Accumulation of 8-Hydroxy-2′-Deoxyguanosine and Mitochondrial DNA Deletion in Kidney of Diabetic Rats

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Oxidative stress may contribute to the pathogenesis of diabetic nephropathy. However, the detailed molecular mechanism remains uncertain. Here, we report oxidative mitochondrial DNA (mtDNA) damage and accumulation of mtDNA with a 4,834-bp deletion in kidney of streptozotocin-induced diabetic rats. At 8 weeks after the onset of diabetes, levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), which is a marker of oxidative DNA damage, were significantly increased in mtDNA from kidney of diabetic rats but not in nuclear DNA, suggesting the predominant damage of mtDNA. Semiquantitative analysis using PCR showed that the frequency of 4,834-bp deleted mtDNA was markedly increased in kidney of diabetic rats at 8 weeks, but it did not change at 4 weeks. Intervention by insulin treatment starting at 8 weeks rapidly normalized an increase in renal 8-OHdG levels of diabetic rats, but it did not reverse an increase in the frequency of deleted mtDNA. Our study demonstrated for the first time that oxidative mtDNA damage and subsequent mtDNA deletion may be accumulated in kidney of diabetic rats. This may be involved in the pathogenesis of diabetic nephropathy.
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Diabetic nephropathy is the major cause of morbidity and mortality in diabetic patients. It is more rapidly liable to functional deterioration compared with other types of chronic renal disease and finally progresses to renal failure requiring dialysis therapy. Several mechanisms have been proposed for the pathogenesis of diabetic vascular complications that include nephropathy, such as hyperfiltration (1), increased production of advanced glycation end products (AGEs) (2), activation of protein kinase C (3–5), enhanced polyol pathway (6,7), and enhanced oxidative stress (8–10). A number of in vitro and in vivo studies suggest that oxidative stress is increased in diabetic patients and animal models of diabetes (8–14). Although enhanced oxidative stress may contribute to the initiation and development of diabetic nephropathy, the detailed molecular mechanism remains uncertain.

In general, oxidative stress, including reactive oxygen species (ROS), can damage cellular macromolecules. Among the oxidative damages, base modifications, such as oxidation of deoxyguanosine to 8-hydroxy-2′-deoxyguanosine (8-OHdG) and subsequent mutations of mitochondrial DNA (mtDNA), have received increasing attention in recent years. It is widely accepted that mtDNA is 10–20 times more vulnerable to oxidative damage and subsequent mutations than nuclear DNA (15–17). More than 50 pathogenic mtDNA mutations associated with or responsible for specific human diseases have been reported. Congenital mtDNA mutations as well as oxidative stress–induced mtDNA mutations may be related to the pathophysiology of various diseases. It has been shown that oxidative stress–induced mtDNA mutations may be related to aging-related organ dysfunction (18–23) and several degenerative diseases (24,25). We speculated that enhanced oxidative stress might induce mtDNA damage and subsequent mtDNA mutations in kidney of diabetic subjects, and this might be involved in the pathogenesis of diabetic nephropathy.

In this study, we first examined the oxidative DNA damage in kidney of streptozotocin (STZ)-induced diabetic rats by measuring the levels of 8-OHdG in kidney and urine. Second, we surveyed the frequency of mtDNA with a common deletion of 4,834 bp (23,26–28) in kidney of STZ-induced diabetic rats. In addition, we evaluated the effect of intervention by insulin treatment on 8-OHdG levels in mtDNA and the frequency of mtDNA with a 4,834-bp deletion in kidney of diabetic rats.

RESEARCH DESIGN AND METHODS

Animals. Male Wister rats were bred under pathogen-free conditions at Kyushu University Animal Center, Fukuoka, Japan. Two experimental groups were formed: STZ-induced diabetic rats and age-matched control rats. The animals had free access to tap water and standard chow (Clea Japan, Tokyo).

At 8 weeks of age, the rats were injected intraperitoneally with STZ (Sigma, St. Louis, MO) at 80 mg/kg body wt. One day after injection, the development of diabetes was verified by the presence of hyperglycemia (plasma glucose level ≥300 mg/dl). The rats were used for experiments at 4 or 8 weeks after the onset of diabetes. This study was approved by the Committee of the Ethics on Animal Experiments, Graduate School of Medical Sciences, Kyushu University.

Measurement of urinary 8-OHdG. Two-milliliter samples of well-mixed 24-h urine were collected from rats. The samples were purged of air with a steam of nitrogen to prevent the artificial formation of 8-OHdG and stored frozen at −80°C until analyzed. Urine samples were centrifuged at 2,000*g* for 20 min, and after proper dilution, the supernatant was used for the determination of 8-OHdG by a competitive enzyme-linked immunosorbent assay (ELISA) kit (8-OHdG Check; Japan Institute for the Control of Aging, Fukuroi,

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8-OHdG, 8-hydroxy-2′-deoxyguanosine; AGE, advanced glycation end product; COX, cytochrome C oxidase; ELISA, enzyme-linked immunosorbent assay; mtDNA, mitochondrial DNA; ROS, reactive oxygen species; STZ, streptozotocin.

