Original Paper

Ophthalmic Research

Ophthalmic Res 2006;38:95–104
DOI: 10.1159/000090451

Received: June 9, 2005
Accepted after revision: August 29, 2005
Published online: December 20, 2005

Maintaining the Redox-Balance Intact: Gosha-Jinki-Gan but Not Insulin Activates Retinal Soluble Guanylate Cyclase in Diabetic Rats

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Key Words
Diabetic retinopathy · Japanese phytotherapy (Kampo) · Gosha-jinki-gan · Peroxynitrite · Lipid peroxide · Soluble guanylate cyclase

Abstract
Strategies to prevent hyperglycemia-induced cytotoxic reactive oxygen species in the retina include the prevention of free radical production, activation of radical-scavenging capacities and inhibition of aldose reductase. This study examined the effect of the standardized Japanese herbal extract product gosha-jinki-gan (GJG) in comparison to insulin treatment in the rat retina. Diabetes was induced in male Wistar rats by single injection of streptozotocin (50 mg/kg i.p.). At 6 and 12 weeks, eyecups were removed for immunohistochemistry. At 12 weeks, lipid peroxidation (tested with the antiacrolein antibody, Ab5F6) was enhanced significantly in the untreated diabetic group. This effect was absent in both treatment groups, notably in the outer retina. A similar result was obtained for nitrotyrosine overproduction. As an early treatment effect, GJG – but not insulin – enhanced soluble guanylate cyclase (sGC) activation (using the function-sensing antibody, MoAb 3221). GJG not only reduces nitroxidative stress and lipid peroxidation in the retina, it also ameliorates glucose metabolism within the cells. We propose that the high glucose turnover in the insulin-treated model disturbs the intracellular redox equilibrium, one result of which might be the impaired sGC activation.

Introduction

Prolonged hyperglycemia is the key feature of diabetes mellitus, which leads to the diabetic complications including retinopathy, nephropathy and neuropathy. The primary treatment is to normalize glucose levels. Large-scale intervention trials such as the UK Prospective Diabetes Study [1] and the Diabetes Control and Complications Trial [2] have shown that intensive blood glucose control is important in reducing diabetic complications. However, there does not appear to be a level of glycemia below which the risk of progression of diabetic complications is eliminated [3].

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Very recently there has been evidence that attention needs to be paid to the glucose metabolism within the cell, which affects the intracellular redox equilibrium. In the retina, glucose metabolism depends on adequate glucose delivery [4], and it has been hypothesized that insulin-sensitive glucose transport in the retina is involved in the manifestation of diabetic retinopathy [5–7].

Müller (glial) cells express functional insulin receptors, and they possess the main glycogen stores and the main glycolytic capacity within the retina and support the carbohydrate demands of surrounding neurons [8]. In close relation to their function of nourishing retinal neurons stands their ability to metabolize neurotoxic agents: Müller cells convert glutamate into glutamine which can be taken up and metabolized by photoreceptor neurons [9]. The glutamate uptake by Müller cells is impaired in diabetes [10], leading to glutamate excitoxicity. The maintenance of adequate Müller cell function in the context of oxidative stress in the retina is thus important. The nitric oxide (NO) overproduction in diabetic conditions has to be considered in this context [11]. NO activates soluble guanylate cyclase (sGC), which catalyzes the dephosphorylation of GTP to cGMP. In the retina, sGC is present in Müller cells and on-type bipolar cells [12]. The process of K^+ siphoning, the redistribution of excess extracellular potassium [13], seems in part to depend on the adequate regulation of the cGMP-sensitive ion channels of retinal Müller cells [14]. cGMP has therefore been described as second messenger in Müller cells, integrating neuronal and glial responses [14]. Adequate regulation of cGMP levels is hence important for the maintenance of retinal function [15, 16].

To visualize alterations in sGC activation, we applied a novel monoclonal antibody which recognizes a regiospecific epitope, critical for the NO-mediated activation of the enzyme [17]. Previously we have shown that in diabetic conditions, inducible NO synthase is upregulated in a glial component of the retinal tissue as early as 6 weeks after the onset of hyperglycemia [18]. At this stage (6 weeks), free NO for the activation of sGC however was limited, due to its rapid reaction with local superoxide anions (O_2^-). sGC activation could be restored by treatment with superoxide dismutase [18], an enzyme which eliminates O_2^- [19]. We concluded that diabetes-induced oxidative stress mechanisms impair cGMP homeostasis and lead to dysfunction of glial activities.

Several studies have been conducted which have focused on hyperglycemia-induced redox stress mechanisms. Besides the amelioration of blood glucose control, different pharmaceuticals with radical-scavenging ability and aldose reductase inhibitors have been tested to prevent diabetic retinopathy [20–22]. In the rat retina, the presence of aldose reductase has been confirmed in the retinal glial cells and pericytes of retinal capillaries [20]. In streptozotocin (STZ)-diabetic rats, sorbitol levels in the retina have been found to be significantly increased suggesting that sorbitol accumulation plays a role in the development of diabetic retinopathy [20].

