Curcuma Aromatica Inhibits Diabetic Nephropathy in the Rat

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ABSTRACT: To test the possible involvement of reactive oxygen species (ROS) in the etiology of diabetic complications and therapeutic potential of antioxidant biofactors, we studied the effects of *Curcuma aromatica (C. aromatica)* on the pathologic events in streptozotocin-treated diabetic rats. Administration of streptozotocin (100 mg/kg, i.p.) increased plasma levels of glucose, triglyceride, cholesterol, urea nitrogen (BUN), creatinine, and lipid peroxidation products but decreased plasma albumin levels and suppressed the growth of animals. Histological examination revealed a marked injury in renal glomeruli and proximal tubules with concomitant occurrence of 8-hydroxy-2′-deoxyguanosine (8-OHdG) and mitochondrial 4-hydroxy-2-nonenal (4-HNE). Urinary excretion of 8-OHdG was also increased in streptozotocin-treated animals. Administration of streptozotocin decreased the mitochondrial localization of both Cu/Zn-superoxide dismutase (SOD) and cytochrome C in the kidney without affecting the localization of Mn-SOD. When animals were given 1.5% *C. aromatica*-containing diet for 1 wk before and 8 wk after administration of streptozotocin, all the events induced by streptozotocin except for hyperglycemia decreased markedly. Thus, *C. aromatica* may have therapeutic potential for the prevention of hyperglycemia-associated diabetic complications.

Keywords: *C. aromatica*, diabetes, diabetic nephropathy, mitochondria, oxidative stress

Introduction

Hyperglycemia induces nonenzymatic glycation of various proteins through the Maillard reaction, and the resulting Amadori products and advanced glycation end products (AGE) generate reactive oxygen species (ROS) (Sakurai and Tsuchiya 1988; Njoroge and Monnier 1989; Aragno and others 2001). Hyperglycemia-associated oxidative stress has been postulated to play important roles in the development of diabetic complications, including nephropathy (Baynes 1991; Kowluru and others 2000, 2001). Thus, regulation of ROS metabolism may have therapeutic potential in patients with diabetes mellitus. Since diabetes mellitus is a long-lasting chronic disease, some biofactors in daily foods have been expected to play important roles in the suppression of oxidations, tissue injury, and prevention of diabetic complications (Haidara and others 2004).

Several herbal drugs, including those from *Curcuma* species, are such examples. Genus *Curcuma* of the Zingiberaceae family consists of about 70 species in the world. Among 10 species of the family widely distributed in China and Japan, Gajutsu (*C. zedoaria*), Ukon (*C. longa*), and Haruukon (*C. aromatica*), have been used as stomachics, cholagogics, and a healthy food, respectively. Curcumin (1,7-*bis* (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is one of the major components of turmeric, which has antioxidant activity. Recent studies revealed that curcumin has a potent activity to scavenge superoxide and hydroxyl radicals (Reddy and Lokesh 1992; Ruby and others 1995), and exhibits anticarcinogenic and anti-inflammatory activities (Conney and others 1991; Huang and others 1991). Thus, the antioxidant property of curcumin in *C. longa* has been postulated to play a role in the suppression of ROS-induced tissue injury (Ammon and Wahl 1991). In fact, Suryanarayana and others (2005) reported that administration of *C. longa* delayed the progression and maturation of cataracts in streptozotocin-treated hyperglycemic rats. Although the concentration of curcumin in *C. aromatica* (about 0.3%) is significantly lower than that of *C. longa* (about 3.6%), the former turmeric has been used as a traditional medicine for a long time (Ohigashi and others 1997). To test the possible effects of *C. aromatica* on the development of diabetic complications, we studied its effects on pathologic events in streptozotocin-treated hyperglycemic rats.

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Materials and Methods

Reagents

Determination of blood glucose, creatinine, BUN, albumin, triglyceride, and total cholesterol was carried out using kits obtained from Wako Pure Chemical (Osaka, Japan). ELISA kits for the determination of 8-OHdG and monoclonal antibodies against 8-OHdG and 4-HNE were obtained from the Japan Institute for the Control of Aging (Shizuoka, Japan). Rabbit polyclonal antibodies against human Cu/Zn-SOD, Mn-SOD, and mouse cytochrome C were obtained from Calbiochem (San Diego, Calif., U.S.A.), Stressgen Biotech (Ann Arbor, Mich., U.S.A.), and Pharmingen (Franklin Lake, N.J., U.S.A.), respectively. *Curcuma aromatica* was provided by Okinawa Fermentative Chemistry (Okinawa, Japan). Other reagents used were of the highest grade, commercially available, and obtained from Wako Pure Chemical.

Animal experiments

Male Wistar rats, weighting 200 to 220 g, were allowed free access to water and either standard diet or 1.5% *C. aromatica*-containing diet for 1 wk. The constituents of *C. aromatica* were the same as described previously (Wu and others 2000). One week after feeding with the 2 types of diets, 100 mg/kg of streptozotocin was administered intraperitoneally to induce diabetes mellitus. Control animals were given equal volume of saline (1 mL/animal). At the indicated

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times after the injection of streptozotocin, blood samples were obtained from the orbital venous plexus after fasting for overnight. Seven weeks after the administration of streptozotocin, the urine samples were collected for 24 h and used for biochemical analysis.

