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LABORATORY INVESTIGATION

# Inducible Nitric Oxide Synthase Mediates Retinal DNA Damage in Goto-Kakizaki Rat Retina

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**Abstract**

**Purpose:** To examine the nitrosative and oxidative DNA damage induced by 8-nitroguanine and 8-hydroxy-2-deoxy guanosine (8-OHdG), and to determine the role played by inducible nitric oxide synthase (iNOS) in damage to DNA in the retina of the Goto-Kakizaki (GK) rat.

**Methods:** Experiments were performed on GK rats, an animal model of spontaneous type 2 diabetes without obesity or visible diabetic vascular lesions. Immunohistochemistry was used to determine the retinal distribution of 8-nitroguanine, 8-OHdG, and iNOS in GK rats and control rats. The change in the expression of 8-nitroguanine and 8-OHdG in GK rats was also determined following an intravitreal injection of 1400W, an inhibitor of iNOS activity.

**Results:** Immunohistochemical analysis showed that 8-nitroguanine and 8-OHdG were expressed strongly in the inner nuclear layer of GK retinas but only weakly in control retinas. This expression was correlated with an increase in the expression of iNOS in GK retinas, which was confirmed by the inhibition of iNOS activity by 1400W.

**Conclusion:** These findings demonstrate that iNOS plays a crucial role in nitrosative and oxidative DNA damage in GK rats, suggesting a retinal neurotoxic role of nitric oxide and superoxide in diabetic retinas. **Jpn J Ophthalmol** 2008;52:314–322 © Japanese Ophthalmological Society 2008

**Key Words:** diabetes mellitus, Goto-Kakizaki rat, 8-hydroxy-2-deoxy guanosine, inducible nitric oxide synthase, 8-nitroguanine

## Introduction

Diabetic retinopathy (DR) is a major cause of blindness, and considerable attention has been given to the vascular changes in eyes of patients with DR.[1] Pathological changes, such as apoptosis and progressive loss of neurons and altered glutamate metabolism, also develop in other retinal elements of diabetic retinas.[2] In addition, there is evidence that the neurons in the inner nuclear layer (INL) and the retinal ganglion cell layer (RGCL) undergo apoptosis in eyes of patients with DR.[3] These changes manifest clinically as a progressive loss of visual function independent of the presence of neovascularization.[4] Very little is known about the mechanisms inducing neuronal apoptosis in the retina of diabetic patients, which may, in part, explain why no major advances in the treatment of this disease have been achieved.

In diabetes the plasma concentrations of both glucose and proinflammatory cytokines increase,[5,6] which leads to an increase in the expression of inducible nitric oxide synthase (iNOS) through the activation of NF-κB.[7–10] This increase is accompanied by an increase in the generation of nitric oxide (NO). It has recently been suggested that NO and proinflammatory cytokines such as interleukin (IL)-1β

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and tumor necrosis factor (TNF)-α are the mechanisms underlying insulin resistance, type 1 (insulin-dependent) and type 2 (insulin-independent) diabetes, and diabetic complications.[9,11–15] NO is known to influence apoptosis in a variety of animal models of diabetes, and its effect can be pro- or antiapoptotic.[16] NO reacts with superoxide ($O^{2\bullet-}$) to form peroxynitrite ($ONOO^-$), a powerful oxidant causing oxidative and nitrosative DNA damage.[17,18] $ONOO^-$ can mediate the formation of 8-hydroxy-2-deoxy guanosine (8-OHdG)[19] and 8-nitroguanine, markers of nitrosative DNA damage.[20]

Sennlaub et al.[21,22] reported that retinal apoptosis in a murine model of ischemic proliferative retinopathy is dependent on iNOS, not only in retinal neovascular disease but also in retinal degeneration. Carmo et al.[23] examined Goto-Kakizaki (GK) rats by Western blot analysis and immunohistochemistry and found that iNOS is expressed in the retina, and that the expression is blocked by nitric oxide synthase (NOS) inhibitors. Kowluru and Odenbach[24] showed that the level of 8-OHdG is elevated more than twofold in streptozotocin (STZ)-induced diabetic retinas compared with in control retinas. However, previous studies of NO in DR have not examined 8-nitroguanine as a nitrosative marker of NO activity, nor have they addressed the pathogenic link between excessive iNOS activity and nitrosative and oxidative cell damage.

Thus, the purpose of this study was to determine whether nitrosative and oxidative DNA damage play a role in the pathological changes in the retina of GK rats, a spontaneous animal model of insulin-independent diabetes without obesity or diabetic retinopathy. An important advantage of using this animal model is that pharmacologic agents do not need to be injected to induce the diabetes, and the diabetic state is moderate, resulting in a relatively long follow-up time during which the plasma insulin response to glucose is impaired.[25] We investigated the DNA damage by examining the expression of 8-nitroguanine and 8-OHdG in GK retinas by immunohistochemistry. In addition, we investigated the influence of iNOS on retinal DNA damage by using the iNOS inhibitor 1400W.[26]

## Materials and Methods

### *Animals*

Male GK and Wistar rats were handled according to the principles of the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. The animals were obtained from Pharmacological Research Laboratories (SLC Japan, Hamamatsu, Japan). The GK rats were created by repeated selective breeding of normal Wistar rats using glucose intolerance as the selective breeding index.[25] GK rats develop glucose intolerance at 2 weeks of age, and significant hyperglycemia is found as early as 4 weeks of age.[25,27]

For our experiments, three 4-month-old and five 6-month-old GK rats were used. Age- and sex-matched Wistar rats were used as controls. The animals were housed in metal cages and given food and water ad libitum. The ambient temperature was set at 21°C with a 12 h light: 12 h dark light cycle. The blood glucose level was measured twice a week with a portable blood sugar measurement system (Xtra; Abbott Laboratories, Abbott Park, IL, USA).