Aldose reductase inhibitors prevent the formation of sorbitol, which would otherwise lead to an elevated $NADH/NAD^+$ ratio and favor increased levels of reactive sugars. Those sugars undergo autoxidation. The result is nonenzymatic glycation, a process which induces the formation of reactive oxygen or nitrogen species [23]. The O_2^- thus generated reacts with NO to produce considerable amounts of peroxynitrite ($ONOO^-$), followed by lipid peroxidation [24]. We confirmed these mechanisms in our animal model of diabetic retinopathy and could show that as a consequence, the availability of NO for the activation of sGC is reduced [18].

Since cytotoxic reactive gases play a major role in the development of early diabetic complications, we now ask how insulin and the standardized Japanese herbal extract product gosha-jinki-gan (GJG) interfere with these processes in the diabetic rat retina.

GJG is a combination product of 10 different herbs, which has been widely tested for the indication of diabetic neuropathy [25, 26]. Experimental research on the single herb ingredients as well as the 10-herb formula has revealed the following physiological activities: Antioxidative actions [27, 28], vasodilation, mediated through NO production [29], anticoagulatory effects [30], aldose reductase inhibition [31, 32] and improvement of glucose utilization in both in vitro and animal models [33–35].

For comparison, the main effect of insulin treatment is to lower blood glucose levels. Insulin hence minimizes the direct pro-oxidant effects of elevated blood glucose. However, it has no direct radical-scavenging abilities.

In this paper the different acting mechanisms of GJG are discussed in the context of diabetic preretinopathy damage and compared to the effects of insulin treatment.

Materials and Methods

Study Medication

The extract product GJG (TJ-107 Tsumura gosha-jinki-gan extract granules) is a standardized prescription agent made from 10 East Asian medicinal herbs. Figure 1 shows the three-dimensional HPLC of the extract product. The manufacturing process meets all requirements of the Japanese and International GMP guidelines.

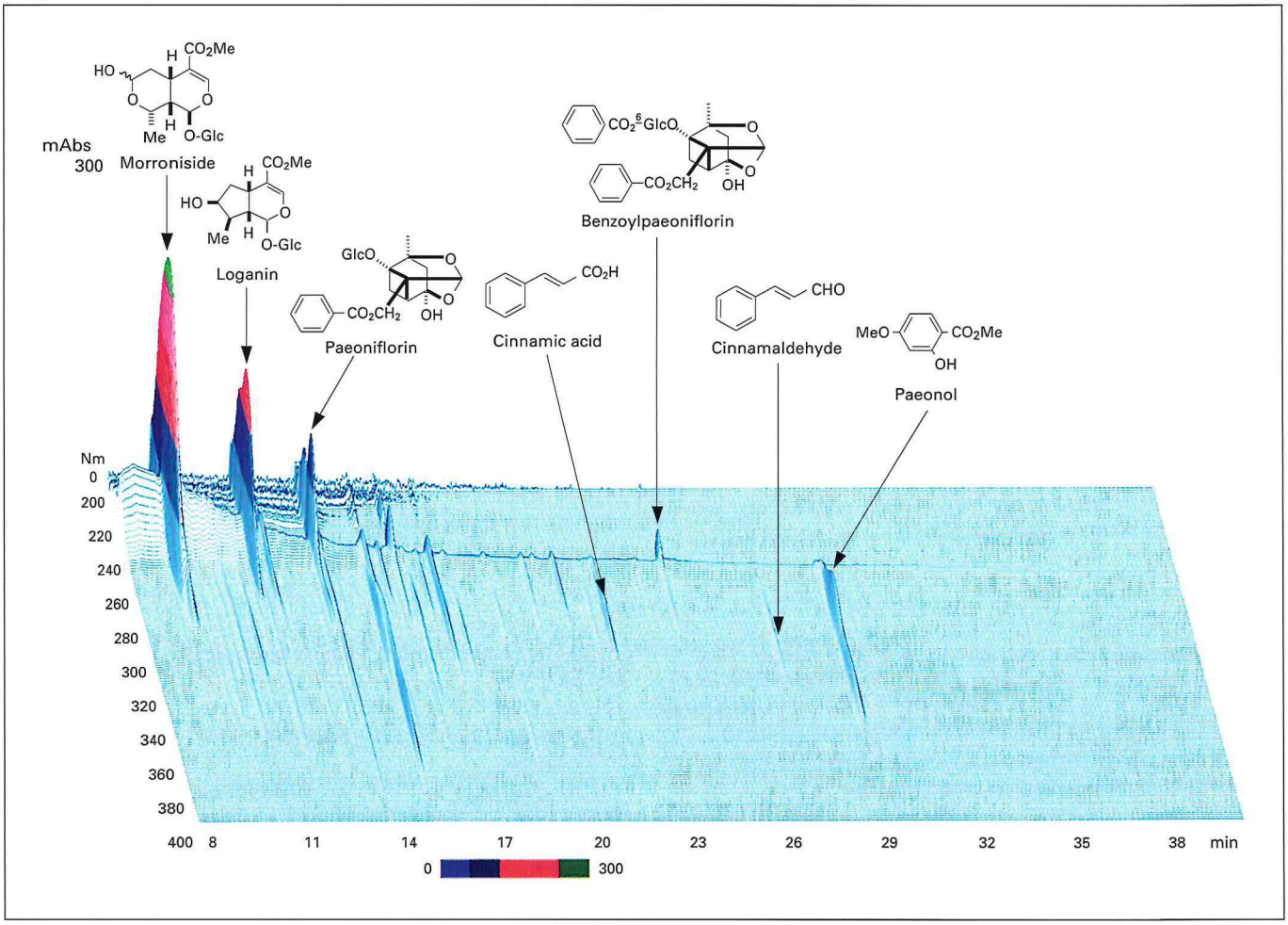

Fig. 1. Three-dimensional HPLC of GJG (TJ-107). For separation of the biopolymers, a TSK-Gel 80_{TS} column (4.6 × 250 mm; Tosoh, Japan) was used. AcOH-$AcONH_4$ (50 m*M*) and acetonitrile (CH_3CN) were used as buffers. Samples were detected by SPD-$M10A_{VP}$ (Shimadzu, Japan) and analyzed with Class LC-10 Ver.1.62 (Shimadzu). The figure was kindly contributed by Tsumura & Co., Japan.

