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Oxidative DNA Damage in Rats with Diabetes Induced by Alloxan and Streptozotocin

Young-Jin Lee, Young-Mee Park[†] and Eun-Mi Choi*

Department of Chemistry and [†]Biology, University of Inchon, Inchon 402-749, Korea

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The role of oxidative stress in the initiation and the complication of diabetes was examined by monitoring blood glucose increase and oxidative DNA damage in rats treated with alloxan or streptozotocin (STZ). Oxidative DNA damage was assessed by quantitating 8-oxo-2′-deoxyguanosine (oxo^{8}dG) excreted in urine and the oxo^{8}dG accumulated in pancreas DNA. Both alloxan and STZ treatments resulted in an abrupt increase in blood glucose and significant increases in urinary and pancreatic oxo^{8}dG. Pretreatment of buthionine sulfoximine (BSO), a glutathione-depleting agent, slightly potentiated the increase of blood glucose and urinary oxo^{8}dG in the alloxan- and STZ-treated rats. Furthermore, the BSO pretreatment caused significant amplification of pancreatic oxo^{8}dG increase in the rats. On the other hand, pretreatment with 1,10-phenanthroline (*o*-phen), a chelator of divalent cations, showed different results between alloxan- and STZ-treated rats. The *o*-phen pretreatment completely blocked diabetes and the increase of oxo^{8}dG by alloxan treatment, while it potentiated the increase of blood glucose and oxo^{8}dG by STZ treatment. The results demonstrate that the causative effect of alloxan on diabetes may be the generation of reactive oxygen species through a Fenton type reaction, but that of STZ may not.

Keywords: Alloxam, DNA damage, Diabetes, STE.

Introduction

Diabetes mellitus is a syndrome initially characterized by a loss of glucose homeostasis. Current evidence suggests that reactive oxygen species (ROS) may participate in the destruction of pancreatic beta cells leading to the type 1 (insulin-dependent) diabetes. ROS, which are the major contributors to oxidative stress, have been known to cause damage to cell components and depletion of antioxidants (Halliwell, 1987; Koh *et al.*, 1997). Oxidative stress is defined as a disturbance in the prooxidant-antioxidant balance in favor of the former leading to oxidative damage to the cell components, which in turn contributes to a host of diseases. Alloxan and streptozocin (STZ) are used to induce experimental diabetes mellitus by destroying pancreatic islet beta cells in animals. Okamoto (Okamoto, 1981; Yamamoto *et al.*, 1981) proposed a model in which alloxan and STZ cause DNA strand breaks in rat pancreatic islets and stimulate nuclear poly (ADP-ribose) synthetase, thereby depleting intracellular NAD levels and inhibiting proinsulin synthesis. It has been established that the initial event in alloxan-induced cell damage is the generation of ROS (Cohen and Heikkila, 1974; Takasu *et al.*, 1991). Superoxide dismutase and catalase, the scavengers of ROS, were found to protect against islet DNA strand breaks and inhibition of proinsulin synthesis induced by alloxan (Uchigata *et al.*, 1982). However, the mechanism by which STZ exerts its diabetogenic action is controversial. The initial action of STZ, an N-nitrosourea derivative of glucosamide, has been hypothesized to be the alkylation of specific sites on DNA bases (Bennett and Pegg, 1981; LeDoux *et al.*, 1986). Superoxide dismutase and catalase did not affect islet DNA strand breaks or inhibition of proinsulin synthesis induced by STZ (Uchigata *et al.*, 1982; Wilson *et al.*, 1984), and STZ did not produce chemiluminescence when exposed to islets (Asayama *et al.*, 1984), which indicates that STZ does not act through the generation of ROS. However, there are other reports suggesting that STZ stimulated the generation of reactive oxygen species in pancreatic islets *in vitro* (Takasu *et al.*, 1991; Heller *et al.*, 1994). Likewise, protection against STZ-induced diabetes by dimethyl urea, a hydroxyl radical scavenger was reported (Sandler and Andersson, 1982).

