Green tea (*Camellia sinensis*) Attenuates Nephropathy by Downregulating Nox4 NADPH Oxidase in Diabetic Spontaneously Hypertensive Rats[1–3]

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Abstract

Green tea (GT), through its antioxidant properties, may be useful to treat or prevent human diseases. Because several lines of evidence suggest that oxidative stress contributes to the pathogenesis of diabetic nephropathy, we tested the hypothesis that GT prevents diabetes and hypertension-related renal oxidative stress, attenuating renal injury. Spontaneously hypertensive rats (SHR) with streptozotocin-induced diabetes and nondiabetic SHR were treated daily with tap water or freshly prepared GT (13.3 g/L). After 12 wk, the systolic blood pressure did not differ between treated and untreated nondiabetic or diabetic rats. However, body weight was less ($P < 0.05$) and glycemia was greater in diabetic SHR rats than in nondiabetic rats. Renal oxidative stress variables such as 8-hydroxy-2′-deoxyguanosine (8-OHdG) and nitrotyrosine expression, NADPH oxidase-dependent superoxide generation, and the expression of renal cortex Nox4 were greater ($P < 0.05$) in diabetic rats that received water (DW) than in nondiabetic rats that received water (CW). The 8-OHdG and NADPH oxidase-dependent superoxide generation were significantly less in rats treated with GT. Nitrotyrosine and Nox4 expression were significantly less in diabetic rats that received GT (DGT) than in DW. Likewise, the indices of renal injury, albuminuria, and renal expression of collagen IV were significantly greater in DW than in CW. These differences were significantly less in DGT than in DW. GT reestablished the redox state and reduced the indicators of nephropathy without altering glycemia and blood pressure levels in diabetic SHR. These findings suggest that the consumption of GT may ameliorate nephropathy in diabetic hypertensive patients. J. Nutr. 139: 96–100, 2009.

Introduction

Hypertension and diabetes frequently coexist, and the presence of these 2 conditions significantly magnifies the risk of nephropathy (1). Accordingly, blood glucose and blood pressure are among the most effective maneuvers for the treatment and prevention of diabetic renal disease (2–5). Likewise, the presence of hypertension in experimental diabetes mellitus (DM)[6] aggravates and anticipates renal abnormalities (6) that can be prevented by antihypertensive therapy (7).

Diabetic nephropathy is primarily metabolic and hemodynamic in origin and results from interaction between genetic susceptibility and environmental factors (8). There is considerable evidence that oxidative stress has a role in the pathogenesis of diabetic nephropathy (9). Hyperglycemia and hypertension can activate multiple pathways that lead to increased generation of superoxide anions and other reactive oxygen species (ROS) (reviewed in 10,11). Some of these pathways include enhanced activity of the mitochondrial electron transport chain induced by hyperglycemia (12), increased expression, and uncoupling of endothelial nitric oxide synthase, leading to greater production of superoxide relative to nitric oxide (9) and activation of the reduced forms of NADPH (13–15). The latter system is present in different renal cell types (16,17) and may contribute to renal damage in the presence of diabetes or hypertension (18,19). In addition, we have recently shown that hypertension increases renal oxidative stress by elevating NADPH-dependent superoxide production and decreasing antioxidant defenses in spontaneously hypertensive rats (SHR) with streptozotocin (STZ)-induced diabetes (19).

[6] Abbreviations used: 8OHdG, 8-hydroxy-2′-deoxyguanosine; AER, albumin excretion rate; AUC, area under the curve; CW, nondiabetic rats that received water; CGT, nondiabetic rats that received daily green tea; DM, diabetes mellitus; DW, diabetic rats that received water; DGT, diabetic rats that received green tea; GT, green tea; ROS, reactive oxygen species; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; STZ, streptozotocin.

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Strategies that reduce superoxide formation via NADPH oxidase and/or increase the activity of antioxidant defense systems can attenuate hyperglycemia-induced renal injury (18,20–22). For instance, phosphorothioated antisense oligonucleotides for Nox4, a homolog of the gp91phox subunit of NADPH oxidase, inhibits NADPH-dependent ROS generation in the renal cortex and reduces glomerular hypertrophy and the expression of renal fibronectin (20).