For the application in humans, a daily dose of the study medication (7.5 g) contains 4.5 g of a spray-dried mixed extract made from the following herbs: rhemanniae radix (5.0 g), achyranthis radix (3.0 g), corni fructus (3.0 g), dioscoreae rhizoma (3.0 g), plantaginis semen (3.0 g), alismatis rhizoma (3.0 g), Poria (Hoelen) (3.0 g), moutan cortex (3.0 g), cinnamomi cortex (1.0 g) and heat-processed aconiti tuber powder (1.0 g). By means of a special heat-processing procedure, the total content of aconitine alkaloid is reduced to a nontoxic daily dose of less than 90 μg [36].

Animal Experiments

All animal experiments were performed with the approval and in accordance with the recommendations of the Animal Care and Utilization Committee of Keio University School of Medicine. Conventional male adult Wistar rats (280 ± 10 g) were kept under a 24-hour light and dark cycle at an ambient room temperature of 22–23°C, with free access to water and standard rat chow. Following an overnight fast, diabetes was induced by a single intraperitoneal injection of STZ (50 mg/kg body weight; Sigma, Tokyo, Japan), dissolved in 0.1 *M* citrate buffer (pH 4.5). Diabetic blood glucose levels (fig. 2) were found on the following day, using glucose test strips (BM-Accutest; Roche Diagnostics, Tokyo, Japan) on blood taken after puncturing the tail vein [37]. A control group of 5 rats received the citrate buffer alone. Treatment was started 24 h after diabetes induction. The diabetic rats were randomly assigned to 1 of 3 groups (of 3 rats for each the 6- and 12-week experiments): one group remained untreated, one group received GJG (1 g TJ-107 extract granules/kg/day dissolved in the drinking water), and another group received insulin (2–4 IU Actraphane 30/70) by subcutaneous injection once daily administered in the evening. The initial dose of 4 units Actraphane had to be reduced in 3 rats within the first week of diabetes to prevent hypoglycemia. Blood glucose levels (using an internationally approved system of blood test strips; Accutest) and body weight were measured at regular intervals.

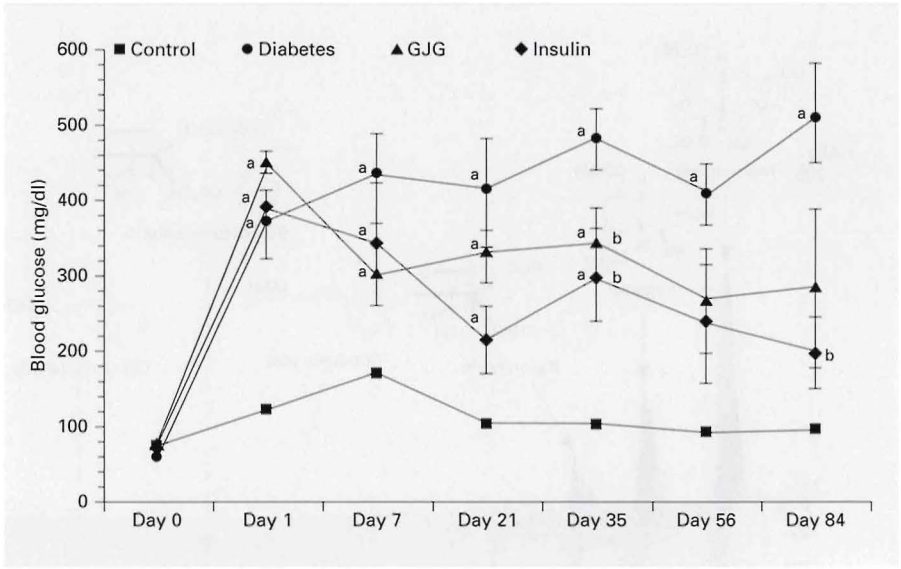

Fig. 2. Development of blood glucose. Note the significant increase in blood glucose in the untreated diabetic rats: [a] $p < 0.05$, compared to controls; [b] $p < 0.05$, compared to untreated diabetic rats. There is a decrease in blood glucose in both treatment groups, which becomes significant as against the diabetic rats. The mean blood glucose level of all diabetic rats remains above 200 mg/dl. Values are expressed as means ± SE.