Not only are ROS involved in the cause of diabetes, but

* To whom correspondence should be addressed.
Tel: 82-32-770-8233; Fax: 82-32-770-8238
E-mail: eunmi@lion.inchon.ac.kr

they also appear to play a role in some of the complications seen in long-term treatment of diabetes. Markers of free radical damage are elevated in patients with diabetes and various antioxidants are decreased. (Jennings *et al.*, 1991; Griesmacher *et al.*, 1995). There are many pathways known to cause oxidative stress to cells including environmental factors, which are tending to increase these days. Therefore, elucidating the mechanisms by which alloxan and STZ induce diabetes will help us to understand the significance of ROS in the establishment of the disease as well as in long-term complications.

To examine and differentiate the role of oxidative stress in the initiation and the complication of diabetes, we monitored the time course of blood glucose increase and changes in 8-oxo-2′-deoxyguanosine (oxo^{8}dG) excretion in urine, which comes from oxidatively damaged DNA as a repaired product, after the treatments of alloxan and STZ. We also assessed the oxidative DNA damage accumulated in the pancreas by quantitating oxo^{8}dG accumulation. In addition, we examined the effects of pretreatment with buthionine sulfoximine, a glutathione-depleting agent, and 1,10-phenanthroline, a chelator of divalent cations, on the diabetogenic activity of alloxan and STZ to investigate the role of oxidative stress in diabetes.

Materials and Methods

Materials Alloxan, streptozotocin (STZ), Nuclease P1, proteinase K, alkaline phosphatase, 2′-deoxyguanosine (dG), reduced (GSH) and oxidized (GSSG) glutathione, L-buthionine sulfoximine (BSO), 1,10-phenanthroline (*o*-phen), 2,4-dinitrofluorobenzene (FDNB), and N-ethylmaleimide (NEM) were purchased from Sigma (St. Louis, USA). Deoxy[1′,2′-^{3}H]guanosine 5′-triphosphate (30.0 Ci/mmol) was purchased from Amersham (Buckinghamshire, England). Authentic 8-oxo-2′-deoxyguanosine (oxo^{8}dG) was prepared following the procedure of Kasai and Nishimura (1984). [1′,2′-^{3}H]8-oxo-2′-deoxyguanosine ([^{3}H]oxo^{8}dG) was prepared as described by Shigenaga *et al.* (1989). Monoclonal antibody to oxo^{8}dG was a generous gift from Dr. B. N. Ames, University of California (Berkeley, USA). Spherisorb NH_2 column (particle size 5 μm, 250 × 4.6 mm) was from ISCO (Lincoln, USA) and Supelcosil LC-18 column (particle size 5 μm, 250 × 4.6 mm) was from Supelco (Bellefonte, USA).

Animals Male Sprague-Dawley rats (160~200 g) were used for all experiments. Rats were fed a standard laboratory diet and water *ad libitum* and maintained under a 12-h light/dark cycle. They were housed individually in metabolic cages. Diabetes was induced by injection of either alloxan (70 mg/kg) dissolved in phosphate buffered saline (PBS) into the tail vein or STZ (60 mg/kg) dissolved in citrate buffered saline (CBS) into the peritoneal cavity. Control rats received PBS or CBS only. BSO (220 mg/kg) and *o*-phen (15 mg/kg) were dissolved in PBS and injected intraperitoneally 30 min and 3 h, respectively, before the injection of alloxan or STZ. Blood samples were obtained from tail veins 1, 2, and 3 days after the treatment, and plasma glucose was measured by the glucose oxidase method (Bergmeyer and Bernt, 1974). Twenty-four-hour urine outputs were collected over three consecutive days after the treatment and stored at −20°C until analyzed. The rats were sacrificed by CO_2 and decapitated on the final day of blood and urine collection, 3 days after the treatment. The pancreases were removed, washed, and frozen immediately by clamping with liquid nitrogen-cooled tongs, and stored at −75°C until analyzed.