TABLE 1
Primers used for PCR amplification of deleted mtDNA and wild-type mtDNA

Name	Sequence 5′→3′	Binding site on rat mtDNA
L588	AGCCGTCCTACTACTTCTCTCACTG	5881 to 5906
L768	GCTTAGAGCGTTAACCTTTTAAG	7687 to 7709
L782	TTTCTTCCCAAACCTTTCCT	7825 to 7844
L797	GTTCCCATCAATTCTATTCC	7978 to 7997
L1576	GGTTCTTACTTCAGGGGCCATC	15768 to 15789
H1296	CAGCAGTTATGGATGTGGCG	12981 to 12962
H1309	AAGCCTGCTAGGATGCTTC	13117 to 13099
H1511	GGCCTCCGATTCATGTTAAGACTA	15142 to 15118
H1626	GTGGAATTTTCTGAGGGTAGGC	16288 to 16267

Japan). The determination range was 0.5–200 ng/ml. The urinary 8-OHdG was expressed as total amounts excreted in 24 h.

Mesaurement of 8-OHdG levels in kidney. At 8 weeks after the onset of diabetes, rats were anesthetized by intraperitoneal injection of pentobarbital (80 mg/kg) before performance of surgical procedures. The kidney was rapidly excised and separated into cortices and papillae. Then, the samples were frozen in liquid nitrogen and kept at −80°C until analyzed. On the day of assay, the samples were homogenized in 5 ml of 50 mmol/l Tris-HCl (pH 7.4) with 20 strokes of Dounce homogenizer. The homogenates were centrifuged at 800*g* for 10 min to precipitate nuclear fraction. The supernatant was again centrifuged at 7,000*g* for 10 min to yield mitochondrial fraction. One milliliter of solution containing 10 mmol/l EDTA, 10 mmol/l Tris-HCl (pH 8.0), 150 mmol/l NaCl, and 0.2% SDS was added to each fraction. After homogenization, the mixture was incubated at 56°C for 70 min with proteinase K (700 μg/ml) and then heated at 95°C for 10 min. DNA was extracted with equal volumes of phenol, chloroform, and isoamyl alcohol (25:24:1) and then with chloroform. DNA was precipitated with a one-tenth volume of 2 mol/l NaCl and two volumes of 70% ethanol at −20°C for 2 h and rinsed with ethanol. DNA was resuspended in 10 mmol/l Tris-HCl and 0.1 mmol/l EDTA (pH 8.0). Five microliters of 200 mmol/l sodium acetate buffer (pH 4.8) and 5 μg nuclease P1 (USB, Cleveland, OH) (1 mg/ml in 10 mmol/l sodium acetate) were added to 45-μl DNA samples. After purging with a nitrogen steam, the mixtures were incubated at 37°C for 1 h to digest the DNA to nucleotides. Then, 5 μl of 500 mmol/l Tris-HCl (pH 8.0), 10 mmol/l $MgCl_2$, and 0.6 units *Escherichia coli* alkaline phosphatase (Toyobo, Osaka, Japan) were added to the samples. After purging with a nitrogen steam, the mixtures were incubated at 37°C for 1 h to hydrolyze the nucleotides to nucleosides. The nucleoside samples were used for the determination of 8-OHdG by competitive ELISA kit as described above.

Semiquantitation of mtDNA deletion using PCR. Total DNA was isolated from kidneys as described above at 4 or 8 weeks after the onset of diabetes. Primers for the PCR amplifications used are shown in Table 1. The primer sets for amplification of 4,834-bp deletion mtDNA were L782 and H1309, L768 and H1296, and L797 and H1309. The primer set for control amplification of wild-type mtDNA was L1576 and H1626. For amplification of a longer mtDNA fragment (5,881–15,142), a primer set of L588 and H1511 (29) was used. All primers were synthesized by Invitrogen (Tokyo). Sequence and numbering are based on the published rat mtDNA sequence (23,26).

PCRs contained 0.2 mmol/l dNTP, 0.3 μmol/l of each primer, 1.0 unit *Taq* polymerase (Toyobo), and various amounts (0.01–0.2 μg) of starting total DNA as template in a 50 μl reaction solution. The thermal cycling condition was started with one cycle at 94°C for 2 min. This was followed by 35 cycles at 94°C for 15 s, 60°C for 30 s, 68°C for 1 min, and 68°C for final extension for 5 min. Amplification was carried out in a Thermal Cycler system (Astec, Fukuoka, Japan). PCR products were electrophoresed on a 5% polyacrylamide gel and visualized by bistragreen staining. The number of mtDNA deletion products was determined for each sample by densitometry.

Using primer sets of L588 and H1511, multiple PCR products were amplified from mtDNA templates isolated from diabetic kidney but not from mtDNA templates isolated from control kidney (Fig. 1*A*). These results suggest that multiple mtDNA deletions might exist in diabetic kidney. Therefore, in the present study, the frequency of 4,834 mtDNA deletion, which was reported to be one of the most frequent deletions (26–28), was evaluated for quantitation. Three different sets of primers were used on all samples to ensure that the PCR products seen were not the result of primer misannealing. The 4,834 mtDNA deletion products shifted appropriately in size when different sets of primers were used, as shown in Fig. 1*B*. Levels of the 4,834-deleted mtDNA were expressed as the ratio of the signal intensity relative to that for the wild-type mtDNA.