After 6 and 12 weeks, the rats were sacrificed. They were anesthetized by intraperitoneal injection of pentobarbital sodium (Somnopentyl, KS Tokyo, Japan) followed by perfusion fixation with phosphate-buffered saline and paraformaldehyde (4%) [18]. The eyes were then prepared and the parietotemporal pole was marked with ophthalmic silk (0.4metric, Mani Sutures, Japan) for orientation. They were enucleated, and retinal cups were prepared carefully under the microscope. Additional immersion fixation was done for 1 h in 4% paraformaldehyde on ice. Afterwards, the eyecups were passed through different solutions of sucrose to achieve cryoprotection [18]. The following day, the eyecups were embedded in OCT compound (Tissue-tek, Sakura, Japan), frozen with liquid nitrogen and stored at –80°C.

Immunohistochemistry

For immunostaining, 7-μm-thick slices were cut sagittally, using a hand-driven cryostat (Leica), and mounted on APS-superfrost slides (Matsunami, Japan) by thaw fixation. Immunohistochemistry was carried out using an avidin-biotinylated enzyme complex system (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, Calif., USA) [18]. Staining was performed 3–5 times for all the antibodies on different eyecups. One product of lipid peroxidation was assessed using the monoclonal antiacrolein (Ab5F6, 2 μg/ml) antibody. The antibody is specific to the acrolein-lysine adduct of oxidized proteins [38]. The formation of peroxynitrite was indirectly tested using the commercially available anti-nitrotyrosine polyclonal antibody (NO_2-Y, 4 μg/ml; Upstate Biotechnology, Lake Placid, N.Y., USA). This antibody binds to proteins which have been posttranslationally modified by the NO-derived oxidant peroxynitrite [39]. The gas-mediated activation of sGC was examined, using a function-sensing antibody against sGC (MoAb 3221, 30 μg/ml). This antibody binds to a specific region of the α- and β-subunit and thus recognizes the conformational change of the enzyme upon binding of NO to the prosthetic heme of the β-subunit. sGC 3221 antibody staining was compared with the sGC 28131 antibody (50 μg/ml) which binds to the β-subunit of the enzyme alone and is therefore independent of enzyme activation [12, 17, 18].

Evaluation of Site-Specific Changes of Immunoreactivities in the Retina

For statistical evaluation, the stained tissue sections were photographed at a magnification of ×40 using a digital camera set for low gain, 48 bit (16 bit per red-green-blue color), at a resolution of 1,600 × 1,200 pixels. For each antibody used, 3 pictures were taken from each of 3 different cuts for each group. In the following analyses, the data from 5 eyes (control and diabetic group) and 3 different rats (GJG- and insulin-treated groups) were used. Site-specific changes in immunoreactivities among retinal cell layers were evaluated according to previously established protocols [40] with modifications [18]. For each day of staining, several images were taken with a blank slide in order to allow us to 'flatfield' the images. The idea is that variations in the blank-slide image are due to variations of the lighting across the image or camera responsivity. Whilst these effects were small, it is still in principle desirable to remove them. To use the intensities of the chromogen formed by the diaminobenzidine-horseradish peroxidase reaction as an index of immunoreactivities, nuclear counterstaining with methyl green was removed using the fact that its color composition is different to the horseradish peroxidase staining in the images. (For acrolein, counterstaining with methyl green was omitted, since staining with the antiacrolein antibody falls into the cell nuclei, allowing orientation.) We then have a digital image of the amount of immunostaining present. For each image, the staining intensity was measured in 5 different layers of the retina: these layers were the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer nuclear layer (ONL) and external limiting membrane (ELM). To avoid bias in image analysis, an algorithm was developed to detect the structure of the layers and place 5 boxes automatically along each evaluated layer of the retina. For each box, the average staining intensity was calculated. In the case of antiacrolein and NO_2-Y staining the uniform background was first