Quantitation of oxo^{8}dG in urine Urine samples (1~5 ml), diluted with an equal volume of 1 M NaCl, were spiked with [^{3}H]oxo^{8}dG to determine the recovery of oxo^{8}dG and then applied to a preconditioned C18/OH SPE column. The column was washed and oxo^{8}dG was eluted with 15% methanol (MeOH) in 50 mM KH_2PO_4 buffer, pH 7.5, as described by Shigenaga *et al.* (1989). The eluate was applied to an anti-oxo^{8}dG immunoaffinity column, whereafter the column was washed and the antibody binding compounds were eluted with MeOH as described by Park *et al.* (1992). The resulting MeOH eluate was concentrated to dryness under a stream of nitrogen and resuspended in H_2O. The samples were analyzed for oxo^{8}dG by HPLC with the electrochemical (EC) detection method as described previously (Shigenaga *et al.*, 1990). Compounds were separated by reversed phase HPLC on a Supelcosil LC-18 column. The mobile phase consisted of 10% MeOH/10 mM KH_2PO_4 and the flow rate was 0.8 ml/min. Samples were analyzed by EC detection employing an ESA (Belford, USA) model 5200A Coulochem II detector with a 5011 analytical cell. The oxidation potentials set for the dual Coulochem detector were 0.15 and 0.30 V for electrodes 1 and 2, respectively.

Quantitation of oxo^{8}dG in DNA DNA was isolated from tissues as described by Gupta (1984). Samples containing 200~400 μg DNA in 200 μl of 20 mM sodium acetate, pH 4.8, were digested to nucleotides with 20 μg of nuclease P1 at 37°C for 30 min. Samples were then treated with 20 μl of 1 M Tris·HCl, pH 7.5, and 4 units of alkaline phosphatase at 37°C for 1 h to liberate the corresponding nucleosides from the phosphate residues (Shigenaga *et al.*, 1990). Twenty to fifty μl of DNA hydrolyzate was filtered and analyzed for dG and oxo^{8}dG by the HPLC method as described above. Samples were analyzed by separate UV (245 nm) and EC detection systems linked in series for detection of dG and oxo^{8}dG, respectively. The amount of oxo^{8}dG in DNA was expressed as the number of oxo^{8}dG per 10^6 dG.

Quantitation of glutathione Glutathione was analyzed by a modification of the HPLC method of Reed *et al.* (1980). Five percent perchloric acid extracts of tissues were derivatized with FDNB as described by Collison *et al.* (1986). Reduced glutathione and oxidized glutathione were separated by HPLC on a Spherisorb NH_2 column. To avoid the effect of artificial oxidation of glutathione during treatment, extracts with 5% perchloric acid containing 50 mM NEM were used to determine oxidized glutathione. Reduced glutathione was determined by the difference between total glutathione and oxidized glutathione. Total glutathione was expressed as GSH equivalent to the sum of GSH and GSSG, (GSH + 2 GSSG), and the oxidation state of glutathione was expressed as percentage of GSH equivalent of GSSG, (100% × 2 GSSG/total glutathione).

Results

Diabetes was induced in rats by injection with alloxan or streptozotocin (STZ). The content of oxidative DNA damage in the rats was monitored for 3 days by quantitating oxo^{8}dG in 24 h urine output, and then oxo^{8}dG accumulated in the pancreas DNA was measured 3 days after the treatment. The effects of pretreatment of buthionine sulfoximine (BSO), a glutathione-depleting agent, and 1,10-phenanthroline (*o*-phen), a chelator of divalent cations, on the diabetogenic activity of alloxan and STZ were also examined.

Figures 1–3 show the results of alloxan treatment. Figure 1 shows changes in the level of blood glucose in alloxan-treated rats. Alloxan resulted in about a three-fold increase of blood glucose within 24 h. Pretreatment of BSO potentiated the alloxan-induced increase of blood glucose. We noticed that the blood glucose level in BSO-pretreated rats dropped on the third day. We could not explain the reason for the phenomenon, yet we consistently observed the drop of blood glucose after reaching a certain level (~400 mg/dl) regardless of the nature of the treatment. On the other hand, pretreatment with *o*-phen completely blocked alloxan-induced diabetes.