Green tea (GT; *Camellia sinensis*) is a rich source of polyphenols, particularly flavonoids that have beneficial effects in the treatment of certain forms of cancer, arthritis, and cardiovascular disorders (reviewed in 23). The administration of epigallocatechin gallate, a polyphenol that accounts for ~30% of the dry weight of GT, to SHR improves endothelial function and insulin sensitivity and reduces blood pressure (24). In rats with STZ-induced diabetes, the administration of GT in the drinking water improves renal function and reduces the blood glucose and glycated protein concentrations (25). GT also attenuates the retinopathy and renal mitochondrial ROS production in this same model (27). Although most of the biological actions of GT have been attributed to its antioxidant properties (23), there has been no systematic study of its efficacy in preventing oxidative stress caused by hypertension and hyperglycemia, thereby protecting against renal injury.

In this work, we tested the hypothesis that GT attenuates renal injury in diabetic hypertensive rats. In addition, to explore the underlying molecular mechanisms, we examined the effects of GT on the expression of NADPH oxidase Nox4, which is a major source of ROS production in the kidney (20).

Materials and Methods

Rats and experimental protocol. This study complied with the guidelines of the Brazilian College for Animal Experimentation and was approved by the institutional Committee for Ethics in Animal Experimentation (CEEA/IB/UNICAMP, protocol no. 1119–1). SHR (Taconic) bred in our animal facility were used in this study. Experimental diabetes was induced in 12-wk-old hypertensive male SHR (~250 g) by a single i.v. (tail vein) injection of STZ (50 mg/kg; dissolved in sodium citrate buffer, pH 4.5; Sigma) after overnight food deprivation. Nondiabetic SHR received only vehicle (citrate buffer). Seventy-two hours after the injection of STZ or citrate buffer, blood glucose concentrations were measured with a colorimetric enzymatic GOD-PAP assay (Merck) in rats deprived of food for 4 h. Rats with blood glucose concentrations ≥15 mmol/L were considered diabetic for these experiments. Diabetic and nondiabetic SHR were randomly assigned to groups of 8 rats each that received only water [nondiabetic water (CW; $n = 8$) and diabetic water (DW; $n = 8$)] or GT [nondiabetic GT (CGT; $n = 8$) and diabetic GT (DGT; $n = 8$)] as their sole source of drinking water for 12 wk. All of the rats were housed in groups of 4 and were fed nonpurified, pelleted rat diet containing 22% protein, 55% carbohydrate, and 4.5% fat (Nuvital Nutrientes S/A). Diabetic rats received 2 U of long-acting insulin (Novo Industry A/S) 3 times each week. Japanese GT (Midori Indústria de Chá) was prepared daily as described by the manufacturer: 10 g of dry tea was added to 750 mL of deionized boiled tap water cooled to 90°C and then brewed for 3 min, decanted, filtered, placed on ice, and protected from light with aluminum foil.

Variables measured. Body weight, plasma glucose, systolic blood pressure (SBP), and albumin excretion rate (AER) were measured at 0, 4, 8, and 12 wk. The rats were killed by CO_2 asphyxia 12 wk after the induction of diabetes. The abdomen was opened via a midline incision and the right kidney was immediately removed, decapsulated, weighed, and processed for homogenization of the cortical tissue. The left kidney was similarly removed and cut longitudinally into 2 halves; one half was frozen in liquid nitrogen and stored at −80°C and the other half was fixed by immersion in methacarn (60% methanol, 30% chloroform, and 10% glacial acetic acid).

SBP measurements. SBP was measured by tail-cuff plethysmography with an MK III physiograph (Narco Bio-System) in unanesthetized rats before randomization (5 determinations per rat) and every 4 wk throughout the experiment.