Immunoblotting of cytochrome c oxidase. Powdered frozen tissues from kidney, as described above, were homogenized on ice with a Polytron for 20 s in a buffer containing 50 mmol/l Tris-HCl (pH 7.4), 2% SDS, 1 mmol/l phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin and then homogenized again with 20 strokes of a Dounce homogenizer. The homogenates were centrifuged at 10,000*g* for 20 min to precipitate cell debris. The samples were separated on 12% SDS gel under reducing conditions and then immunoblotted with monoclonal anti–cytochrome C oxidase (COX) subunit I or anti-COX subunit IV antibody (Molecular Probe, Eugene, OR). The blots were then incubated with horseradish peroxidase–linked second antibody followed by chemiluminescence detection, according to the manufacture's instructions (Pierce, Rockford, IL)

Intervention by insulin treatment. Starting at 8 weeks after the onset of diabetes, STZ-induced diabetic rats were treated with insulin. Intermediate-acting insulin (Novolin N; Novo Nordisk, Tokyo) was subcutaneously administered twice a day until the animals were killed. The doses of insulin to be given were determined each time from a sliding scale, according to blood glucose levels, to achieve strict glucose control. At 1 and 2 weeks after the induction of insulin treatment, the 8-OHdG levels in urine and kidney and the frequency of mtDNA deletion in kidney were measured.

Statistical analysis. All values shown are expressed as means ± SE. Statistical analysis was performed by ANOVA followed by Fisher's comparison test.

RESULTS

Urinary 8-OHdG excretion. The total amounts of urinary 8-OHdG excretion were significantly greater in STZ-induced diabetic rats than in age-matched control rats at both 4 weeks (2,089 ± 259 ng/day, $n = 6$ vs. 793 ± 44 ng/day, $n = 6$, $P < 0.01$) and 8 weeks after the onset of diabetes (2,280 ± 230 ng/day, $n = 6$ vs. 632 ± 56 ng/day, $n = 6$, $P < 0.001$).

8-OHdG contents in kidney tissues. The levels of 8-OHdG in the mtDNA were significantly increased in both renal cortices and papillae of diabetic rats as compared with those from the control rats at 8 weeks after the onset of diabetes (cortices: 11.8 ± 1.1 ng/mg DNA, $n = 6$ vs. 4.2 ± 0.9 ng/mg DNA, $P < 0.01$; papillae: 19.2 ± 3.3 ng/mg DNA, $n = 6$ vs. 5.2 ± 2.1 ng/mg DNA, $P < 0.01$) (Fig. 2*A*). In contrast, the levels of 8-OHdG in nuclear DNA were not significantly increased in either renal cortices or papillae from diabetic rats (Fig. 2*B*).

mtDNA deletion in kidney tissues. PCR amplification showed that the 4,834 mtDNA deletion products from diabetic kidney were increased in a dose-dependent manner from 0.01 to 0.2 μg of template DNA, but those from control kidney had very low levels at the same doses of starting template DNA (Fig. 3). The mtDNA deletion products from diabetic kidney were increased in a cycle-dependent manner from 25 to 37 cycles as well (Fig. 3). The wild-type mtDNA products were also increased in a