Figure 2 shows the content of oxo^{8}dG in the 24-h urine output of alloxan-treated rats. The oxo^{8}dG in the first 24-h urine in all alloxan-treated rats was the same as the controls. However, oxo^{8}dG in the second and the third day urine was significantly higher than controls. Urinary oxo^{8}dG excretion in control rats showed some increase with days. We speculate that this could be due to the stressful conditions of restraint in metabolic cages. BSO-pretreated rats showed almost the same degree of increase

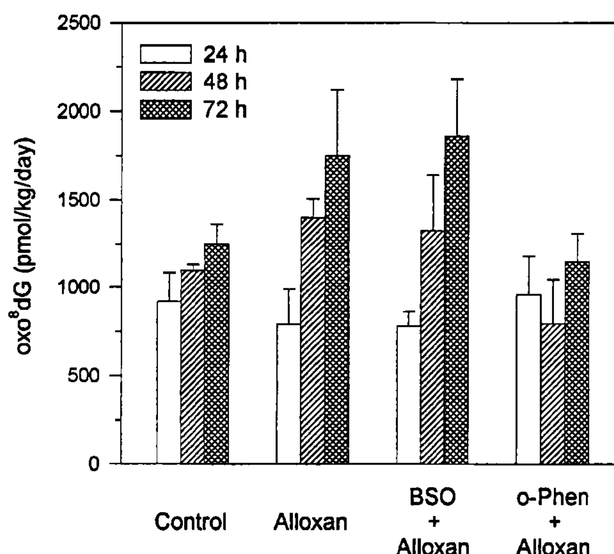

Fig. 2. Effect of alloxan on the rate of oxo^{8}dG excretion in urine. Rats were treated as described in Fig. 1. Twenty-four-hour urine outputs were collected. SPE- and then anti-oxo^{8}dG immunoaffinity column-processed urine samples were analyzed for oxo^{8}dG by HPLC-EC methods. Results are expressed as mean ± SE (n = 5).

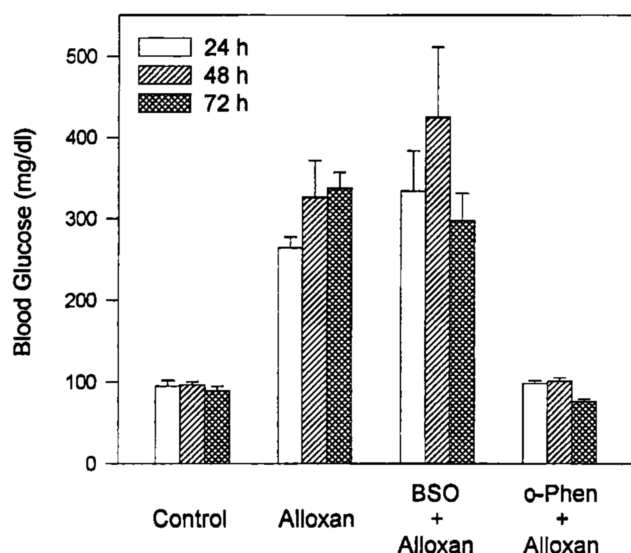

Fig. 1. Effect of alloxan on the concentration of blood glucose. The tail vein of rats were injected with alloxan (70 mg/kg) dissolved in phosphate buffered saline (PBS). Control rats received PBS only. Buthionine sulfoximine (BSO, 220 mg/kg) and 1,10-phenanthroline (*o*-phen, 15 mg/kg) were dissolved in PBS and injected into the peritoneal cavity 30 min and 3 h, respectively, before the injection of alloxan. Results are expressed as mean ± SE (n = 5).