TABLE 1 Physiological variables measured monthly from nondiabetic and diabetic SHR that consumed water or GT for 12 wk[1]

	Wk				Effect[2]	P
	0	4	8	12		
Body weight, g					DM	<0.0001
CW	256 ± 20.4	338.6 ± 44.3	350.8 ± 33.5	354.3 ± 37.1		
CGT	259.9 ± 16.1	315.9 ± 16.4	333.8 ± 18.3	349.3 ± 21.8		
DW	256.4 ± 23.6	210.2 ± 47.4	229.6 ± 30.9	252.3 ± 24.6		
DGT	261.5 ± 23.7	249 ± 25.5	242.9 ± 56.8	277.5 ± 52.2		
SBP, *mm Hg*					DM X GT	0.038
CW	188.2 ± 15.4	198.7 ± 13.9	203.2 ± 13.8	202.2 ± 13.3		
CGT	178.2 ± 11.3	185.9 ± 12.4	186.9 ± 14.5	197.9 ± 7.9		
DW	178.4 ± 11.1	180.7 ± 9.4	192.5 ± 16.4	192.1 ± 16.3		
DGT	186.3 ± 13.3	189.6 ± 17.5	188.9 ± 22.3	193.2 ± 16.5		
Blood glucose, *mmol/L*					DM	<0.0001
CW	8.6 ± 1.0	10.4 ± 3.0	8.2 ± 2.0	8.6 ± 1.2		
CGT	8.8 ± 0.6	9.6 ± 2.5	8.7 ± 1.7	8.9 ± 1.4		
DW	25.1 ± 3.0	26.4 ± 3.6	34.8 ± 4.8	25.8 ± 3.8		
DGT	24.1 ± 4.1	27.9 ± 6.5	32.3 ± 8.7	27.0 ± 4.5		
Urine volume, *mL/24 h*					DM	<0.0001
CW	25.9 ± 12.1	32.0 ± 8.8	40.9 ± 6.5	37.3 ± 22.6		
CGT	23.0 ± 1.4	36.4 ± 14.5	34.8 ± 9.9	30.3 ± 7.1		
DW	24.1 ± 7.4	75.4 ± 15.0	86.6 ± 19.0	65.6 ± 25.5		
DGT	22.4 ± 8.6	86.3 ± 13.4	83.4 ± 18.0	69.6 ± 35.2		

[1] Values are expressed as means ± SD, $n = 8$.
[2] Significant effects detected in repeated-measures 2-way ANOVA.

AER. Before the induction of diabetes mellitus and every 4 wk throughout the experiment, urine samples were collected by placing each rat in a metabolic cage for 24 h. The urine samples were analyzed by single radial immunodiffusion to determine the AER, as previously described (7,19).

NADPH oxidase activity. NADPH oxidase activity was measured by the lucigenin-enhanced chemiluminescence method as previously described by Gorin et al. (20) with some modifications (19). Superoxide production was measured every 20 s for 10 min and expressed as relative luminescence units·20 s^{-1}·mg^{-1} of protein. We used diphenyleneiodonium, an inhibitor of flavin-containing oxidases, and rotenone, an inhibitor of complex I of the mitochondrial respiratory chain, to identify the source of superoxide production. Protein concentrations were measured using the Bradford dye-binding method (26) with bovine serum albumin as the standard.

Western blot. Renal cortical homogenates were prepared as described elsewhere (19) and were used to quantify nitrotyrosine (an indicator of oxidative/nitrosative stress-induced protein modifications), collagen IV, and Nox4 (a homolog of the gp91phox subunit of NADPH oxidase) by Western blot analysis (19). Molecular weight markers (PageRuler, Fermentas Life Sciences) were used as standards. The proteins were separated on 10% polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. After blocking with 5% nonfat milk, the membranes were incubated with the following primary antibodies: mouse monoclonal anti-nitrotyrosine antibody (1:2000; clone 1A6, Upstate), goat anti-type IV collagen antibody (1:500; Southern Biotech), or rabbit polyclonal anti-Nox4 (1:2000) (20). After washing with PBS containing 0.1% Tween-20, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz). Immunoreactive bands were visualized using the enhanced chemiluminescence method (Super Signal CL-HRP Substrate System; Pierce). The uniformity of protein loading and the transfer efficiency were assessed by reprobing the membranes for actin (goat polyclonal anti-actin antibody, 1:1000, Santa Cruz) or β-tubulin (anti-β-tubulin mouse mAb DM1B, 1:1000, Calbiochem). Exposed films were scanned with a laser densitometer (Bio-Rad) and were analyzed quantitatively with Multi-Analyst Macintosh Software for Image Analysis Systems (Bio-Rad).

