Effects of Insulin and Antioxidant on Plasma 8-Hydroxyguanine and Tissue 8-Hydroxydeoxyguanosine in Streptozotocin-Induced Diabetic Rats

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Cumulating evidence suggests that enhanced oxidative stress may contribute to diabetic angiopathy. The levels of 8-hydroxydeoxyguanosine (8-OHdG) and 8-hydroxyguanine (8-OHG), indicators of oxidative DNA damage, in tissue or body fluid are increased in diabetic patients. However, it is unclear whether plasma 8-OHG correlates with tissue 8-OHdG and whether insulin or antioxidant treatment reduces plasma 8-OHG in diabetic state. In this study, we measured the 8-OHG levels in plasma as well as the 8-OHdG levels in liver and kidney in streptozotocin-induced diabetic rats (DR) treated with insulin (DR+I), insulin and probucol (DR+I/P), or insulin and vitamin E (DR+I/E). There was a correlation between plasma 8-OHG levels and tissue 8-OHdG levels (plasma 8-OHG vs. liver 8-OHdG: $r = 0.64$, $P < 0.001$; plasma 8-OHG vs. kidney 8-OHdG: $r = 0.38$, $P = 0.06$). DR had levels of plasma 8-OHG that were three times higher than control rats (CR), whereas they had levels of tissue 8-OHdG that were ~1.5–2 times higher. Plasma 8-OHG levels in DR were almost normalized by insulin treatment, although insulin partially corrected hyperglycemia (plasma 8-OHG: CR 3.3 ± 2.7 pmol/ml; DR 10.4 ± 2.3 pmol/ml, $P < 0.05$ vs. CR; DR with insulin 3.6 ± 1.0 pmol/ml, $P < 0.05$ vs. DR). However, tissue 8-OHdG levels in DR were significantly decreased by combined treatment with insulin and antioxidant (probucol or vitamin E), but not by insulin treatment alone. This data suggests that plasma 8-OHG could be a useful biomarker of oxidative DNA damage in diabetic subjects. The mechanism of differential response of plasma 8-OHG and tissue 8-OHdG to insulin and antioxidant treatment remains to be elucidated. *Diabetes* 50: 2837–2841, 2001

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CR, control rats; dG, deoxyguanosine; DR, diabetic rats; DR+I, DR with insulin treatment; DR+I/E, DR+I with vitamin E treatment; DR+I/P, DR+I with probucol treatment; HPLC, high-performance liquid chromatography; I/E, insulin and vitamin E treatment; 8-OHdG, 8-hydroxydeoxyguanosine; 8-OHG, 8-hydroxyguanine; ROS, reactive oxygen species; STZ, streptozotocin.

A role of reactive oxygen species (ROS) has been demonstrated as a cause of type 1 diabetes induced by chemicals such as alloxan and streptozotocin (STZ) in experimental animals (1). However, not only are oxygen radicals involved in the cause, but diabetic status itself is associated with increased production of ROS, and this condition in turn has been suggested as one of the pathogenic mechanisms of diabetic complications (2,3). A recent study has shown that blockade of hyperglycemia-induced ROS production reverses the pathways implicated in diabetic angiopathy: activation of protein kinase C, formation of advanced glycation end product, and elevated sorbitol content in cultured endothelial cells (4). It suggests that enhanced ROS production in diabetes may be a common pathway linking diverse pathogenic mechanisms of diabetic vascular complications. Therefore, assessment of oxidative stress in diabetic patients may be important for the prediction and prevention of diabetic complications.

A direct measurement of ROS production in target organs is not currently possible in vivo. However, it can be indirectly assessed by measurement of oxidative products because ROS, which is an inherently unstable molecule because of an unpaired electron, undergoes a series of interactions with biological macromolecules such as lipids, proteins, and DNA (5). In the diabetic state, the levels of markers for lipid peroxidation (e.g., malondialdehyde and thiobarbituric acid–reactive substances) and protein oxidation [e.g., *N*-(carboxymethyl)-lysine, *N*-(carboxymethyl)-hydroxylysine, and pentosidine] are elevated (6).