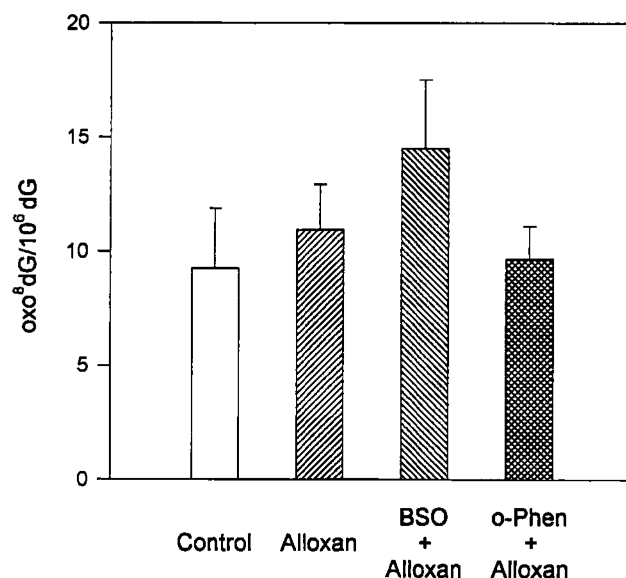

Fig. 3. Effect of alloxan on the content of oxo^{8}dG in DNA of the pancreas. Rats were treated as described in Fig. 1. Rats were sacrificed three days after the treatment. DNA was isolated from the pancreas, and hydrolyzed and analyzed for oxo^{8}dG by HPLC-EC methods. Results are expressed as mean ± SE (n = 5).

in urinary oxo^{8}dG as alloxan-only treated rats. Rats pretreated with *o*-phen, however, did not show the oxo^{8}dG increase. Rats treated with BSO or *o*-phen alone showed the same results as the controls (data not shown).

Figure 3 shows the contents of oxo^{8}dG in the pancreas DNA of alloxan-treated rats. Alloxan resulted in a small increase in oxo^{8}dG. BSO pretreatment caused a significant amplification of the alloxan-induced oxo^{8}dG increase. On the other hand, *o*-phen pretreatment completely inhibited alloxan-induced oxo^{8}dG increase. Rats treated with BSO or *o*-phen alone showed the same results as the control (data not shown).

Figures 4–6 show the results of STZ treatment. Figure 4 shows the level of blood glucose in STZ-treated rats. Streptozotocin also caused an abrupt increase of blood glucose within 24 h, and BSO pretreatment slightly potentiated the increase as in the alloxan experiment. However, *o*-phen pretreatment resulted in completely different results from the alloxan experiment in that it did not prevent the induction of diabetes by STZ. In fact, we observed even higher blood glucose levels than for the STZ-only treatment on the 2nd day. We observed again the drop of blood glucose after reaching about 400 mg/dl at the third day.

Excretion of oxo^{8}dG into urine in STZ-treated rats was significantly higher than in the controls, and BSO pretreatment caused further increase in oxo^{8}dG excretion (Fig. 5). In contrast to the result of alloxan treatment (Fig. 2), *o*-phen pretreatment enhanced the STZ-induced increase in oxo^{8}dG excretion rather than inhibit it. Likewise, the level of oxo^{8}dG accumulated in the pancreas DNA of STZ-treated rats was also slightly higher than in the controls (Fig. 6). The increase in DNA oxo^{8}dG by STZ

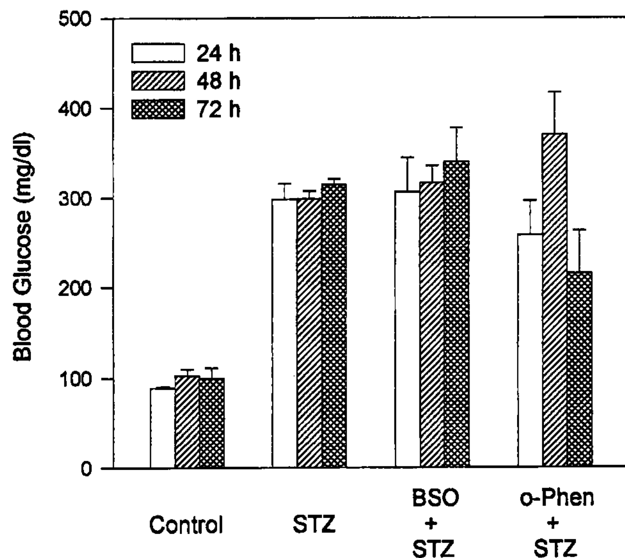

Fig. 4. Effect of streptozotocin (STZ) on the concentration of blood glucose. Rats were injected with STZ (60 mg/kg) dissolved in citrate buffered saline (CBS) into the peritoneal cavity. Control rats received CBS only. Buthionine sulfoximine (BSO, 220 mg/kg) and 1,10-phenanthroline (*o*-phen, 15 mg/kg) were dissolved in PBS and injected into the peritoneal cavity 30 min and 3 h, respectively, before the injection of STZ. Results are expressed as mean ± SE (n = 5).