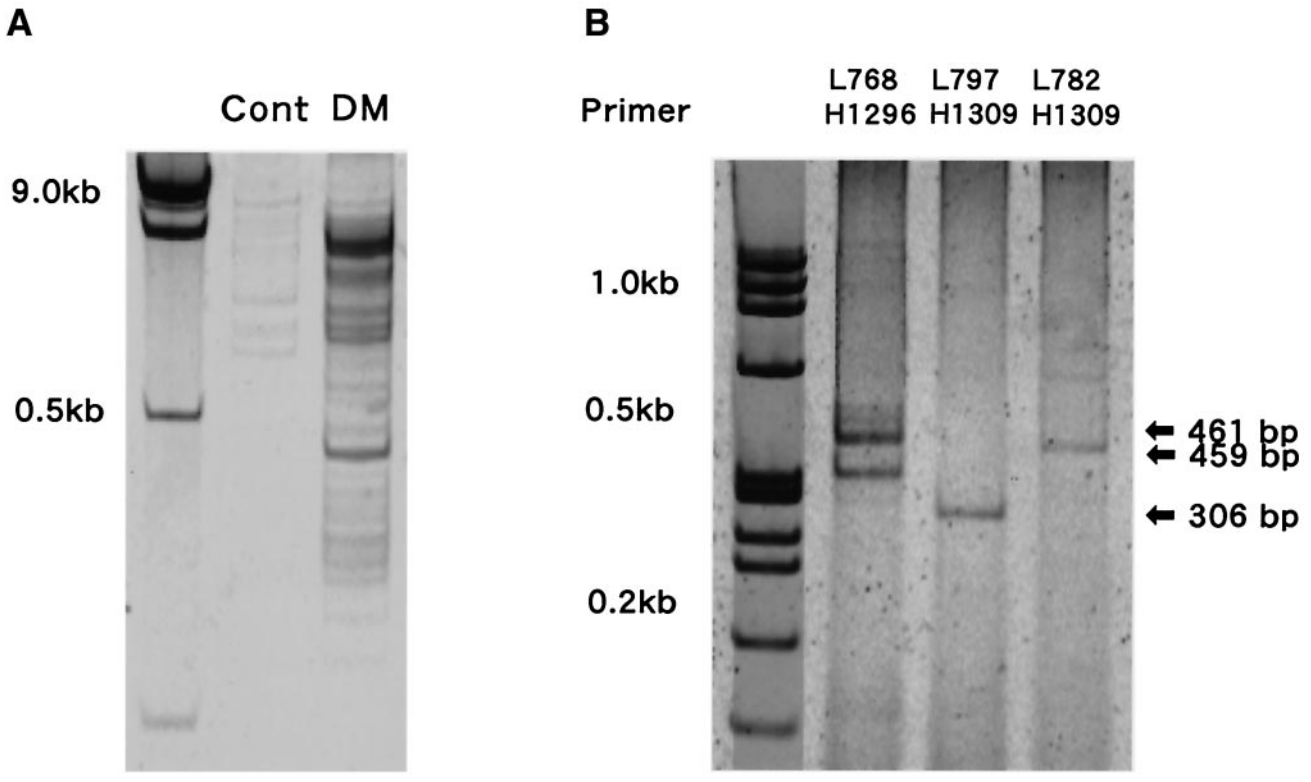

FIG. 1. *A*: **Multiple PCR products amplified from mtDNA using primer sets of L588 and H1511. The total DNA extracted from kidney of control and diabetic rats (DM) were used as a template.** *B*: **PCR products amplified from mtDNA with a 4,834-bp deletion using three different sets of primers. The total DNA extracted from kidney of diabetic rats was used as a template. PCR products amplified from mtDNA with a 4,834-bp deletion were the 459-, 306-, and 461-bp fragments corresponding to three different primer sets of L768 and H1296, L782 and H1309, and L797 and H1309, respectively. PCR amplification was carried out as described in RESEARCH DESIGN AND METHODS. PCR products were electrophoresed on a 5% polyacrylamide gel and visualized by bistragreen staining. Similar results were obtained on all samples.**

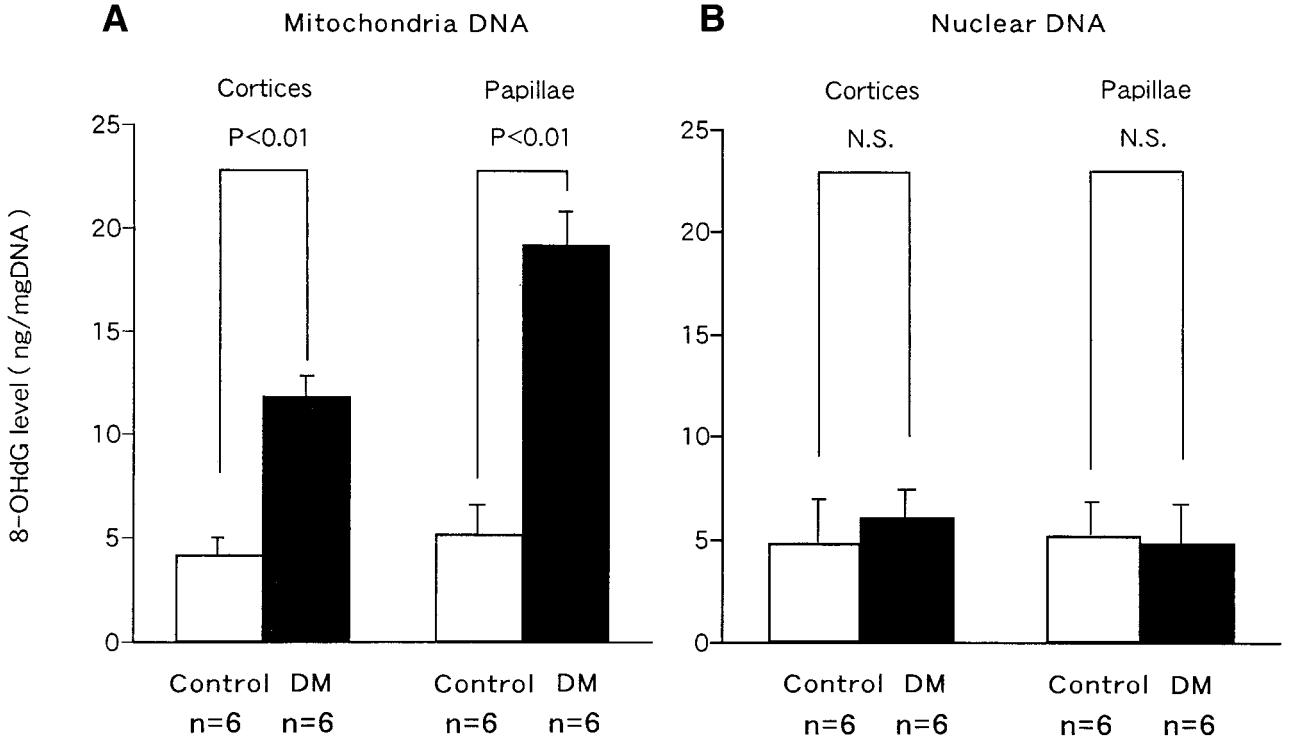

FIG. 2. Levels of 8-OHdG in mtDNA (*A*) and nuclear DNA (*B*) from kidney of control and STZ-induced diabetic rats (DM). mtDNA and nuclear DNA were extracted separately from renal cortices and papillae of control and diabetic rats. Both extracted DNAs were enzymatically hydrolyzed into nucleosides, and levels of 8-OHdG were measured by ELISA kit. NS, not significant. Data are means ± SE.

