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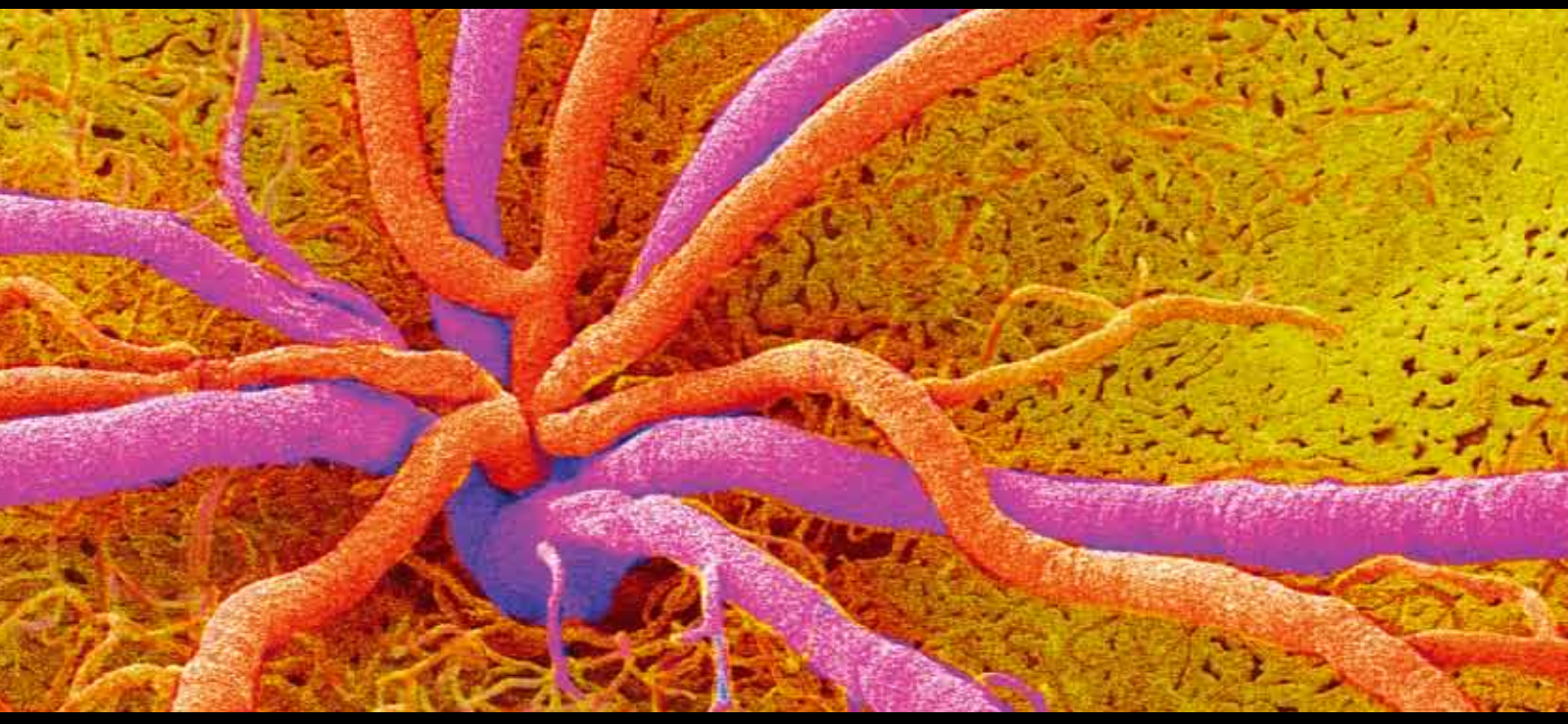
New Horizons in Research on Diabetic Complications of the Eye: Special Emphasis on Diabetic Cataracts and Retinopathy

Guest Editors: Kavita R. Hegde, Renu A. Kowluru, Susanne Mohr, Ram H. Nagaraj, and J. Mark Petrash



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Editorial Board

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Editorial

New Horizons in Research on Diabetic Complications of the Eye: Special Emphasis on Diabetic Cataracts and Retinopathy

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The incidence of diabetes is increasing at an alarming rate all over the world. As a consequence, the complications related to diabetes, including those affecting the eye (cataracts and retinopathy), nerve, and kidneys, are also becoming more prevalent. In the eye, diabetes affects both lens and retina resulting in devastating effects on vision. Although significant advances have been made towards understanding their pathophysiology using animal models of diabetes, progress in the area of development of pharmacological means for their prevention and treatment is still very minimal, if any. The pathogenetic mechanisms underlying both of these ocular complications of diabetes are considered multifactorial. The major factors considered to be involved in diabetic cataractogenesis are the hyperglycemia-induced excessive generation of free radicals and consequent oxidative stress as well as aberrations in tissue metabolism. Free radicals have also been implicated in the pathogenesis of diabetic retinopathy (DR). In addition, DR is considered to be associated with aberrations in several other cellular signaling pathways mediated via inflammatory intermediators, pigment epithelium-derived factor, various kinases, and Rho and Ras GTPases. Identification of pathways/molecules that can be targeted for modification by appropriate compounds is highly essential for the development of pharmacological therapy for these highly prevalent blinding diseases. In this special issue intended to present new research on diabetic cataracts and retinopathy, we have invited papers that address these issues.

The first four articles in this issue are focused on cataract formation in diabetes. The first one provides an overview of the pathogenesis of diabetic cataracts, clinical studies investigating the association between diabetes and the development of cataract, and current treatment of cataracts in the diabetic population. The anticataractogenic potential of aldose reductase inhibitors and antioxidants has also been discussed. The article by Petrash et al. focuses on the role of aldo-ketoreductases in the eye. The expression profiles of the AKR1B1 and AKRB10 have been characterized in various human ocular tissues and their implications in the pathogenesis of diabetic cataract have been discussed. The next article examines the role of glyoxalase I in the inhibition of methylglyoxal advanced glycation end products-AGEs formation in lenses of transgenic mice overexpressing human glyoxalase I, and the fourth one reviews the pathophysiological characteristics of ocular complications in the Spontaneously Diabetic Torii rat such as the formation of cataracts which can become hypermature. It also suggests the suitability of this model for the study of DR. Subsequent articles in this issue focus on the pathogenesis of DR and examine possible targets for pharmacological intervention. The article by Daniel Ng reviews the genetic aspects of DR, and the next article identifies genes associated with DR among Mexican Americans from Starr County in Texas using genome-wide association study. The seventh paper examines the possibility of treatment with the angiotensin-converting enzyme inhibitor, trandolapril, for regression of DR, and

the 8th paper discusses the role of toxic AGEs receptor in the pathogenesis of DR. This is followed by a review implicating Rho/ROCK (Rho Kinase) pathway in DR and suggests the possibility of this pathway being a target for therapeutic intervention. The article by Wu et al. investigates the role of extracellular signal-regulated kinase 5 (ERK5) mediated signaling in modulating glucose-induced expression of VEGF. The final paper in this issue compares NADPH oxidase versus mitochondria-derived reactive oxygen species in glucose-induced apoptosis of pericytes in early DR.

This special issue will therefore provide the readers an insight into some of the latest research being pursued to elucidate the mechanisms underlying DR and cataracts. It also presents some interesting targets which could be manipulated pharmacologically for potential clinical use.

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Review Article

Diabetic Cataract—Pathogenesis, Epidemiology and Treatment

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Cataract in diabetic patients is a major cause of blindness in developed and developing countries. The pathogenesis of diabetic cataract development is still not fully understood. Recent basic research studies have emphasized the role of the polyol pathway in the initiation of the disease process. Population-based studies have greatly increased our knowledge concerning the association between diabetes and cataract formation and have defined risk factors for the development of cataract. Diabetic patients also have a higher risk of complications after phacoemulsification cataract surgery compared to nondiabetics. Aldose-reductase inhibitors and antioxidants have been proven beneficial in the prevention or treatment of this sightthreatening condition in in vitro and in vivo experimental studies. This paper provides an overview of the pathogenesis of diabetic cataract, clinical studies investigating the association between diabetes and cataract development, and current treatment of cataract in diabetics.

1. Introduction

Worldwide more than 285 million people are affected by diabetes mellitus. This number is expected to increase to 439 million by 2030 according to the International Diabetes Federation.

A frequent complication of both type 1 and type 2 diabetes is diabetic retinopathy, which is considered the fifth most common cause of legal blindness in the United States [1]. In 95% of type 1 diabetics and 60% of type 2 diabetics with disease duration longer than 20 years, signs of diabetic retinopathy occur. More severe cases of proliferative diabetic retinopathy are seen in patients suffering from type 1 diabetes. Tight control of hyperglycemia, blood lipids, and blood pressure has been shown to be beneficial to prevent its development or progression [2–4].

Cataract is considered a major cause of visual impairment in diabetic patients as the incidence and progression of cataract is elevated in patients with diabetes mellitus [5, 6]. The association between diabetes and cataract formation has been shown in clinical epidemiological and basic research studies. Due to increasing numbers of type 1 and type 2 diabetics worldwide, the incidence of diabetic cataracts steadily rises. Even though cataract surgery, the most common surgical ophthalmic procedure worldwide, is an effective cure, the elucidation of pathomechanisms to delay or prevent the development of cataract in diabetic patients remains a challenge. Furthermore, patients with diabetes mellitus have higher complication rates from cataract surgery [7]. Both diabetes and cataract pose an enormous health and economic burden, particularly in developing countries, where diabetes treatment is insufficient and cataract surgery often inaccessible [8].

2. Pathogenesis of Diabetic Cataract

The enzyme aldose reductase (AR) catalyzes the reduction of glucose to sorbitol through the polyol pathway, a process linked to the development of diabetic cataract. Extensive research has focused on the central role of the AR pathway as the initiating factor in diabetic cataract formation.

It has been shown that the intracellular accumulation of sorbitol leads to osmotic changes resulting in hydropic lens fibers that degenerate and form sugar cataracts [9, 10]. In the lens, sorbitol is produced faster than it is converted to fructose by the enzyme sorbitol dehydrogenase. In addition,

the polar character of sorbitol prevents its intracellular removal through diffusion. The increased accumulation of sorbitol creates a hyperosmotic effect that results in an infusion of fluid to countervail the osmotic gradient. Animal studies have shown that the intracellular accumulation of polyols leads to a collapse and liquefaction of lens fibers, which ultimately results in the formation of lens opacities [9, 11]. These findings have led to the "Osmotic Hypothesis" of sugar cataract formation, emphasizing that the intracellular increase of fluid in response to AR-mediated accumulation of polyols results in lens swelling associated with complex biochemical changes ultimately leading to cataract formation [9, 10, 12].

Furthermore, studies have shown that osmotic stress in the lens caused by sorbitol accumulation [13] induces apoptosis in lens epithelial cells (LEC) [14] leading to the development of cataract [15]. Transgenic hyperglycemic mice overexpressing AR and phospholipase D (PLD) genes became susceptible to develop diabetic cataract in contrast to diabetic mice overexpressing PLD alone, an enzyme with key functions in the osmoregulation of the lens [16]. These findings show that impairments in the osmoregulation may render the lens susceptible to even small increases of AR-mediated osmotic stress, potentially leading to progressive cataract formation.

The role of osmotic stress is particularly important for the rapid cataract formation in young patients with type 1 diabetes mellitus [17, 18] due to the extensive swelling of cortical lens fibers [18]. A study performed by Oishi et al. investigated whether AR is linked to the development of adult diabetic cataracts [19]. Levels of AR in red blood cells of patients under 60 years of age with a short duration of diabetes were positively correlated with the prevalence of posterior subcapsular cataracts. A negative correlation has been shown in diabetic patients between the amount of AR in erythrocytes and the density of lens epithelial cells, which are known to be decreased in diabetics compared to nondiabetics suggesting a potential role of AR in this pathomechanism [20].

The polyol pathway has been described as the primary mediator of diabetes-induced oxidative stress in the lens [21]. Osmotic stress caused by the accumulation of sorbitol induces stress in the endoplasmic reticulum (ER), the principal site of protein synthesis, ultimately leading to the generation of free radicals. ER stress may also result from fluctuations of glucose levels initiating an unfolded protein response (UPR) that generates reactive oxygen species (ROS) and causes oxidative stress damage to lens fibers [22]. There are numerous recent publications that describe oxidative stress damage to lens fibers by free radical scavengers in diabetics. However, there is no evidence that these free radicals initiate the process of cataract formation but rather accelerate and aggravate its development. Hydrogen peroxide (H_2O_2) is elevated in the aqueous humor of diabetics and induces the generation of hydroxyl radicals (OH–) after entering the lens through processes described as Fenton reactions [23]. The free radical nitric oxide ($NO^{\bullet}$), another factor elevated in the diabetic lens [24] and in the aqueous humor [25], may lead to an increased peroxynitrite formation, which in turn induces cell damage due to its oxidizing properties.

Furthermore, increased glucose levels in the aqueous humor may induce glycation of lens proteins, a process resulting in the generation of superoxide radicals (O_2^-) and in the formation of advanced glycation endproducts (AGE) [26]. By interaction of AGE with cell surface receptors such as receptor for advanced glycation endproducts in the epithelium of the lens further O_2^- and H_2O_2 are generated [27].

In addition to increased levels of free radicals, diabetic lenses show an impaired antioxidant capacity, increasing their susceptibility to oxidative stress. The loss of antioxidants is exacerbated by glycation and inactivation of lens antioxidant enzymes like superoxide dismutases [28]. Copper-zink superoxide dismutase 1 (SOD1) is the most dominant superoxide dismutase isoenzyme in the lens [29], which is important for the degradation of superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen [30]. The importance of SOD1 in the protection against cataract development in the presence of diabetes mellitus has been shown in various in vitro and in vivo animal studies [31–33].

In conclusion, a variety of publications support the hypothesis that the initiating mechanism in diabetic cataract formation is the generation of polyols from glucose by AR, which results in increased osmotic stress in the lens fibers leading to their swelling and rupture.

3. Clinical Studies Investigating the Incidence of Diabetic Cataract

Several clinical studies have shown that cataract development occurs more frequently and at an earlier age in diabetic compared to nondiabetic patients [34–36].

Data from the Framingham and other eye studies indicate a three to fourfold increased prevalence of cataract in patients with diabetes under the age of 65, and up to a twofold excess prevalence in patients above 65 [34, 37]. The risk is increased in patients with longer duration of diabetes and in those with poor metabolic control. A special type of cataract—known as snowflake cataract—is seen predominantly in young type 1 diabetic patients and tends to progress rapidly. Cataracts may be reversible in young diabetics with improvement in metabolic control. The most frequently seen type of cataract in diabetics is the age-related or senile variety, which tends to occur earlier and progresses more rapidly than in nondiabetics.

The Wisconsin Epidemiologic Study of Diabetic Retinopathy investigated the incidence of cataract extraction in people with diabetes. Furthermore, additional factors associated with higher risk of cataract surgery were determined. The 10-year cumulative incidence of cataract surgery was 8.3% in patients suffering from type 1 diabetes and 24.9% in those from type 2 diabetes. Predictors of cataract surgery included age, severity of diabetic retinopathy and proteinuria in type 1 diabetics whereas age and use of insulin were associated with increased risk in type 2 diabetics [38].