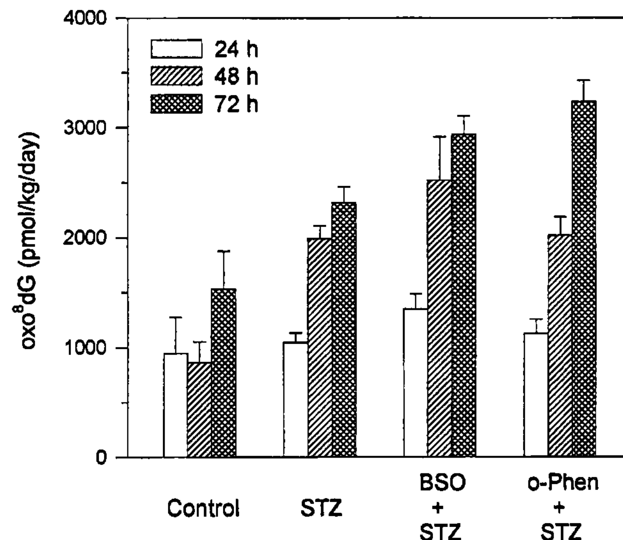

Fig. 5. Effect of streptozotocin (STZ) on the rate of oxo^{8}dG excretion in urine. Rats were treated as described in Fig. 4. Twenty-four-hour urine outputs were collected. SPE- and then anti-oxo^{8}dG immunoaffinity column-processed urine samples were analyzed for oxo^{8}dG by HPLC-EC. Results are expressed as mean ± SE (n = 5).

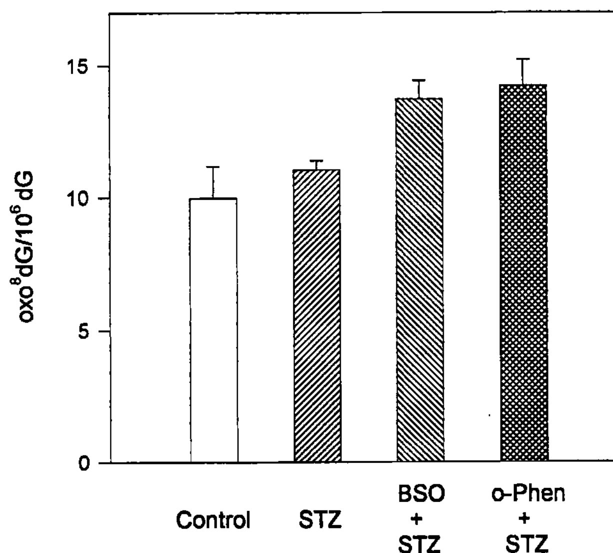

Fig. 6. Effect of streptozotocin (STZ) on the content of oxo^{8}dG in DNA of the pancreas. Rats were treated as described in Fig. 4. Rats were sacrificed three days after the treatment. DNA was isolated from the pancreas, and hydrolyzed and analyzed for oxo^{8}dG by HPLC-EC. Results are expressed as mean ± SE (n = 5).

treatment was greatly amplified by BSO-pretreatment. In contrast to the result of the alloxan treatment, *o*-phen pretreatment amplified the STZ-induced increase of oxo^8dG in pancreas DNA rather than inhibit it.