### *Production of Anti-8-Nitroguanine Antibody*

Anti-8-nitroguanine polyclonal antibody was produced by a modified method.[28] Initially, 8-nitroguanosine was incubated with sodium metaperiodate for 20 min at room temperature, and then conjugated with rabbit serum albumin (RSA) for 1 h, followed by incubation with sodium borohydride for 1 h. The conjugate was dialyzed against 150 mM NaCl overnight. The 8-nitroguanine-aldehyde–RSA conjugate was mixed with Freund's complete adjuvant and injected intracutaneously into a rabbit. Four weeks after the initial immunization of the rabbits, the same antigen was given and the blood was collected 10 days later. The 8-nitroguanine was immobilized in a Cellulofine GCL-2000 m column (Seikagaku Kogyo, Tokyo, Japan), and the antibody was purified by affinity chromatography. The specificity of the purified antibody was examined by dot immunobinding assays and adsorption tests.[29]

### *Histological Processing*

Immunohistochemistry was used to determine whether 8-nitroguanine, 8-OHdG, and iNOS were expressed in the rat retina. Rats were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital and perfused through the heart for 30 s with 0.2 mol/l phosphate-buffered saline (PBS). The animals were then perfused through the heart with a fixative consisting of 4.0% paraformaldehyde in 0.1 mol/l PBS. The eyes were immediately enucleated, and the cornea and lens were removed. The eyecups were immersed overnight in the same fresh fixative. The retina was cut into small wedges using a razor blade, and pieces were embedded in paraffin and cut with a microtome. The paraffin sections (6 μm thick) were deparaffinized in xylene and then placed in 0.3% $H_2O_2$ in methanol for 5 min to block the endogenous peroxidase activity.

The sections were incubated with a rabbit polyclonal anti-8-nitroguanine antibody (2 μg/ml), mouse monoclonal anti-8-OHdG antibody (5 μg/ml, Japan Institute for the Control of Aging, Shizuoka, Japan), or mouse monoclonal anti-iNOS antibody (1:400, Sigma, St. Louis, MO, USA) overnight at room temperature. Next, the sections were incubated with biotinylated secondary antibody solution and VECTASTAIN ABC reagent from a VECTASTAIN ABC kit according to the manufacturer's protocol (Vector Laboratories, Burlingame, CA, USA). Then, the sections were incubated in peroxidase substrate solution until the

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desired stain intensity developed. Negative control experiments with 4-month-old GK retinas were performed by omitting the first antibody, and no staining was found. Additional sections were stained with hematoxylin and eosin and used for morphological studies.

The 8-nitroguanine, 8-OHdG, iNOS, and 8-OHdG immunoreactivity in GK retinas was also assessed by a double-immunofluorescence labeling method. The sections were incubated with a rabbit polyclonal anti-8-nitroguanine antibody (2 μg/ml) and a mouse monoclonal anti-8-OHdG antibody (5 μg/ml), or a rabbit polyclonal anti-iNOS antibody (1:200; Sigma), and a mouse monoclonal anti-8-OHdG antibody (5 μg/ml) overnight at room temperature. Then, the sections were incubated for 3 h with an Alexa 594-labeled goat antibody against rabbit IgG (1:400) and an Alexa 488-labeled goat antibody against mouse IgG (1:400; Molecular Probes, Eugene, OR, USA). The stained sections were examined under an inverted laser scan microscope (LSM 410, Zeiss, Gottingen, Germany).

### *Statistical Analysis of 8-Nitroguanine and 8-OHdG Immunostaining*

To determine whether the number of 8-nitroguanine- and 8-OHdG-positive cells in the INL and the RGCL of 6-month-old GK rats ($n = 5$) was significantly different from that in age-matched control rats ($n = 5$), the cells were counted in sections from both types of rats. The average number of the cells in the INL/mm$^2$ of retina and the RGCL/retinal cross section was determined. The significance of the differences in the number of cells between the 6-month-old GK rats and the control rats was assessed by the Mann-Whitney *U* test.

### *Effect of 1400W, an iNOS Inhibitor*

The right eyes of three 4-month-old GK rats were injected intravitreally twice (separated by a 5-day interval) with 2 μl of 15 mg/ml 1400W (Calbiochem, France Biochem, Merdon, France), a highly specific inhibitor of iNOS.[21,26] The left eyes of GK rats were injected intravitreally with 2 μl of 1% PBS as controls. Both eyes were enucleated 1 week later, and the treated eyes were assessed by immunohistochemical methods with anti-8-nitroguanine and anti-8-OHdG antibodies.

## Results

### *Animals*

The glucose concentration in the blood of the GK rats was significantly higher than that in the control rats at 4 to 6 months of age ($P < 0.01$; Fig. 1). During this period, cataracts and signs of DR were absent.

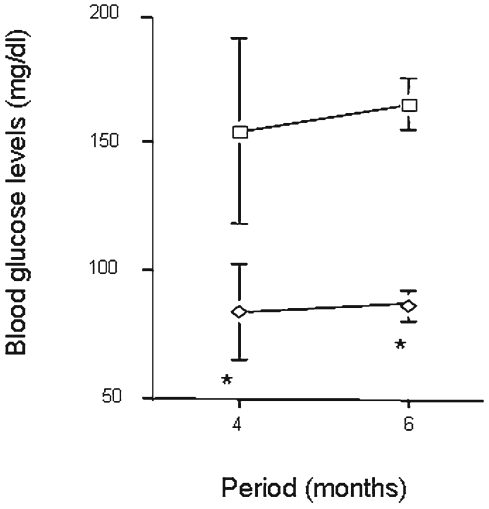


**Figure 1.** Relationship between age and blood glucose level in Goto-Kakizaki (GK) (□) and control rats (◇). Blood glucose levels were significantly higher in GK rats than in age-matched control rats at 4 to 6 months of age. *$P < 0.01$. Points and error bars represent means ± SD.