A follow-up examination of the Beaver Dam Eye Study cohort, consisting of 3684 participants 43 years of age and older, performed 5 years after the baseline evaluation showed an association between diabetes mellitus and cataract formation [39]. In the study, the incidence and progression of cortical and posterior subcapsular cataract was associated with diabetes. In addition, increased levels of glycated hemoglobin were shown to be associated with an increased risk of nuclear and cortical cataracts.

In a further analysis of the Beaver Dam Eye study the prevalence of cataract development was studied in a population of 4926 adults [40]. Diabetic patients were more likely to develop cortical lens opacities and showed a higher rate of previous cataract surgery than nondiabetics. The analysis of the data proved that longer duration of diabetes was associated with an increased frequency of cortical cataract as well as an increased frequency of cataract surgery.

The aim of the population-based cross-sectional Blue Mountains Eye Study was to examine the relationship between nuclear, cortical, and posterior subcapsular cataract in 3654 participants between the years 1992 to 1994 [41]. The study supported the previous findings of the harmful effects of diabetes on the lens. Posterior subcapsular cataract was shown to be statistically significantly associated with diabetes. However, in contrast to the Beaver Dam Eye Study, nuclear cataract showed a weak, not statistically significant, association after adjusting for other known cataract risk factors.

A population-based cohort study of 2335 people older than 49 years of age conducted in the Blue Mountains region of Australia investigated associations between diabetes and the 5-year incidence of cataract. The results of this longitudinal study conducted by the same group of investigators as the Blue Mountains Eye Study demonstrated a twofold higher 5-year incidence of cortical cataract in participants with impaired fasting glucose. Statistically significant associations were shown between incident posterior subcapsular cataract and the number of newly diagnosed diabetic patients [42].

The Visual Impairment Project evaluated risk factors for the development of cataracts in Australians. The study showed that diabetes mellitus was an independent risk factor for posterior subcapsular cataract when present for more than 5 years [43].

A goal of the Barbados Eye study was to evaluate the relationship between diabetes and lens opacities among 4314 black participants [44]. The authors found that diabetes history (18% prevalence) was related to all lens changes, especially at younger ages.

4. Cataract Surgery in Diabetic Patients

Phacomulsification is nowadays the preferred technique in most types of cataract. This technique was developed by Kelman in 1967 and was not widely accepted until 1996 [45]. It results in less postoperative inflammation and astigmatism, more rapid visual rehabilitation and, with modern foldable lenses, a lower incidence of capsulotomy than with the outdated extracapsular surgery. There has been a recent shift in emphasis towards earlier cataract extraction in diabetics. Cataract surgery is advisable before lens opacity precludes detailed fundus examination.

While the overall outcomes of cataract surgery are excellent, patients with diabetes may have poorer vision outcomes than those without diabetes. Surgery may cause a rapid acceleration of retinopathy, induce rubeosis or lead to macular changes, such as macular edema or cystoid macular edema [46, 47]. The worst outcomes may occur in operated eyes with active proliferative retinopathy and/or preexisting macular edema [48, 49].

In diabetics with or without evidence of diabetic retinopathy the blood-aqueous barrier is impaired leading to an increased risk of postoperative inflammation and development of a sight-threatening macular edema, a process that is exacerbated by cataract surgery [50–52]. Factors that influence the amount of postoperative inflammation and the incidence of clinical and angiographic cystoid macular edema are duration of surgery, wound size and posterior capsular rupture or vitreous loss. Liu et al. showed that phacoemulsification surgery affects the blood-aqueous barrier more severely in diabetic patients with proliferative diabetic retinopathy than in patients with nonproliferative diabetic retinopathy or nondiabetic patients [53]. An analysis of Medicare beneficiaries ($n = 139759$) from the years 1997 through 2001 revealed that the rate of cystoid macular edema diagnosis after cataract surgery was statistically significantly higher in diabetic patients than in nondiabetics [54].

Several clinical studies investigated the role of phacoemulsification cataract surgery on the progression of diabetic retinopathy. One year after cataract surgery, the progression rate of diabetic retinopathy ranges between 21% and 32% [55–58]. Borrillo et al. reported a progression rate of 25% after a follow-up period of 6 months [59]. A retrospective review of 150 eyes of 119 diabetic patients undergoing phacoemulsification surgery showed a similar progression of diabetic retinopathy in 25% of cases within the follow-up period of 6–10 months [56].

A prospective study evaluating the onset or worsening of macula edema at 6 months following cataract surgery in patients with mild or moderate nonproliferative diabetic retinopathy reported an incidence of 29% (30 of 104 eyes) of macula edema based on angiographic data [60]. Krepler et al. investigated 42 patients undergoing cataract surgery and reported a progression of diabetic retinopathy of 12% in operated versus 10.8% in nonoperated eyes during the follow-up of 12 months [61]. During the same follow-up period of 12 months, Squirrell et al. showed that out of 50 patients with type 2 diabetes undergoing unilateral phacoemulsification surgery 20% of the operated eye and 16% of the nonoperated had a progression of diabetic retinopathy [62]. Liao and Ku found in a retrospective study that out of 19 eyes with preoperative mild to moderate nonproliferative diabetic retinopathy 11 eyes (57.9%) showed progression of diabetic retinopathy 1 year after surgery, while 12 eyes (63.2%) had progressed 3 years postoperatively. The progression rates were statistically significant when compared to eyes without preoperative retinopathy [63]. A

recently published prospective study evaluated eyes from 50 diabetic patients with and without retinopathy after cataract surgery by optical coherence tomography [64]. The authors reported an incidence of 22% for macula edema following cataract surgery (11 of 50 eyes) while macula edema did not occur in eyes without retinopathy. When only eyes with confirmed diabetic retinopathy were evaluated ($n = 26$), the incidence for postoperative macula edema and cystoid abnormalities increased to 42% (11 of 26 eyes). Minimal changes from baseline values in center point thickness were observed in eyes with no retinopathy. Eyes with moderate nonproliferative diabetic retinopathy or proliferative diabetic retinopathy developed an increase from baseline of 145 μm and 131 μm at 1 month and 3 month, respectively. The difference in retinal thickening between the 2 groups at 1 and 3 months was statistically significant and among patients with retinopathy inversely correlated with visual acuity improvements.

5. Anticataract Treatment

5.1. Aldose-Reductase Inhibitors. Aldose reductase inhibitors (ARI) comprise a variety of structurally different compounds like plant extracts, animal tissues or specific small molecules. In diabetic rats, plant flavonids, such as quercitrin or the isoflavone genistein, have delayed diabetic cataract formation [65–68]. Examples of natural products with known AR inhibitory activity are extracts from indigenous plants like Ocimum sanctum, Withania somnifera, Curcuma longa, and Azadirachta indica or the Indian herbal Diabecon [69, 70]. Levels of polyol in the lenses of rats have been reduced by injection of intrinsic ARI containing extracts from human kidney and bovine lenses [71]. Nonsteroidal anti-inflammatory drugs, such as sulindac [72, 73], aspirin [74, 75] or naproxen [76] have been reported to delay cataract in diabetic rats through a weak AR inhibitory activity.

Several experimental studies support the role of ARI in preventing and not only delaying diabetic cataract formation. In a rat model of diabetes, animals were treated with the AR inhibitor Renirestat [77]. The study reported a reduction of sorbitol accumulation in the lens as compared to untreated diabetic rats. Furthermore, in Ranirestat treated diabetic rats there were no signs of lens damage like degeneration, swelling, or disruption of lens fibers throughout the treatment period in contrast to the untreated group.

In a similar study, diabetic rats were treated with a different ARI, Fidarestat [78]. Fidarestat treatment completely prevented cataractous changes in diabetic animals. In dogs the topically applied ARI Kinostat has been shown to reverse the development of sugar cataracts [79].

Other ARI with a beneficial effect on diabetic cataract prevention encompass Alrestatin [80], Imrestat [81], Ponalrestat [82], Epalrestat [83], Zenarestat [84], Minalrestat [85], or Lidorestat [86].

These studies provide a rationale for a potential future use of ARI in the prevention or treatment of diabetic cataracts.

5.2. Antioxidant Treatments of Diabetic Cataracts. As oxidative damage occurs indirectly as a result of polyol accumulation during diabetic cataract formation, the use of antioxidant agents may be beneficial.

A number of different antioxidants have been reported to delay cataract formation in diabetic animals. These include the antioxidant alpha lipoic acid, which has been shown to be effective in both delay and progression of cataract in diabetic rats [87].

Yoshida et al. demonstrated that the combined treatment of diabetic rats with vitamin E, a lipid-soluble and antioxidant vitamin, and insulin synergistically prevented the development and progression of cataracts in the animals [88].

Pyruvate, an endogenous antioxidant, has recently gained attention for its inhibitory effect on diabetic cataract formation by reducing sorbitol formation and lipid peroxidation in the lens [89]. A study performed by Varma et al. showed that the incidence of cataract in diabetic rats was lower in the pyruvate-treated group than in the untreated control group [90]. Additionally, the severity of opacities in the pyruvate-treated rats was minor than in the control animals. The beneficial effect of pyruvate in the prevention of cataract is mainly attributed to its effective scavenging ability for reactive oxygen species generated by increased levels of sugars in diabetic animals [91].

However, clinical observations in humans suggest that the effect of antioxidant vitamins on cataract development is small and may not prove to be clinically relevant [92].

5.3. Pharmacological Agents for the Treatment of Macular Edema Following Cataract Surgery. Proinflammatory prostaglandins have been shown to be involved in the mechanisms leading to fluid leakage from perifoveal capillaries into the extracellular space of the macular region [93]. Due to the ability of topical nonsteroidal anti-inflammatory drugs (NSAIDs) to block the cyclooxygenase enzymes responsible for prostaglandin production, studies suggested that NSAIDs may also reduce the incidence, duration and severity of cystoid macular edema [94–97] by inhibiting the release and breakdown of the blood-retina barrier [98, 99].

Nepafenac, a topical NSAID indicated for the prevention and treatment of anterior segment pain and inflammation after cataract surgery, has been used recently in clinical trials to test its efficacy in reducing the incidence of macular edema after cataract surgery. The active ingredient is a prodrug that rapidly penetrates the cornea to form the active metabolite, amfenac, by intraocular hydrolases particularly in the retina, ciliary body epithelium and choroid [100].

A retrospective study compared the incidence of macular edema after uneventful phacoemulsification between 240 patients treated for 4 weeks with topical prednisolone and 210 patients treated with a combination of prednisolone and nepafenac for the same time. The authors concluded that patients treated with topical prednisolone alone had a statistically significantly higher incidence of macular edema than those treated with additional nepafenac [101].

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Research Article

Aldo-Keto Reductases in the Eye

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Aldose reductase (AKR1B1) is an NADPH-dependent aldo-keto reductase best known as the rate-limiting enzyme of the polyol pathway. Accelerated glucose metabolism through this pathway has been implicated in diabetic cataract and retinopathy. Some human tissues contain AKR1B1 as well as AKR1B10, a closely related member of the aldo-keto reductase gene superfamily. This opens the possibility that AKR1B10 may also contribute to diabetic complications. The goal of the current study was to characterize the expression profiles of AKR1B1 and AKR1B10 in the human eye. Using quantitative reverse transcriptase-PCR and immunohistochemical staining, we observed expression of both AKR genes in cornea, iris, ciliary body, lens, and retina. Expression of AKR1B1 was the highest in lens and retina, whereas AKR1B10 was the highest in cornea. Lenses from transgenic mice designed for overexpression of AKR1B10 were not significantly different from nontransgenic controls, although a significant number developed a focal defect in the anterior lens epithelium following 6 months of experimentally induced diabetes. However, lenses from AKR1B10 mice remained largely transparent following longterm diabetes. These results indicate that AKR1B1 and AKR1B10 may have different functional properties in the lens and suggest that AKR1B10 does not contribute to the pathogenesis of diabetic cataract in humans.

1. Introduction

Diabetes mellitus is recognized as a leading cause of new cases of blindness among Americans between the ages of 20 and 74. At least 5,000 new cases of legal blindness result each year from diabetic retinopathy alone [1]. The incidence of cataract is also much higher in diabetic than in nondiabetic individuals [2]. Many theories have been advanced to explain the pathogenesis of diabetic eye disease. These include excess formation of advanced glycation end-products [3], activation of PKC isoforms [4], activation of the polyol pathway [5], and excessive oxidative stress [6]. Considerable evidence points to excess conversion of glucose to sorbitol, mediated by aldose reductase (AKR1B1), as a key factor in diabetic cataract formation. AKR1B1-mediated polyol accumulation causes osmotic imbalances that lead to fiber cell swelling, liquefaction, and eventually cataract [5]. Compelling evidence to support this hypothesis came from Lee and coworkers, who created a transgenic mouse model that expressed high levels of AKR1B1 in lens fiber cells [7]. These mice developed cataracts following diabetes induction, demonstrating an essential role for AKR1B1 in mediating high glucose-dependent cataract formation.

The role of AKR1B1 during euglycemia is still unclear. The aldo-keto reductase (AKR) gene superfamily includes several enzymes and proteins with similar structures and/or enzymatic activities. The AKR1B subfamily contains two genes that are expressed at relatively high levels in human tissues. AKR1B1, which is equivalent to aldose reductase, is expressed in many tissues throughout the body. AKR1B10, which has been given the trivial names human small intestine reductase (HSIR) and AKR1B1-like protein 1 (ARL-1), is

also expressed in many tissues [8, 9]. Based on a blot analysis of multiple tissue RNAs, gene transcript levels of AKR1B10 closely parallel those of AKR1B1 [8]. The broad catalytic similarities between AKR1B1 and AKR1B10 make it difficult to map the distribution of these proteins in human tissues using enzyme activity assays. The enzymes utilize an overlapping array of substrates, and many so-called aldose reductase inhibitors effectively block both AKR1B1 and AKR1B10 [10]. Therefore, studies conducted over 2 decades ago to demonstrate expression of AKR1B1 in tissues of the human eye may have lacked sufficient specificity to distinguish between these two closely related gene products [11, 12]. In the current study, we have reexamined the expression pattern of these enzymes, taking into account the possibility that AKR1B10 may contribute to the aldo-keto reductase profile of ocular tissues and thus may participate in the pathogenesis of diabetic eye disease. The current study also addressed the question of whether AKR1B10 contributes to the onset and progression of cataracts in a mouse model of diabetes.

2. Materials and Methods

2.1. Human Eyes and Specimens. Human postmortem eyes were obtained from certified eye banks through the National Disease Research Interchange. The time interval between death to enucleation (<8 hours) and then to fixation (usually 8–12 hours) was rigorously controlled. Once received in the laboratory, tissues were handled under RNAse-free conditions. The cornea, iris, ciliary body, lens, and retinas were carefully dissected and used to prepare protein lysates.