Discussion

The development of insulin-dependent diabetes results from the destruction of pancreatic β cells. Genetic factors, various immune alterations, and environmental factors have been studied as possible causes of the diabetes. The aim of the present study was to verify the role of oxidative stress in diabetes mellitus. It has been suggested that oxidative stress is involved in the initiation of diabetes and in the development of complications of the disease. The increase of markers for oxidative stress was observed in the test animals treated with diabetogenic agents and also in diabetic patients. However, in most of the studies with test animals, the measurement was performed at the stage of full-blown diabetes, i.e., a few weeks after the initiation of the treatment, which makes it difficult to distinguish the effect of oxidative stress in the causation from the effect of it in the complication of diabetes. For the purpose of differentiation of the two effects of oxidative stress, we followed the time course of diabetes and oxidative stress by monitoring blood glucose and excretion of 8-oxo-2′-deoxyguanosine (oxo^8dG) into urine, respectively, for the first three days after treatment with alloxan or streptozotocin (STZ). We also measured oxo^8dG accumulated in pancreas DNA at the earliest stage possible, i.e., three days after treatment.

When DNA is damaged, nonspecific DNA repair enzymes excise lesions to release deoxynucleotides and base-specific repair glycosylases excise corresponding bases. The deoxynucleotides are enzymatically hydrolyzed to stable deoxynucleosides, and these repair products are transported through the blood and excreted in the urine (Fraga *et al.*, 1990; Choi, 1993). The damaged lesions escaping repair accumulate in DNA. Therefore, when evaluating the extent of oxidative DNA damage, both the accumulated lesions and the repaired lesions should be considered. 8-Oxo-2′-deoxyguanosine is an excellent marker for oxidative DNA damage because it has been shown that it is one of the major products produced by a wide array of oxidant-generating conditions (Kasai and Nishimura, 1986), and it can be measured with very high sensitivity by HPLC with electrochemical detection (Shigenaga *et al.*, 1990). Basal levels of oxo^8dG excretion in urine were 800~900 pmol/kg/day and oxo^8dG in pancreas DNA was 9~10 $oxo^8dG/10^6$ dG in the present study. The basal levels of oxo^8dG in rat tissues reported in the literatures range from 8 to 73 $oxo^8dG/10^6$ dG, but are typically 10~20 $oxo^8dG/10^6$ dG (Fraga *et al.*, 1990; Degan *et al.*, 1991; Park *et al.*, 1994). This indicates that oxidative damage to DNA is a ubiquitous process occurring under normal physiological conditions.

Our results clearly demonstrate that alloxan induces diabetes by generating oxygen free radicals through a Fenton-type reaction, which involves metal-catalyzed redox reactions, because pretreatment with *o*-phen, a divalent metal ion chelator, completely blocked the induction of diabetes and the increase of oxo^8dG by alloxan at the same time (Figs. 1–3). On the other hand, the causative effect of STZ in the induction of diabetes does not seem to be the generation of free radicals as some investigators have suggested because *o*-phen did not give protection against the induction of diabetes by STZ (Fig. 4).

The results show that blood glucose levels are closely correlated with urinary oxo^8dG excretion and oxo^8dG in pancreas DNA in both alloxan- and STZ-induced diabetes. Because STZ did not seem to produce oxygen free radicals, it is certain that the oxo^8dG found in STZ-treated rats resulted from the oxidative stress accompanying the diabetes. On the other hand, alloxan itself is an oxidative stress agent, so oxo^8dG in urine output and in pancreas DNA could be the result of oxidative stress either by alloxan itself or by the pathology of the diabetes. When we compared the time course of blood glucose increase with that of oxo^8dG excretion in urine, it was found that the blood glucose increase preceded the oxo^8dG increase. 8-Oxo-2′-deoxyguanosine comes from the repair of oxidatively damaged DNA, therefore the output of oxo^8dG in urine depends on the activity of repair systems, in both constitutive and inducible forms. It has been reported that 8-oxoguanine glycosylase, one of the oxidatively damaged DNA repair enzymes, was very rapidly induced in rat by treatment with oxidative stress agents and peaked at 6 h after the treatment (Lee *et al.*, 1993). Therefore, if we assume that the repair systems excising oxo^8dG from oxidatively damaged DNA have a similar time course of induction, the oxo^8dG observed in the urine of alloxan-treated rats might be contributed mostly by the oxidative stress accompanying the disease and not by the alloxan itself, because the first 24-h urine shows no increase in oxo^8dG. In fact, the time course of the increase of oxo^8dG in the urine of alloxan-induced diabetic rats was similar to that of STZ-induced diabetic rats. This suggests that the alloxan at the amount used in this study was enough to initiate diabetes but was not enough to cause detectable amount of oxo^8dG increase by its oxidative effect. However, the conclusion for this issue should wait until more concrete evidence is provided by experiments such as insulin-intervention (to compensate for blood glucose elevation) and the antioxidant treatment after the development of diabetes.