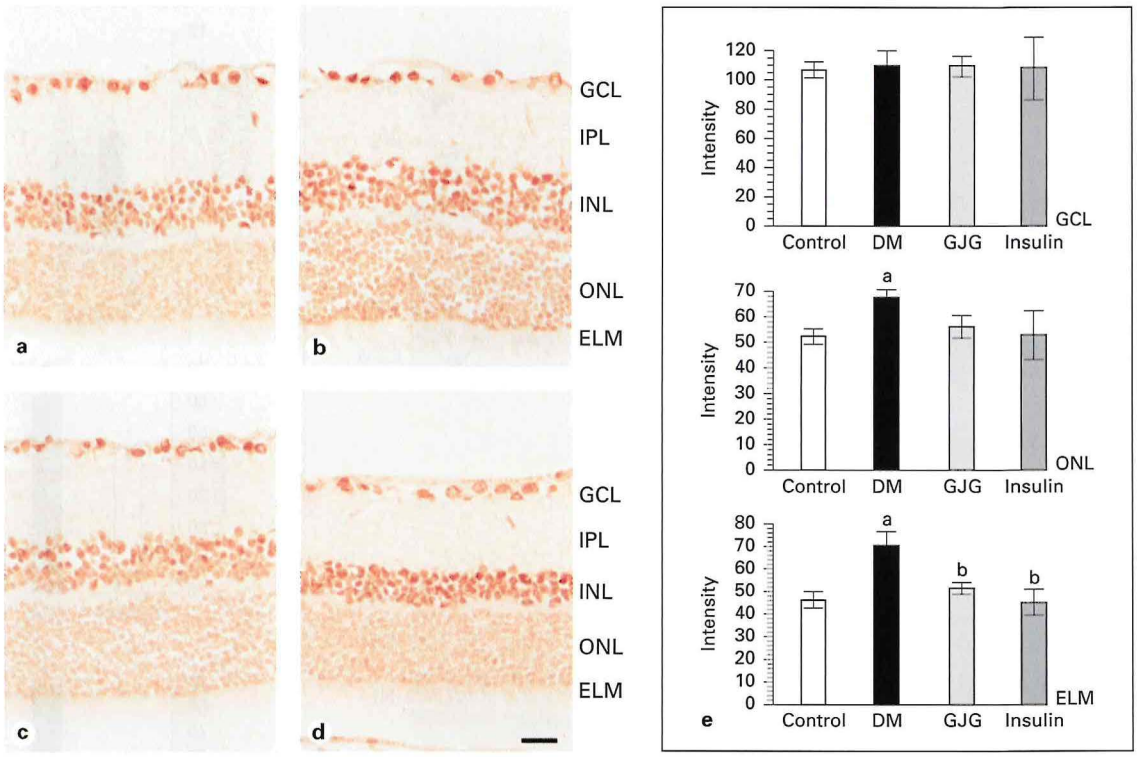

Fig. 3. The increased lipid peroxidation in the diabetic rat retina as detected with the antiacrolein antibody is reduced by GJG and insulin treatment after 12 weeks of diabetes. (Counterstaining with methyl green has not been performed for this antibody.) **a** Vehicle-treated control. **b** Diabetic rat group. **c** Diabetic rat group treated with GJG. **d** Diabetic rat group treated with insulin. **e** Layer-specific changes of acrolein immunoreactivity expressed as gray levels in the control, untreated diabetic (DM), GJG-treated diabetic (GJG) and insulin-treated diabetic groups (insulin). Scale bar = 25 μm. [a] $p < 0.05$, compared to controls; [b] $p < 0.05$, compared to untreated diabetic rats. Values are expressed as means ± SE.

removed. The mean of the 5 boxes was used in the subsequent analysis of layer-specific immunoreactivity (gray levels).

With NO_2-Y staining, vertical stripe-like structures appeared and were clearly visible in the IPL. In addition to the above computer analysis, those neurofilaments which crossed the upper line of the box in the IPL were counted manually.

Statistics

Values are expressed as means ± SE. Significant differences between the means were evaluated using ANOVA followed by Student's t test. Differences of $p < 0.05$ were considered to be statistically significant.

Results

Development of Blood Glucose

As shown in figure 2, the blood glucose levels of the healthy control group remained normal throughout the study. As is to be expected, the untreated diabetic group's blood sugar levels were significantly higher than the control group's throughout the entire study. The average of the means (excluding day 0 which is before the STZ injection) was 435 mg/dl. The GJG- and insulin-treated groups also had elevated blood glucose levels, with the average of their shown means being 329 and 280 mg/dl, respectively. Both groups began with significantly higher blood glucose levels than the control group; however, at week 8 the significance was lost, although the mean blood glucose level remained above 200 mg/dl. Due to the sacrifice of rats at 6 weeks, the variability had increased.

As figure 2 indicates, the standard error of the measured glucose levels of the diabetic, insulin-treated and GJG-treated groups was high. The individual rats in these groups show large variations in their glucose levels measured at the different time points. This time variability in part reflects our experimental setup, where the rats had free access to rat chow during the night and the blood