2.2. Quantitative Real-Time PCR (qRT-PCR). Total RNA was extracted from human ocular tissues using an RNase kit (Qiagen). After digesting genomic DNA using DNase I (Roche), cDNA was synthesized from 1 μg total RNA using Retroscript Kit (Ambion) in 20 μL volume. Quantitative real-time PCR for AKR1B1 and AKR1B10 were done using an iCycler iQ Detection System (Bio-Rad, Hercules, CA). Reaction mixtures contained iQ SYBR Green Supermix (Bio-Rad) and primers 5′for-CCCAAAGATGATAAAGGTAATGCCATCGGT-3′ and 5′rev-CGATCTGGAAGTGGCTGAAATTGGAGA-3′ for AKR1B10, 5′for-TGAGTGCCACCCATATCTCA-3′ and 5′rev-TGTCACAGACTTGGGGATCA-3′ for AKR1B1, or 5′for-AGAAGGAGATCACTGCCCTGGCACC and 5′rev-CCTGCTTGCTGATCCACATCTGCTG for β-actin. PCR condition was 1 cycle of 95°C for 3 minutes followed by 40 cycles at 95°C for 20 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. All the samples were run in triplicate, and the results were averaged. Specific amplification of AKR1B1, AKR1B10, and β-actin (244 bp for AKR1B1, 133 bp for AKR1B10 and 162 bp for β-actin) was confirmed by gel electrophoresis and melting curve analysis after PCR. In order to compare expression patterns among tissues, relative quantification of gene expression was performed using the standard curve method. The quantification data of AKR1B1 and AKR1B10 was indicated as a relative ratio of its signal to that of β-actin to normalize the starting amount of template cDNA.

2.3. Western Blot Analysis. Proteins were isolated from human ocular tissues by treatment in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, pH 8.0, 1 mM EGTA, pH 7.0, 0.2 mM sodium orthovanadate, 1 mM PMSF, 0.5% NP-40) with freshly added aprotinin to a final concentration of 5 μg/mL. Protein concentration was determined with the bicinchoninic acid methods, using BSA as standard (Micro BCA Protein Assay Kit; Pierce, Rockford, IL). Equivalent amounts of protein (40 μg) from total cell lysates or tissue lysates were boiled in Nupage LDS sample buffer (Invitrogen) for 5 minutes and analyzed by 10% SDS-PAGE. Separated proteins were transferred to Hybond-P PVDF membrane and were blocked with TBS-0.1% Tween-20 containing 5% nonfat milk for overnight. Membranes were incubated with antibodies for AKR1B1 (1 : 3000) or AKR1B10 (1 : 3000 dilution), probed with horseradish peroxidase-conjugated antirabbit secondary antibody (1 : 8000) for 2 hours, and washed. Immune complexes were visualized with the ECL plus system and scanned on a STORM 860 phosphorimager. Membranes were washed and reprobed with anti-β-actin antibody. Recombinant AKR1B1 or AKR1B10 proteins were used as size standards.

2.4. Antibody Preparation. Antibodies to AKR1B1 and AKR1B10 were prepared through a commercial service (Bethyl Laboratories, Montgomery, TX). Antibodies to human AKR1B1 were made by immunizing rabbits with recombinant human AKR1B1, purified as described previously [13]. Antibodies to AKR1B10 were prepared using synthetic peptides derived from AKR1B10 encompassing residues 120 to 134 (CDDLFPKDDKGNAIGG). In both cases, antibodies were purified by column chromatography using the immunogen bound to a solid phase support as the affinity ligand. Antibody specificity was verified using purified recombinant AKR1B1 and AKR1B10 in a western blotting format (data not shown).

2.5. Immunohistochemistry and Immunofluorescence. Immunohistochemical analysis for AKR1B1 and AKR1B10 was done with the formalin-fixed, paraffin-embedded tissue. The sections were deparaffinized in xylene, incubated for 30 minutes in methanol containing 3% H_2O_2 to inhibit endogenous peroxidase activity, rehydrated through a series of graded alcohols, and stained for AKR1B1 or AKR1B10 via the immunoperoxidase technique. The tissue was covered with 20% inactivated normal donkey serum in Tris-buffered saline, pH 7.6, incubated for 30 minutes at room temperature, and blotted and incubated overnight with a 1 : 500 dilution of AKR1B1 antiserum or a 1 : 500 dilution of rabbit anti-AKR1B10 peptide antibody overnight at 4°C. Goat antirabbit antibody was used as a secondary antibody after 500-fold dilution. Preimmune serum was used on sections serving as negative controls. Immunostaining was visualized using diaminobenzidine tetrahydrochloride (DAB), a horseradish

peroxidase system (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin. Areas of positive reactivity are stained brown.

2.6. Transgenic Mice. Transgenic mice on C57BL6 background were produced for lens-enriched expression of AKR1B10. The transgene construct was prepared by ligating the hybrid α/δ-crystallin promoter [14] to a complete cDNA sequence encoding AKR1B10. Further details on the preparation and characterization of five independent founder lines of AKR1B10 transgenic mice will be presented elsewhere. Animals derived from founder line PAR30 used in this study were maintained by outbreeding to the C57BL6 strain obtained from Jackson Laboratories (Bar Harbor, ME).

2.7. Diabetes Induction. Experimental diabetes was induced in transgenic and nontransgenic control mice by treatment with a low-dose regimen of streptozotocin as described [15]. Fasting blood sugars were measured starting 2 weeks after the final streptozotocin treatment and monthly after hyperglycemia was established. For blood sugar measurements, animals were fasted for 6 hours prior to collection of a drop of blood from the saphenous vein. Glucose levels were measured immediately using a glucometer (AlphaTRAK, Abbot Laboratories, Chicago, IL). Animals were included in the study if fasting glucose levels were 250–350 mg/dL.

3. Results

3.1. Aldo-Keto Reductases in the Human Eye. We used a quantitative real-time PCR-based assay (qRT-PCR) for measuring the expression profiles of the AKR1B1 and AKR1B10 genes in human eye tissues. The RT-PCR method was chosen because it provided the specificity necessary to distinguish between AKR1B1 and AKR1B10 gene transcripts, unlike the case with standard nucleic acid hybridization methods such as Northern blotting.

The expression profiles for AKR1B1 and AKR1B10 mRNA levels were measured in cornea, iris, ciliary body, lens, and retina. Data on the apparent abundance of gene-specific transcripts were computed relative to β-actin and are shown in Figure 1. Transcripts derived from the AKR1B1 gene are present in all tissues examined, and are the highest in lens followed by retina and cornea. In the case of AKR1B10 gene transcripts, the highest transcript levels are found in cornea, with substantially lower levels found in iris, ciliary body, lens, and retina.

To examine the distribution of the AKR1B1 and AKR1B10 at the protein level, we carried out immunohistochemical staining of paraffin sections produced from human eyes using affinity purified antibodies prepared as described in Section 2. The results are shown in Figure 2 and can be summarized as follows.

Cornea. Both AKR1B10 and AKR1B1 are expressed in the corneal epithelium. Staining appeared to be stronger in the basal as compared to the superficial cell layer. Intense staining for AKR1B10 was observed in the corneal stroma whereas AKR1B1 staining was relatively limited in this region.

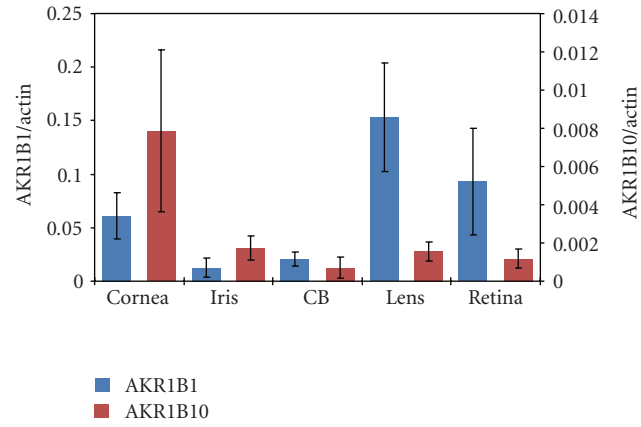


Figure 1: Expression of AKR1B1 and AKR1B10 in human eye tissues. Gene transcript levels were measured by quantitative real-time PCR as described in Section 2. Data are mean ± SD among 5 nondiabetic male donors aged 65. ± 9.2 years. Data for AKR gene transcripts levels are normalized to RT-PCR for β-actin.

Lens. Staining for AKR1B1 and AKR1B10 was observed in the lens epithelium and fiber cells located in the superficial cortex. The staining was the greatest at the equator region and diminished in cells located deeper in the cortex and nucleus.

Retina. AKR1B1 and AKR1B10 stained heavily in cell nuclei in the inner nuclear layer as well as in some ganglion cells, especially near the perinuclear cytoplasm and inner limiting membrane. Intense staining was also observed in the inner and outer plexiform layers. No significant immunohistochemical staining of AKR1B10 could be observed around the retinal vessels. In all cases, no staining positivity was observed when the primary antibody was omitted.

Transgenic Mice. Based on results from RT-PCR and immunostaining experiments, it appears that both AKR1B1 and AKR1B10 are expressed in the human lens. To assess whether high levels of AKR1B10 can predispose the lens toward diabetic cataract, we produced transgenic mice designed for overexpression of the enzyme in the lens. As shown in Figure 3, AKR1B10 was readily detected by western blotting of lens homogenates from transgenic animals but was absent in nontransgenic control lenses. Immunohistochemical staining showed intense positivity in the outer cortical fiber cells of transgenic animals and no detectable staining of nontransgenic controls (Figure 3). Thus, expression of AKR1B10 in the transgenic lens had a similar regional distribution as endogenous AKR1B10 in the human lens. On a gross level, overexpression of AKR1B10 did not have a measureable impact on lens development, as the wet weight and appearance of transgenic lenses was not significantly different from nontransgenic controls (data not shown).

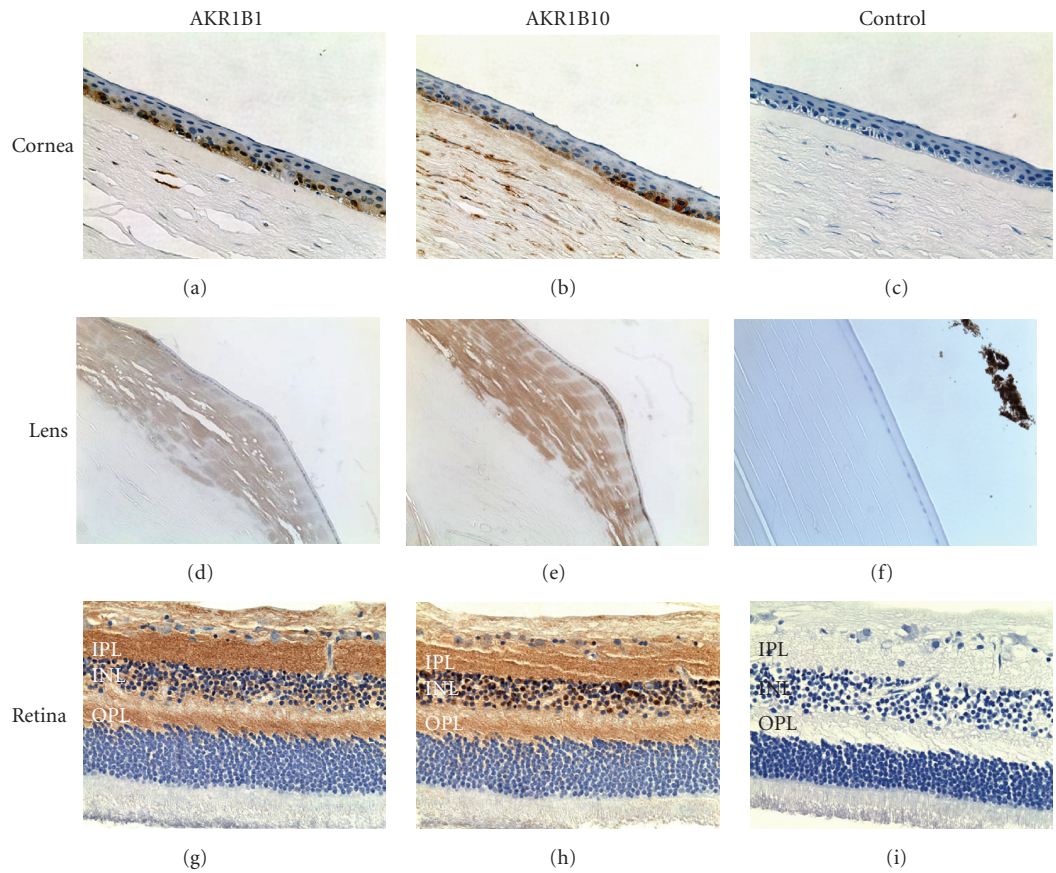


Figure 2: Immunostaining for AKR1B1 and AKR1B10 in human eye tissues. A donor eye (77-year-old male) was treated with antibodies to AKR1B1 and AKR1B10 or preimmune control serum. Immune complexes were visualized by treatment with a horseradish peroxidase-conjugated secondary antibody and signal developed using diaminobenzidine tetrahydrochloride (DAB) to give a brown color. Tissues examined include cornea (a)–(c), lens (d)–(f), and retina (g)–(i). Immunostaining was particularly strong in the inner plexiform (IPL) and outer plexiform (OPL) layers. The inner nuclear layer is shown (INL).

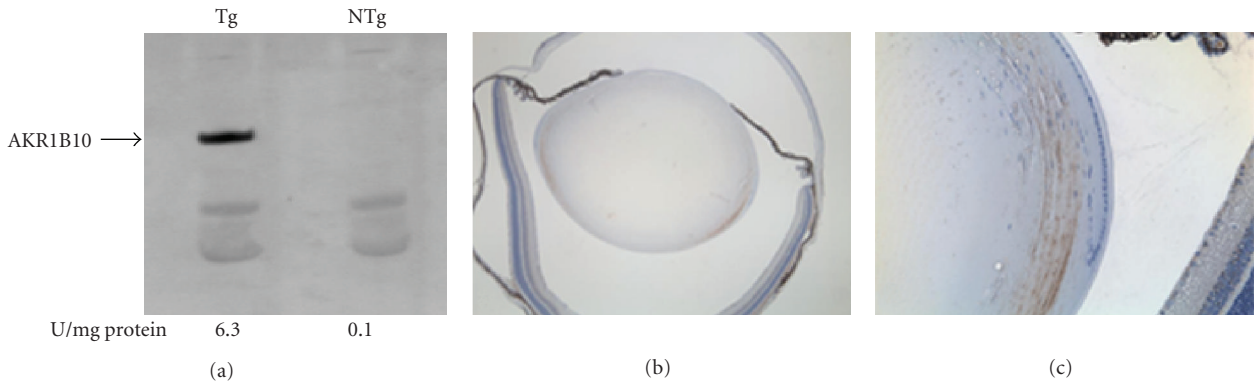
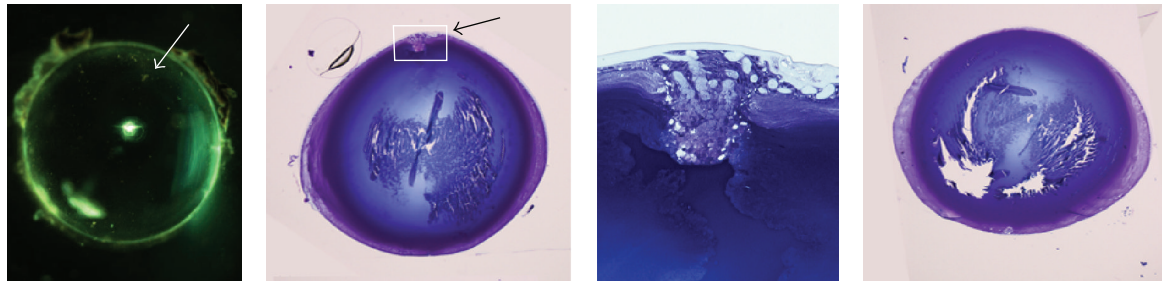


Figure 3: AKR1B10 expression in the transgenic lens. (a) Western blot demonstrating expression of AKR1B10 in lens of transgenic (Tg) mice; the characteristic band was not observed in lenses from nontransgenic (NTg) controls. Aldo-keto reductase enzyme activity in lenses is shown below each lane. (b) and (c) Immunohistochemical stain for AKR1B10 expression in the transgenic lens.

