCG eliminates ROS in vitro.[30] In vitro studies have suggested that green tea polyphenols inhibit LPS-mediated increases in TNF-*α* mRNA levels by blocking NF-*κ*B activation in macrophage RAW264.7 cells.[31] In addition, animal and human studies have demonstrated that consuming EGCG or green tea extract reduces 8-hydroxydeoxyguanosine (OHdG), which is a product of DNA oxidation by ROS.[32,33] Supplementation with 0.1% EGCG may reduce the activation of NF-*κ*B in adipose tissue of GK rats. In addition, it has been reported in animal studies that a higher intake of EGCG causes oxidative DNA damage in the hamster pancreas and liver.[34] Furthermore, a higher consumption of green tea or catechins,

in particular by supplementation, can exert liver injury.[35] These results indicate that a higher intake of EGCG can induce oxidative stress. EGCG metabolites may induce oxidative stress in adipose tissue as well as in other tissues. Therefore, whether intake of EGCG at a relatively low concentration (0.1%) or at a higher concentration (0.2−0.5% supplementation) alters NF-κB activity in adipose tissue of GK rats needs to be examined. In addition, it should be studied whether EGCG metabolites such as oxidized EGCG or EGCG itself are different from the serum to the adipose tissue of GK rats fed diets containing EGCG at concentrations of 0.1, 0.2, and 0.5%.

To extrapolate our results to humans, the doses used in this study should be mentioned. From the average daily food intake of the GK rat (17 g), the GK rats fed a diet containing 0.1% EGCG consumed 17 mg of EGCG, which is equivalent to 50 mg/kg for a rat of 340 g. Converting this dose from rats consuming 68 kJ/day to humans consuming 1120 kJ/day using an isocaloric calculation method,[36,37] we roughly estimate that the dose of 50 mg/kg for GK rats corresponds to 280 mg of EGCG per day consumed by a 70 kg person (4 mg/kg/day for humans). Similarly, consumption of a diet containing 0.5% EGCG for rats in this study is approximately comparable to daily consumption of 1400 mg in humans. Because a cup of green tea contains 150−180 mg of EGCG and commercially available EGCG supplements contain up to 350 mg EGCG/tablet, the low dose (0.1%) used for GK rats in the current study is achievable in humans consuming EGCG as a supplement or by drinking (2−3 cups) of green tea. Our results suggest that these doses may reduce inflammation in the adipose tissue and represent a practical range for human consumption of EGCG.

The substrate used as EGCG in this study is not completely purified (>90% EGCG). Using the same EGCG substrate, many studies have demonstrated effects of green tea catechins in animals and humans.[15,38,39] In addition, a previous study showed that the plasma concentration of EGCG in C57BL/10-*mdx* mice fed a diet containing 0.1% EGCG, which is the same product used in this study, for 5 weeks was 29.7 ± 3.2 ng/mL.[38] However, other catechins, tea flavins, and caffeine could be included in the EGCG substrate. Thus, further studies should examine whether supplementation with completely purified EGCG at a concentration of 0.1% reduces expression of inflammation-related genes in adipose tissue of GK rats.

In conclusion, we have demonstrated that dietary supplementation with EGCG at a relatively low concentration (0.1%), but not at higher concentrations, suppresses hyperglycemia-induced inflammatory responses by reducing the expression of chemokines and inflammatory cytokines such as TNF-α, MCP-1, IL-1β, and IL-18 in adipose tissue.

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Notes

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ABBREVIATIONS USED

CVD, cardiovascular disease; EGCG, (−)-epigallocatechin gallate; GK, Goto-Kakizaki; HbA1c, hemoglobin A1c; HPRT, hypoxanthine guanine phosphoribosyl transferase; IL, interleukin; MCP, monocyte chemotactic protein; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OGTT, oral glucose tolerance test; OHdG, hydroxydeoxyguanosine; PAI, plasminogen activator inhibitor; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; SEM, standard error of the mean; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; T2DM, type 2 diabetes mellitus

REFERENCES

(1) Esposito, K.; Nappo, F.; Marfella, R.; Giugliano, G.; Giugliano, F.; Ciotola, M.; Quagliaro, L.; Ceriello, A.; Giugliano, D. Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans: role of oxidative stress. *Circulation* **2002**, *106*, 2067−2072.

(2) Knowler, W. C.; Barrett-Connor, E.; Fowler, S. E.; Hamman, R. F.; Lachin, J. M.; Walker, E. A.; Nathan, D. M. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N. Engl. J. Med.* **2002**, *346*, 393−403.