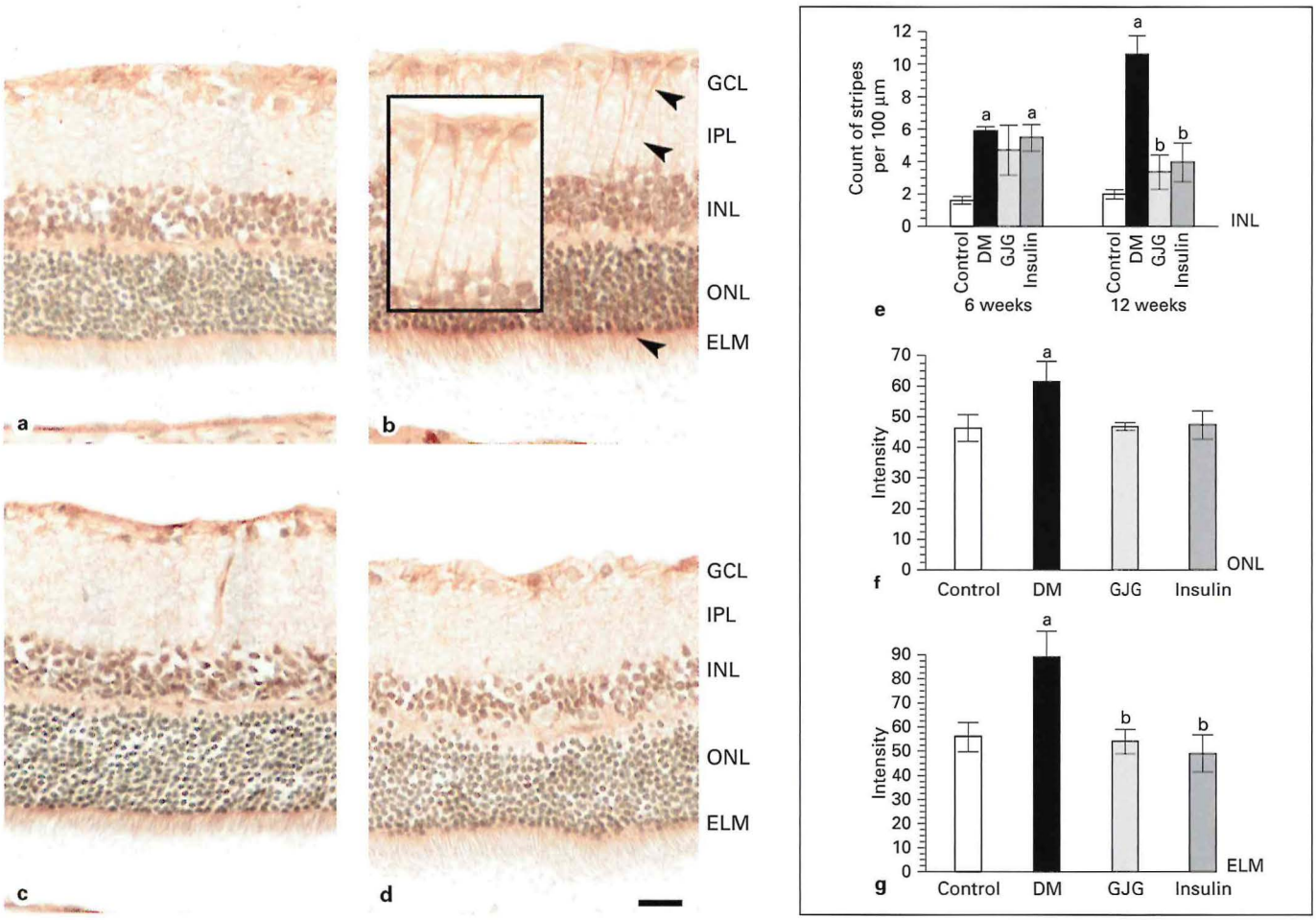

Fig. 4. The increased peroxynitrite formation (arrowheads) in the diabetic rat retina is significantly reduced at 12 weeks in the GJG and insulin-treated groups. **a** Vehicle-treated control. **b** Diabetic rat group. **c** Diabetic rat group treated with GJG. The inset shows a detail image of the GCL and IPL layers. **d** Diabetic rat group treated with insulin. **e** NO_2-Y immunoreactivity expressed as the number of stripe-like structures in the IPL. **f**, **g** Layer-specific changes of NO_2-Y immunoreactivity at 12 weeks expressed as gray levels in the control, untreated diabetic (DM), GJG-treated diabetic (GJG) and insulin-treated diabetic groups (insulin). Scale bar = 25 µm. [a] $p < 0.05$, compared to controls; [b] $p < 0.05$, compared to untreated diabetic rats. Values are expressed as means ± SE.

glucose was measured in the mornings. In these circumstances the individual rat's glucose level varies depending on when and how much the rat ate and drank. This variation accounts for a substantial part of the standard errors.

Lipid Peroxidation and Peroxynitrite Formation in the Diabetic Rat Retina

Figure 3 illustrates the distribution of antiacrolein MoAb5F6 staining in the different rat groups at 12 weeks. Compared to the control rat retina (fig. 3a), the retina of the diabetic group (fig. 3b) showed a strong immunoreactivity for this antibody, especially in the ONL and ELM. The statistical analysis revealed a significant increase of stain strength in these layers for the diabetic rat group (fig. 3e). In contrast, this increase was not seen in the GJG- and insulin-treated diabetic rats (fig. 3c, d). Immunohistochemical densitometry confirmed this difference to be statistically significant (fig. 3e).

Using the anti-NO_2-Y antibody, we previously concluded that peroxynitrite formation is enhanced in the diabetic rat retina [18]. Figure 4b shows the immunopositive stripe-like structures of Müller-cell-associated processes extending throughout the whole retina. These pro-