We induced experimental diabetes in our transgenic mice to determine if over-expression of AKR1B10 influences the susceptibility of the mouse lens to cataracts. Both transgenic and nontransgenic animals with and without experimental diabetes were monitored for up to six months for the appearance of lens opacities. In all cases, the lenses remained essentially clear and developed only minor focal areas of light scattering in the lens nucleus, typical of the normal aging mouse lens [16]. A refractive abnormality localized on the anterior epithelium was observed in 50% (3 of 6) of the



(a) (b) (c) (d)

Figure 4: Lens defect in AKR1B10 lens after long-term diabetes. (a) Brightfield microscopy of transgenic lens demonstrating light scattering defect (arrow). (b) AKR1B10 transgenic lens showing defect at the anterior aspect of the lens (arrow). (c) Magnification of the boxed area from panel (b). (d) Lens from nontransgenic control with equivalent duration of diabetes. Panels (b)–(d) are from toluidine blue-stained lenses.

AKR1B10 mice with diabetes. This defect gave rise to light scattering when viewed through a slit lamp ophthalmoscope or after dissection and brightfield illumination (Figure 4). Histological examination showed that this abnormality was associated with a localized disorganization of epithelial cells, formation of large vacuoles, and disrupted contact between epithelial cells and the lens capsule. This defect was not observed in age-matched nontransgenic controls with equivalent duration of experimental diabetes ($n = 4$) or in nondiabetic transgenic controls ($n > 6$). The epithelial defect we observed is fundamentally different from cortical opacities that characterize the majority of diabetic cataracts.

4. Discussion

Cataract formation is a major complication of diabetes. Osmotic stress to lens fiber cells resulting from excessive production and/or accumulation of sorbitol has been proposed as a mechanism leading to diabetic cataracts in humans. Varma and coworkers previously demonstrated a strong correlation between the abundance of polyol pathway metabolites sorbitol and fructose and blood glucose levels in cataracts extracted from diabetic patients [17]. Our gene expression profiling of AKR1B1 and AKR1B10 using gene-specific RT-PCR clearly demonstrated that both of these aldo-keto reductases are expressed not only in lens but also in cornea, retina, and ciliary body. This raised the possibility that diabetes-induced cataract and retinopathy, as well as increased risk for glaucoma and corneal abnormalities, may develop through multiple AKR-linked mechanisms.

We employed a genetic strategy to determine if AKR1B10 contributes to the pathogenesis of diabetic cataract in a mouse model. Mouse lenses contain insignificant levels of AKR1B3, the mouse ortholog of human AKR1B1. Other members of the AKR1B subfamily, such as AKR1B7 (major vas deferens protein, MVDP; 18) and AKR1B8 (fibroblast growth factor-induced protein 1; FR-1; 19), are virtually undetectable in the mouse lens. Previous studies by Lee et al. demonstrated that transgenic mice that overexpress AKR1B1 in the lens develop cataracts after induction of galactosemia or experimental diabetes [7]. Therefore, transgenic expression of the human AKR1B10 in the mouse lens allowed us to assess the impact of this enzyme on diabetic cataract formation using a transgenic mouse model that had been validated for diabetic cataract in a previous study.

In our diabetic animal model studies, we intentionally sought to achieve modest (250–350 mg/dL) levels of hyperglycemia so as to closely mimic the situation experienced by human patients with poorly controlled diabetes. Since the AKR1B10 transgenic mice remained cataract-free throughout 6 months of experimentally induced diabetes, it seems reasonable to conclude that AKR1B10 likely has a limited role in the pathogenesis of diabetic cataract.

5. Conclusions

Both AKR1B1 and AKR1B10 are produced in many tissues of the eye affected by diabetes, including cornea, iris, ciliary body, lens, and retina. Because lens transparency was maintained in AKR1B10 transgenic mice following 6 months of experimental diabetes, we conclude that AKR1B10 has a limited role in the pathogenesis of cataract in human patients with diabetes.

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Research Article

Inhibition of Methylglyoxal-Mediated Protein Modification in Glyoxalase I Overexpressing Mouse Lenses

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Objective. Here we tested the role of Glo I in the prevention of advanced glycation end product (AGE) formation in transgenic mouse lenses. *Methods*. A transgenic animal line that expressed high levels of human Glo I in the lens was developed from the C57B6 mouse strain. The role of Glo I in the inhibition of MGO-AGE formation was tested in organ-cultured lenses. *Results*. Organ culture of Wt and Glo I lenses with 5 mM D, L-glyceraldehyde (GLD) enhanced MGO by 29-fold and 17-fold in Wt lenses and Glo I lenses, respectively. Argpyrimidine levels were 192 ± 73 pmoles/mg protein, and hydroimidazolone levels were 22 ± 0.7 units/μg protein in GLD-incubated Wt lenses. In Glo I lenses, formation of AGEs was significantly inhibited; the argpyrimidine levels were 82 ± 18 pmoles/mg protein, and the HI levels were 2.6 ± 2.3 units/μg protein. Incubation of Wt lens proteins with 5 mM ribose for 7 days resulted in the formation of pentosidine. However, the levels were substantially higher in Glo I lens proteins incubated with ribose. *Conclusion*. Our study provides direct evidence that Glo I activity plays an important role in the regulation of AGE synthesis in the lens; while Glo I activity blocks the formation of MGO-AGEs, it might promote the formation of sugar-derived AGEs.

1. Introduction

Lens proteins undergo numerous physicochemical changes during aging and cataract formation. Some of the prominent changes are protein crosslinking, chromophore and fluorophore adduct formation on proteins, deamidation and truncation [1]. Several mechanisms have been proposed for such changes, including the Maillard reaction. The Maillard reaction is a nonenzymatic reaction of reactive carbonyls, such as glucose and ascorbate oxidation products, with the amino groups of proteins. The reaction proceeds through the formation of an Amadori product which, by a series of reactions, produces advanced glycation end products (AGEs) on proteins [2]. Many AGEs have been detected in the human lens, that include, glucosepane [3], pentosidine [4], N^{ε}-carboxymethyllysine [5], pyrraline [6], K2P [7], and vesperlysine [8]. While vesperlysine and K2P are lysine-lysine crosslinking adducts, pentosidine and glucosepane are lysine-arginine adducts. These AGEs progressively accumulate in aging lenses and accumulate at a higher rate in cataractous lenses.

Methylglyoxal (MGO) is an additional AGE precursor in the lens. It is an α-dicarbonyl compound produced nonenzymatically from the triose phosphate intermediates of glycolysis [9]. MGO reacts rapidly with arginine residues on proteins to form hydroimidazolone (HI, there are three isomers of this product) and argpyrimidine adducts [10]. While argpyrimidine is a blue fluorescent product, HI isomers are nonfluorescent and nonchromophoric adducts. MGO also reacts with the lysine residues of proteins to produce N^{ε}-carboxyethyl lysine [5], MOLD, and MODIC [3, 11]. MOLD is an imidazolium salt that is formed from a crosslinking adduct between two lysine residues, and MODIC is a lysine-arginine crosslinking structure. These AGEs are present in relatively high concentrations in aged and cataractous human lenses [11–13].

MGO is metabolized by glyoxalase and aldo-keto reductases in the lens. However, glyoxalase constitutes the major route of metabolism [14]. Glyoxalase is comprised of two enzymes, glyoxalase I (Glo I), which converts hemithioacetal (formed nonenzymatically from the reaction of glutathione and MGO) to S-D-lactoyl glutathione, and glyoxalase II (Glo II), which catalyzes the conversion of S-D-lactoyl glutathione to D-lactate [15].

Glo I appears to be critical for reducing MGO concentrations and subsequent AGE formation in micro- and macrovascular endothelial cells [16, 17] and for the survival of human retinal capillary pericytes in high glucose environments [18]. Its activity in the lens is enhanced during diabetes, possibly as a means to cope with increased MGO concentrations [19]. In the rat lens, Glo I inhibition led to an increase in MGO and AGE content [20]. These findings suggest that Glo I is critical for the reduction of MGO accumulation and AGE formation. To gain further insight into the role of Glo I in the lens, we developed a transgenic mouse line that specifically overexpresses human Glo I in lens epithelial and fiber cells. Using lenses from this animal model, we show that Glo I inhibits MGO-derived AGE formation. Surprisingly, enhanced Glo I activity led to higher levels of a sugar-derived AGE, pentosidine, in ribose incubated-lens proteins when compared to the levels observed for Wt lens proteins. The findings in this study clearly implicate Glo I in the prevention of MGO-mediated AGE synthesis but at the same time suggest that Glo I may enhance sugar-mediated AGE synthesis in the lens.

2. Materials and Methods

M-199 medium with Earle's salts, reduced glutathione (GSH), 5,5′-dithiobis 2-nitro-benzoic acid (DTNB), ethylenediaminetetraacetic acid (EDTA), DL-glyceraldehyde, heptafluorobutyric acid, 6-hydroxy-2, 4, 5-triaminopyrimidine (TRI), HEPES, trichloroacetic acid (TCA), sodium carbonate, streptozot-1ocin, and phenylmethyl sulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co, St. Louis, MO. HI monoclonal antibody was made in mice by immunizing with HI coupled to KLH (Kanade et al., unpublished).

3. Generation of Glyoxalase I Transgenic Animals

The studies complied with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research and were approved by the Case Western Reserve University Institutional Animal Care and Use Committee. A transgenic mouse line was produced by the standard pronuclear microinjection technique. The details of the transgene DNA construct are illustrated in Figure 1. The human glyoxalase I (Glo I) gene was inserted between EcoR1 sites of a minigene construct that contained a chick δ1-crystallin lens enhancer upstream of the αA-crystallin promoter and a rabbit β-globin intron. Human growth hormone polyA was inserted downstream

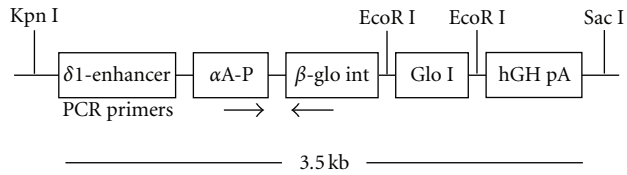

Figure 1: Human Glo I minigene construct. The human Glo I gene was linked to the chimeric promoter that contains the mouse αA-crystallin promoter (αA-P) and chick δ1-crystallin lens enhancer (δ-en), which drives the expression of Glo I specifically in the lens epithelium and fiber cells.

from the αA-crystallin promoter. At 2 to 3 weeks after birth, tail biopsies were obtained, and genomic DNA was screened for transgene integration by PCR using the forward primer 5′-TCT GAG AGC CTC TGC TGC TC -3′ and the reverse primer 5′-GGT CCA TGG TGA TAC AAG GGA C -3′. The identified founders were crossed with wild type C57BL6 to establish a hemizygous line. Homozygous transgenic mouse lines were established by breeding the hemizygous mice within the same line. All experiments were performed using lenses from the homozygous line.

4. In Situ Hybridization

In situ hybridization was performed with a ^{35}S-labeled riboprobe homologous to the human growth hormone (hGH) sequences as described previously in [21].

5. Morphological Changes

Immediately after dissection, the eyes were fixed in 10% neutral-buffered formalin, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin using the standard procedure. After rehydration in xylene and ethanol series, paraffin-embedded eye sections were treated with citrate buffer (pH 6.0) for 20 min at 70°C, cooled and then incubated in 3.0% hydrogen peroxide to block endogenous peroxidase. The sections were incubated with streptavidin D and biotin blocking solution for 15 min each at room temperature and then in mouse-on-mouse (M.O.M, Vector Laboratories, CA) Ig blocking solution for 1 hr at room temperature. After washing in PBS, the sections were incubated in M.O.M diluent, followed by incubation in mouse anti-Glo I mAb [22] diluted to 24 μg/ml in PBS overnight at 4°C. After being thoroughly washed in PBS, the slides were incubated with M.O.M biotinylated antimouse IgG reagent and rinsed thoroughly in PBS. The slides were then incubated in ABC Vectastain Elite Peroxidase (Vector Laboratories) and rinsed in PBS. The sections were stained by incubating in 3, 3′-diaminobenzidine substrate, rinsed thoroughly in deionized water, and counterstained with hematoxylin. The slides were viewed with an Olympus BX-60 upright microscope (Tokyo, Japan). Color images were captured using a SPOT RT Slider camera (Diagnostic Instruments, MI) connected to a Macintosh computer using Spot software version 3.5.5.

6. Lens Organ Culture

The mouse lenses from 8-week-old Wt and transgenic animals were dissected out of the eye by a posterior approach without the dissecting tools coming into direct contact with the lens. The lenses were cultured in modified TC-199 media according to Shamsi et al. [20]. Briefly, the lenses were placed in a 24-well plate filled with 2 ml/well of media containing 25 mM HEPES (pH 7.4) and 0.9% sodium carbonate, 30 μg/ml streptomycin and 30 U/ml penicillin. The osmolarity of the media was measured to be ~320 mOsm. The medium was incubated for 2 hr in a 37°C incubator with 5% CO_2 and 95% air prior to the addition of the lenses. The lenses were incubated for 24 hr, and those that developed haziness were discarded. The lenses were maintained for 48 hr in media containing GLD, and the media was changed after 24 hr. Lenses incubated with media alone served as the control. After the incubation, lenses were washed with 2 ml PBS twice and frozen at −80°C.

7. Assay for Glo I Activity

Lenses were homogenized in 0.1 M Tris-HCl buffer, pH 7.4, containing 100 μM PMSF. After homogenization, the extract was centrifuged at 18,000 *g* for 30 min at 4°C. Glo I activity was measured by monitoring the formation of *S*-D-lactoylglutathione at 240 nm over a period of 5 min. The enzyme activity was calculated from the molar extinction coefficient of *S*-D-lactoylglutathione (3370 cm^{-1}at 240 nm) and expressed as μmoles of *S*-D-lactoylglutathione formed per min per mg of protein.

8. MGO Estimation

MGO was estimated according to the method of Espinosa-Mansilla et al. [23]. The lenses were homogenized in 150 μl of 10% TCA and centrifuged. One hundred microliters of supernatant from the TCA extraction was mixed with 1 mM 6-hydroxy-2, 4, 5-triaminopyrimidine (TRI) in 250 μl of sodium acetate buffer at pH 4.05. The mixture was incubated at 60°C for 45 min. The sample was filtered through a 0.45 μm centrifugal filter and injected into a C18 reversed phase HPLC column as per the previously reported procedure in [19]. The MGO content in the samples was calculated by comparison with known quantities of similarly processed MGO standards, and the results were expressed as pmoles/lens.