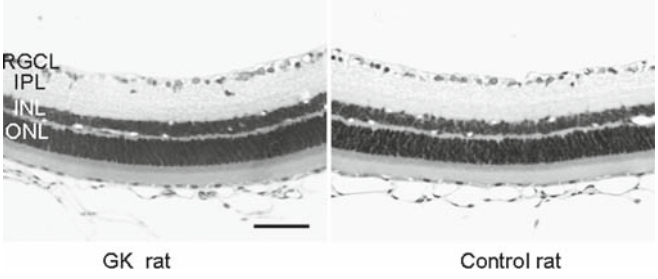


**Figure 2.** Hematoxylin and eosin-stained sections through retinas of 6-month-old GK and control rats. Examination of retinas of 6-month-old GK and control rats showed that all retinal layers were intact, and no remarkable pathologic changes were seen in either type of rat. *RGCL*, retinal ganglion cell layer; *IPL*, inner plexiform layer; *INL*, inner nuclear layer; *ONL*, outer nuclear layer. Bar = 50 μm.

### *Hematoxylin and Eosin-Stained Sections*

Takeo-Goto et al.[30] observed no remarkable pathological differences between GK and control retinas in 7-month-old rats. We also found that sections across the retina of 4- and 6-month-old GK rats did not differ morphologically from those of control rats (Fig. 2).

### *Formation of 8-Nitroguanine and 8-OHdG in Eyes of GK and Control Rats*

Retinas of GK and control rats stained immunohistochemically for 8-nitroguanine are shown in Fig. 3A. In control retinas, only weak immunoreactivity for 8-nitroguanine was detected in the RGCL and no immunoreactivity was detected in the INL. On the other hand, many 8-nitrogua-

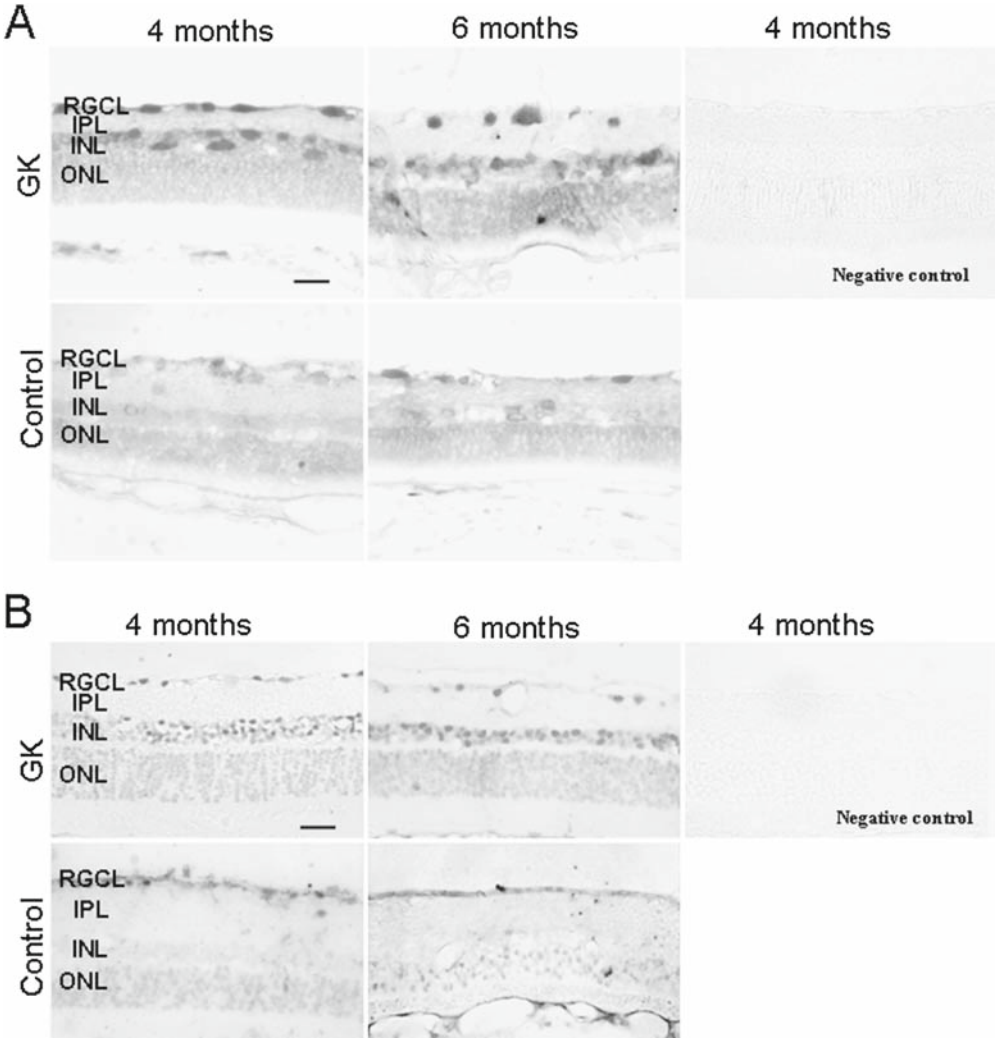


**Figure 3A, B.** Immunohistochemical retinal sections for 8-nitroguanine (**A**) and 8-hydroxy-2-deoxyguanosine (8-OHdG) (**B**). **A** 8-Nitroguanine-positive cells are seen in the RGCL and the INL of 4- and 6-month-old GK retinas. Control retinas show weak immunoreactivity for 8-nitroguanine in a few cells of the RGCL and no immunoreactivity in the INL. **B** 8-OHdG-positive cells are present in the RGCL, INL, and ONL of 4- and 6-month-old GK rat retinas. Control retinas show weak immunoreactivity for 8-OHdG in a few cells of the RGCL and no immunoreactivity in the INL. Negative control experiments on 4-month-old GK retinas, omitting the first antibody, showed no staining. Bars = 25 μm.

nine-positive cells were found in the RGCL and INL in retinas of 4- and 6-month-old GK rats.