Immunohistochemistry. Oxidative stress-induced DNA base modification was assessed by immunohistochemistry for 8-hydroxy-2′-deoxyguanosine (8-OHdG; a DNA base-modified product) in 4-μm-thick sections of methacarn-fixed, paraffin-embedded renal tissue using a mouse monoclonal anti-8-OHdG antibody (N45.1; Japan Institute for the Control of Aging, Japan) as previously described (19). Tubulointerstitial cells containing 8-OHdG-positive nuclei were counted in 50 sequential high power microscopic fields (×400) by an observer unaware of the study group from which the section was derived.

Statistical analysis. The results were expressed as the means ± SD. Two-way ANOVA was used to analyze the data. For body weight, SBP, glycemia, and urine volume 2-way ANOVA for repeated measures was used. Nonparametric data such as the number of positive cells for 8-OHdG were analyzed after conversion to rank. When the diabetes × GT interaction was significant, Fisher's protected least significant difference test was performed. Differences in albuminuria between groups were assessed as the area under the curve (AUC) and the results were compared using Kruskal-Wallis and Mann-Whitney U tests (28). Statistical significance was set at $P < 0.05$. All analyses were conducted using StatView software (SAS Institute).

Results

Physiological variables. Body weight gain was less ($P < 0.0001$) in diabetic SHR than in control rats. The relative kidney weight was markedly greater ($P < 0.0001$) in DW (0.52 ± 1.0 g/100 g body) than in both CW (3.8 ± 0.3) and CGT (3.7 ± 0.2). The SBP was similar in all groups. The blood glucose concentration was greater in diabetic rats than in controls but was not affected by GT (**Table 1**).

NADPH-dependent superoxide generation, Nox4 expression, and oxidative stress markers. NADPH-dependent superoxide production was greater ($P = 0.01$) in renal cortical homogenates of DW rats compared with the control rats and was less ($P = 0.01$) in GT-treated groups (**Fig. 1**). Preincubation of renal cortical homogenates with diphenyleneiodonium (final concentration, 20 μmol/L) completely blocked NADPH-induced superoxide production, whereas preincubation with rotenone (final concentrations of 20 and 100 μmol/L) did not affect superoxide production (data not shown). These results indicated that NADPH oxidase was the most likely source of superoxide anion.

The expression of Nox4 was greater ($P = 0.04$) in the DW group than in the CW group and it was reduced to the levels of both control groups in the DGT (DM × GT; $P = 0.03$) (**Fig. 2A,B**).

The nitrotyrosine concentration was greater in the DW group than in the CW and CGT groups ($P = 0.01$) and it was reduced ($P = 0.04$) to both nondiabetic levels in DGT SHR (DM × GT; $P = 0.03$) (Fig. 2C,D).

The number of renal cortical cells with 8-OHdG-positive nuclei was greater ($P < 0.01$) in the DW group than in the CW group and it was reduced by GT treatment ($P < 0.01$) (**Supplemental Fig. 1**).