Assay for oxidative stress

At the indicated times, blood samples were obtained from animals and subjected to D-ROMs test (Cesarone and others 1999; Alberti and others 2000), using a Free Radical Analysis System (FRAS, Diacron, Grosseto, Italy). Plasma samples (10 μL) was added to 1 mL of an assay mixture, gently mixed, and incubated for 1 min at 37 °C. The optical density at 505 nm was measured using a spectrophotometer.

Histological analysis

The kidney was perfused with ice-cold 0.9% NaCl solution, fixed in 5% formalin, and embedded in paraffin. Thin sections of tissue specimens were stained with hematoxylin and eosin. Immunohistochemical staining of 8-OHdG was performed as described previously (Ihara and others 1999). Histological examination was carried out under a light microscope.

Analysis of mitochondrial respiration

Renal cortical mitochondria were isolated according to the method of Jung and Pergande (1985). Briefly, kidneys were perfused with ice-cold 0.9% NaCl solution containing 0.5 mM EDTA and their cortex was homogenized in 50 mL of 210 mM mannitol

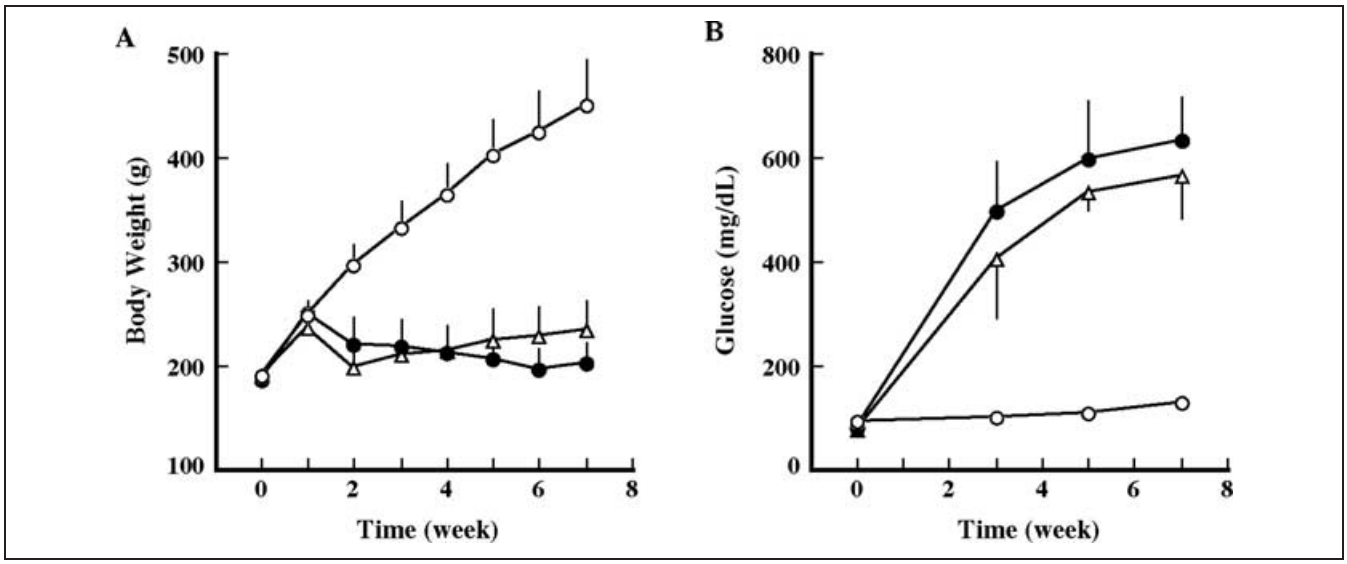

Figure 1—Effect of *C. aromatica* on the body weight and plasma glucose levels in streptozotocin-treated rat. One week after giving 1.5% *C. aromatica*-containing diet, rats were intraperitoneally administered with 100 mg/kg of streptozotocin. They were fed either normal or *C. aromatica*-containing diet for 7 wk. During the experiments, body weight and plasma glucose levels were determined. Open circles, control group; closed circles, streptozotocin-treated group; triangles, streptozotocin + *C. aromatica*-treated group.