As a biomarker for oxidative DNA damage, 8-hydroxydeoxyguanosine (8-OHdG) in tissue or body fluid is known as a sensitive indicator (7–9). 8-OHdG, a major product of oxidative DNA damage, is produced by enzymatic cleavage after 8-hydroxylation of the guanine base of DNA (10–12). Because 8-OHdG is an unstable molecule, it is further hydrolyzed to 8-hydroxyguanine (8-OHG), and both 8-OHdG and 8-OHG are released into systemic circulation and secreted in urine. Previous studies have shown that diabetic patients have higher levels of 8-OHdG in the mononuclear cells and urine (13–15). Moreover, kidney 8-OHdG levels in STZ-induced diabetic rats (DR) is increased and reduced by insulin treatment (16). However, measurement of 8-OHdG has been hampered by the risk of auto-oxidation of deoxyguanosine (dG) during the prepa-

TABLE 1
Clinical characteristics of STZ-induced DR, DR+I, DR+I/P, or DR+I/E

	n	Body weight (g)	24-h urine volume (ml)	Blood glucose (mmol/l)	Average dose of insulin (unit)
CR	7	346.3 ± 9.4	19.1 ± 0.8	4.7 ± 0.2	—
DR	6	237.1 ± 18.3*	120.4 ± 20.1*	26.9 ± 0.6*	—
DR + I	7	342.2 ± 5.8†	49.2 ± 11.1†	14.3 ± 1.3*†	5.5 ± 0.8
DR + I/P	6	318.8 ± 8.3†	25.7 ± 4.0†	12.7 ± 2.1*†	6.5 ± 0.7
DR + I/E	7	323.4 ± 6.4†	31.9 ± 6.0†	16.5 ± 2.5*†	6.3 ± 0.4

Values are means ± SE. Diabetes in rats was induced by intraperitoneal injection of 60 mg/kg STZ. For insulin treatment, daily subcutaneous injection of intermediate-acting insulin (target blood glucose 11.1 to ~16.7 mmol/l) was given at 4:00 P.M. from the 3rd day after administration of STZ for 4 weeks. DR + I/P had subcutaneous insulin and probucol (1% wt/wt) in diet. DR + I/E had subcutaneous insulin and vitamin E–enriched diet (100 IU/kg food wt). *$P < 0.005$ vs. CR, †$P < 0.005$ vs. DR.

ration of a sample as well as during the work-up procedure for high-performance liquid chromatography (HPLC), resulting in false-high background and low sensitivity (17). Measurement of plasma 8-OHG may provide a more accurate and stable assay for oxidative DNA damage because 8-OHG is not affected by the work-up procedure (18). However, it is unclear whether serum 8-OHG could be a good indicator reflecting oxidative DNA damage in tissue. Moreover, there is little data regarding 8-OHG in the diabetic state.

In this study, we investigated a correlation between plasma 8-OHG and tissue 8-OHdG in male Sprague-Dawley rats. For the measurement of 8-OHG and 8-OHdG, we applied HPLC with electrochemical detection, which is the most sensitive and reliable method (18,19). We also investigated the effects of insulin and antioxidant (vitamin E and probucol) treatment on oxidative DNA damage in STZ-induced DR, using plasma 8-OHG and tissue 8-OHdG as biomarkers.

RESEARCH DESIGN AND METHODS

Animals. Male 7-week-old Sprague-Dawley rats were obtained from Daehan Laboratory Animal Research (Seoul, Korea). Animals were maintained under controlled temperature (21–23°C) and light (12:12 h light:dark cycle, lights on at 7:00 A.M.), with ad libitum access to food and water.