The retinal sections of GK and control rats similarly stained immunohistochemically for 8-OHdG are shown in Fig. 3B. In control retinas, weak immunoreactivity against 8-OHdG was detected in a few cells of the RGCL but no immunoreactivity was detected in the INL. 8-OHdG-positive cells were detected in the RGCL, the INL, and the outer nuclear layer (ONL) of 4- and 6-month-old GK rat retinas, and 8-nitroguanine and 8-OHdG immunoreactivity was colocalized in the nuclei of cells in the INL and the RGCL (Fig. 4A, yellow in merged image).

A significant increase in the number of 8-nitroguanine- and 8-OHdG-positive cells was observed in the INL of 6-month-old GK rat retinas (Fig. 5A, B). The number of 8-nitroguanine-positive cells was increased more than four-fold, and the number of 8-OHdG-positive cells more than 2.5-fold, in the INL of 6-month-old GK rat retinas compared with age-matched control retinas ($P < 0.05$). Although a slight increase in the number of 8-nitroguanine- and 8-OHdG-positive cells was found in the RGCL of GK retinas, the increase was not significantly different from that found in control retinas (8-nitroguanine, $P = 0.059$; 8-OHdG, $P = 0.093$; Fig. 5D, E).

Because the 8-nitroguanine- and 8-OHdG-positive cells were located primarily in the INL and the RGCL, we further assessed the diabetes-induced neuronal loss by counting the nuclei in the INL and RGCL in GK and age-matched control retinas (Fig. 5C, F). We found that the numbers of 8-nitroguanine- and 8-OHdG-positive cells in the INL and RGCL in the two types of rats were not significantly different; the INL typically had about 500–600 cells/mm$^2$ and the RGCL about 190–210 cells/retinal cross section.

Together, these results indicate that nitrosative and oxidative DNA damage was present in the INL and RGCL in GK rat retinas but that these changes did not lead to significant loss of neurons at 6 months of age.

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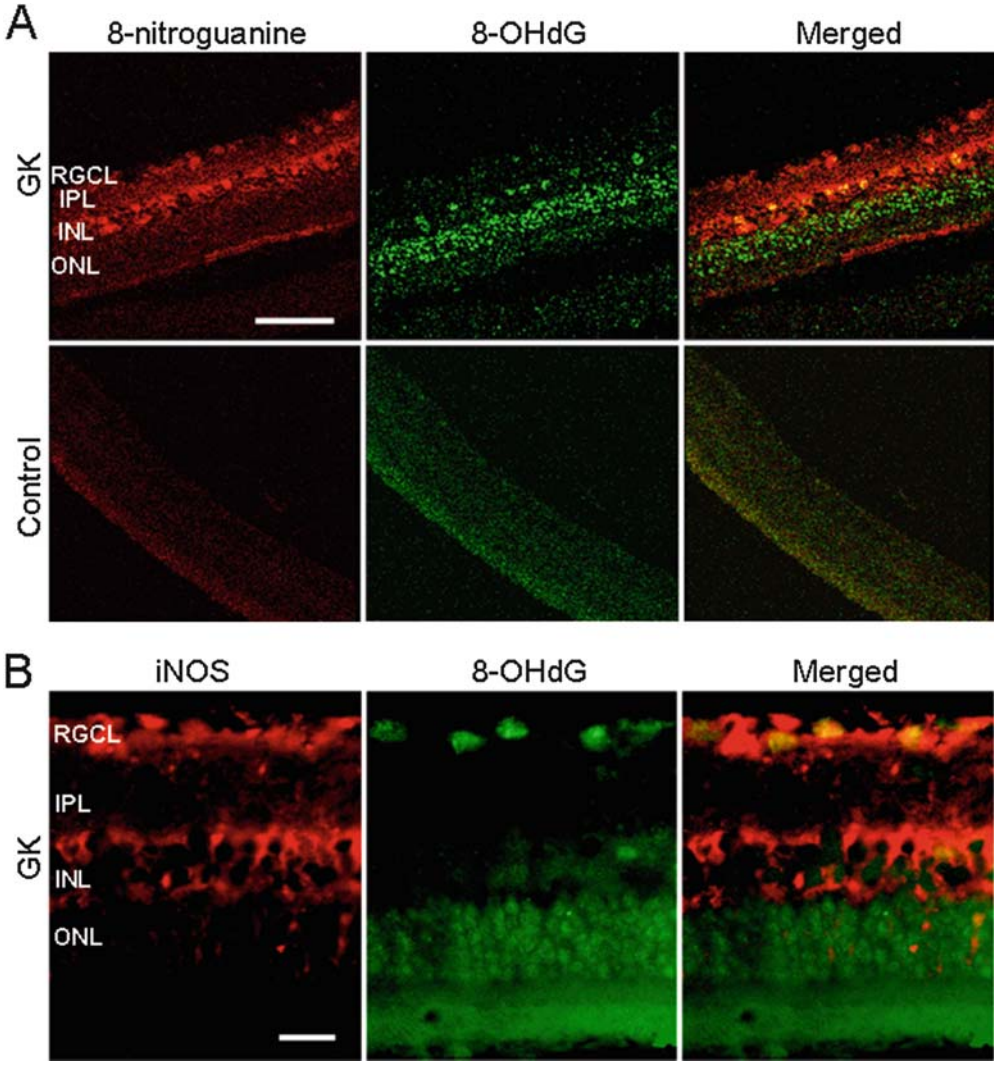


**Figure 4. A** Double-immunostaining for 8-nitroguanine (*red*) and 8-OHdG (*green*) in GK and control rats observed with an epifluorescence microscope. In 4-month-old GK retinas, the immunoreactivity of 8-nitroguanine and 8-OHdG was colocalized in the nuclei of cells of the INL and RGCL. On the other hand, in control retinas, little or no immunoreactivity against 8-nitroguanine or 8-OHdG was observed. Bar = 100 μm. **B** Double-immunostaining for iNOS (*red*) and 8-OHdG (*green*) examined with an epifluorescence microscope. In 4-month-old GK rat retinas, iNOS expression was observed mainly in Müller cells, the INL, and the RGCL, whereas 8-OHdG was observed in the nuclei of cells of the RGCL, INL, and ONL. Bars = 10 μm.