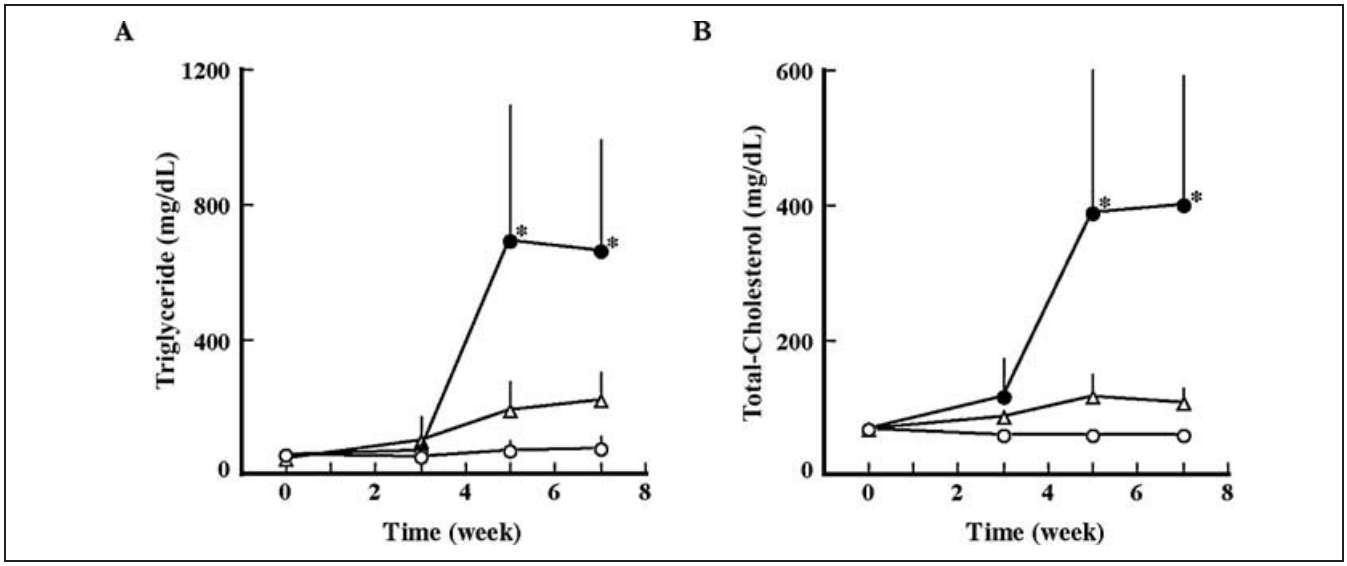

Figure 2—Effect of *C. aromatica* on streptozotocin-induced hyperlipidemia. Plasma levels of triglyceride and total cholesterol were measured as described in the methods. All values are given as the mean ± SD. Statistical analysis was performed using Student's *t*-test. Open circles, control group; closed circles, streptozotocin-treated group; triangles, streptozotocin + *C. aromatica*-treated group. *, $P < 0.05$ compared with curcuma-fed diabetic rats (mean ± SD).

containing 70 mM sucrose, 0.5 mM EDTA, and 4 mM Tris-HCl buffer (pH 7.4) using a Teflon™ homogenizer. The homogenate was centrifuged at 800 × *g* at 4 °C for 10 min. The supernatant was centrifuged at 12000 × *g* for 5 min. The pellet (mitochondrial fraction) was then suspended in the homogenization medium (10 to 20 mg protein/mL) and stored on ice until use. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard. Mitochondrial respiration was analyzed polarographically using a Clark-type oxygen electrode at 25 °C (Packer and others 1966). The freshly prepared renal mitochondria were suspended in a reaction medium consisting of 210 mM sucrose, 10 mM KCl, 10 mM KH_2PO_4, 0.5 mM EDTA, and 60 mM Tris-HCl (pH 7.4) at a concentration of 0.2 mg protein/mL. Oxygen consumption was monitored in the presence of 5 mM succinate and 300 μM ADP. Respiratory control index (RCI) was analyzed to test the function of mitochondria (Chang and others 2002).

Assay for ROS generation by renal mitochondria

Mitochondrial samples (10 μL) were incubated in a reaction medium (475 μL) consisting of 210 mM sucrose, 10 mM KCl, 10 mM KH_2PO_4, 0.5 mM EDTA, and 60 mM Tris-HCl buffer (pH 7.4) in the presence of 400 μM L-012. After incubation of the mixture for 3 min at 25 °C, the reaction was started by adding 5 mM of succinate. During the incubation, chemiluminescence (CHL) intensity of L-012 was recorded continuously for 10 min using a Luminescence Reader BLR-201 (Aloka, Tokyo) as described previously (Imada and others 1999).

Western blot analysis

Mitochondrial specimens were subjected to polyacrylamide gel (15%) electrophoresis (PAGE) in the presence of 0.1% SDS. The electrophoresed proteins in the gel were transferred to an Immobilon membrane (Millipore). The membrane was blocked with 5% skim milk overnight at 4 °C, and subsequently incubated with primary antibodies at 25 °C for 1 h and then with horseradish peroxidase-conjugated secondary antibodies. Immune complexes thus formed were detected with ECL reagents (Amersham Bioscience, Calif., U.S.A.).

Statistical analysis

The data were expressed as the mean ± SD derived from at least 6 animals. Group comparisons were performed using Student's *t* test or ANOVA. Differences were considered significant at $P < 0.05$.