Renal injury indices. The urinary AER and the accumulation of kidney collagen IV were used as markers of functional and morphological renal injury, respectively. The AER estimated as the AUC was greater ($P = 0.048$) in the DW group than in the CW group and was less in the DGT group than in the DW group (**Fig. 3**). Western blotting showed that renal cortical expression of collagen IV was greater ($P = 0.01$) in DW rats than in nondiabetic SHR and was reduced to the levels of both nondiabetic groups in the DGT (DM × GT; $P = 0.03$) (**Fig. 4A,B**).
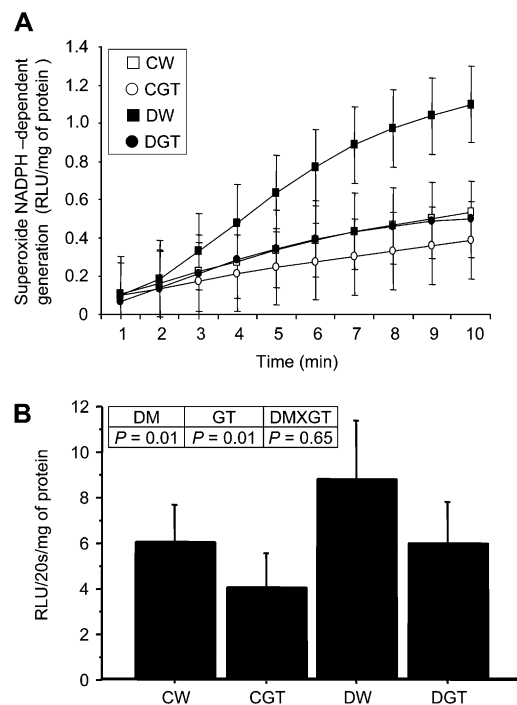

FIGURE 1 NADPH oxidase activity in renal cortical homogenates from nondiabetic and diabetic SHR that consumed water or GT for 12 wk. (*A*) Superoxide anion generation measured every 20 s for 10 min. (*B*) Mean NADPH oxidase activities during the last 5 min were used for comparison among the groups. Bars represent the means ± SD, $n = 5$–8. Means without a common letter differ, $P < 0.05$.
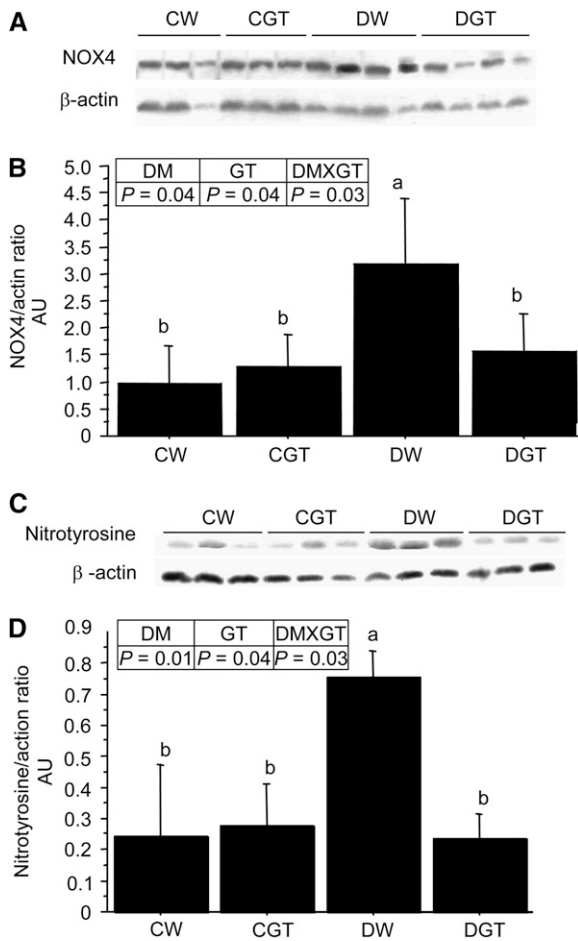
FIGURE 2 Representative Western blots of the renal cortical expression from nondiabetic and diabetic SHR that consumed water or GT for 12 wk of Nox4 (*A*) and nitrotyrosine (*C*). Densitometric analysis of both bands was used to determine the Nox4:actin ratio (*B*) and nitrotyrosine:actin ratio (*D*), presented as arbitrary units (AU). Bars represent the means ± SD of at least 3 independent experiments ($n = 3–4$ rats per group). Means without a common letter differ, $P < 0.05$.