### *Expression of iNOS in Eyes of GK Rats and Control Rats*

The expression of iNOS in eye sections of GK and control rats is shown in Fig. 6. In GK retinas, iNOS expression was observed mainly in Müller cells and cells of the INL and RGCL. The immunoreactivity in the ONL most likely indicated staining of the distal processes of the Müller cells (labeled M in the figure) because of the staining pattern; that is, the stained processes extended through the ONL between the nuclei of the photoreceptor cells. In control retinas, little or no iNOS expression was observed in the Müller cells, the INL, or the RGCL. iNOS expression and 8-OHdG formation in GK retinas are shown in Fig. 4B. iNOS was expressed (red) mainly in the Müller cells and cells in the INL and RGCL, whereas 8-OHdG (green) was observed in the nuclei of cells in the RGCL, the INL, and the ONL. The immunoreactivity of iNOS and 8-OHdG was colocalized in the nuclei of cells in the RGCL and the INL (Fig. 4B, yellow in merged image).

### *Intravitreal Injections of 1400W in GK Rats*

To evaluate the potential therapeutic use of iNOS inhibitors in DR, we tested the effect of a highly potent iNOS inhibitor, 1400W,[26] in GK rats. 1400W was injected intravitreally twice (separated by a 5-day interval) into 4-month-old rats to achieve more complete iNOS inhibition in GK retinas than with subcutaneous administration. The eyes from these rats were enucleated 1 week after the last injection and prepared for the immunohistochemical detection of 8-nitroguanine and 8-OHdG. The expression of 8-nitroguanine and 8-OHdG was significantly depressed in the retinas of 1400W-treated GK rats compared with in vehicle-injected rats (Fig. 7).

## Discussion

Our immunohistochemical studies showed that the numbers of 8-nitroguanine- and 8-OHdG-positive cells were signifi-