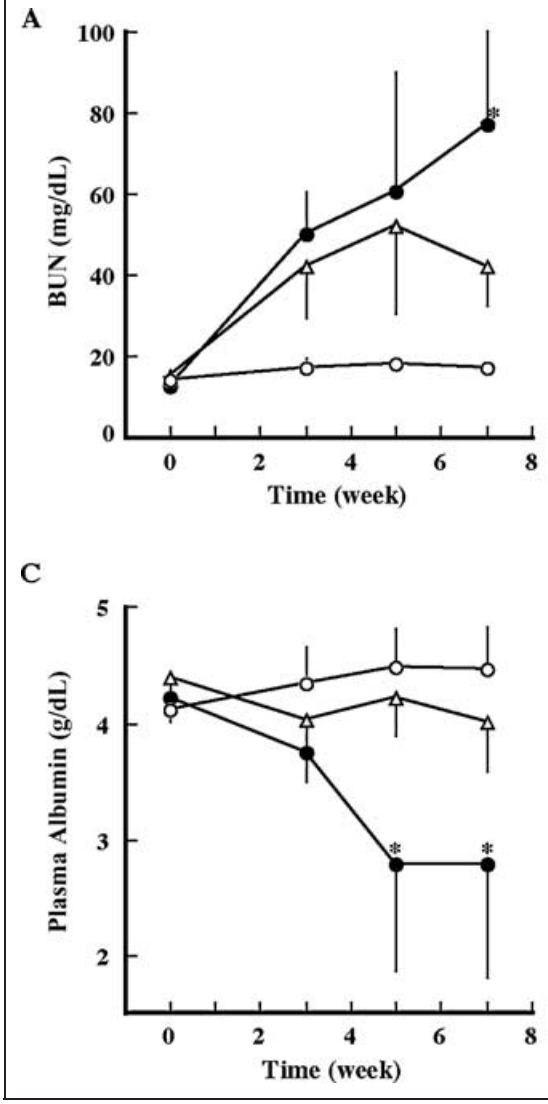

Figure 3—Effect of *C. aromatica* on streptozotocin-induced renal failure. Plasma levels of BU, creatinine, and albumin were measured as described in the methods. At 7 wk after the treatment, protein concentration in urine samples collected for 24 h were determined. All values are given as the mean ± SD. Statistical analysis was performed using Student's *t*-test. Open circles, control group; closed circles, streptozotocin-treated group; triangles, streptozotocin + *C. aromatica*-treated group. *, $P < 0.05$ compared with *C. aromatica*-fed diabetic rats (mean ± SD).

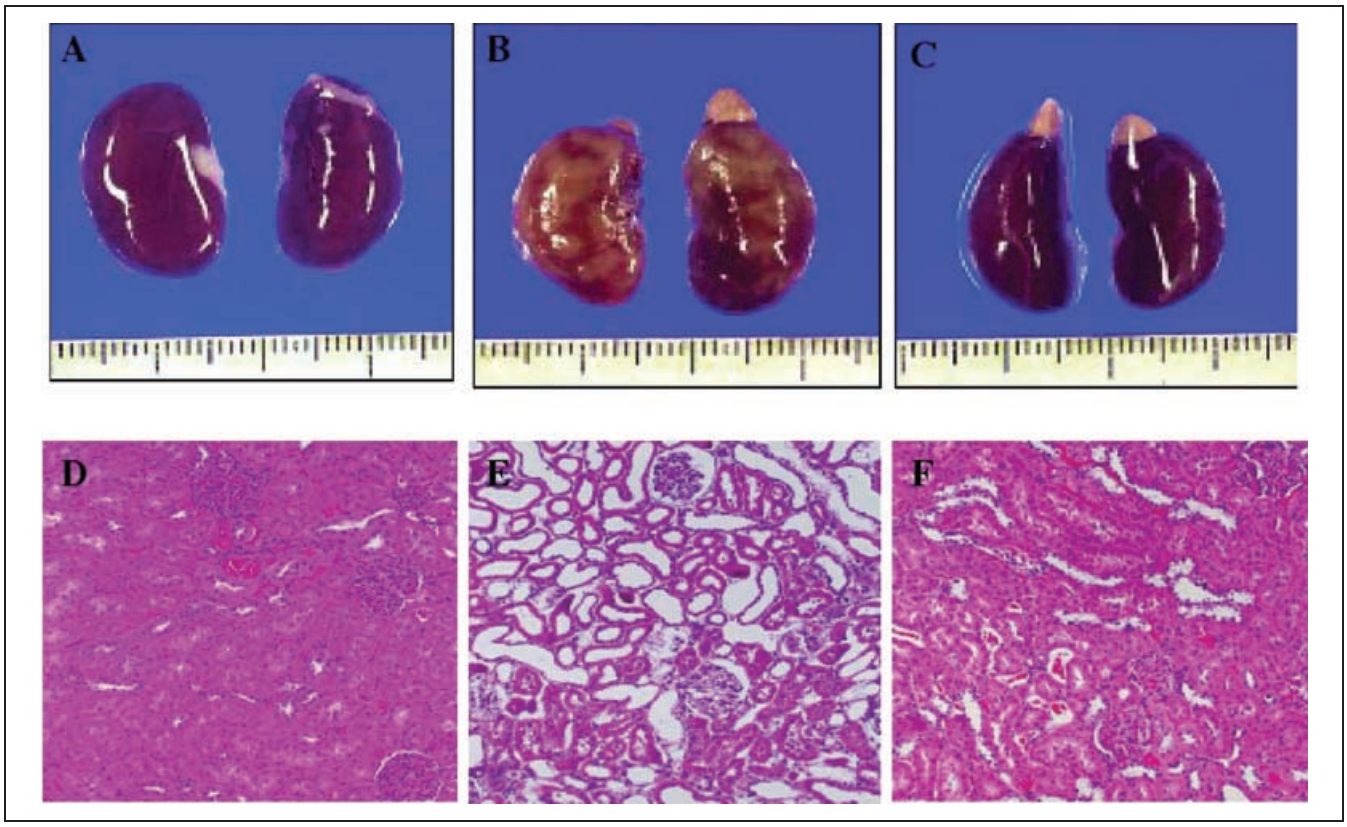

Figure 4—Effect of *C. aromatica* on streptozotocin-induced renal injury. Seven weeks after streptozotocin-treatment, animals were killed and kidneys were fixed with 10% formalin, paraffin-embedded renal sections were stained with HE (D, E, F). A and D, control group; B and E, streptozotocin-treated group; C and F, streptozotocin + *C. aromatica*-treated group. Magnification (D–F) × 100.