Our results demonstrate that diabetes is accompanied by increased oxidative stress at a very early stage of the disease, therefore complications could develop early if not treated properly. The increased oxidative stress in diabetic

Table 1. Effect of buthionine sulfoximine (BSO) and 1,10-phenanthroline (*o*-phen) on the content of glutathione in the pancreas.

Treatment	Glutathione (nmol/mg protein)			Oxidation (%)
	GSH	GSSG	Total	
Control	11.51 ± 1.08	0.01 ± 0.01	11.51 ± 1.08	0.17
BSO	3.25 ± 0.67	0.15 ± 0.06	3.55 ± 0.67	8.45
o-Phen	6.80 ± 1.00	0.05 ± 0.02	6.90 ± 1.05	1.45

Rats were injected with BSO (220 mg/kg) or *o*-phen (15 mg/kg) dissolved in PBS into the peritoneal cavity, and sacrificed 3 days after. Glutathione in the pancreas was analyzed by a modification of the HPLC method of Reed *et al.* (1980). Results are expressed as mean ± SE ($n = 5$).

patients seems to be related to the underlying metabolic abnormalities in diabetes. Protein glycation and glucose oxidation were suggested to be possible sources of free radicals (Wolff *et al.*, 1991; Ceriello *et al.*, 1996).

Pretreatment with BSO, a glutathione-depleting agent, resulted in a marginal increase in oxo^{8}dG excretion in both the alloxan- and STZ-treated rats. However, accumulation of oxo^{8}dG in DNA of diabetic pancreas was significantly increased by BSO pretreatment. Therefore, little increase in urinary oxo^{8}dG could probably be due to a limited capacity of DNA repair systems. Pancreatic β cells were shown to be low in antioxidant enzyme gene expression; indeed, superoxide dismutase and glutathione peroxidase gene expressions were 30~40% and 15% of those in liver, respectively, and catalase gene expression was not detectable at all in pancreatic islets (Lenzene *et al.*, 1996). Therefore, the disease progression may be retarded by supplementing antioxidant defenses.

Pretreatment of *o*-phen produced controversial results. While it completely prevented alloxan-induced diabetes, it aggravated the STZ-induced increase of blood glucose. 8-Oxo-2′-deoxyguanosine increase was also enhanced by *o*-phen in STZ rats, while it was inhibited in alloxan rats. The results indicate that *o*-phen prevented ROS-originated induction of diabetes, but it rather enhanced the oxidative stress accompanying the disease. In fact, we found that not only BSO but also *o*-phen resulted in oxidation and depletion of glutathione in the pancreas (Table 1), which may be one of the causes for enhanced oxidative stress by *o*-phen in STZ-induced diabetic rats. Our results are consistent with the previous report that an increase in oxidized glutathione caused by H_2O_2 was enhanced by *o*-phen in cultured Chinese hamster V79 cells (Ochi, 1995).

In summary, our results demonstrate that the causative effect of alloxan in the induction of diabetes was ROS generation via a Fenton-type reaction, while the causative effect of STZ was not. The levels of oxo^{8}dG increased at the very early stage of the disease in both alloxan- and STZ-induced diabetic rats, which seems to be caused mainly by the oxidative stress accompanying the diabetes. Depletion of glutathione aggravated the progress of the disease and also the increase of oxo^{8}dG. Therefore, antioxidants should provide beneficial inhibitory effects on the progress of the diabetes and the development of complication.

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