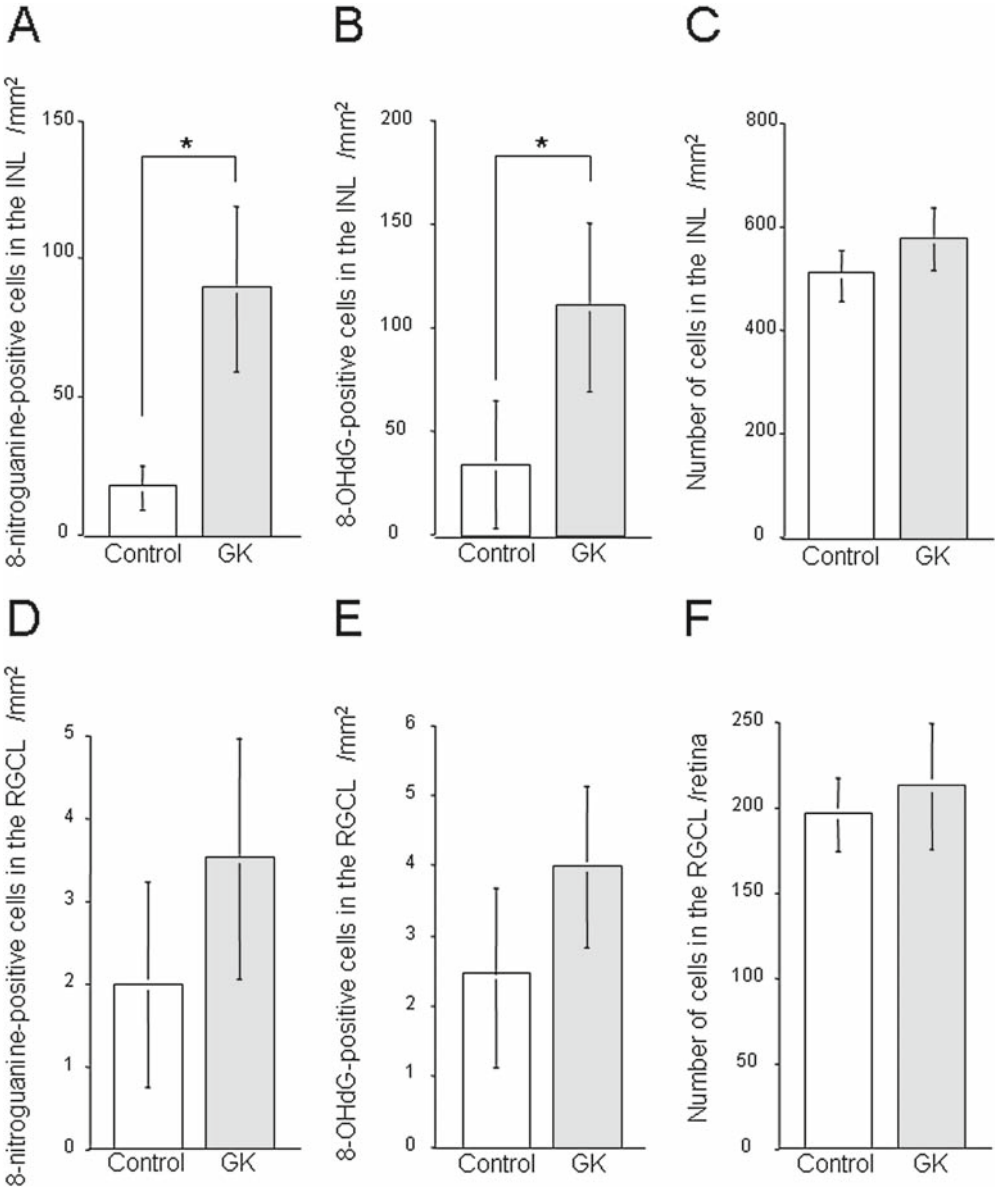


**Figure 5A–F.** Numbers of 8-nitroguanine-positive (**A**) and 8-OHdG-positive (**B**) cells, and the total number of cells (**C**) in the INL, and numbers of 8-nitroguanine-positive (**D**) and 8-OHdG-positive (**E**) cells, and the total number of cells (**F**) in the RGCL of 6-month-old GK rats ($n$ = 5) versus age-matched control rats ($n$ = 5). There were significantly more 8-nitroguanine-positive (**A**) and 8-OHdG-positive cells (**B**) in the INL of GK retinas than in that of age-matched control retinas (*$P < 0.05$). Although a slight increase in the number of 8-nitroguanine-positive (**D**) and 8-OHdG-positive cells (**E**) was seen in the RGCL of GK retinas compared with the numbers found in control retinas, the differences were not statistically significant (8-nitroguanine-positive: $P = 0.059$; 8-OHdG-positive: $P = 0.093$). No significant difference in the total number of cells in the INL (**C**) or the RGCL (F) was observed in the two types of retina. The INL typically had about 500–600 cells/mm$^2$, and the RGCL about 190–210 cells/retinal cross section.

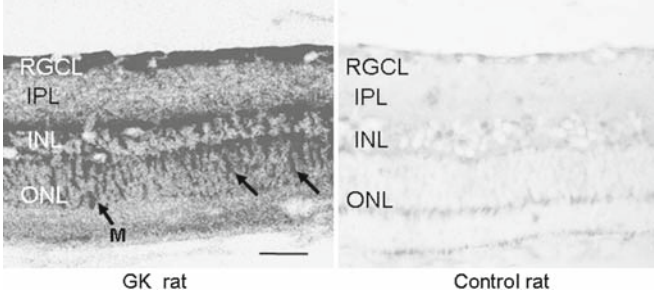


**Figure 6.** Immunohistochemical retinal sections for inducible nitric oxide synthase (iNOS). In GK rat retinas, iNOS expression was observed mainly in the processes of Müller cells (*M, arrows*), the INL, and the RGCL. In control retinas, little or no iNOS expression was observed in Müller cells, the INL, or the RGCL. Bar = 25 μm.

cantly higher in the INL of GK retinas than in that of control retinas. Although these findings indicate that significant neuronal DNA damage occurred in the retinas of GK rats, morphometric studies showed that the numbers of cells in the INL and the RGCL of 6-month-old GK rat retinas were not significantly different from those in age-matched control rat retinas. These findings indicate that the significant neuronal DNA damage did not lead to a significant loss of neurons, and they agree with the results of Takeo-Goto et al.,[30] who found no histopathological changes in GK rats at 7 months of age.

Another major finding of this study was the significantly higher levels of iNOS protein in GK retinas than in control retinas. Immunohistochemical studies showed that iNOS protein was expressed mainly in Müller cells, the INL, and the RGCL of GK retinas, whereas iNOS protein was

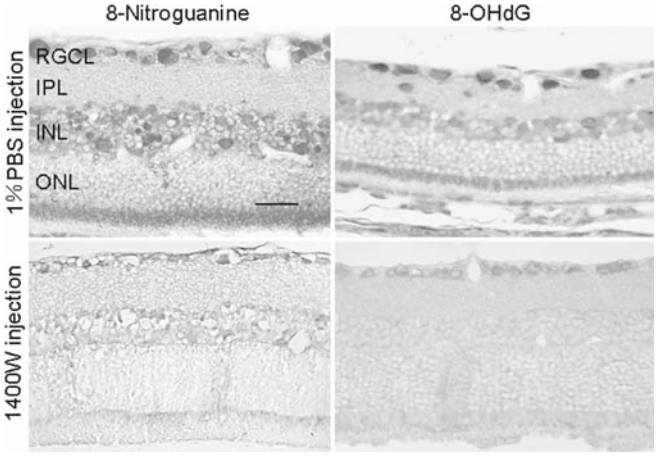


**Figure 7.** Intravitreal injections of 1400W (a specific iNOS inhibitor) in GK rats. Two microliters of vehicle [1% phosphate-buffered saline (*PBS*)] or 15 mg/ml 1400W was injected intravitreally twice (separated by a 5-day interval) into 4-month-old GK rats to achieve more complete inhibition of iNOS in GK retinas than is possible with subcutaneous administration. One week after the last injection, the retinas were isolated, and the formation of 8-nitroguanine and 8-OHdG was investigated immunohistochemically. The expression of 8-nitroguanine and 8-OHdG was significantly depressed in the retinas of 1400W-treated GK rats compared with that in vehicle-injected rats. Bar = 25 μm.

expressed only weakly in Müller cells, the INL, and the RGCL of control retinas. These observations are in agreement with the findings of an earlier study in GK rats, which showed that iNOS protein was localized in the RGCL, INL, and ONL of GK retinas, whereas iNOS protein was expressed only in the INL of control retinas.[23]

Our results showed clear staining of Müller cells. Similar results were reported by Kobayashi et al.,[31] who found higher iNOS expression in Müller cells in ischemic retinas, induced by bilateral common carotid artery occlusion, than in control retinas. Other studies have shown that iNOS is activated in both the acute and chronic phases of diabetes.[32,33] Taken together, these results suggest that DNA damage as assessed by the increased expression of 8-nitroguanine and 8-OHdG in the GK rats is mediated by NO production through iNOS.

Increased levels of 8-OHdG have been reported in STZ-induced diabetic rats,[24] and we also showed increased nitrosative and oxidative DNA damage in GK rats. Increased oxidative stress in diabetes is considered to be a factor contributing to the development of diabetic complications, including DR,[34] and reactive oxygen species are generated by high glucose levels and inflammatory cytokines. Thus, diabetes shares many similarities with chronic inflammatory diseases.