9. Estimation of GSH

GSH was determined according to Cui and Lou [24]. Each lens was homogenized in 150 μl of 10% TCA, and the homogenate was centrifuged at 10,000 g for 10 min. The supernatant was used for MGO estimation, and the pellet was used for AGE estimation (see below). Twenty microliters of lens TCA supernatant was mixed with 10 μl of DTNB (2 mg/2.5 ml methanol). The volume was adjusted to 200 μl with 1.0 M Tris-HCl buffer, pH 8.2 containing 0.02 M EDTA. The absorbance of the reaction product was measured at 412 nm, and the GSH level was quantified by comparing to GSH standards.

10. HPLC Assay for Argpyrimidine and Pentosidine

TCA-pelleted lens protein from the MGO estimation was washed with ether and air dried overnight. The pellet was suspended with 6 N HCl and incubated for 16 hr at 110°C. The acid was evaporated in a Savant SpeedVac system, and the pellet was resuspended in 250 μl of water filtered through a 0.45 μm centrifugal filter. Aliquots of all samples were analyzed by HPLC for argpyrimidine and pentosidine as previously described in [25, 26].

11. ELISA for Hydroimidazolone (HI)

Microplate wells were coated overnight with 5 μg of soluble lens protein per well in 50 mM carbonate buffer (pH 9.6) in triplicate. The wells were then washed three times with phosphate-buffered saline-Tween-20 (PBS-T) and incubated with 50 μl of diluted HI monoclonal antibody for 1 hr at 37°C. Following this step, the wells were washed three times with PBS-T and incubated with 50 μl of goat antimouse IgG diluted in PBS-T (1:5000) for 1 hr at 37°C. After the wells were washed with PBS-T, they were incubated with 100 μl of 3, 3′, 5, 5′-tetramethylbenzidine substrate (Sigma). The enzyme reaction was stopped by the addition of 50 μl of 2N H_2SO_4, and the absorbance was measured at 450 nm in a Dynex MRX 5000 Microplate Reader. One unit of HI was defined as an increase in 0.01 O.D (at 450 nm)/μg protein.

12. Statistical Analysis

Fisher's PLSD test (Statview 5.0; SAS Institute, Inc., Cary, NC) was used to evaluate the differences among treatment groups. We considered $P \leq .05$ to be statistically significant.

13. Results and Discussion

Histological examination of lenses from 6-month-old homozygous transgenic animals did not show any morphological changes when compared to the lens of a Wt mouse of a similar age (Figure 2(a)). Immunohistological examination revealed that Glo I was overexpressed both in epithelial cells and in outer cortical fiber cells (Figure 2(b)). Glo I overexpression was further confirmed by in situ hybridization, which showed high levels of Glo I mRNA in epithelial cells and fiber cells (Figure 2(c)). Glo I activity in transgenic lenses was approximately 86-fold higher than in Wt lenses (Figure 2(d)).

To determine if the overexpression of Glo I prevents MGO-AGE formation, the lenses were incubated with 5 mM GLD. Direct exposure of the lenses to MGO was avoided, as MGO becomes cytotoxic above 500 μM and causes opacity of the lens. We have previously shown that incubation of lenses with GLD results in high levels of MGO and MGO-AGEs in organ-cultured rat lenses [20]. Incubation of Wt lenses

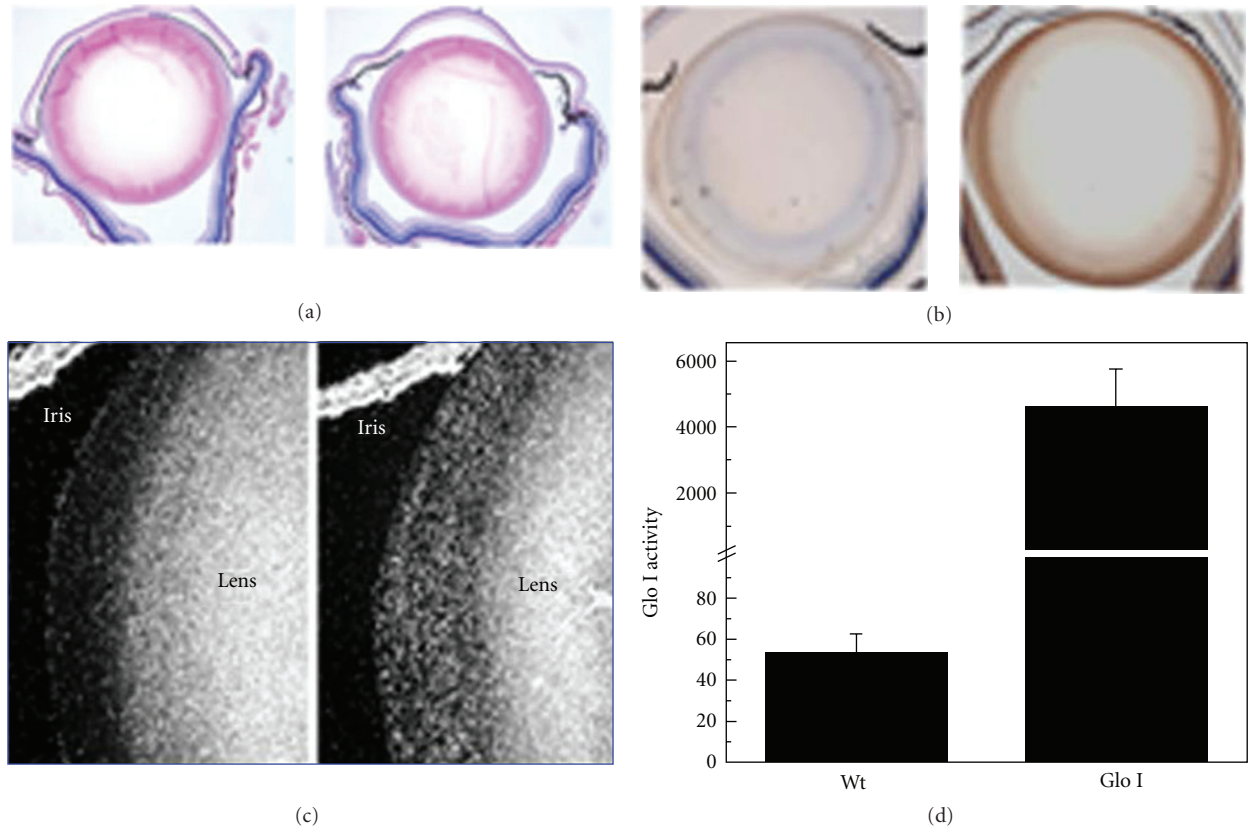

Figure 2: Glo I expression and activity in transgenic mouse lenses. (a) H&E staining of Wt and Glo I transgenic lenses showed no morphological changes in 6-month old animals. No difference was noticeable between the Wt and transgenic lens. (b) Immunohistochemistry using a monoclonal antibody for human Glo I showed human Glo I expression in epithelial cells and outer cortical fiber cells. Glo I immunoreactivity was absent in Wt lenses. (c) In situ hybridization shows Glo I mRNA in outer cortical and epithelial cells in Glo I transgenic animals. (d) Glo I activity was measured in the water-soluble lens proteins. Glo I-catalyzed formation of *S*-D-lactoylglutathione from MGO and GSH was monitored at 240 nm. The data are the mean ± SD from 4 lenses.

with GLD resulted in a profound accumulation of MGO. This was significantly reduced in Glo I lenses (Figure 3(a)), suggesting that the overexpression of Glo I inhibited MGO accumulation in the lens. These results are in line with the previous reports showing that Glo I overexpression reduced intracellular MGO levels in human umbilical vein endothelial cells [16] and reduced MGO-AGEs in rat renal tubular epithelial cells [27]. In addition, it has been shown that a reduction of Glo I activity results in MGO-AGEs in cells [17, 28], which is compatible with the present findings.

GSH is a cofactor of Glo I. Our previous study has shown that incubation with GLD reduces GSH levels in rat lenses, possibly because of enhanced oxidation [20]. We investigated whether GLD also reduced GSH in mouse lenses. Upon incubation with GLD, GSH levels were reduced nearly 10-fold in both Wt and Glo I lenses (Figure 3(b)). Even though the residual GSH levels were far smaller than those present in lenses incubated without GLD, the levels found in lenses incubated with GLD could have been sufficient to support Glo I activity. This assertion is supported by the fact that MGO levels in GLD-incubated Glo I lenses were approximately 50% lower than those present in GLD-treated Wt lenses (Figure 3(a)). These results are in agreement with our previous studies with rat lenses where we found that GSH levels, even though precipitously diminished upon incubation with GLD, were sufficient to reduce the levels of an MGO-AGE in the lens [20].

The MGO-AGE levels were reduced as a consequence of the increased Glo I activity and decreased MGO levels. Incubation of the lenses with GLD resulted in nearly 200 pmoles/μmole amino acid argpyrimidine in Wt lenses. This was reduced by nearly 2-fold ($P < .001$) in GLD-treated Glo I lenses (Figure 4(a)). Similarly, the HI content was reduced nearly 10-fold ($P < .0001$) in Glo I lenses when compared to Wt lenses (Figure 4(b)). These results suggest that Glo I regulates MGO-AGE formation in the lens. Our results are compatible with previous studies that have shown similar effects of Glo I overexpression on the reduction of MGO-AGEs [16, 27].

Pentosidine is an AGE that is formed as a crosslinking adduct between a lysine and an arginine residue in proteins [29]. We have previously shown that MGO inhibits pentosidine synthesis from ribose and ascorbate [26]. We reasoned that MGO occupies arginine residues because of its extreme

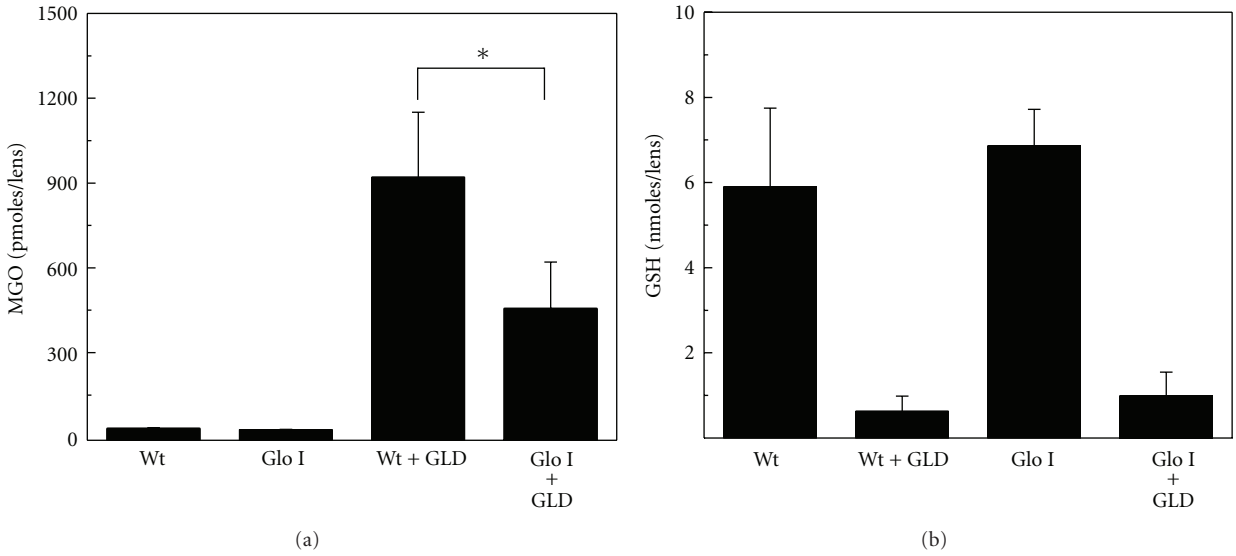

Figure 3: MGO and GSH levels in organ-cultured lenses. Lenses were organ cultured for 48 hr in the absence or presence of 5 mM GLD. Lenses were homogenized in 10% TCA, and supernatants were used for MGO and GSH estimation as described in the Materials and Methods. MGO levels (a) are expressed as pmoles/lens ($^{*}P < .005$), and GSH levels (b) are expressed as nmoles/lens. The average wet weight of each lens was 5.5 mg. The results are the mean ± SD from 6 lenses.

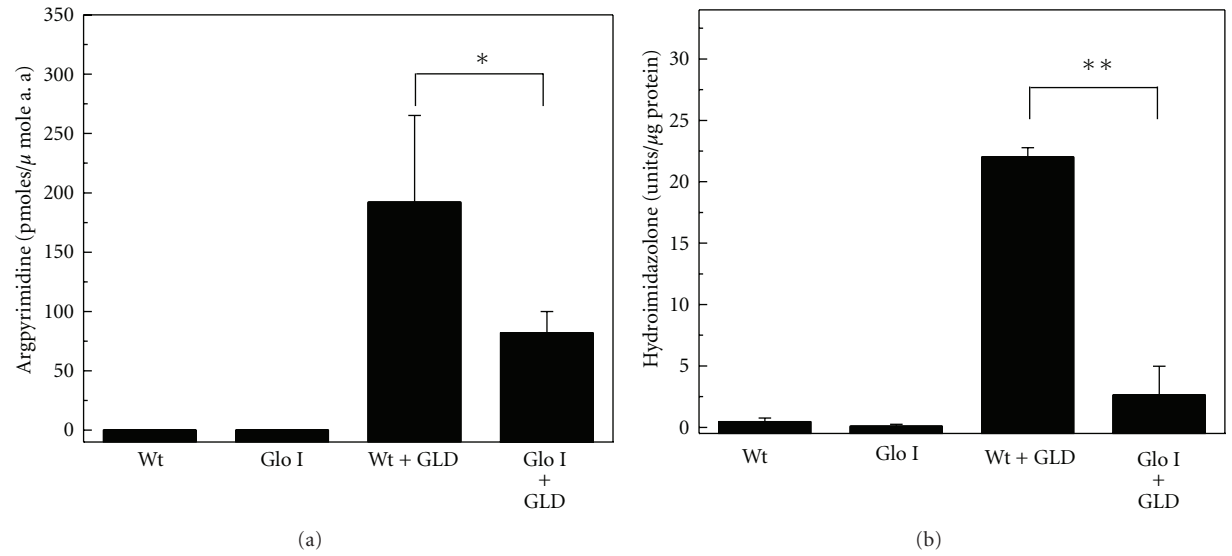

Figure 4: MGO-derived AGEs are inhibited by Glo I overexpression. (a) Argpyrimidine content in lenses cultured in the presence of GLD. TCA precipitated lens protein was hydrolyzed by 6 N HCl and subjected to HPLC analyses as described in the Materials and Methods. The results are the mean ± SD of 6 lenses. ($^{*}P < .001$). (b) HI estimation by ELISA. Microplate wells were coated overnight with 5 μg of soluble lens protein per well and incubated with a monoclonal antibody for HI followed by goat antimouse IgG. The results are the mean ± SD from 4 lenses. ($^{**}P < .0001$).

reactivity with it and thereby blocks pentosidine formation. To test whether Glo I overexpression prevented MGO-mediated inhibition of pentosidine synthesis, we incubated water soluble proteins (without dialysis, to preserve GSH) from Wt and Glo I lenses with 5 mM ribose for 7 days at pH 7.4. While the incubation of ribose with Wt lens proteins resulted in the accumulation of pentosidine, as expected, incubation of ribose with Glo I lens proteins resulted in even higher concentrations (30% more) of pentosidine (Figure 5). This suggests that MGO is generated during ribose-mediated glycation and that MGO is metabolized by Glo I in isolated lens proteins. In fact, in a previous study, we have shown that MGO-mediated argpyrimidine synthesis occurs during glycation by ribose [30], implying that MGO is produced during the reaction. Thus, the present study confirms those results and provides a basis for the argument that Glo I

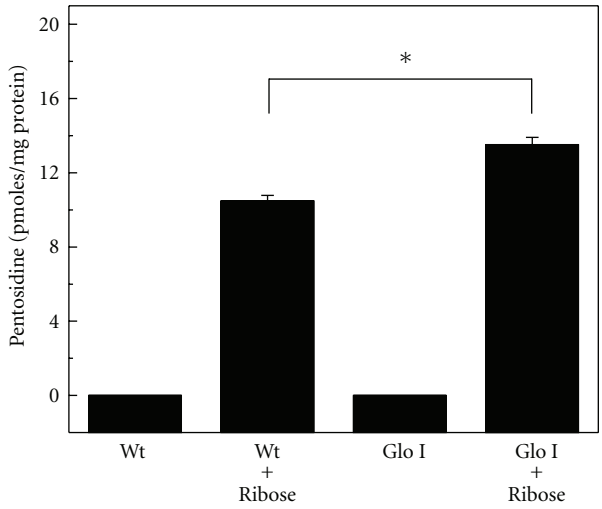

Figure 5: Promotion of pentosidine synthesis by Glo I overexpression. Wt and Glo I lens homogenates (5 mg/ml) were incubated with 5 mM ribose for 7 days and analyzed by HPLC for pentosidine. The results are the mean ± SD from 3 experiments. ($^*P < .0001$).

overexpression may not be the best strategy to reduce AGEs in cells and tissues.