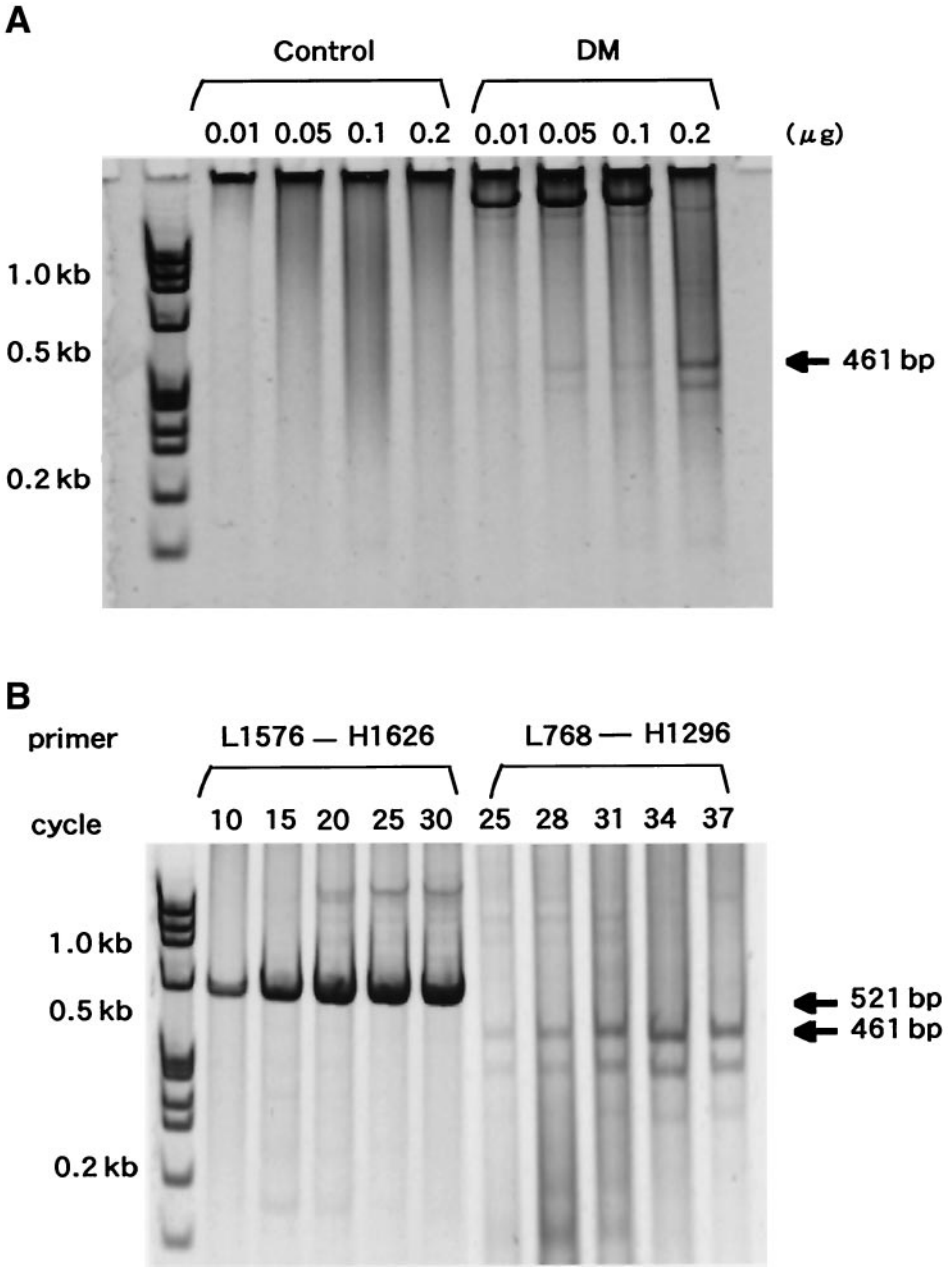

FIG. 3. PCR amplification of mtDNA with a 4,834 deletion from kidney of control and STZ-induced diabetic rats (DM). PCR products from mtDNA with a 4,834 deletion were electrophoresed on a 5% polyacrylamide gel containing bistragreen and analyzed by densitometry. *A*: PCR amplification of mtDNA with a 4,834 deletion was carried out using various doses (0.01, 0.05, 0.1, and 0.2 μg) of starting DNA template from kidney of control and diabetic rats. *B*: PCR amplification of wild-type mtDNA and deleted mtDNA was carried out in a condition of various cycles (10, 15, 20, 25, and 30 cycles for wild-type mtDNA and 25, 28, 31, 34, and 37 cycles for deleted mtDNA). A primer set of L768 and H1296 was used for amplification of deleted mtDNA, and a primer set of L1576 and H1626 was used for wild-type mtDNA.

cycle dependency from 10 to 30 cycles (Fig. 3). Similar results were obtained on all samples.

To compare the frequency of mtDNA deletion between diabetic and control rats, the PCR products amplified in a condition of 0.2 μg template DNA and 35 cycles were quantified by densitometoric analysis. These semiquantitative analysis showed that the kidneys from diabetic rats had low levels of the mtDNA deletion products at 4 weeks after the onset of diabetes and that the difference between diabetic and control rats was not statistically significant. In contrast, at 8 weeks, the kidneys from diabetic rats exhibited markedly higher levels of mtDNA deletion products compared with those from control rats, as shown in Fig. 4.