It has been reported that NO can react with $O^{2\bullet-}$ to subsequently induce DNA damage by deaminating DNA bases, resulting in mutations.[35,36] Shibutani et al.[37] illustrated mutagenic replication of 8-OHdG as a template causing G→T transversion. 8-Nitroguanine formed in DNA is also chemically unstable, and thus can be spontaneously released, resulting in the formation of an apurinic site.[17,18,20] The apurinic site can pair with adenine during DNA synthesis, leading to G→T transversion.[38]

We did not detect any significant loss of retinal neurons in the RGCL or the INL of GK rats despite increasing nitrosative and oxidative DNA damage, unlike the neuronal cell loss in the RGCL and the INL observed in the retinas of diabetic humans[3] and rats.[39–41] Martin et al.[40] suggested that diabetes-induced neuronal loss in STZ-induced diabetic rats occurs in the RGCL through an apoptotic pathway. Barber et al.[39] reported significant changes in the thickness of the INL as a consequence of diabetes. Park et al.[41] reported a thinning of the INL and marked thinning of the ONL by 24 weeks after onset of diabetes. Although no pathologic changes are observed in GK rat retinas until 7 months of age,[30] functional neuronal changes, including electroretinogram abnormalities[42] and altered glutamate metabolism,[30] can be identified, suggesting that neuronal degenerative changes occur in GK retinas apart from the vascular cells of the retina. Our data also showed neuronal abnormalities in GK retinas and suggest that although diabetes induces nitrosative and oxidative DNA damage, the damage is probably not strong enough to evoke significant loss of retinal neurons in GK retinas.

In support of the possible role of iNOS in the pathogenesis of DR, it has been shown that administration of aminoguanidine, an inhibitor of iNOS, has many beneficial effects on DR. Kern et al.[43] reported that aminoguanidine inhibits capillary apoptotic cell death and development of DR. Hammes et al.[44] also reported that secondary intervention with aminoguanidine retarded the progression of DR in a STZ-induced diabetic model. However, it is not possible at present to conclude that aminoguanidine inhibits retinopathy solely by the inhibition of NO production, because that agent also inhibits other diabetes-induced biochemical abnormalities, notably the formation of advanced glycation end products.[45]

Thus, in this study, GK eyes were injected intravitreally with 1400W at 4 months of age, when iNOS is expressed. Among the inhibitors of nitric oxide synthases, 1400W is by far the most selective in inhibiting the activity of iNOS; its ratio of selectivity for iNOS versus endothelial NOS is more than 4000-fold, in contrast to that of aminoguanidine (11-fold), $N^5$-iminoethyl-L-ornithine (49-fold),[46] and isothioureas (two- to sixfold).[47] In addition, the in vitro potency of 1400W in inhibiting iNOS is 135 and 19 times that of aminoguanidine and $N^5$-iminoethyl-L-ornithine, respectively.[46] Our experimental approach using 1400W is thus more useful than those using aminoguanidine for definitive evaluation of the role of NO in diabetic retinal damage.

Sennlaub et al.[21] reported that 1400W (2 μl of 15 mg/ml, intravitreally injected) protected the hypoxic retina from degeneration in ischemic proliferative retinopathy. Our study also showed that 8-nitroguanine and 8-OHdG were significantly suppressed in 1400W-treated eyes compared with in vehicle-treated eyes. This is good evidence that nitrosative and oxidative DNA damage in the retina of type 2 diabetes patients is closely associated with NO via iNOS activation.

In conclusion, our study demonstrated the neurotoxic effects of NO and $O^{2-}$ on retinal neurons, and these findings may implicate the iNOS pathway in the retinal DNA damage observed during the diabetic retinal pathological process.