Result

Effect of *C. aromatica* on streptozotocin-treated rats

When animals were treated with streptozotocin, the increase of their body weight was suppressed significantly as compared with the control group (Figure 1). Administration of *C. aromatica* had no appreciable effect on the inhibitory effect of streptozotocin. Under identical conditions, hyperglycemia became apparent with streptozotocin-treated animals. Administration of *C. aromatica* also showed no appreciable effect on streptozotocin-induced hyperglycemia. Administration of streptozotocin increased the plasma levels of triglyceride and cholesterol by a mechanism that was inhibited by *C. aromatica*-containing diet (Figure 2).

Effect of *C. aromatica* on streptozotocin-induced changes

Administration of streptozotocin time dependently increased the plasma levels of BUN and creatinine with concomitant decrease in albumin levels. Administration of *C. aromatica*-containing diet inhibited the streptozotocin-induced increase of BUN and creatinine and the decrease in albumin (Figure 3). Administration of streptozotocin increased the urinary proteins by a *C. aromatica*-inhibitable mechanism. Histological examination revealed that streptozotocin strongly impaired the structure of glomeruli and proximal tubules in the kidney (Figure 4). Administration of *C. aromatica* effectively inhibited the streptozotocin-induced renal injury.

Effect of *C. aromatica* on oxidative stress

To evaluate the possible involvement of oxidative stress in streptozotocin-induced change in plasma constituents, we analyzed

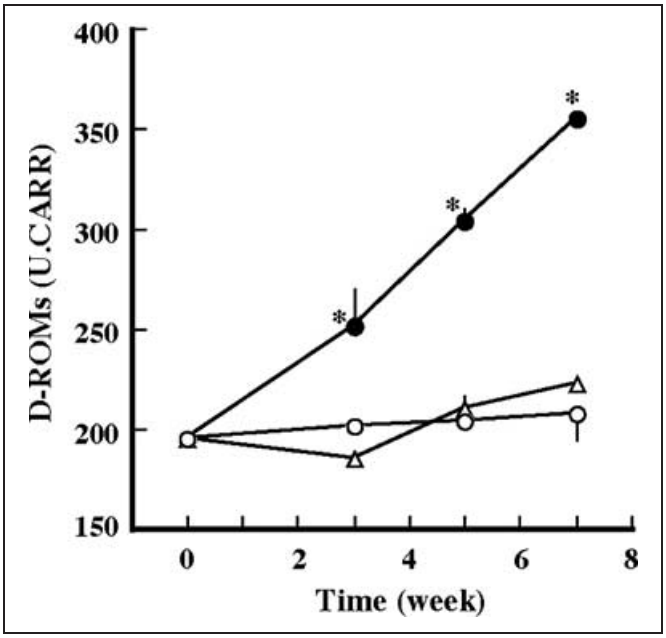

Figure 5—Effect of *C. aromatica* on oxidative stress in streptozotocin-treated animals. Levels of oxidative stress in the circulation of diabetic rats were determined using a free radical determination system (D-ROMs test). All values are given as the mean ± SD. Statistical analysis was performed using Student's *t*-test. Open circles, control group; closed circles, streptozotocin-treated group; triangles, streptozotocin + *C. aromatica*-treated group. *, $P < 0.05$ compared with curcuma-fed diabetic rats (mean ± SD).

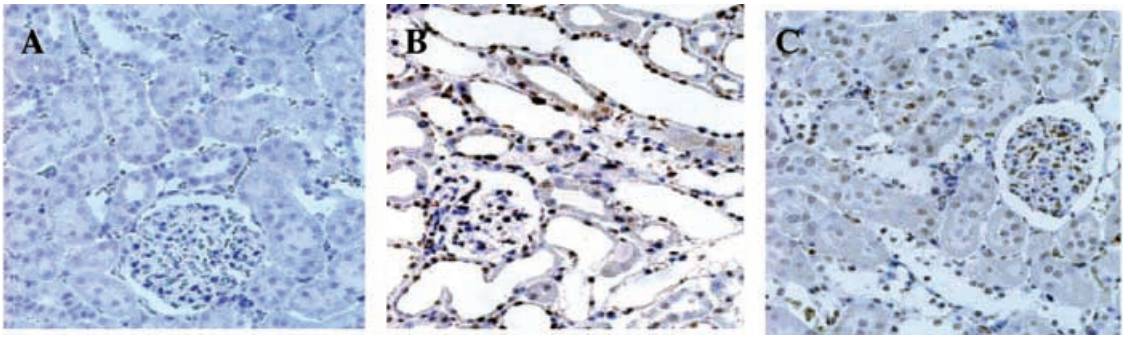

Figure 6—Effect of *C. aromatica* on oxidative DNA damage in the kidney of streptozotocin-treated rats. Seven weeks after streptozotocin treatment, renal specimens were stained with anti-8OHdG antibody. The kidney of streptozotocin-treated animals (B) shows intense nuclear staining as compared to that with *C. aromatica*-treated group (C). Other conditions were as in Figure 5. Magnification: × 200.

lipid peroxides using the free radicals determination system (D-ROMs test). This test has been used to analyze a balance between free-radical generation and antioxidant defense systems (Cesarone and others 1999; Alberti and others 2000). Oxidized metabolites in plasma increased with time in streptozotocin-treated diabetic animals, suggesting the involvement of oxidative stress in tissue injury. Administration of *C. aromatica*-containing diet inhibited the streptozotocin-induced increase in oxidized metabolites in plasma (Figure 5). To investigate the possible involvement of oxidative stress in the pathogenesis of streptozotocin-induced hyperglycemia and renal injury, immunohistochemical analysis of 8-OHdG in the kidney was performed (Figure 6). The kidney of streptozotocin-treated animals showed strong staining with anti-8OHdG that was inhibited by treating animals with *C. aromatica*. Streptozotocin also increased the urinary excretion of 8-OHdG by a mechanism that was inhibited by *C. aromatica* (Figure 7).