14. Conclusions

In conclusion, our study provides direct evidence for the modulation of AGE synthesis by Glo I. Determining whether MGO-mediated AGE inhibition is beneficial or harmful requires further work, as our studies have shown that mild modification of lens αA-crystallin makes it a better chaperone protein [31, 32]. The chaperone property of αA-crystallin has been proposed to play an important role in maintaining the transparency of the aging lens. The other finding that Glo I-mediated removal of MGO could promote synthesis of AGEs from sugars suggests that overexpression of Glo 1 to deplete MGO may be counterintuitive for prevention of AGE synthesis in the lens.

Abbreviations

AGEs: advanced glycation end products
TCA: trichloroacetic acid
HI: HI
DTNB: 5, 5′-dithiobis (2-nitro-benzoic acid)
GLD: DL-glyceraldehyde
GSH: glutathione reduced form
MGO: methylglyoxal
TRI: 6-hydroxy-2,4,5-triaminopyrimidine
HFBA: heptafluorobutyric acid
DAB: 3,3′-diaminobenizidine.

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Review Article

Pathophysiological Characteristics of Diabetic Ocular Complications in Spontaneously Diabetic Torii Rat

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The Spontaneously Diabetic Torii (SDT) rat, a nonobese type 2 diabetes model, develops severe diabetic retinopathy as result of chronic severe hyperglycemia. Although existing diabetes animal models also develop ocular complications, severe retinal lesions frequently observed in human diabetes patients such as preretinal neovascularization or retinal detachment are not found. Distinctive features in SDT rat are hypermature cataract, tractional retinal detachment with fibrous proliferation, and massive hemorrhaging in the anterior chamber. These pathophysiological changes are caused by sustained hyperglycemic condition and subsequent increased expression of vascular endothelial growth factor (VEGF) in retina, iris, and ciliary body. Although some differences in diabetic retinopathy exist between SDT rats and humans (e.g., a low incidence of neovascular formation and poor development of nonperfused area are found in this animal), SDT rat will be a useful model in studies of the pathogenesis and treatment of diabetic retinopathy.

1. Introduction

Many animal models have been used in research into diabetes mellitus (DM) and its complications. Animal models of chemically induced DM such as streptozotocin (STZ) or alloxan-induced diabetic animals are widely used [1]. Genetic models of DM such as Nonobese diabetic (NOD) mice [2], Bio-Breeding (BB) rats [3], *ob/ob* mice [4], *db/db* mice [5], Goto-Kakizaki (GK) rats [6], Zucker diabetic fatty (ZDF) rats [7], and Otsuka Long-Evans Tokushima fatty (OLETF) rats [8] are also used commonly. Although these model animals develop either type 1 (T1D) or type 2 (T2D) diabetes and subsequent ocular complications, severe retinal lesions frequently observed in human diabetes patients such as preretinal neovascularization or retinal detachment are not found; early pathological changes such as pericyte loss [9, 10], early biophysiological changes such as retinal leukostasis [11], and abnormal pattern in electroretinogram (ERG) [12] are observed, at most.

The Spontaneously Diabetic Torii (SDT) rat, a nonobese T2D model is an inbred rat strain established from Sprague-Dawley (SD) rat by Shinohara et al. [13, 14]. As a result of chronic severe hyperglycemia, SDT rats develop diabetic retinopathy (DR) [13–20], diabetic peripheral neuropathy [19, 20], and diabetic nephropathy [21]. Of these, severe DR such as cataract, tractional retinal detachment with fibrous proliferation, and massive hemorrhaging in the anterior chamber is a distinctive feature of SDT rat [13, 14]. Since the establishment and the first report of SDT rat by Shinohara et al. [13], dozens of papers have been published. In the present short paper, pathophysiological characteristics of ocular complications in SDT rat are outlined.

2. General Characteristics

Male SDT rats exhibit noticeable hyperglycemia, polyuria, and glucosuria concomitant with diminished blood insulin levels and decreased body weight by 15–20 weeks of age (Figure 1). The cumulative incidence of diabetes reaches 100% up to 40 weeks of age. In contrast, the incidence is 33% in female SDT rats [13]. Preceding the onset of diabetes, glucose intolerance with impaired insulin secretion [13, 22] and impaired lipid catabolism [23] are also observed. Genetic analysis for diabetes in SDT rats identified significant

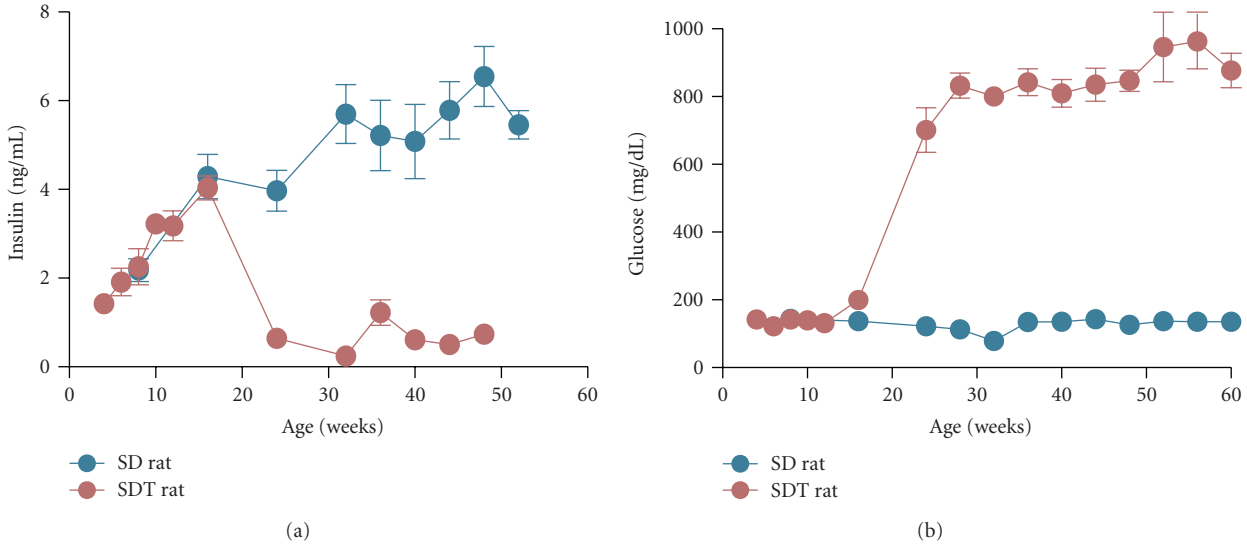

Figure 1: Nonfasting plasma insulin and glucose levels in Spontaneously Diabetic Torii (SDT) rats and control Sprague-Dawley (SD) rats. Diminish of pancreatic β-cells evokes hypoinsulinemia (a) and subsequent severe hyperglycemia (b) in SDT rats. Plasma glucose levels sharply increase at 15–20 weeks of age and eventually reach a plateau, approximately 800 mg/dL. Each value represents mean ± S.E.M. (N = 6–8).

quantitative trait loci (QTL) (*Gisdt1*, *Gisdt2*, and *Gisdt3*) for glucose intolerance on rat chromosomes 1, 2, and X, respectively, indicating that the diabetic features in SDT rat are polygenically inherited [24]. Hyperglycemia in SDT rat is spontaneously developed, predominantly due to an insulin secretory defect resulting from pathological damage to the pancreatic islets, especially β-cells [22, 25]. Following primary microvascular events in the pancreatic islet such as congestion and hemorrhage (8–10 weeks), inflammation, progressive fibrosis (10–20 weeks), and atrophy with diminished β-cells (38 weeks) are observed [22]. These inflammations are different from autoimmune-mediated inflammation observed in autoimmune diabetes. A major locus on chromosome 3 (*Dmsdt1*) was identified as a dominantly acting SDT allele that induces islet inflammation and fibrosis [26].

3. Ocular Pathology

3.1. Lens. Cataract is the most frequently occurring ocular complication in DM and is also often observed in diabetic animal models with certain disease duration (e.g., STZ-induced diabetic rats, ZDF rats). In male SDT rats, macroscopic opacity of lens is observed by 40 weeks of age (Figures 2(a) and 2(b)) [13]. Lens clouding begins at posterior pole of lens and finally progresses to mature cataract. Histopathologically, hypermature cortical cataract is suggested by severe swelling, vacuolation, liquefaction, disintegration of the lens fibers, and Morgani's globules in the lens cortex. Nuclear sclerosis and opacified lens cortex are observed, and lens rupture is also found at this stage [18]. Because of this mature cataract, fundus could not be examined by ophthalmoscopy. Cataract in SDT rats was completely prevented by glycemic control such as chronic insulin treatment [17, 19, 20] and pancreatic transplantation [27]. In addition, the histopathological changes of lens were preceded by an increase in lens sorbitol content (our unpublished data). These findings clearly indicate that cataract in SDT rat is due to sustained hyperglycemia.

3.2. Vitreous Body. Proliferative diabetic retinopathy (PDR) concerns new vessels extending into the vitreous cavity and causing fibrovascular proliferation, retinal detachment, and vitreous hemorrhage. In SDT rats, the vitreous body was shrunken and cortex was detached from the retina. Proliferative fibrovascular membrane was formed between the folded retina and the distorted lens (Figures 2(c) and 2(d)) [13, 16, 18]. Fibrovascular membrane was infiltrated with inflammatory cells, and capillary vessels found in the fibrovascular membrane had thin walls. Vitreous hemorrhages were observed in some severe cases [18].

3.3. Retina. Among the many diabetic animal models, severe retinal abnormality is a prominent feature of SDT rat. In SDT rats, retina was locally thickened and formed retinal folds and was detached from retinal pigmented epithelium. These tractional changes of retina were observed only at the center of retina, never at the peripheral retina (Figures 2(c) and 2(d)) [13, 15, 16, 18]. Immunostaining for albumin showed marked leakage at the site of the tractional retinal detachment, suggesting hyperpermeability of the retinal vessels around the detachment site [16]. Fluorescein retinal flatmount of SDT rats showed abnormal vascular formation, including venous dilation and meandering vascular networks (Figures 2(e) and 2(f)) [16, 17]. Dilated retinal vessels and a newly formed capillary network were also evident pathologically. Slight hemosiderin deposition was found in the retinal ganglion cell layer [16, 18]. Acellular capillaries and pericyte

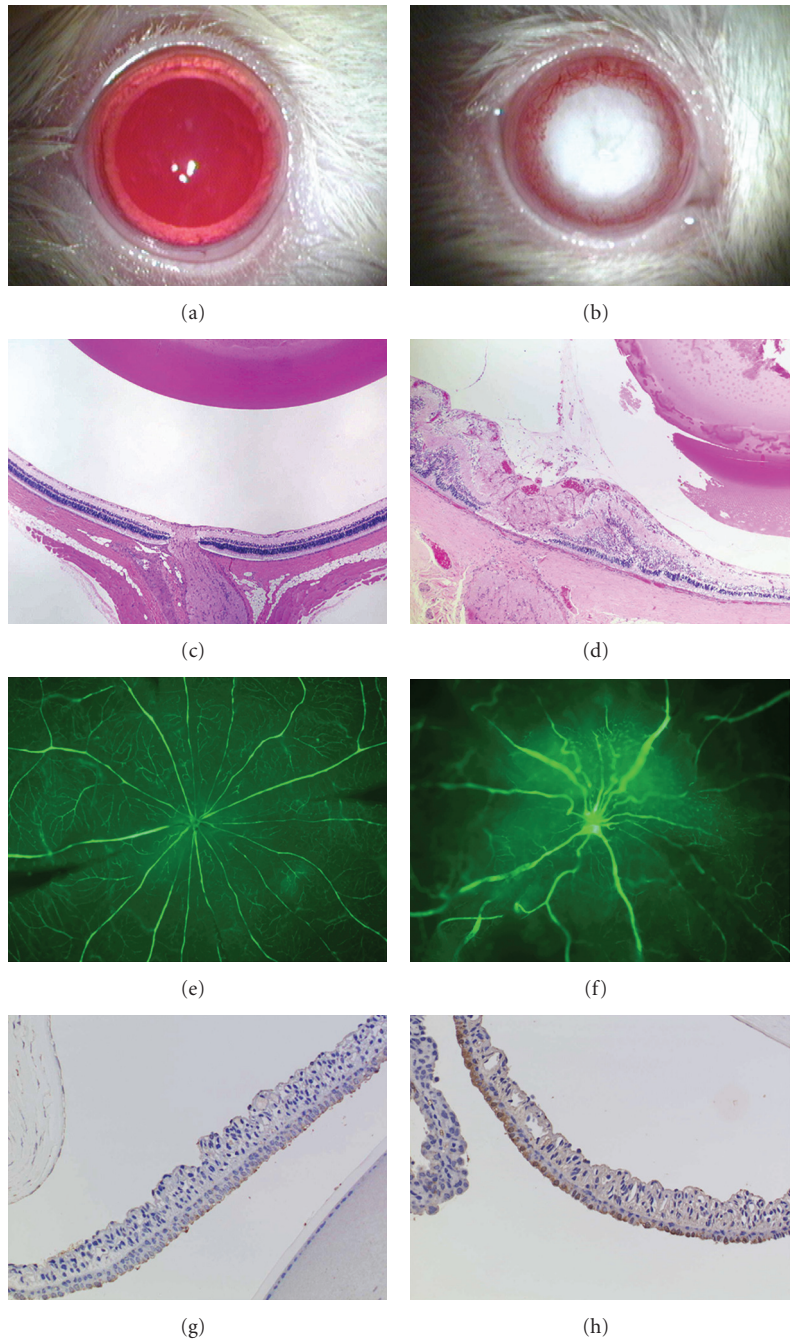

Figure 2: Typical ocular observations in control male SD rats (a, c, e, and g) and SDT rats (b, d, f, and h). (a) and (b) macroscopic photographs of eyes. Hypermature cataract is observed 100% in male SDT rats by 40 weeks of age. (c) and (d) histopathological changes in lens and retina at 80 weeks old. Proliferative fibrovascular membrane in vitreous is found around the optic disk. Retina is locally thickened and formed a fold. Disintegration of lens is also observed. H&E stain. (e) and (f) fluorescein angiomicroscopy at 80 weeks of age. Abnormal vascular formation, including venous dilation and meandering vascular networks, is characteristically observed in SDT rats. Extensive fluorescein leakage is found around the optic disk. (g) and (h) anti-VEGF staining of iris at 80 weeks of age. VEGF immunoreactivity is increased in iris of SDT rats. This may cause a massive hemorrhage on iris in some severe cases.

loss were observed in trypsin digestion preparation; however, capillary microaneurysms were not evident [16, 17]. Vascular nonperfusion area, bleeding, or hard/soft exudates were not observed in flat-mount preparations of retina from SDT rats even at over 80 weeks of age [15].