(3) Surmi, B.; Hasty, A. Macrophage infiltration into adipose tissue: initiation, propagation and remodeling. *Future Lipidol.* **2008**, *3*, 545−556.

(4) Brunetti, P. The lean patient with type 2 diabetes: characteristics and therapy challenge. *Int. J. Clin. Pract. Suppl.* **2007**, *153*, 3−9.

(5) Mochizuki, K.; Shimada, M.; Tanaka, Y.; Fukaya, N.; Goda, T. Reduced expression of β2 integrin genes in rat peripheral leukocytes by inhibiting postprandial hyperglycemia. *Biosci., Biotechnol., Biochem.* **2010**, *74*, 2470−2474.

(6) Tanaka, Y.; Mochizuki, K.; Fukaya, N.; Shimada, M.; Goda, T. The α-glucosidase inhibitor miglitol suppresses postprandial hyperglycaemia and interleukin-1β and tumour necrosis factor-α gene expression in rat peripheral leucocytes induced by intermittent sucrose loading. *Br. J. Nutr.* **2009**, *102*, 221−225.

(7) Fukaya, N.; Mochizuki, K.; Shimada, M.; Goda, T. The α-glucosidase inhibitor miglitol decreases glucose fluctuations and gene expression of inflammatory cytokines induced by hyperglycemia in peripheral leukocytes. *Nutrition* **2009**, *25*, 657−667.

(8) Evans, J. L.; Goldfine, I. D.; Maddux, B. A.; Grodsky, G. M. Are oxidative stress-activated signaling pathways mediators of insulin resistance and beta-cell dysfunction? *Diabetes* **2003**, *52*, 1−8.

(9) Babu, P. V.; Liu, D. Green tea catechins and cardiovascular health: an update. *Curr. Med. Chem.* **2008**, *15*, 1840−1850.

(10) Moon, H. S.; Lee, H. G.; Choi, Y. J.; Kim, T. G.; Cho, C. S. Proposed mechanisms of (−)-epigallocatechin-3-gallate for anti-obesity. *Chem. Biol. Interact.* **2007**, *167*, 85−98.

(11) Wolfram, S. Effects of green tea and EGCG on cardiovascular and metabolic health. *J. Am. Coll. Nutr.* **2007**, *26*, 373S−388S.

(12) Wolfram, S.; Raederstorff, D.; Preller, M.; Wang, Y.; Teixeira, S. R.; Riegger, C.; Weber, P. Epigallocatechin gallate supplementation alleviates diabetes in rodents. *J. Nutr.* **2006**, *136*, 2512−2518.

(13) Ueda, M.; Ashida, H. Green tea prevents obesity by increasing expression of insulin-like growth factor binding protein-1 in adipose tissue of high-fat diet-fed mice. *J. Agric. Food Chem.* **2012**, *60*, 8917−8923.

(14) Wolfram, S.; Raederstorff, D.; Wang, Y.; Teixeira, S. R.; Elste, V.; Weber, P. TEAVIGO (epigallocatechin gallate) supplementation prevents obesity in rodents by reducing adipose tissue mass. *Ann. Nutr. Metab.* **2005**, *49*, 54−63.

(15) Goto, Y.; Suzuki, K.; Ono, T.; Sasaki, M.; Toyoda, T. Development of diabetes in the non-obese NIDDM rat (GK rat). *Adv. Exp. Med. Biol.* **1988**, *246*, 29−31.