Effect of intervention by insulin treatment. STZ-induced diabetic rats were treated with insulin, starting 8 weeks after the onset of diabetes. Glycemic control was achieved as follows: mean plasma glucose levels and fructosamine levels on the last day were 113.5 ± 6.7 mg/dl and 169.0 ± 1.2 μmol/l in control rats, 474.5 ± 33.9 mg/dl and 292.0 ± 15.32 μmol/l in nontreated diabetic rats, and 109.5 ± 2.0 mg/dl and 160.3 ± 2.5 μmol/l in insulin-treated diabetic rats, respectively. In nontreated diabetic rats, total amounts of urinary 8-OHdG were gradually in-

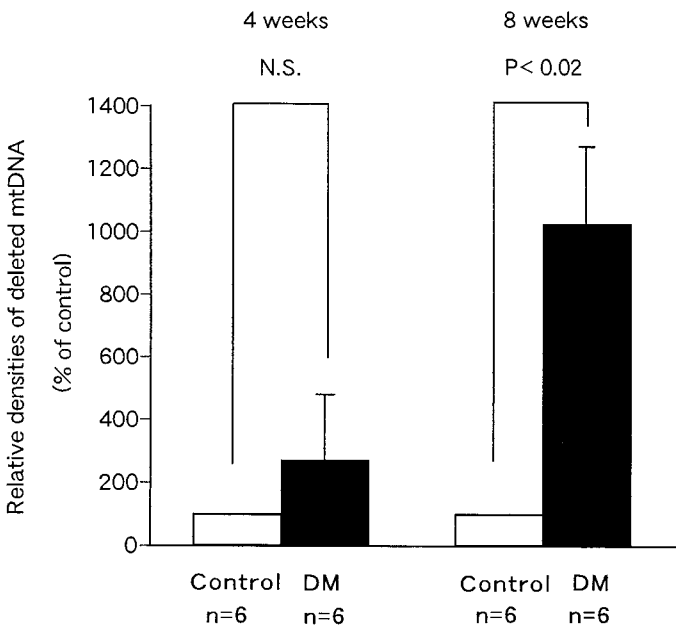

FIG. 4. Semiquantitive analysis of mtDNA with a 4,834 deletion in kidney of control rats and STZ-induced diabetic rats (DM). PCR products from mtDNA with a 4,834 deletion were electrophoresed on a 5% polyacrylamide gel containing bistragreen and analyzed by densitometry. The columns show the relative densities of PCR products from deleted mtDNA from kidney of control and diabetic rats at 4 or 8 weeks after the onset of diabetes. Data are means ± SE.

creased, at least up to 10 weeks after the onset of diabetes, as shown in Fig. 5. Insulin treatment completely reversed the increased amounts of urinary 8-OHdG to control levels after 1 and 2 weeks.

In parallel, the levels of 8-OHdG in the mtDNA from kidney were also completely normalized to control levels after 2 weeks of insulin treatment (Fig. 6*A* and *B*). However, the increased frequency of deleted mtDNA in kidney of diabetic rats remained elevated to a similar degree as that in nontreated diabetic rats, even after 2 weeks of insulin treatment (Fig. 6*C*). Therefore, to exclude the effect of STZ on this deletion, insulin treatment was also performed between 4 and 8 weeks after the onset of diabetes. The institution of glycemic control with insulin treatment achieved near euglycemia between 4 and 8 weeks after the onset of diabetes and completely prevented the increased frequency of deleted mtDNA as well as increased the 8-OHdG levels in kidney of diabetic rats to control levels.

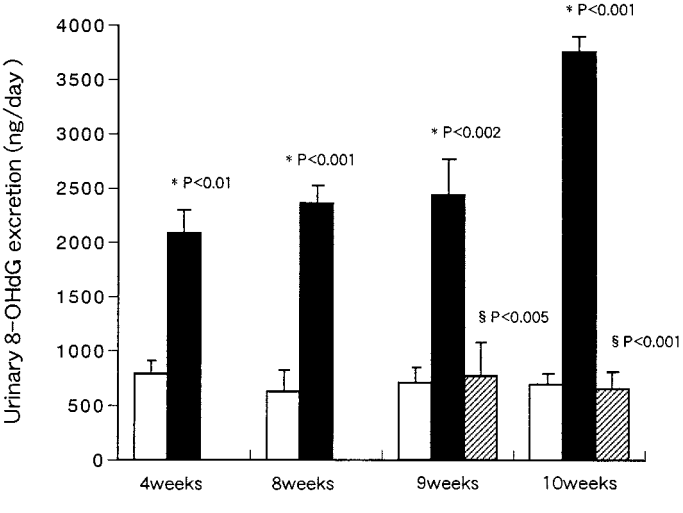

FIG. 5. Effect of intervention by insulin treatment on the increase in urinary 8-OHdG excretion in diabetic rats. Diabetic rats were treated by insulin starting at 8 weeks after the onset of diabetes. The amounts of urinary 8-OHdG were measured by ELISA kit as described in RESEARCH DESIGN AND METHODS. □, control rats (n = 4); ■, nontreated diabetic rats (n = 4); ▨, insulin-treated diabetic rats (n = 4). *P value versus control rats; §P value versus nontreated diabetic rats. Data are means ± SE.

Western blot analysis of COX. According to Western blot analysis, the amounts of mtDNA-encoded COX subunit I were significantly decreased ($P < 0.01$) in kidney from diabetic rats compared with those from control rats (Fig. 7). In contrast, the amounts of nuclear DNA–encoded COX subunit IV did not differ between diabetic and control rats (Fig. 7). In parallel with the changes of mtDNA deletion, increased amounts of COX subunit I in diabetic rats were completely normalized to control levels by preventive insulin treatment, and they remained elevated to a similar degree as those in nontreated diabetic rats after 2 weeks of interventive insulin treatment.