3.4. Iris. Although the neovascular glaucoma is frequently observed in human DR, iris neovascularization has not been reported among diabetic animal models. Iris neovascularization was found in some cases in SDT rats. Fibrovascular tissue covering the pupil or anterior lens capsule may

induce pupillary block or angle-closure glaucoma. In a severe case, massive hemorrhage was found in the anterior chamber [13, 16, 18]. Increased aqueous humor vascular endothelial growth factor (VEGF) level [17], which may be derived from anti-VEGF antibody positive ciliary epithelium [28] and iris (Figures 2(g) and 2(h)), presumably causes iris neovascularization and subsequent bleeding from the neovasculature into the anterior chamber.

4. Electroretinogram (ERG)

ERG is used to detect abnormal function of the retina. Prolongation of the peak latency in ERG is a very early alteration in diabetic patients, even in patients having no ophthalmoscopically visible alterations at this stage [29, 30]. STZ-induced diabetic rats show prolongation of peak latency in ERG, and the prolongation is prevented with insulin treatment [31, 32]. SDT rats also showed retinal dysfunction in ERG earlier than histopathological changes [17, 33]. Both amplitudes and implicit times of the ERG in SDT rats were not significantly different from those of SD rats at prediabetic stage. However, at postdiabetic 44 weeks of age, amplitudes of the $a-$ and $b-$waves and the oscillatory potentials (OPs) were reduced with prolonged implicit times in SDT rats. Because OPs are preferentially decreased in human DR, this is a differing characteristic between DR of humans and SDT rats. The prolonged implicit times and decreased amplitudes of OPs were clearly prevented with long-term treatment of insulin [17], PKCβ-specific inhibitor, JTT-010 [20], or an angiotensin II receptor blocker (ARB), candesartan [34]. Therefore, depressed ERG in SDT rat is caused by chronic hyperglycemia and may reflect vascular and neuronal damage of retina, as frequently observed in human DR.

5. Vascular Endothelial Growth Factor (VEGF) and Pigment Epithelium-Derived Factor (PEDF)

VEGF plays an important role in retinal neovascularization and hyperpermeability [35, 36]. Clinical observations indicate that VEGF concentration in ocular fluid positively correlates with neovascularization activity in DR [37, 38]. In patients with DR and macular edema, increase of vitreous and/or aqueous humor VEGF concentration has been reported [38, 39]. Increased VEGF mRNA expression and anti-VEGF staining area (e.g., retinal vessels, ganglion cell layer, inner plexiform layer, outer plexiform layer, and retinal pigment epithelium cells) in the retina of SDT rat are reported [15, 40]. Increased aqueous humor VEGF level in SDT rats tended to reduce by glycemic control with insulin treatment [17]. Candesartan decreased the expression of VEGF mRNA by reducing the accumulation of advanced glycation end products (AGEs) [34] and NADPH oxidase [41] in SDT rats. Gene transfer of the soluble form of VEGF receptor Flt-1 (*sflt-1*), the endogenous specific inhibitor of VEGF, into retina with adeno-associated viral (rAAV5) vectors prevented the progress of DR in SDT rats [42]. Therefore, the increased expression of VEGF induced by sustained hyperglycemia is thought to be causally related to DR include retinal neovascularization in SDT rat.

On the other hand, expression of pigment epithelium-derived factor (PEDF), a potent inhibitor of ocular angiogenesis [43], is also upregulated in plasma of SDT rat [44], and anti-PEDF antibody positive cells are detected in retinal vessels, nerve fiber layer, ganglion cell layer, inner plexiform layer, inner nuclear cell layer, outer plexiform layer, and retinal pigment epithelium cells in SDT rats [40]. These findings that VEGF and PEDF expressions were both up-regulated in SDT rat retinae are different from the human DR with low levels of PEDF [45], and the high PEDF levels in retina may have contributed to a low incidence of neovascular formation and poor development of nonperfused area in DR of SDT rat.

6. Conclusion

Human DR is characterized by microaneurysms, intraretinal punctate hemorrhages, and macular edema. At advanced stage, nonperfusion area and subsequent neovascularization of the retina, which may extend into the vitreous cavity, proliferation of fibrous tissue, vitreous hemorrhage, and retinal detachment, are frequently found. To study and develop treatment for DR, animal models with DR resembling human DR are desperately needed. The earliest histopathological signs of DR such as selective loss of intramural pericytes from retinal capillaries, capillary dilation, and varicose loop formation are frequently observed in some diabetic animal models (e.g., STZ-induced diabetic rats) [46]. Meanwhile, SDT rats develop severe ocular complications such as neovascularization and tractional changes in retina. These findings are the main differences between SDT rat and other rodent diabetes models (Table 1). The tractional changes caused by vitreoretinal interaction are similar to those that occur in human PDR. There is no rodent model with DR of such severity; therefore, SDT rat may be the best candidate for research into DR. One of the possible reasons why retinal neovascularization is observed characteristically in SDT rat is the survival period without glycemic control. In our laboratory, SDT rats survived more than 90 weeks without insulin therapy. Under chronic hyperglycemic circumstances, retina is exposed to high concentration of VEGF over a long period. These factors may cause thickening of the posterior vitreous cortex or modified vitreo-retinal interaction [47] in SDT rats. Meanwhile, in contrast to patients with DR, SDT rats did not develop vascular nonperfusion, bleeding, or hard/soft exudates in retina [15]. These may be crucial differences between humans and rodents. Upregulation of PEDF may be a possible cause of low incidence of neovascular formation and nonperfusion area in SDT rat [40].

In addition to retinal pathology, there are some other similarities and differences in ocular pathology between diabetic patients and SDT rats (Table 1). Senile cataract is accelerated in diabetics; however, true diabetic cataract is rare condition, occurring typically in young people with acute diabetes. On the other hand, SDT rats and other diabetic

Table 1: Ocular pathological findings in diabetic human, SDT rats, and STZ-induced diabetic rats.

	Human	SDT rats	STZ rats
Retina			
Hyperpermeability	Yes	Yes	Yes
Retinal detachment	Yes	Yes	No
Retinal thickness	Yes	Yes	Yes
Avascular area	Yes	No	No
Neovascularization	Yes	Yes	Yes
Retinal microaneurysm	Yes	No	No
Pericyte loss	Yes	Yes	Yes
Vessel abnormality	Yes	Yes	Yes
VEGF expression	High	High	High
PEDF expression	Low	High	High
Vitreous body			
Proliferative membrane	Yes	Yes	No
Iris			
Neovascularization	Yes	Yes	No
Lens			
Cataract	Yes (senile cataract $\gg$ true diabetic cataract)	Yes (true diabetic cataract)	Yes (true diabetic cataract)

animals such as STZ-induced diabetic rats show typical true diabetic cataract. The difference may be caused by rapid progression of extreme hyperglycemia in these diabetic animal models.

When the aqueous humor does not drain properly by neovasculatures, increased intraocular pressure results in glaucoma. Iris neovascularization and subsequent development of neovascular glaucoma are serious consequences for patients with PDR. Although diabetes may act as a risk factor of open-angle glaucoma [48], there are no diabetic animal models of spontaneously progress iris neovascularization. However hyperpermeability in iris vessels and decreased iris blood perfusion caused by iris vascular endothelial dysfunction are reported [49, 50], iris neovascularization was not found in STZ-induced diabetic rats. Since SDT rat shows iris neovascularization and bleeding from the neovasculature in some severe cases, SDT rat is a useful model of diabetic rubeosis and is expected as a model of diabetic glaucoma [13, 14].

Although DR in SDT rat differs from human DR in some respects, these ocular lesions are much more severe than those observed in other diabetic models and were clearly prevented by glycemic control [17, 19, 20, 27]. To accelerate the development of diabetes and its complications in SDT rats, Masuyama et al. established SDT.Cg-*Lepr*^{fa} congenic rats (SDT fatty rats) by introducing an *fa* allele of the leptin receptor gene of Zucker Fatty rat into the genome of SDT rats [51]. Onset of diabetes in SDT fatty rat is accelerated to 5 weeks of age by developing adiposity and insulin resistance. Diabetic complications also develop at younger age than in SDT rat [52].

In conclusion, severe ocular complications are distinctive features of SDT rat and were "diabetic", although there are some differences from human DR. In view of the present situation, that there are no ideal animal models of human DR, SDT rat will be useful animal model in studies of the pathogenesis of DR.

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Review Article

Human Genetics of Diabetic Retinopathy: Current Perspectives

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Diabetic retinopathy (DR) is a most severe microvascular complication which, if left unchecked, can be sight-threatening. With the global prevalence of diabetes being relentlessly projected to rise to 438 million subjects by 2030, DR will undoubtedly pose a major public health concern. Efforts to unravel the human genetics of DR have been undertaken using the candidate gene and linkage approaches, while GWAS efforts are still lacking. Aside from evidence for a few genes including aldose reductase and vascular endothelial growth factor, the genetics of DR remain poorly elucidated. Nevertheless, the promise of impactful scientific discoveries may be realized if concerted and collaborative efforts are mounted to identify the genes for DR. Harnessing new genetic technologies and resources such as the upcoming 1000 Genomes Project will help advance this field of research, and potentially lead to a rich harvest of insights into the biological mechanisms underlying this debilitating complication.

1. Introduction

Diabetic retinopathy (DR) is a most severe microvascular complication which if left unchecked can be sight-threatening. DR ranks as a common cause of blindness worldwide, particularly among adults [1–3]. With the global prevalence of diabetes being projected to rise to 438 million subjects by 2030, DR will certainly pose a major public health concern [4].

The presence of diabetic retinopathy is evidenced by the appearance of retinal microvascular lesions. Early changes include microaneurysms, hemorrhages, hard exudates, cotton wool spots, intraretinal microvascular abnormalities, and venous beading and characterize nonproliferative diabetic retinopathy (NPDR). The more severe state of proliferative diabetic retinopathy (PDR) is marked by the formation of abnormal fragile new blood vessels that are prone to hemorrhage. Finally, visual impairment results in secondary to pre-retinal or vitreous hemorrhage and diabetic maculopathy.

2. Familial Clustering of DR

Epidemiological studies have shown that the prevalence of DR increases with diabetes duration and various clinical measures, primarily intensive glycaemic control, can delay the development of DR [5, 6]. It is however noteworthy that some patients may still develop DR even with good glycaemic control. Conversely, some patients with poor glycaemic control are spared from this complication and notably, in long surviving patients with type 1 diabetes, the association between diabetic retinopathy and glycaemic control is less well supported [7]. Genetic susceptibility may underlie this observation, a proposal that was supported by twin analysis conducted more than three decades ago [8]. Of late, this early evidence for a role of genetic factors in DR has been corroborated by familial aggregation studies among patients with either type 1 or type 2 diabetes (Table 1). Familial clustering also extends across different ethnicities. This effect of genes likely influences the various stages of DR including NPDR, PDR, and macular edema although different genes may impact specific stages of disease [9–13].

3. Candidate Genes for DR

The search for DR genes has predominantly been undertaken using the candidate gene approach. The case-control study design is generally employed and is appropriate for detecting both major and minor genes. The candidate gene approach requires a fair knowledge of the pathogenic mechanisms underlying DR and this has benefitted from the many years of research in this field [14–16]. Several pathways and processes have been strongly implicated including the renin-angiotensin system, polyol pathway, nonenzymatic glycation, endothelial dysfunction, vascular tone maintenance, extracellular matrix remodeling, and angiogenesis which is dysregulated in diabetes leading to proliferation of new fragile retinal capillaries that culminate in PDR [17, 18]. Correspondingly, a host of genes involved in these pathways/processes have been treated as potential candidate genes. These genes include angiotensin-I converting enzyme (*ACE*), angiotensin II type 1 receptor (*AGTR1*), angiotensinogen (*AGT*), vascular endothelial growth factor (*VEGF*), aldose reductase (*AR2*), receptor for advanced glycation endproducts (*RAGE*), glucose transporter 1 (*GLUT1*), inducible and constitutive nitric oxide synthases (*NOS2A*, *NOS3*), transforming growth factor beta (*TGFbeta*), endothelin isoforms, and its cellular receptors, among others [19–28].

As is the current thought in the field of complex genetics, the effect sizes of these genetic factors are likely to be modest although major genes have been postulated to exist. Consequently, individual studies have, more often than not, yielded inconsistent and even conflicting findings [29]. To circumvent this issue, meta-analyses have been undertaken to pinpoint the few genes for which there might be cumulative evidence for an association with DR. Three of these genes are highlighted in the following sections.

4. Aldose Reductase (AKR1B1, Human Chromosome 7q35)

Aldose reductase (AKR1B1) is the rate-limiting enzyme of the polyol pathway, which catalyzes NADPH-dependent reduction of glucose to sorbitol. AKR1B1 has been reported in human pericytes, and activation of this pathway has been strongly implicated in the pathogenesis of DR. Notably, retinal vascular changes such as microaneurysm formation and degeneration of retinal pericytes may be induced in rats and dogs that have been made hyperglycemic by a diet rich in galactose, the latter being reduced by AKR1B1 to form galactitol [14, 15]. The search for pharmacological inhibitors of this enzyme for use in the treatment of DR is ongoing [30]. In a recent meta-analysis of the various polymorphisms in *AKR1B1*, the Z-2 allele of the $(CA)_n$ microsatellite located at the 5′ end of the gene showed the most significant association with diabetic retinopathy (OR = 2.33, 95% CI = 1.49–3.64, $P = .0002$), independently of the type of diabetes present. This association was present regardless of whether cases had NPDR (OR = 1.64, 95% CI = 1.14–2.35, $P = .0075$) or PDR (OR = 1.51, 95% CI = 1.16–1.97, $P = .0023$) [31]. Conversely, the Z + 2 and Z alleles conferred protection against DR [31]. Beside the $(CA)_n$ microsatellite, the association of the promoter SNP rs759853 and DR has also been reported in a number of studies. Meta-analysis suggested that the T allele conferred protection against DR in type 1 diabetes (OR = 0.49, 95% CI 0.36–0.68, $P < .0001$) while there was no statistically significant association in patients with type 2 diabetes [31].