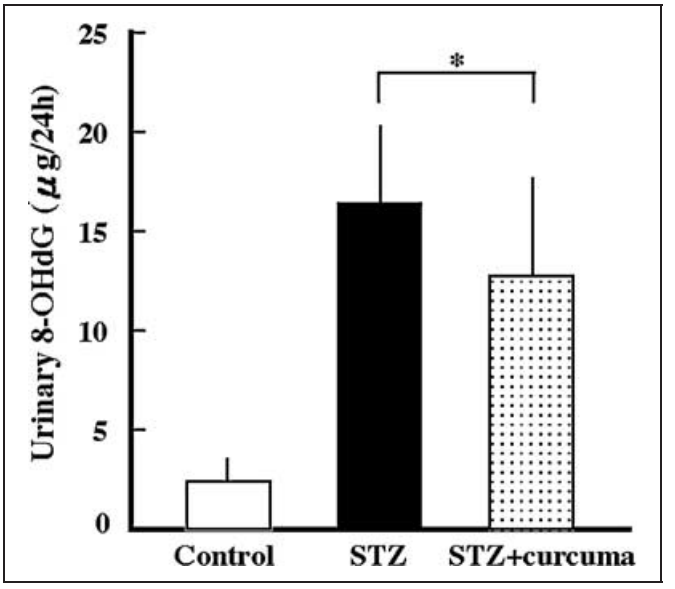

Figure 7—Effect of *C. aromatica* on oxidative stress in streptozotocin-treated animals. On week 7, levels of 8-OHdG in urine sample collected for 24 h were measured as described in the text. All values are given as the mean ± SD. Statistical analysis was performed using Student's *t*-test. *, $P < 0.05$ compared with *C. aromatica*-treated rats (mean ± SD).

Effect of *C. aromatica* on streptozotocin-induced mitochondrial injury

Since *C. aromatica* strongly inhibited the streptozotocin-induced renal injury, we also analyzed the changes in renal mitochondria in diabetic animals (Figure 8). ROS generation by isolated mitochondria from streptozotocin-treated animals increased significantly as compared with that from control animals. The RCI of renal mitochondria markedly decreased with streptozotocin-treated animals as compared with the control group. Administration of *C. aromatica* significantly inhibited the increase in mitochondrial generation of ROS and the decrease in RCI in streptozotocin-treated hyperglycemic animals. Mn-SOD and Cu/Zn-SOD are important factors for the protection of mitochondria from oxidative stress. Release of cytochrome C from mitochondria is a critical step leading to oxidative injury and cell death (Green and Reed 1998). Thus, we analyzed the localization of Cu/Zn-SOD, Mn-SOD, and cytochrome C in renal mitochondria from control and streptozotocin-treated animals (Figure 9A). Mitochondrial localization of cytochrome C and Cu/Zn-SOD, but not Mn-SOD, strongly decreased in the kidney of streptozotocin-treated animals. Since mitochondria are one of the major sites for ROS generation, we analyzed the possible occurrence of 4-HNE in renal mitochondria (Figure 9). Western blot analysis reveled that renal mitochondrial 4-HNE increased markedly in streptozotocin-treated animals by a mechanism that was inhibited by *C. aromatica*.

Discussion

The present work shows that administration of streptozotocin increased oxidative stress and elicited hyperglycemia that caused renal dysfunction associated with hypoalbuminemia and hyperlipidemia and finally decreased the body weight of animals. Feeding animals with *C. aromatica*-containing diet inhibited the occurrence of renal injury and hyperlipidemia in streptozotocin-treated animals without suppressing hyperglycemia and loss of body weight.

Since the plasma level of BUN, a marker for renal proximal tubule injury, increased more rapidly than that of creatinine and urinary proteins, markers for glomerular injury, the streptozotocin-induced renal injury, might occur initially at proximal tubule cells and then glomerular constituent cells. It should be noted that mitochondrial density is higher with renal proximal tubule cells than with glomerular cells (Inoue and others 1979). Because mitochondria are one of the major sites for ROS generation in cells, oxidative injury would occur more readily in and around mitochondria than in other organelles. Consistent with this notion is the present findings that oxidative stress in the circulation (determined by DROM test) increased and the RCI of renal mitochondria decreased continuously soon after the administration of streptozotocin with concomitant generation of 8-OHdG in renal proximal tubule cells. In this

context, ROS generation and oxidative cell injury were more marked with mitochondria-enriched cells than those with mitochondria-deficient cells (Qian and others 2005). Furthermore, hyperglycemia has been shown to enhance mitochondrial generation of ROS (Sakai and others 2003). Thus, it is not surprising that streptozotocin-induced hyperglycemia preferentially elicited oxidative injury of renal proximal tubules.