DISCUSSION

Enhanced oxidative stress has been considered to contribute to the pathological processes of diabetic complications, including nephropathy. However, the detailed biomolecular mechanism remains to be elucidated. In general, oxidative stress can affect nucleic acids and generate various modified bases in DNA. Among the latter, 8-OHdG is the most abundant and appears to play a crucial role in mutagenesis (30). Increased levels of 8-OHdG have been reported in urine (11,31), mononuclear cells (11,32), and skeletal muscles (12) of diabetic patients. Ha et al. (13) have also shown that the levels of 8-OHdG are increased in kidney tissues of STZ-induced diabetic rats. In the present study, we further revealed that levels of 8-OHdG were increased in mtDNA from both cortices and papillae of diabetic rats at 8 weeks after the onset of diabetes. In contrast, the levels of 8-OHdG in nuclear DNA were not significantly increased in either cortices or papillae of diabetic rats, consistent with the previous finding that mtDNA is more sensitive to oxidative stress compared with nuclear DNA. Changes in cortical mtDNA as well as papillary mtDNA might reflect those occurring in tubular cells, given the relative mass of the tubular cells versus glomerular cells in renal cortices. Changes in glomerular mtDNA remain to be elucidated. In parallel with the results of renal tissues, the amounts of urinary 8-OHdG excretion were markedly increased in diabetic rats, and those increases were reversed by insulin treatment. Although urinary 8-OHdG (33) and serum 8-hydroxyguanine levels (34) are supposed to be markers of the total systemic oxidative stress in vivo, the relative contribution of renal 8-OHdG to urinary 8-OHdG should be evaluated in future studies.

As the possible consequence of increased formation of 8-OHdG, we showed the increase in mtDNA with a 4,834 deletion in kidney of diabetic rats. The kidneys of diabetic rats exhibited markedly higher levels of deleted mtDNA compared with those of control rats at 8 weeks after the onset of diabetes, but not at 4 weeks. These results suggest that deleted mtDNA may be accumulated with duration of

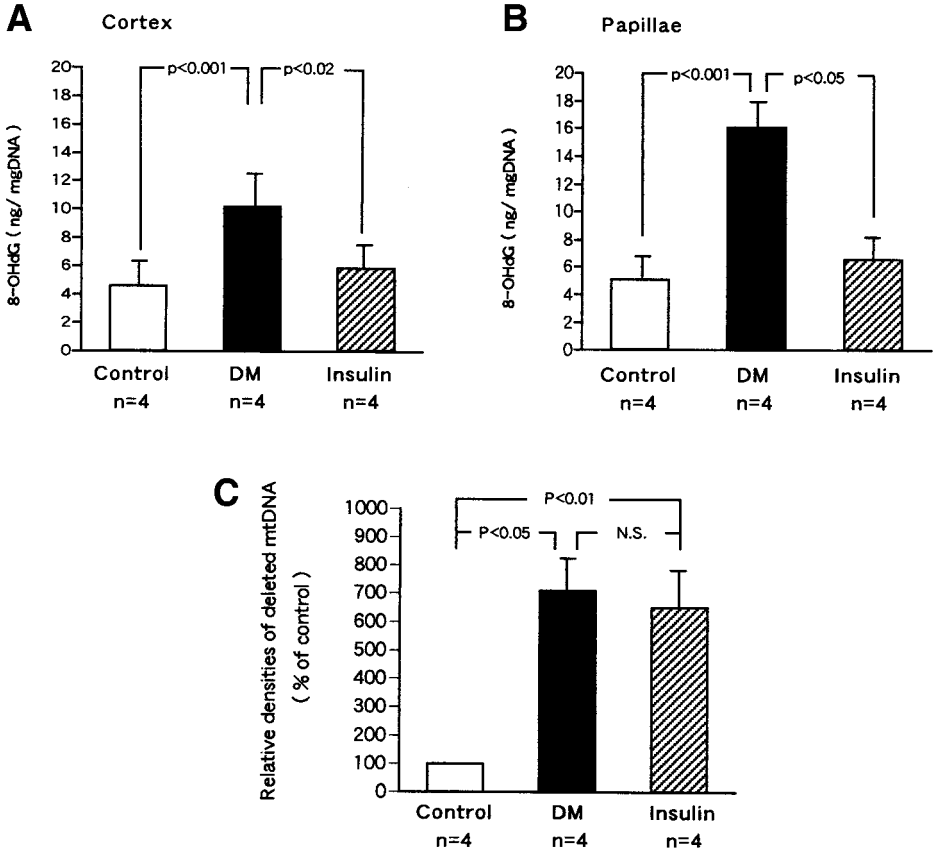

FIG. 6. Effect of intervention by insulin treatment on 8-OHdG levels and mtDNA deletion in diabetic kidney. Diabetic rats were treated by insulin starting at 8 weeks after the onset of diabetes. The amounts of 8-OHdG levels in mtDNA from renal cortices (*A*) and papillae (*B*) were measured by ELISA kit, as described in RESEARCH DESIGN AND METHODS. *C*: PCR products from mtDNA with a 4,834 deletion were electrophoresed on a 5% polyacrylamide gel containing bistragreen and analyzed by densitometry. The columns show the relative densities of PCR products from deleted mtDNA from kidney of control, nontreated diabetic rats (DM), and insulin-treated diabetic rats (insulin) at 8 weeks after the onset of diabetes. Data are means ± SE.