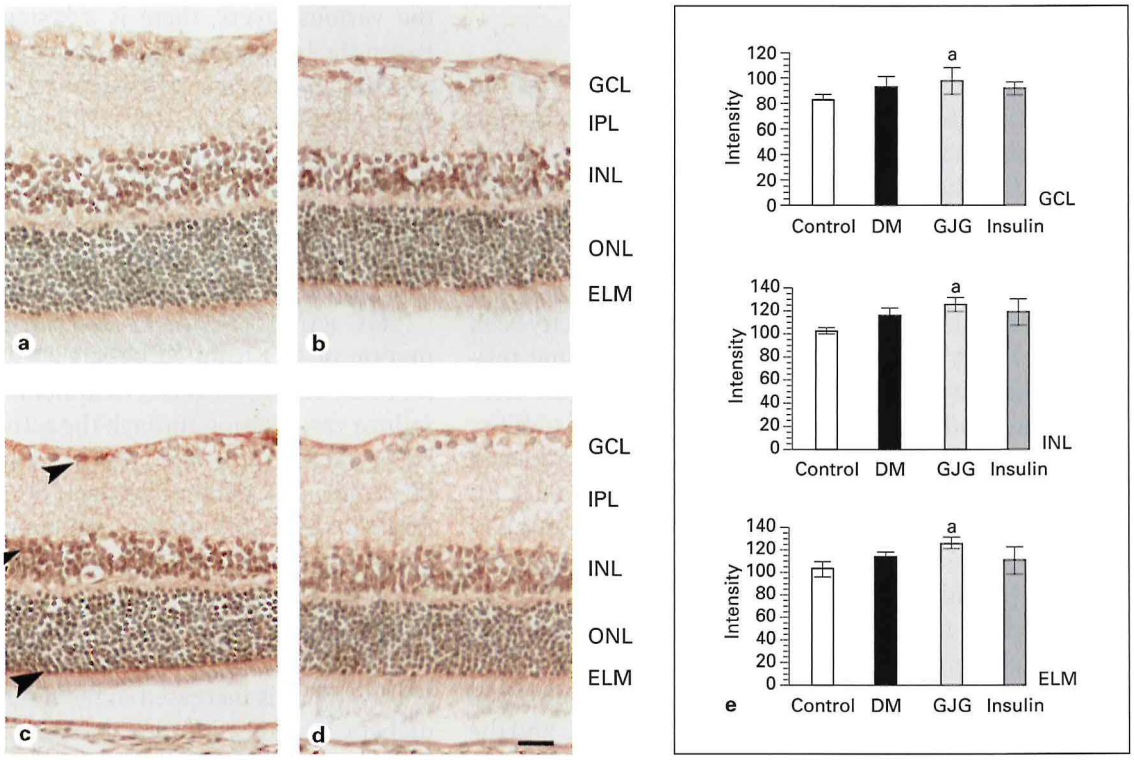

Fig. 5. Visualization of sGC activities at 6 weeks after the induction of diabetes. Note the increase (arrowheads) in the GJG-treated group as compared to the control. **a** Vehicle-treated control. **b** Diabetic rat group. **c** Diabetic rat group treated with GJG. **d** Diabetic rat group treated with insulin. **e** Layer-specific changes of sGC 3221 immunoreactivity expressed as gray levels in the control, untreated diabetic (DM), GJG-treated diabetic (GJG) and insulin-treated diabetic groups (insulin). Scale bar = 25 μm. * $p < 0.05$, compared to controls. Values are expressed as means ± SE.

cesses are clearly visible in the IPL of the untreated diabetic rat group at 12 weeks after STZ administration. Statistical analysis showed a significant enhancement also in the ONL and the ELM (fig. 4e). Similar to the antiacrolein staining, the enhancement in this diabetic group was most apparent in the outer retina. Again, the increase in stain intensity was not seen in either treatment group at 12 weeks. When the stripe-like processes were counted in the IPL, we found their density to be significantly increased in the untreated diabetic group. This increase became apparent at 6 weeks and was further enhanced at 12 weeks. In both treated diabetic groups, we found an increase in stripes at 6 weeks of diabetes, which for the insulin-treated group was significant. However, there is a decrease in stripe density at 12 weeks for both treatment groups. Compared to the untreated diabetic group, this decrease is significant (fig. 4e).

Early sGC Activation in GJG Treatment

Since we found that NO-mediated oxidative changes in the diabetic rat retinas were attenuated by both GJG and insulin, we looked at other NO-mediated processes, such as sGC activation.

No significant changes in the sGC levels were observed for the untreated diabetic group. In the GJG-treated diabetic group however, there was early sGC activation as compared to the control group at 6 weeks (fig. 5c). Statistical analysis of the stain intensity showed the increase in sGC 3221 staining to be statistically significant in the GCL, INL and ELM (fig. 5e). At 12 weeks, no statistically significant difference from the control was observed. The intensity of sGC 28131 staining did not change significantly in our study (data not shown). Insulin treatment did not restore sGC activity at a statistically significant level in any layer of the retina at any time point.

Discussion

The current study showed that both GJG and insulin are able to inhibit the formation of the lipid peroxidation product acrolein and of peroxynitrite in the retina of STZ-induced diabetic rats. We have previously discussed that under diabetic conditions, these oxidative processes are initiated by the formation of excess NO and O_2^- [18]. Locally overproduced O_2^- readily reacts with NO, resulting in peroxynitrite formation [24], which has indirectly been detected by an antibody against nitrated tyrosine residues. Tyrosine residues on the surface of proteins form dityrosine bonds, which influence the structural stability and function of the proteins. Dityrosine bonds are formed through radical reactions including hydroxyl radical, O_2^- and peroxynitrite, but not by oxygen or NO themselves [41]. We have shown that the nitroxidative changes are localized to Müller cells [18]. These cells extend throughout the whole retina, forming zonulae adherentes with adjacent photoreceptors in the ELM.

The lipid peroxidation product acrolein is formed in the outer retina (reaching significance in the ONL and the adjacent ELM [18]). Antiacrolein staining is significantly elevated in the nuclei of the ONL but is also visible in the INL, pointing to its reactivity toward DNA [42]. In this context, very recently it has been shown that acrolein can induce apoptosis through the mitochondrial pathway leading to DNA fragmentation [43].

From these observations we concluded that the outer portion of the retina serves as a primary target of oxidative stress in diabetic preretinopathy damage. This might be related to the low microvascular density in the outer retina which could make these layers most susceptible to shortage of oxygen and nutritional supply, especially since the ONL and ELM contain the photoreceptors, which constitute another major cell compartment for oxygen consumption.