Experimental protocols. Diabetes in rats was induced by a single intraperitoneal injection of 60 mg/kg STZ (Sigma, St Louis, MO), as previously described (16). STZ was dissolved in 2.5 ml/kg citrate buffer (pH 4.5) just before injection. Rats in the control group (CR) ($n = 7$) received an intraperitoneal injection of an equal volume of citrate buffer instead of STZ. At 48 h after STZ injection, blood samples were obtained from the tail vein, and blood glucose concentrations were measured with a Surestep glucometer (Lifescan, Milpitas, CA). Successful induction of diabetes was defined as a blood glucose level of >11.1 mmol/l. On day 3 post–STZ treatment, DR were randomly assigned to four groups ($n = 6$–7 each group): diabetes with no treatment (DR), diabetes with insulin treatment (DR+I), diabetes with insulin and probucol treatment (DR+I/P), or diabetes with insulin and vitamin E treatment (DR+I/E). For insulin-treated groups, intermediate-acting insulin (Insulatard, Novo Nordisk, Copenhagen) was administered subcutaneously once a day (at 4:00 P.M.) until the animals were killed. Blood glucose levels were measured every other day, just before insulin injection. The doses of insulin were adjusted to reach target blood glucose levels of 11.1 to ~16.7 mmol/l. Probucol-treated rats had probucol (1% of food weight)-containing diet, and vitamin E–treated animals had vitamin E (100 IU/kg food weight)-enriched diets for 4 weeks. On day 28, rats were anesthetized with ether, and blood was obtained from the inferior vena cava. Plasma was separated by centrifugation (1,500 rpm) for 15 min at 4°C and stored at −70°C. Liver and kidney were quickly removed and stored at −70°C until DNA was extracted.

Measurement of tissue 8-OHdG. Liver and kidney DNA was extracted using DNA extractor WB kit (Wako, Osaka, Japan). The tissue 8-OHdG level was measured using the method described by Shigenaga et al. (20) and was expressed as a relative amount per 10^5 dG. To avoid the auto-oxidation of 8-OHdG during the experimental procedure, all solutions were saturated with argon gas, and all tubes were flushed with argon gas just before use.

Measurement of plasma 8-OHG. Determination of plasma 8-OHG levels was performed using the method of Park et al. (21). For the measurement of plasma 8-OHG, 1 ml plasma was spiked with 1,000 cpm [^{14}C]-OHG. Plasma protein was precipitated by the addition of an equal volume of acetonitrile, and precipitated protein was separated by centrifugation at 3,000*g* for 15 min at 4°C. Supernatant was transferred to a new tube and mixed with eight volumes of water. The resulting sample was applied to the preconditioned C18/OH solid-phase extraction column. The solid-phase extraction column was washed with 5 ml of 50 mmol/l KH_2PO_4 buffer (pH 7.5), and then retained compounds were eluted with 3 ml of 15% methanol in the same buffer. The elute was loaded into the immunoaffinity column prepared with monoclonal antibody for 8-OHG (kindly provided by Dr. Bruce Ames, University of California, Berkeley, CA). Purified 8-OHG was dissolved in 50 μl water and injected into an HPLC device equipped with a Beckman Ultrasphere ODS column (5 μm, 4.6 mm × 25 cm) and an electrochemical detector (Coulchem Model 5100 A; ESA). The height of the 8-OHG peak and the total radioactivity of the elute were measured. The height of the peak was used to determine the total amount of 8-OHG injected, which was the sum of the plasma 8-OHG and [^{14}C]-OHG added. The amount of 8-OHG was determined by subtracting the amount of [^{14}C]-OHG injected from the total amount of 8-OHG injected. The amount of [^{14}C]-OHG injected was determined from the calibration curve of the peak height of [^{14}C]-OHG. The radioactivity of the elute was used to determine the amount of loss of 8-OHG during the purification procedure using the immunoaffinity column.

Analysis of data. All data presented were expressed as the mean ± SE. Correlations between plasma 8-OHG levels and tissue 8-OHdG levels were analyzed by Spearman's correlation because the distributions of plasma 8-OHG levels and kidney 8-OHdG levels were skewed. The levels of 8-OHG or 8-OHdG between groups were compared by analysis of variance with the post hoc least significance difference test. $P < 0.05$ was considered to be significant.

RESULTS

Metabolic characteristics of the animals. Blood glucose levels in DR were about five times higher than those in CR at 4 weeks post–STZ treatment (DR 26.9 ± 0.6 mmol/l vs. CR 4.7 ± 0.2 mmol/l; $P < 0.005$) (Table 1). DR had significantly higher 24-h urine volume and lower body weight compared with CR (24 h urine volume: 120.4 ± 20.1 vs. 19.1 ± 0.8 ml for DR vs. CR, $P < 0.005$; body weight: 237.1 ± 18.3 vs. 346.3 ± 9.3 g for DR vs. CR, $P < 0.005$) (Table 1). Insulin treatment attenuated the hyperglycemia, weight loss, and polyuria seen in DR, although they were not completely reversed to the levels of CR by insulin treatment (blood glucose: 26.9 ± 0.6 vs. 14.3 ± 1.3 mmol/l for DR vs. DR+I; $P < 0.005$) (Table 1). There was no significant difference in body weight, 24-h urine volume, blood glucose level, and injected insulin dose between DR+I and DR+I/P or between DR+I and D+I/E (Table 1).