diabetes in kidney tissues of diabetic rats. This specific large-scale deletion of mtDNA has been reported to exist in organs of aged rats (26–28). The age-associated large-scale deletions seen most often in humans are the 4,977- and 7,436-bp deletions, which are among the many mtDNA deletions first reported in the muscle of patients with Kearns-Sayer syndrome or chronic progressive external ophthalmoplegia syndrome (35–37). These deletions in rats and humans are flanked by two homologous repeats. These homologous repeats are supposed to induce such specific large-scale deletions by slip-mispairing during DNA replication (38). Because these deletions span a region that encodes five kinds of tRNA, respiratory enzyme subunits in complex I, complex IV, and complex V, the functions of the respiratory enzymes containing deleted mtDNA-encoded proteins subunits may decline gradually in the tissue cells as the deleted mtDNA are accumulated. The present results show that the proportion of deleted mtDNA to the total mtDNA reached ~0.1% at 8 weeks after the onset of diabetes, using a kinetic PCR analysis. This level of deletion observed in kidney tissues of diabetic rats may still be too low to affect overall organ function. However, it is likely that this common deletion in mtDNA may be accompanied by other multiple deletions, as previously shown in aging tissues (39–41). According to Katsumata et al. (41), the total detection system for mtDNA deletions revealed that 235 types of deletions existed with various sizes in the heart of a 78-year-old human. The present study also suggested that multiple deletions might exist in kidney tissues of diabetic rats. Furthermore, the present study showed that the amounts

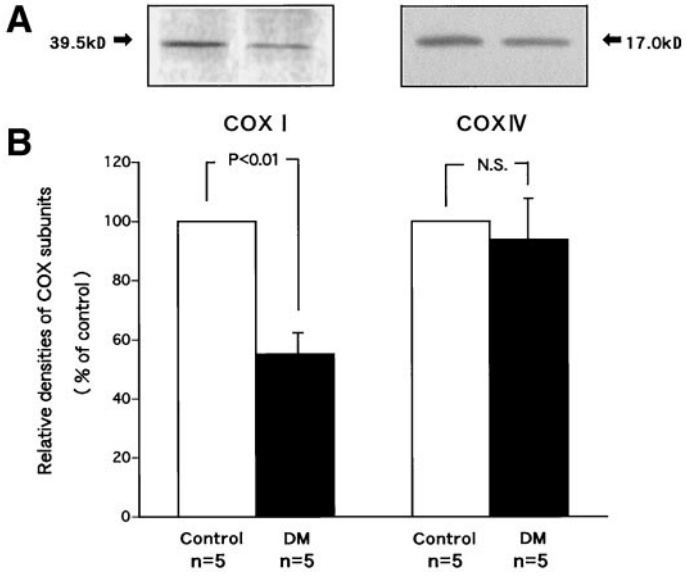

FIG. 7. Western blot analysis of COX I and COX IV in kidney from control and diabetic rats (DM). *A*: Western blots of representative experiment. *B*: densitometry analysis of Western blots. Data are mean percentage of control ± SE.

of mtDNA-encoded COX subunit were significantly decreased in kidney tissues of diabetic rats as one of the possible consequences of mtDNA deletions. Thus, the defective respiratory chain induced by mtDNA mutations may generate more ROS and free radicals, which further enhance the oxidative damage to mtDNA as well as other biomolecules. Finally, such a vicious cycle of oxidative damage may result in deterioration in ATP synthesis and lead to cellular dysfunction and apoptosis (42,43). Oxidative stress and subsequent mtDNA mutations may contribute to the development of diabetic nephropathy. The correlation between cumulative effect of total multiple deletions and functional changes in kidney tissues of diabetes should be evaluated in future studies.

In addition, we examined the effect of intervention by insulin treatment on the increases in 8-OHdG level and deleted mtDNA in kidney tissues of diabetes. It is of great interest that intervention by insulin treatment starting at 8 weeks after the onset of diabetes rapidly normalized both increases in urinary 8-OHdG excretion and 8-OHdG levels in kidney of diabetic rats. This result clearly confirmed that the increases in urinary 8-OHdG excretion and 8-OHdG levels in mtDNA were caused by the diabetic state and not by a direct toxic effect of STZ. Hyperglycemia, a key clinical manifestation of diabetes, is supposed to generate ROS through various mechanisms, such as increased formation of AGEs (2), enhanced polyol pathway (6,7), increased superoxide release from mitochondria (44), and activation of NAD(P)H oxidase (45). Such rapid reversibility at least suggests that the contribution of AGEs to enhanced oxidative stress may not be very strong, considering that it would take longer to reverse the AGE level. The present results also suggest that repair of oxidative mtDNA damage induced by diabetes is very active. This finding is in agreement with recent reports (46,47) showing that the repair system of mtDNA damage may be more active than previously thought. The levels of 8-OHdG may represent the dynamic steady-state levels determined by the balance of oxidative attack and repair in mtDNA rather than simple accumulation of 8-OHdG. In contrast to 8-OHdG levels, an increased level of deleted mtDNA in diabetic kidney was not reversed by interventive insulin treatment. This result suggests that mtDNA mutations are not easily reversed by glycemic control after they have been established. This might be related to the phenomenon known as "the point of no return" observed in the progression of diabetic nephropathy. Further study regarding the effect of insulin treatment for a longer period is needed to prove this hypothesis.

In conclusion, our study demonstrated for the first time that the 4,834-bp deleted mtDNA was accumulated in parallel with an increased level of 8-OHdG in kidney of STZ-induced diabetic rats. This might be involved in the pathogenesis of diabetic nephropathy, and mtDNA might be a new therapeutic target for preventing the development of diabetic nephropathy.

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