Discussion

The results of this study show that GT ameliorates the albuminuria and renal accumulation of collagen IV and reduces markers of renal oxidative stress in diabetic SHR. The underlying mech-
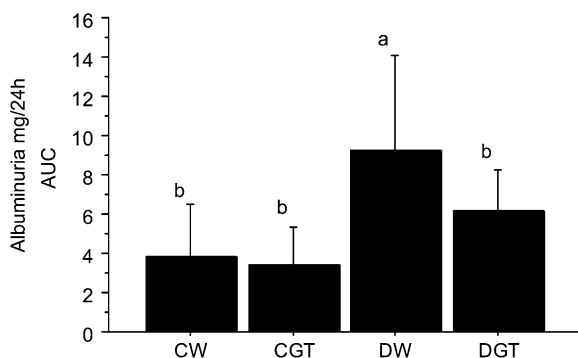

FIGURE 3 AER from nondiabetic and diabetic SHR that consumed water or GT for 12 wk. Histogram representative of albuminuria accessed as the AUC, $n = 8$ per group. Means without a common letter differ, $P < 0.05$.
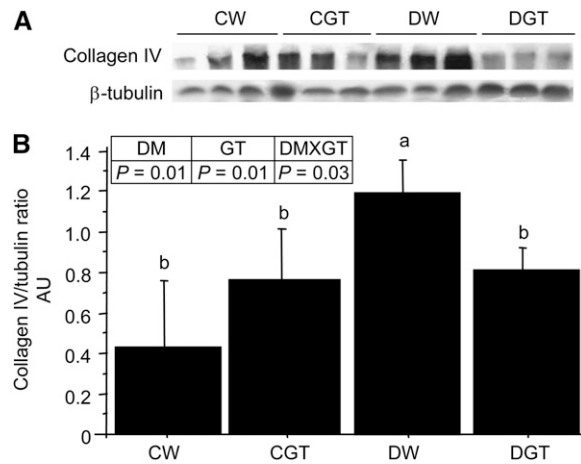

FIGURE 4 Representative Western blots of the renal cortical expression from nondiabetic and diabetic SHR that consumed water or GT for 12 wk of collagen IV (*A*). Densitometric analysis of both bands was used to determine the collagen IV:*β*-tubulin ratio, presented as arbitrary units (AU) (*B*). Bars represent the mean ± SD of at least 3 independent experiments ($n = 3–4$ rats per group). Means without a common letter differ, $P < 0.05$.

anism of these beneficial effects apparently involves the downregulation of NADPH oxidase Nox4 expression.

The adequate control of blood pressure and blood glucose concentration is central to the treatment and prevention of renal lesions in diabetes (2–5). GT and epigalocatechin gallate, the main polyphenolic component of GT, reduce blood glucose in diabetic rats (25,29), although this effect is not always observed (26). Our findings agree with the latter study, because GT did not affect the blood pressure or blood glucose concentrations of SHR. This observation further supports the importance of oxidative stress in the renal injury seen here and strengthens the supposition that the beneficial effects of GT in renal tissue were mediated by restoration of the redox status.

Nox4, a homolog of the gp91phox subunit of NADPH, is highly expressed in the kidney (20,30). Overexpression of Nox4 in the kidney of diabetic rats with STZ-induced diabetes may play an important role in increased ROS production (20,31). Gorin et al. (20) reported that downregulation of Nox4 induced by antisense oligonucleotides completely attenuated oxidative stress in the kidneys of rats with STZ-induced diabetes and that there was a concomitant reduction in glomerular hypertrophy and the accumulation of fibronectin. Similarly, Fujii et al. (32) found that the administration of a statin, pitavastatin, to db/db mice ameliorated the albuminuria and renal mesangial expansion by downregulating Nox4. In agreement with these findings, the downregulation of Nox4 by GT completely attenuated oxidative stress and renal injury in diabetic SHR. The molecular mechanism by which GT regulates Nox4 activity remains to be elucidated.

In conclusion, GT ameliorates renal injury in diabetic hypertensive rats by inhibiting oxidative stress through downregulation of Nox4 expression. These findings suggest that GT consumption may be beneficial in diabetic hypertensive patients.

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