Correlation between plasma 8-OHG levels and tissue 8-OHdG levels. There was a correlation between plasma 8-OHG levels and tissue 8-OHdG levels (plasma 8-OHG vs.

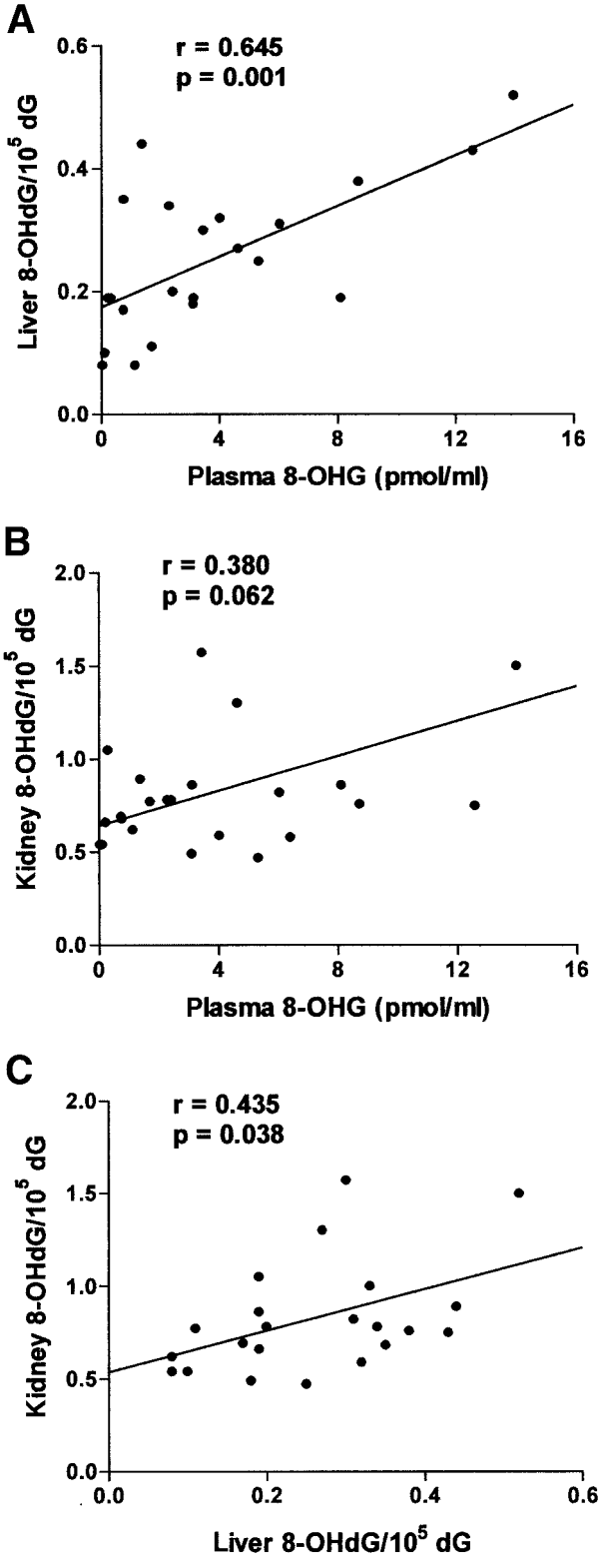

FIG. 1. Correlations between plasma 8-OHG and liver 8-OHdG (*A*), plasma 8-OHG and kidney 8-OHdG (*B*), and liver 8-OHdG and kidney 8-OHdG (*C*). The levels of plasma 8-OHG and tissue 8-OHdG were measured by HPLC-ECD.

liver 8-OHdG: $r = 0.64$, $P < 0.001$; plasma 8-OHG vs. kidney 8-OHdG: $r = 0.38$, $P = 0.06$) (Fig. 1*A* and *B*). Liver 8-OHdG levels also correlated with kidney 8-OHdG levels ($r = 0.435$; $P = 0.038$) (Fig. 1*C*).