The two types of treatment differed in terms of sGC activation. Whereas early sGC activation was observed at a statistically significant level with GJG treatment in the GCL, INL and ELM, no such effect was observed with insulin. The absence of sGC activation with insulin treatment is in accordance with separate findings in endothelial cells under hyperglycemic conditions, in which insulin downregulated cGMP accumulation [44].

Possibly because of our limited sample size, the difference between the sGC activation with insulin treatment and GJG treatment is not significant in any layer of the retina, so future studies are certainly needed before definitive statements can be made. Nonetheless, taken over the various layers, there is a systematic difference between the treatments, and it is interesting to speculate as to what mechanism, consistent with the other observed facts, could produce such a change. In a future study it would be desirable to confirm each of these findings with different methodological settings, as well as to measure the various reaction products in order to have a clearer and more complete picture of the cellular processes in hyperglycemic and treatment conditions.

sGC activation depends upon the presence of NO. Formation of NO from endothelial NO synthase is known for both, insulin and GJG treatment: Compounds of GJG induce vasodilation through the activation of endothelial NO synthase [45]. With regard to insulin, NO has been described as 'second messenger'[46]. In an STZ-induced diabetic rat model, insulin treatment induced vasorelaxation dependent on endothelial NO synthase [47]. However, we do not see NO-induced sGC activation in our insulin-treated rats.

As we know from our previous experiments, inducible NO synthase is increased in the inner retina of untreated diabetic rats. In spite of the increased NO production, we did not find sGC activation. We explained these results as being due to scavenging of NO by O_2^- to form peroxynitrite. Upon cancellation of superoxide generation by intravenous administration of a copper/zinc-type superoxide dismutase [19], sGC activity was restored [18].

In the case of insulin and GJG treatment, the levels of NO_2-Y antibody staining are reduced. This is expected from previous studies where it has been shown that both insulin [48, 49] and GJG [27, 28, 50] suppress the production of superoxide radicals in diabetes. NO should therefore be available for sGC activation. Since both GJG and insulin induce NO production, the differences between GJG and insulin on sGC activation cannot be explained by NO availability.

A more promising mechanism is proposed as follows: glucose metabolism occurs through glycolysis and the polyol pathway, the latter of which is particularly active in diabetic conditions. Both these pathways generate NADH. NADH has to be continually reoxidized, which results in cytosolic reductive stress. During the transient periods of high glucose metabolism the reductive stress might be especially severe in the outer layers of the retina where the oxygen supply is limited. If NADH is generated faster than it can be reoxidized within the mitochondria, the redox equilibrium of the cell is damaged [23]. We hypothesize that the disturbed redox equilibrium of the cell, which accompanies the high glucose influx induced by insulin treatment, impairs the pathway by which NO activates sGC.

Obrosova et al. [51] could show that glycolysis is depressed in galactose-fed rats and that this phenomenon is consistent with a decrease in NAD^+/NADH ratios and an increase in $NADP^+$/NADPH which is also seen in diabetic conditions. Aldose reductase is the enzyme which catalyzes the rate-limiting step in the degradation of glucose, during which NADPH is converted into NADP. Aldose reductase inhibitors can (partially) prevent the resulting hypoxia-like metabolic changes [52]. They also have been shown to counteract diabetes-induced nitrosative stress [53]. GJG is believed to have aldose reductase inhibitory function, whilst insulin does not. For future studies it would be desirable to administer aldose reductase inhibitors to our untreated and insulin-treated diabetic rats for comparison.

The clinical relevance of this process might be seen in the outcome of large-scale intervention trials such as the Diabetes Control and Complications Trial [2]. This trial showed worsening of diabetic retinopathy in patients at the beginning of intensive insulin treatment compared to the conventional treatment group [54]. The temporary deterioration of retinopathy was absent when aldose reductase inhibitors – which inhibit the increased glucose metabolism through the polyol pathway – were administered [22]. The administration of aldose reductase inhibitors to prevent the development of diabetic microangiopathy and basement membrane thickening had previously been shown in animal models [20, 21]. Kampo medicine but not insulin is effective in this context [31, 32].

Our results suggest that administration of GJG not only reduces nitroxidative stress and lipid peroxidation in the retina, but also ameliorates glucose metabolism within the cells. We tentatively suggest that the high glucose turnover within the insulin-treated model disturbs the intracellular redox equilibrium, one result of which might be the impaired sGC activation.

The use of GJG in conjunction with insulin is worth further study. Recently, a Japanese group [35] has found that GJG increases insulin-induced intracellular glucose levels significantly. GJG might therefore help to reduce the amount of insulin needed to maintain energy metabolism within the retina and reduce diabetes-induced complications.

Acknowledgements

We would like to thank Mayumi Kajimura, Department for Biochemistry and Integrative Medical Biology, for her helpful comments during the discussion of the data. Furthermore, we thank Robert Cameron, Max Planck Institute for Solar System Research, Katlenburg-Lindau, Germany, for his contribution in relation to the computer analysis program. This work was supported by a joint grant of the German Alexander von Humboldt Foundation and the Japan Society for the Promotion of Science. This work was further supported by a grant for the 21st Century Center-of-Excellence Program and partly by a Leading Project for Biosimulation and Grant-in-Aid for Creative Scientific Research 17GS0419 from the Ministry of Education and Sciences Technology in Japan. The standardized extract product GJG (Tsumura gosha-jinki-gan) for scientific use was kindly provided by Tsumura & Co., Tokyo, Japan.

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