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## References

1. Engerman RL, Kern TS. Retinopathy in animal models of diabetes. Diabetes Metab Rev 1995;11:109–120.
2. Barber AJ. A new view of diabetic retinopathy: a neurodegenerative disease of the eye. Prog Neuropsychopharmacol Biol Psychiatry 2003;27:283–290.
3. Bek T. Transretinal histopathological changes in capillary-free areas of diabetic retinopathy. Acta Ophthalmol (Copenh) 1994;72:409–415.
4. Palmowski AM, Sutter EE, Bearse MA Jr, et al. Mapping of retinal function in diabetic retinopathy using the multifocal electroretinogram. Invest Ophthalmol Vis Sci 1997;38:2586–2596.
5. Abu el Asrar AM, Maimone D, Morse PH, et al. Cytokines in the vitreous of patients with proliferative diabetic retinopathy. Am J Ophthalmol 1992;114:731–736.
6. Yuuki T, Kanda T, Kimura Y, et al. Inflammatory cytokines in vitreous fluid and serum of patients with diabetic vitreoretinopathy. J Diabetes Complications 2001;15:257–259.
7. Ueda S, Kato S, Matsuoka H, et al. Regulation of cytokine-induced nitric oxide synthesis by asymmetric dimethylarginine: role of dimethylarginine dimethylaminohydrolase. Circ Res 2003;92: 226–233.
8. Zheng L, Howell SJ, Hatala DA, et al. Salicylate-based anti-inflammatory drugs inhibit the early lesion of diabetic retinopathy. Diabetes 2007;56:337–345.
9. McDaniel ML, Kwon G, Hill JR, et al. Cytokines and nitric oxide in islet inflammation and diabetes. Proc Soc Exp Biol Med 1996;211:24–32.
10. King GL, Loeken MR. Hyperglycemia-induced oxidative stress in diabetic complications. Histochem Cell Biol 2004;122:333–338.
11. Skopinski P, Rogala E, Duda-Krol B, et al. Increased interleukin-18 content and angiogenic activity of sera from diabetic (Type 2) patients with background retinopathy. J Diabetes Complications 2005;19:335–338.
12. Sjoholm A. Aspects of the involvement of interleukin-1 and nitric oxide in the pathogenesis of insulin-dependent diabetes mellitus. Cell Death Differ 1998;5:461–468.
13. Kowluru RA, Odenbach S. Role of interleukin-1beta in the development of retinopathy in rats: effect of antioxidants. Invest Ophthalmol Vis Sci 2004;45:4161–4166.
14. Kowluru RA, Odenbach S. Role of interleukin-1 beta in the pathogenesis of diabetic retinopathy. Br J Ophthalmol 2004;88: 1343–1347.
15. Vincent JA, Mohr S. Inhibition of caspase-1/interleukin-1 beta signaling prevents degeneration of retinal capillaries in diabetes and galactosemia. Diabetes 2007;56:224–230.
16. Brune B, von Knethen A, Sandau KB. Nitric oxide and its role in apoptosis. Eur J Pharmacol 1998;351:261–272.
17. Kawanishi S, Hiraku Y. Oxidative and nitrative DNA damage as biomarker for carcinogenesis with special reference to inflammation. Antioxid Redox Signal 2006;8:1047–1058.
18. Kawanishi S, Hiraku Y, Pinlaor S, et al. Oxidative and nitrative DNA damage in animals and patients with inflammatory diseases in relation to inflammation-related carcinogenesis. Biol Chem 2006;387:365–372.
19. Inoue S, Kawanishi S. Oxidative DNA damage induced by simultaneous generation of nitric oxide and superoxide. FEBS Lett 1995;371:86–88.
20. Yermilov V, Rubio J, Becchi M, et al. Formation of 8-nitroguanine by the reaction of guanine with peroxynitrite in vitro. Carcinogenesis 1995;16:2045–2050.
21. Sennlaub F, Courtois Y, Goureau O. Inducible nitric oxide synthase mediates retinal apoptosis in ischemic proliferative retinopathy. J Neurosci 2002;22:3987–3993.
22. Sennlaub F, Courtois Y, Goureau O. Inducible nitric oxide synthase mediates the change from retinal to vitreal neovascularization in ischemic retinopathy. J Clin Invest 2001;107:717–725.
23. Carmo A, Cunha-Vaz JG, Carvalho AP, et al. Nitric oxide synthase activity in retinas from non-insulin-dependent diabetic Goto-Kakizaki rats: correlation with blood-retinal barrier permeability. Nitric Oxide 2000;4:590–596.
24. Kowluru RA, Odenbach S. Effect of long-term administration of alpha-lipoic acid on retinal capillary cell death and the development of retinopathy in diabetic rats. Diabetes 2004;53:3233–3238.
25. Goto Y, Kakizaki M, Masaki N. Production of spontaneous diabetic rats by repetition of selective breeding. Tohoku J Exp Med 1976;119:85–90.
26. Garvey EP, Oplinger JA, Furfine ES, et al. 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo. J Biol Chem 1997;272:4959–4963.
27. Portha B, Serradas P, Bailbe D, et al. Beta-cell insensitivity to glucose in the GK rat, a spontaneous nonobese model for type II diabetes. Diabetes 1991;40:486–491.
28. Erlanger BF, Beiser SM. Antibodies specific for ribonucleosides and ribonucleotides and their reaction with DNA. Proc Natl Acad Sci U S A 1964;52:68–74.
29. Hawkes R, Niday E, Gordon J. A dot-immunobinding assay for monoclonal and other antibodies. Anal Biochem 1982;119: 142–147.
30. Takeo-Goto S, Doi M, Ma N, et al. Immunohistochemical localization of amino acids in the diabetic retina of Goto-Kakizaki rats. Ophthalmic Res 2002;34:139–145.
31. Kobayashi M, Kuroiwa T, Shimokawa R, et al. Nitric oxide synthase expression in ischemic rat retinas. Jpn J Ophthalmol 2000;44:235–244.
32. Bardell AL, MacLeod KM. Evidence for inducible nitric-oxide synthase expression and activity in vascular smooth muscle of streptozotocin-diabetic rats. J Pharmacol Exp Ther 2001;296: 252–259.
33. Tannous M, Rabini RA, Vignini A, et al. Evidence for iNOS-dependent peroxynitrite production in diabetic platelets. Diabetologia 1999;42:539–544.
34. Baynes JW, Thorpe SR. Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. Diabetes 1999;48:1–9.
35. Nguyen T, Brunson D, Crespi CL, et al. DNA damage and mutation in human cells exposed to nitric oxide in vitro. Proc Natl Acad Sci U S A 1992;89:3030–3034.
36. Wink DA, Kasprzak KS, Maragos CM, et al. DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. Science 1991;254:1001–1003.
37. Shibutani S, Takeshita M, Grollman AP. Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. Nature 1991;349:431–434.
38. Loeb LA, Preston BD. Mutagenesis by apurinic/apyrimidinic sites. Annu Rev Genet 1986;20:201–230.
39. Barber AJ, Lieth E, Khin SA, et al. Neural apoptosis in the retina during experimental and human diabetes. Early onset and effect of insulin. J Clin Invest 1998;102:783–791.
40. Martin PM, Roon P, Van Ells TK, et al. Death of retinal neurons in streptozotocin-induced diabetic mice. Invest Ophthalmol Vis Sci 2004;45:3330–3336.
41. Park SH, Park JW, Park SJ, et al. Apoptotic death of photoreceptors in the streptozotocin-induced diabetic rat retina. Diabetologia 2003;46:1260–1268.

42. Matsubara H, Kuze M, Sasoh M, et al. Time-dependent course of electroretinograms in the spontaneous diabetic Goto-Kakizaki rat. Jpn J Ophthalmol 2006;50:211–216.
43. Kern TS, Tang J, Mizutani M, et al. Response of capillary cell death to aminoguanidine predicts the development of retinopathy: comparison of diabetes and galactosemia. Invest Ophthalmol Vis Sci 2000;41:3972–3978.
44. Hammes HP, Strodter D, Weiss A, et al. Secondary intervention with aminoguanidine retards the progression of diabetic retinopathy in the rat model. Diabetologia 1995;38:656–660.
45. Brownlee M. Nonenzymatic glycosylation of macromolecules. Prospects of pharmacologic modulation. Diabetes 1992;41 Suppl 2:57–60.
46. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J 2001;357:593–615.
47. Garvey EP, Oplinger JA, Tanoury GJ, et al. Potent and selective inhibition of human nitric oxide synthases. Inhibition by non-amino acid isothioureas. J Biol Chem 1994;269:26669–26676.