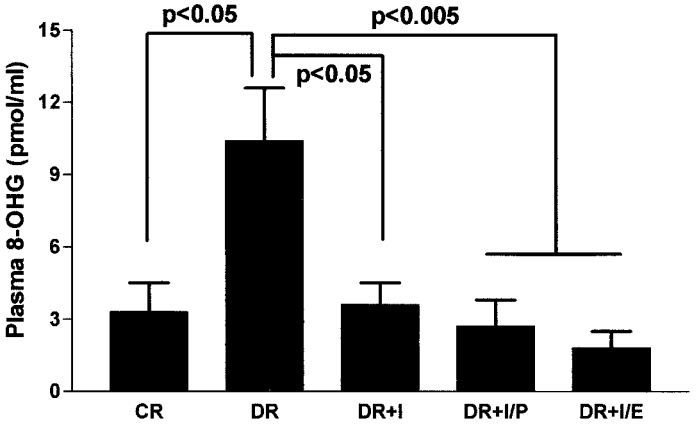

FIG. 2. Effects of insulin and antioxidant treatment on plasma 8-OHG levels in STZ-induced DR. DR+I: target blood glucose 200–300 mg/dl. DR + I/P: insulin and probucol were administered at 1% of food weight. DR + I/E: insulin and vitamin E were administered at 100 IU/kg food wt. Rats were treated for 4 weeks until killed. Plasma 8-OHG levels were significantly increased in DR. Insulin treatment completely reversed elevated levels of plasma 8-OHG in DR. The addition of probucol and vitamin E to insulin tended to decrease plasma 8-OHG.

Plasma 8-OHG levels in DR treated with insulin and antioxidant. Plasma 8-OHG levels in DR were significantly higher than those in CR (10.4 ± 2.2 vs. 3.3 ± 1.2 pmol/ml for DR vs. CR; $P < 0.05$) (Fig. 2). Insulin treatment normalized elevated levels of plasma 8-OHG in DR (plasma 8-OHG: 10.4 ± 2.3 vs. 3.6 ± 1.0 pmol/ml for DR vs. DR+I; $P < 0.05$) (Fig. 2). Adding probucol or vitamin E to the insulin treatment tended to further decrease plasma 8-OHG levels, although it did not reach significance (3.6 ± 1.0, 2.8 ± 1.0, and 1.8 ± 1.9 pmol/ml for DR+I, DR+I/P, and DR+I/E, respectively; P = NS for DR+I vs. both DR+I/P and DR+I/E) (Fig. 2).

Tissue 8-OHdG levels in DR treated with insulin and antioxidant. In DR, liver 8-OHdG levels were significantly higher than those in CR (8-OHdG/10^5 dG: 0.38 ± 0.04 vs. 0.21 ± 0.03 for DR vs. CR; $P < 0.005$) (Fig. 3*A*). Likewise, kidney 8-OHdG levels in DR were significantly higher than those in CR (8-OHdG/10^5 dG: 1.22 ± 0.19 vs. 0.70 ± 0.02 for DR vs. CR; $P < 0.05$) (Fig. 3*B*). In contrast with 8-OHG, 8-OHdG in liver and kidney was not significantly decreased by insulin treatment (liver 8-OHdG/10^5 dG: 0.39 ± 0.04 vs. 0.31 ± 0.04 for DR vs. DR+I, $P = 0.19$; kidney 8-OHdG/10^5dG: 1.21 ± 0.01 vs. 0.92 ± 0.15 for DR vs. DR+I, $P = 0.08$) (Fig. 3*A* and *B*). However, a combined treatment of insulin and probucol normalized tissue 8-OHdG levels (liver 8-OHdG/10^5 dG: 0.38 ± 0.04 vs. 0.15 ± 0.02 for DR vs. DR+I/P, $P < 0.005$; kidney 8-OHdG/10^5 dG: 1.22 ± 0.19 vs. 0.73 ± 0.05 for DR vs. DR+I/P, $P = 0.007$) (Fig. 3*A* and *B*). Similarly, insulin and vitamin E treatment (I/E) significantly decreased tissue 8-OHdG levels (liver 8-OHdG/10^5dG: 0.38 ± 0.04 vs. 0.22 ± 0.03 for DR vs. DR+I/E, $P = 0.004$; kidney 8-OHdG/10^5 dG: 1.22 ± 0.19 vs. 0.61 ± 0.04 for DR vs. DR+I/E, $P = 0.004$) (Fig. 3*A* and *B*).