(16) Livak, K.; Schmittgen, T. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−ΔΔ C(T)) method. *Methods* **2001**, *25*, 402−408.
(17) Lowry, O.; Rosebrough, N.; Farr, A.; Randall, R. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.
(18) Ikeoka, D.; Mader, J.; Pieber, T. Adipose tissue, inflammation and cardiovascular disease. *Rev. Assoc. Med. Bras.* **2010**, *56*, 116−121.
(19) Wang, Z.; Nakayama, T. Inflammation, a link between obesity and cardiovascular disease. *Mediators Inflamm.* **2010**, *2010*, 17.
(20) Dinarello, C. A.; Wolff, S. M. The role of interleukin-1 in disease. *N. Engl. J. Med.* **1993**, *328*, 106−113.
(21) Pachler, C.; Ikeoka, D.; Plank, J.; Weinhandl, H.; Suppan, M.; Mader, J.; Bodenlenz, M.; Regitting, W.; Mangge, H.; Pieber, T.; Ellmerer, M. Subcutaneous adipose tissue exerts proinflammatory cytokines after minimal trauma in humans. *Am. J. Physiol. Endocrinol. Metab.* **2007**, *293*, E690−E696.
(22) Uysal, K. T.; Wiesbrock, S. M.; Marino, M. W.; Hotamisligil, G. S. Protection from obesity-induced insulin resistance in mice lacking TNF-α function. *Nature* **1997**, *389*, 610−614.
(23) Salah, N.; Miller, N.; Paganga, G.; Tijburg, L.; Bolwell, G.; Rice-Evans, C. Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Arch. Biochem. Biophys.* **1995**, *322*, 339−346.
(24) Juhan-Vague, I.; Vague, P. Hypofibrinolysis and insulin-resistance. *Diabetes Metab.* **1991**, *17*, 96−100.
(25) Chen, C.; Jiang, J.; Lü, J.; Chai, H.; Wang, X.; Lin, P.; Yao, Q. Resistin decreases expression of endothelial nitric oxide synthase through oxidative stress in human coronary artery endothelial cells. *Am. J. Physiol. Heart Circ. Physiol.* **2010**, *299*, H193−H201.
(26) Fukuda, S.; Schmid-Schonbein, G. W. Regulation of CD18 expression on neutrophils in response to fluid shear stress. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 13152−13157.
(27) Ceriello, A. New insights on oxidative stress and diabetic complications may lead to a "causal" antioxidant therapy. *Diabetes Care* **2003**, *26*, 1589−1596.
(28) Niedowicz, D. M.; Daleke, D. L. The role of oxidative stress in diabetic complications. *Cell. Biochem. Biophys.* **2005**, *43*, 289−330.
(29) Ginn-Pease, M. E.; Whisler, R. L. Redox signals and NF-κB activation in T cells. *Free Radical Biol. Med.* **1998**, *25*, 346−361.
(30) Rice-Evans, C.; Miller, N.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−956.
(31) Lin, Y. L.; Lin, J. K. (−)-Epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down-regulating lipopolysaccharide-induced activity of transcription factor nuclear factor-κB. *Mol. Pharmacol.* **1997**, *52* (3), 465−472.
(32) Oyama, J.; Maeda, T.; Sasaki, M.; Kozuma, K.; Ochiai, R.; Tokimitsu, I.; Taguchi, S.; Higuchi, Y.; Makino, N. Green tea catechins improve human forearm vascular function and have potent anti-inflammatory and anti-apoptotic effects in smokers. *Intern. Med.* **2010**, *49*, 2553−2559.
(33) Frei, B.; Higdon, J. V. Antioxidant activity of tea polyphenols in vivo: evidence from animal studies. *J. Nutr.* **2003**, *133*, 3275S−3284S.
(34) Takabayashi, F.; Takabayashi, S.; Kaneko, T.; Harada, N. Effect of green tea catechins on oxidative DNA damage of hamster pancreas and liver induced by *N*-nitrosobis(2-oxopropyl)amine and/or oxidized soybean oil. *Biofactors* **2004**, *21*, 335−337.
(35) Mazzanti, G.; Menniti-Ippolito, F.; Moro, P. A.; Cassetti, F.; Raschetti, R.; Santuccio, C.; Mastrangelo, S. Hepatotoxicity from green tea: a review of the literature and two unpublished cases. *Eur. J. Clin. Pharmacol.* **2009**, *65*, 331−341.
(36) Lambert, J. D.; Lee, M. J.; Diamond, L.; Ju, J.; Hong, J.; Bose, M.; Newmark, H. L.; Yang, C. S. Dose-dependent levels of epigallocatechin-3-gallate in human colon cancer cells and mouse plasma and tissues. *Drug Metab. Dispos.* **2006**, *34*, 8−11.
(37) Schneider, K.; Oltmanns, J.; Hassauer, M. Allometric principles for interspecies extrapolation in toxicological risk assessment − empirical investigations. *Regul. Toxicol. Phamacol.* **2004**, *39*, 334−347.
(38) Nakae, Y.; Dorchies, O. M.; Stoward, P. J.; Zimmermann, B. F.; Ritter, C.; Ruegg, U. T. Quantitative evaluation of the beneficial effects in the mdx mouse of epigallocatechin gallate, an antioxidant polyphenol from green tea. *Histochem. Cell Biol.* **2012**, *137*, 811−827.
(39) Wessner, B.; Strasser, E. M.; Koitz, N.; Schmuckenschlager, C.; Unger-Manhart, N.; Roth, E. Green tea polyphenol administration partly ameliorates chemotherapy-induced side effects in the small intestine of mice. *J. Nutr.* **2007**, *137*, 634−640.