The present work also demonstrates that administration of streptozotocin increased the generation of ROS and oxidatively modified proteins in mitochondria of renal proximal tubule cells of hyperglycemic animals. Western blot analysis revealed that the 3 protein bands having molecular size of 55, 50, and 27 kDa were strongly stained by anti-HNE antibody. This observation suggests that these proteins have high affinity for 4-HNE, presumably due

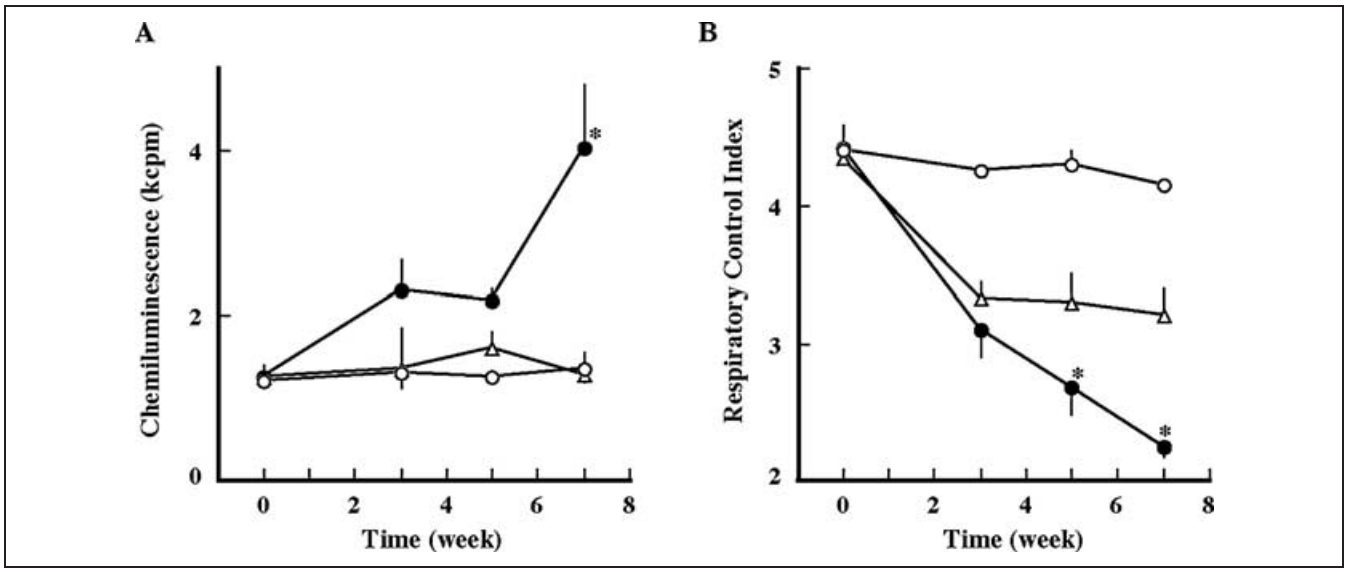

Figure 8—Effect of *C. aromatica* on streptozotocin-induced renal mitochondrial injury. At the indicated times after administration of streptozotocin, ROS generation (A) and respiratory control index (B) of renal cortical mitochondria were determined. ROS generation by mitochondria was measured using 0.4 mM of chemiluminescence probe L-012 in the presence of 5 mM succinate for 10 min. Mitochondrial respiratory control index was analyzed in the presence of 5 mM succinate and 0.1 mM ADP. All values are given as the mean ± SD. Statistical analysis was performed using Student's *t*-test. Open circles, control group; closed circles, streptozotocin-treated group; triangles, streptozotocin + *C. aromatica*-treated group. ∗, $P < 0.05$ compared with *C. aromatica* -fed diabetic rats (mean ± SD).