Correlation between blood glucose levels and plasma 8-OHG or tissue 8-OHdG levels. Neither 8-OHG levels nor 8-OHdG levels were significantly correlated with mean blood glucose levels during insulin treatment, although kidney 8-OHdG levels showed an increasing tendency along with blood glucose levels ($r = 0.32$; $P = 0.07$).

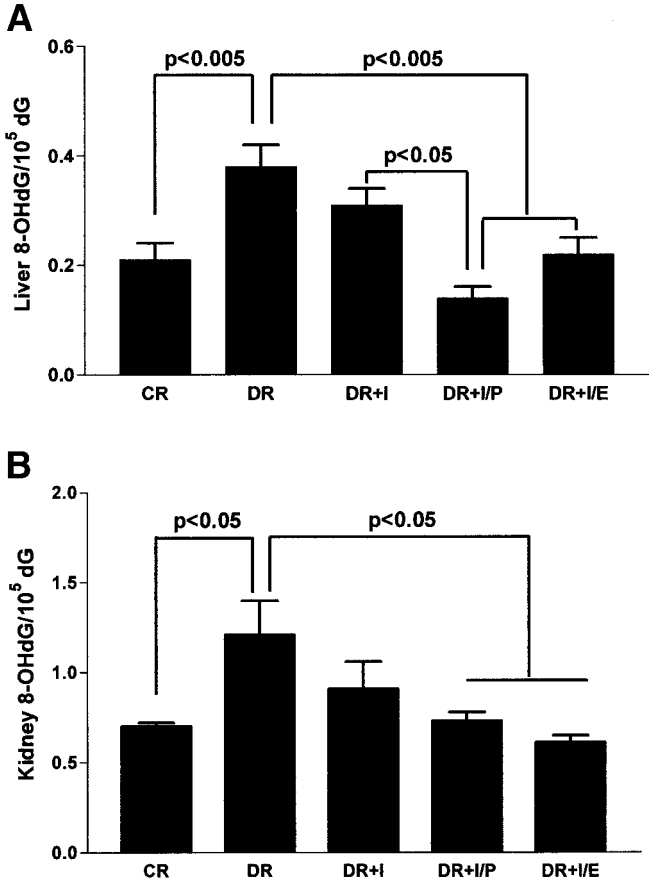

FIG. 3. Effects of insulin and antioxidant treatment on liver 8-OHdG (*A*) and kidney 8-OHdG (*B*) in DR. Compared with CR, DR had higher levels of liver and kidney 8-OHdG. Insulin treatment did not significantly decrease liver and kidney 8-OHdG levels, but combined treatment of insulin and antioxidant normalized liver and kidney 8-OHdG levels.

DISCUSSION

In the diabetic state, increased ROS production and decreased antioxidant capacity has been observed, both resulting in increased oxidative stress (1–3). Enhanced oxidative stress may be closely related to pathogenesis of diabetic macro- and microangiopathy by exerting direct oxidative damage and also by activating the pathways implicated in diabetic angiopathy (3,4).

8-OHdG is produced by oxidative modification of both the purine base in DNA and the repairing process (10–12). The levels of 8-OHdG in urine and tissue are increased in many conditions complicated with enhanced oxidative damage, such as cancer, aging, radiation injury, and smoking (22,23). Although urinary excretion of 8-OHdG has been commonly used as a biomarker for systemic oxidative stress, 8-OHdG assay has been limited by low sensitivity because of auto-oxidation of dG during the sample preparation and work-up procedure (7–9,17). In this study, we measured plasma 8-OHG along with tissue 8-OHdG because 8-OHG is more stable due to resistance to auto-oxidation (18). We found that the plasma 8-OHG level significantly correlated with the tissue 8-OHdG level. Thus, it could be concluded that plasma 8-OHG is a good indicator for oxidative DNA damage in tissue.

Dandona et al. (13) have demonstrated that diabetic patients have higher levels of 8-OHdG in mononuclear cells. An increase in the urinary excretion of 8-OHdG has also been seen in diabetic patients (13–15). We have recently demonstrated that plasma 8-OHG levels are increased in diabetic patients, especially those with advanced diabetic microvascular complications (24). In agreement with previous observations, DR had significantly higher levels of 8-OHdG and 8-OHG compared with normal CR in the present study. Notably, a greater increase was seen in plasma 8-OHG than in liver and kidney 8-OHdG in untreated DR. Animals in the DR group had about three times higher levels of plasma 8-OHG, whereas the tissue 8-OHdG levels were elevated by 1.5- to 2-fold in untreated DR compared with CR. This finding suggests that plasma 8-OHG is a more sensitive marker for oxidative DNA damage.

In the diabetic state, there may be a number of reasons for increased oxidative DNA damage. Hyperglycemia itself contributes to increased generation of ROS by producing reactive ketoimine and ketoamine adducts during oxidative glycosylation and glycoxidation, which further produce ROS such as hydrogen peroxide and superoxide anion in the presence of metal ions (3). Thus, reduction of hyperglycemia may be helpful in decreasing oxidative stress. Interestingly, in this study, insulin treatment almost normalized plasma 8-OHG when hyperglycemia was partially corrected by insulin treatment. However, insulin treatment did not significantly decrease tissue 8-OHdG, although a decreasing tendency in tissue 8-OHdG was seen in insulin-treated DR. This may suggest that normalization of plasma 8-OHG does not mean normalization of oxidative DNA damage in tissue. The reason for the discrepancy in the response to insulin treatment between plasma 8-OHG and tissue 8-OHdG is presently unknown. Insulin might affect the DNA repairing process or conversion of 8-OHdG to 8-OHG independently of the glucose-lowering effect. Further investigation will be needed to explain this phenomenon.

Large-scale epidemiological studies have demonstrated that probucol or dietary supplement of vitamin E reduces the risk of coronary artery disease and restenosis after balloon angioplasty (25–27). In the present study, antioxidant (probucol and vitamin E) treatment, in addition to insulin, normalized elevated tissue 8-OHdG levels. This study supports an important role of antioxidant treatment in the reduction of oxidative stress for the prevention of diabetic vascular complications. Vitamin E, which chelates reactive metal ions, reduces liver and kidney 8-OHdG to a similar degree, whereas probucol is more effective in decreasing liver 8-OHdG. Correction of hyperlipidemia by probucol, in addition to its antioxidant effect, may contribute to reduction of oxidative damage, especially in liver. Although we did not measure the lipid profile in this study, epidemiological studies suggest that hypertriglyceridemia rather than hyperglycemia is more associated with lipid peroxidation (28). However, the mechanism behind this differential effect of probucol in different organs remains to be investigated.

Although there is no doubt that hyperglycemia is a major contributor to oxidative stress, there have been some debate on the association between the glycemic control index and levels of 8-OHdG or 8-OHG. Leinonen et al. (14) showed a positive association of HbA_{1c} and urinary

8-OHdG in diabetic patients. However, in our previous study, there was no significant correlation between HbA_{1c} and plasma 8-OHG (24). In this study, the levels of plasma 8-OHG and tissue 8-OHdG were not significantly correlated with average plasma glucose levels during the treatment period, although kidney 8-OHdG levels showed a tendency of association with blood glucose levels. These discrepancies may imply that various factors other than hyperglycemia involve oxidative DNA damage in the diabetic state.

Oxidative modifications of long-lived proteins such as collagen are associated with the development of diabetic complications (e.g., cataracts, microangiopathy, atherosclerosis, and nephropathy) (3). However, it is as yet uncertain whether oxidative damage of DNA is directly related to the development of chronic diabetic complications. In the present study, we have shown a correlation between plasma 8-OHG and tissue 8-OHdG. This suggests that plasma 8-OHG may be a useful biomarker for the assessment of oxidative DNA damage in diabetic patients. In addition, our data suggests that combined treatment of insulin and antioxidants would be helpful for the reduction of oxidative stress in diabetes.

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