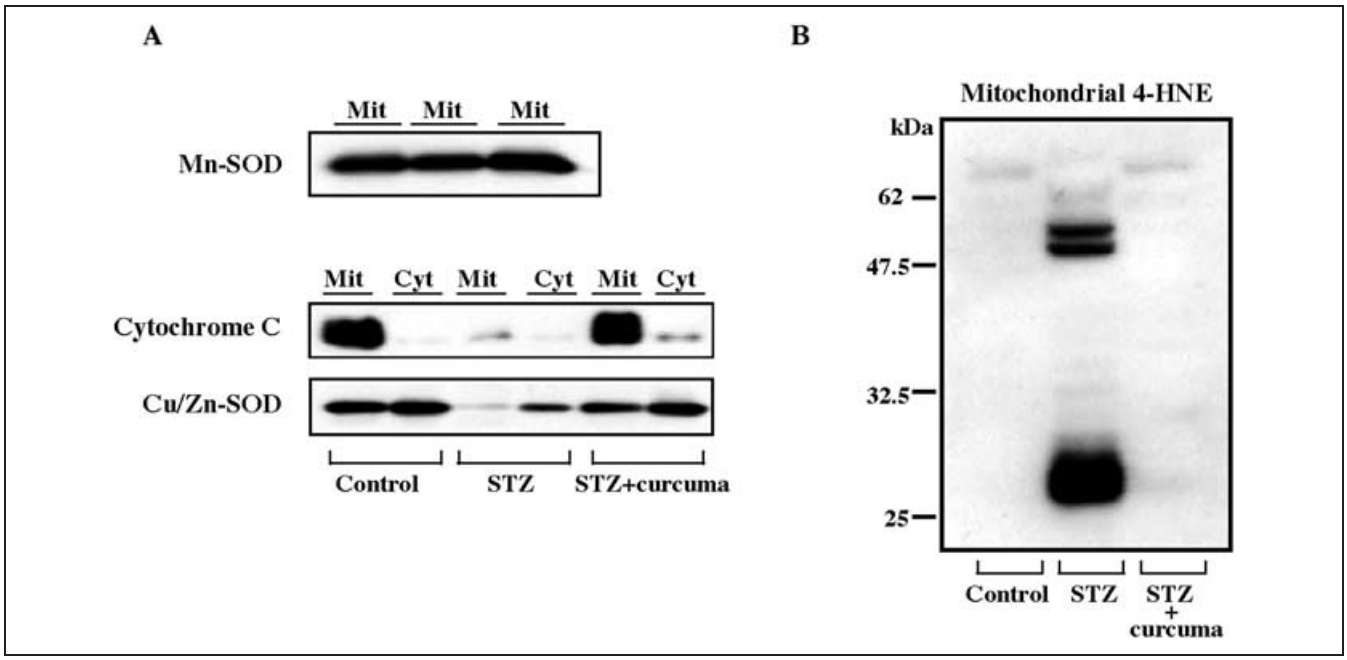

Figure 9—Effect of *C. aromatica* on renal mitochondrial proteins in streptozotocin-treated animals. (A) On week 7, the localization of Mn-SOD, SOD1, and cytochrome C in renal mitochondria and cytosol was analyzed by Western blot analysis. Equal amounts of protein (10 μg/lane) from mitochondria and cytosol were separated on a 15% SDS-PAGE, electrotransferred onto Immobilon-P membranes, and then analyzed with specific anti-Mn-SOD, SOD1 or cytochrome C antibodies. Mit = mitochondria; Cyt = cytosol. (B) Occurrence of 4-HNE in renal mitochondria (10 μg protein/lane) was analyzed immunochemically using specific anti-4HNE antibody. Data show 1 representative result of 3 separate experiments.

to their proximity to the sites of occurrence of lipid peroxidation and/or strong nucleophilicity of their lysyl residues. Identification and characterization of these proteins are under our current investigation.

Oxidative injury of mitochondrial membranes induces membrane permeability transition and releases cytochrome C, a prerequisite reaction to induce apoptosis (Kanno and others 2004). Recent studies revealed that Cu/Zn-SOD is also localized in intermembranous space of mitochondria and effectively inhibits oxidative stress in and around mitochondria (Kira and others 2002). The present work also shows that cytochrome C and Cu/Zn-SOD localized in renal mitochondria markedly decreased in streptozotocin-treated animals. However, the cytosolic level of cytochrome C was below detectable levels and that of Cu/Zn-SOD decreased markedly. In contrast, levels of Mn-SOD in mitochondrial matrix remained unchanged. These results indicate that selective degradation of cytochrome C and Cu/Zn-SOD in renal cytosol would have occurred in streptozotocin-treated hyperglycemic animals. Possible activation of ubiquitin/proteasome system in the kidney of hyperglycemic animals should be studied further.

The present work demonstrates for the first time that administration of *C. aromatica* inhibited the pathologic events induced by streptozotocin except for the occurrence of hyperglycemia and loss of body weight. Although the occurrence of 8-OHdG in the kidney (but not in the urine) was inhibited by *C. aromatica* with concomitant inhibition of renal dysfunction, hypoalbuminemia, proteinuria, and hyperlipidemia, oxidative injury of extrarenal tissue(s) might principally be responsible for the urinary excretion of 8-OHdG. This observation also suggests that protection of mitochondria in renal proximal tubule cells would be the primary site of the protective action of *C. aromatica*. In this context, *C. longa* and its major constituent curcumin have been reported to inhibit various complications in diabetic animals without inhibiting hyperglycemia (Suryanarayana and others 2005). It should be noted that the concentration of curcumin in *C. aromatica* is less than 9% of that in *C. longa*. Preliminary experiments using the same dose of curcumin involved in 1.5% of the dietary *C. aromatica* revealed that such a low dose of curcumin had no appreciable effect on the streptozotocin-induced diabetic complications. Thus, other components than curcumin might be responsible for the inhibitory effects of dietary *C. aromatica* on diabetic complications. Since *C. aromatica* contains various factors, including essential oils enriched with β-elemene, curcumol, and curdione (Wu and others 2000), the roles of these compounds on diabetic complications should be studied further. Apart from the identification and characterization of the effective molecules in *C. aromatica,* this vegetable food has therapeutic potential in the prevention of diabetic complications in hyperglycemic subjects.

Conclusions

Administration of *C. aromatica*-containing diet inhibited the occurrence of streptozotocin-induced renal injury, hyperlipidemia, and loss of body weight without suppressing hyperglycemia. Biochemical analysis revealed that mitochondrial DNA and functions in renal proximal tubule cells were also protected by *C. aromatica*. These results suggest that *C. aromatica* may have therapeutic potential for the prevention of hyperglycemia-associated diabetic complications, including renal injury.

Acknowledgments

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