5. Vascular Endothelial Growth Factor (VEGF, Human Chromosome 6p12)

VEGF is an important growth factor involved in causing vascular permeability. High vitreous levels have been repeatedly detected in eyes of patients undergoing vitrectomy operations for PDR and diabetic macular edema [32–35]. The cellular effects of VEGF are mediated primarily through two closely related receptor tyrosine kinases VEGFR-1 (Flt1) and VEGFR-2 (KDR/Flk1) [36]. Regulation of target genes such as heptocyte growth factor (HGF), urinary and tissue plasminogen activator (uPA, tPA), matrix metalloproteinase-9 (MMP9) is then achieved through complex signaling pathways, including through protein kinase C (PKC) [37]. VEGF inhibition has been shown to ameliorate retinal changes including retinal neovascularization and breakdown of the blood-retinal barrier [38–40]. A total of six polymorphisms (rs25648, rs1570360, rs3095039, rs35569394, rs699947 and rs2010963) in *VEGF* have been examined and of these, the G allele of rs2010963 was significantly associated with a reduced risk of NPDR in patients with type 2 diabetes (OR = 0.62, 95% CI = 0.48–0.81, $P = .0005$) [31]. Considering that VEGF has been implicated in neovascularization, it might appear surprising that none of the polymorphisms so far including rs2010963 has been significantly associated with PDR [31].

6. Angiotensin-I Converting Enzyme (ACE, Human Chromosome 17q23)

Among the DR candidate genes, ACE is the most widely studied. The well-known insertion deletion (I/D) polymorphism in ACE which results from the insertion/deletion of a 287 bp Alu sequence in intron 16 accounts for half the variance of serum enzyme levels. Individuals who are homozygous for the insertion allele (II genotype) have significantly lower levels of ACE compared to carriers of the deletion allele (ID and DD genotypes) [41]. A meta-analysis of six studies on this polymorphism in patients with type 1 diabetes and seven studies in patients with type 2 diabetes suggested that there was no statistically significant association of this polymorphism and the development of any form of diabetic retinopathy [31]. A second recent independent meta-analysis corroborated this finding but suggested that ACE I/D may be associated with PDR (OR = 1.37, 95% CI = 1.02–1.84) under a dominant genetic model assuming either fixed or random effects [42].

Table 1: Familial clustering of DR.

Type of Diabetes	Patients/Study	Evidence for familiar Clustering	Reference
Type 1	DCCT subjects	OR = 3.1, 95% CI = 1.2–7.8	[9]
Type 1	FinnDiane Study	OR = 2.76, 95% CI = 1.25–6.11, Heritability = 52%	[13]
Type 2	Asian Indians	OR = 3.37, 95% CI = 1.56–7.29	[10]
Type 2	Mexican Americans	OR = 1.72, 95% CI = 1.03–2.88	[11]
Type 2	Find-Eye Study	Heritability = 27%	[12]

7. Deficiency of Candidate Gene Studies

Apart from the few genes mentioned above, the overall evidence for the remaining candidate genes investigated to date is weak [31]. Several factors are likely responsible for this situation, a primary one being small sample sizes. Some have as little as 50 subjects while larger studies with more than 100 cases are decidedly uncommon [18, 29]. Meta-analyses have been undertaken to present the overall evidence for an association but this technique has well-known drawbacks, including the possibility of publication bias which has to be carefully assessed.

Another limitation is that most studies failed to take into account the role of haplotype diversity at the candidate gene locus. Studies so far have focused on single (and often random) SNPs and, as such, cannot reasonably exclude a gene as being important in DR since linkage disequilibrium between these SNPs and the true functional SNP may be low [20–23, 26]. To circumvent this problem, it will be potentially useful to examine haplotype-disease associations. The usefulness of this approach was demonstrated recently in the case of ACE where risk haplotypes for diabetic nephropathy (another important microvascular complication of diabetes) were identified in independent studies [43, 44]. Another point of note is that the reported studies have rarely taken into account the potential influence of covariates. One such covariate is diabetes duration, the importance of which has been demonstrated through simulation studies [45] as well as in epidemiological studies seeking to identify genes for another microvascular complication of diabetes (i.e., diabetic nephropathy) [46, 47].

8. Linkage Studies

Besides the candidate gene approach, whole genome linkage studies have also been undertaken to identify chromosomal regions which potentially harbor major genes for DR. Three such studies have been reported and these have been conducted on Mexican Americans and Pima Indians with type 2 diabetes [48–50] (Table 2). With the possible exception of human chromosome 1p36, the linkage evidence for the other regions has not been replicated and this is undoubtedly related to the very modest LOD scores initially reported (Table 2). Genetic linkage appeared to exist for both advanced stages as well as earlier manifestations of DR [49]. The identities of the purported major susceptibility genes in these linkage regions continue to remain elusive.

9. Genomewide Association Studies (GWASs)

Recent advances have made it possible to genotype the human genome at up to a million polymorphic sites in thousands of samples within a reasonable time frame. This has heralded the era of genomewide association studies (GWASs) which have been successful at pinpointing a number of novel genes related to a spectrum of diseases [51]. Unfortunately, no GWAS effort to identify DR genes has been reported to date. Extrapolating from the collective GWAS experience [51, 52], one can nevertheless anticipate that for any DR gene found through GWAS, the implicated risk alleles will have limited effect sizes with OR <1.4 while the risk allele frequencies will be quite frequent in the population (>0.20). Thus, the risk alleles will be low penetrant since many individuals will harbor the risk alleles, but among these, the majority will tend to remain disease-free. In addition, GWAS will not be expected to unmask the identity of the major susceptibility genes for DR even in regions that show linkage to this disease [52]. Finally, the genes identified through GWAS will only account for a very limited proportion of the familial clustering observed for DR. Clinically, the genetic information gleaned from GWAS will have limited utility as potential disease classifiers [53].

However, balancing these modest expectations of GWAS lies the prospect of identifying novel genes which can undoubtedly shed fresh insight into the pathogenic pathways responsible for DR. Indeed, it is realistic to hope that elucidation of these pathways can in the long run lead to new molecular targets for pharmacological intervention. Already, newer therapies based on known pathogenic pathways particularly intravitreal antiangiogenesis agents that act to suppress VEGF are already being evaluated, although the evidence base currently does not yet support their routine use in the clinic [54]. In the interim, good glycaemic and blood pressure control necessarily remains the cornerstone in the prevention of DR [54].

10. Conclusion

From the current survey of the human genetics of DR, it is clear that this field remains poorly developed. However, therein lies the promise of impactful scientific discoveries if concerted and collaborative efforts are mounted to identify the genes responsible for DR. Just as the

Table 2: Potential chromosomal regions linked to DR (LOD scores >1).

Chromosome	Definition of DR	Population	LOD score	Nearest genetic markers	Reference
1p36	Retinopathy score in worst eye	Pima Indians	3.1	D1S3669	[50]
1p36	Any DR	Mexican Americans	1.24	GGAT2A07	[49]
2q37	Severe NPDR/PDR	Mexican Americans	1.11	AFM112yd4	[49]
3p26	Severe NPDR/PDR	Mexican Americans	1.29	GATA22G12	[49]
3q12	Severe NPDR/PDR	Mexican Americans	1.40	GATA68D03	[49]
3q12	Any DR	Mexican Americans	2.41	GATA68D03	[49]
3q26	Presence of at least one microaneruysm, hemorrahage or proliferative DR	Pima Indians	1.36	D3S3053, D3S2427	[48]
7p15	Any DR	Mexican Americans	1.02	GATA41G07	[49]
9q21	Presence of at least one microaneruysm, hemorrhage or proliferative DR	Pima Indians	1.46	D9S1120, D9S910	[48]
12p13	Any DR	Mexican Americans	2.47	GATA49D12	[49]
12q23	Severe NPDR/PDR	Mexican Americans	1.03	GATA85A04	[49]
15q25	Any DR	Mexican Americans	1.07	ATA28G05	[49]
15q26	Any DR	Mexican Americans	1.16	GATA22F01	[49]

International Human Genome and HapMap Projects have propelled genetic discoveries in the past few years, the added resources of the upcoming 1000 Genomes Project (http://www.1000genomes.org/) as well as new genetic technologies may likewise prove beneficial to this field of research and lead to a rich harvest of insights into the biological mechanisms underlying this debilitating complication.

Conflict of Interest

The author has no involvements that might raise the question of bias in the work reported or in the conclusions, implications, or opinions stated.

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Research Article

Identification of Diabetic Retinopathy Genes through a Genome-Wide Association Study among Mexican-Americans from Starr County, Texas

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To identify genetic loci for severe diabetic retinopathy, 286 Mexican-Americans with type 2 diabetes from Starr County, Texas, completed physical examinations including fundus photography for diabetic retinopathy grading. Individuals with moderate-to-severe non-proliferative and proliferative diabetic retinopathy were defined as cases. Direct genotyping was performed using the Affymetrix GeneChip Human Mapping 100 K Set, and SNPs passing quality control criteria were used to impute markers available in HapMap Phase III Mexican population (MXL) in Los Angeles, California. Two directly genotyped markers were associated with severe diabetic retinopathy at a *P*-value less than .0001: SNP rs2300782 ($P = 6.04 \times 10^{-5}$) mapped to an intron region of CAMK4 (calcium/calmodulin-dependent protein kinase IV) on chromosome 5, and SNP rs10519765 ($P = 6.21 \times 10^{-5}$) on chromosomal 15q13 in the FMN1 (formin 1) gene. Using well-imputed markers based on the HapMap III Mexican population, we identified an additional 32 SNPs located in 11 chromosomal regions with nominal association with severe diabetic retinopathy at *P*-value less than .0001. None of these markers were located in traditional candidate genes for diabetic retinopathy or diabetes itself. However, these signals implicate genes involved in inflammation, oxidative stress and cell adhesion for the development and progression of diabetic retinopathy.

1. Introduction

Diabetic retinopathy is a common microvascular complication of diabetes and remains one of the leading causes of blindness throughout the world [1]. It is estimated that 4.1 million Americans have diabetic retinopathy, which causes 12,000 to 24,000 new cases of blindness every year [2]. National Health Interview Survey and US census data lead to projections that the number of Americans 40 years or older having diabetic retinopathy will triple from 5.5 millions in 2005 to 16 millions in 2050 [3]. Although the underlying mechanisms leading to diabetic retinopathy have not been clarified, many risk factors have been reported, including poor glycemic control, longer diabetes duration, hypertension, hyperlipidemia, and albuminuria [4–7]. Evidence from ethnic and family studies has implicated genetic susceptibility for diabetic retinopathy. Mexican-Americans and African-Americans have been reported to have higher prevalence and worse severity of diabetic retinopathy in the US population when compared to non-Hispanic Whites [8, 9]. The Diabetes Control and Complications Trial (DCCT) showed a 3.1 times increased risk of severe retinopathy

for individuals with retinopathy-positive relatives, and a correlation of retinopathy severity of 0.187 for all family members [10]. Similar familial clustering of diabetic retinopathy was also found among South Indians [11] and Mexican-Americans [12] with type 2 diabetes. The FIND-Eye study recently reported the broad sense heritability for diabetic retinopathy as 27% overall and 24% in Mexican-American families [13].

The suggestive genetic contribution to diabetic retinopathy has lead to the search for candidate genes and for genome-wide linkage between genetic markers and this complex disease, but no conclusive loci have been identified or replicated [14, 15]. Affected sibpair analysis in Pima Indians showed some evidence for linkage to diabetic retinopathy on chromosome 3 and 9 [16], and another signal on chromosome 1 was found in a subsequent study of the same population [17]. Our group also performed a linkage scan in Mexican-Americans from Starr County, Texas, and proposed 25 potential candidate genes for diabetic retinopathy under the linkage peaks on chromosome 3, 6, 12, 15, 19, and 20 [18].

As linkage studies lack power to identify alleles with modest effects or those that interact with other genetic or environmental factors on disease risk, genome-wide association studies (GWASs) using dense sets of SNPs across the genome have rapidly advanced our understanding of the genetic background of complex diseases [19]. The Starr County Health Studies has also reported GWAS results for type 2 diabetes among Mexican-Americans [20] with supportive replications from other studies [21–23]. Utilizing stereoscopic fundus photography within this population, we present here the first genome-wide association analysis of diabetic retinopathy among Mexican-Americans with type 2 diabetes from Starr County, Texas.

2. Materials and Methods

2.1. Study Subjects. This study includes 286 individuals with type 2 diabetes representing the earliest onset sibling having fundus photography from sibships with two or more type 2 diabetes-affected siblings from an ongoing genetic study of diabetic retinopathy among Mexican-Americans in Starr County, Texas [18, 20]. Baseline characteristics were obtained from personal interviews and physical examinations. Fasting blood samples and urine specimens were collected for glycemic, lipid, and microalbuminuria measurements, as previously described [24].

2.2. Genotyping. DNA samples extracted from participants were genotyped using the Affymetrix GeneChip Human Mapping 100 K Set [20, 25]. Genotypes were called using the GEL algorithm [26] given its improved call rate and consistent results with other calling methods [20]. According to the annotation file released in March, 2008 from the Affymetrix NetAffx Analysis Center (http://www.affymetrix.com/analysis/index.affx) and the National Center of Biotechnology Information (NCBI) Human Genome Build 36 data, we analyzed 112,666 autosomal SNPs that can be mapped to the NCBI Entrez SNP database and also defined their associated genes in this mapping 100 K set.

2.3. Diabetic Retinopathy Grading. All participants completed detailed ophthalmologic examinations including stereoscopic color fundus photography of the seven standard fields from the Diabetic Retinopathy Study (DRS) for each eye [27]. Photographs were sent to the University of Wisconsin Reading Center for examination and grading [28] using the Early Treatment Diabetic Retinopathy Study (ETDRS) adaptation of the modified Airlie House classification system [29]. The score for the more severely affected eye of an individual was used to classify retinopathy status. Since it has been shown that familial factors seem to especially influence the severity of diabetic retinopathy [10, 12], we focused this analysis on severe diabetic retinopathy and defined our cases and controls as follows. ETDRS grade 10–37: normal to early nonproliferative diabetic retinopathy (NPDR-E) as controls, and 43–85: moderate-to-severe nonproliferative diabetic retinopathy (NPDR-S) and proliferative diabetic retinopathy (PDR) as cases.

2.4. Quality Control and Population Stratification. Fisher's Exact tests of Hardy-Weinberg Equilibrium for controls and for all samples, as well as χ^2 tests for the distribution of missing genotypes between cases and controls, were conducted for all 112,666 autosomal SNPs. SNP quality was also assessed based on genotype call rate and minor allele frequency (MAF). A complete-linkage hierarchical clustering method implemented in PLINK [30] was conducted to explore any possible substructure among study subjects using pairwise identity-by-state (IBS) distance across the genome-wide SNP data. SNPs with a genotyping rate <95%, minor allele frequencies <1% in all study subjects, and P-value of exact Hardy-Weinberg Equilibrium test <.001 both in the entire study subjects and control group were tagged for potential quality control issues. A genomic inflation factor (λ) [31] and mean χ^2 statistics generated from all tested SNPs were also calculated to evaluate the effect of population stratification. The quantile-quantile plot of observed and expected distributions of P-values was used to assess any distortion of observed distribution from the null.

2.5. Imputation of Untyped SNPs. To expand the genomic coverage, we applied a computationally efficient hidden Markov Chain model [32] programmed in MACH (http://www.sph.umich.edu/csg/abecasis/MACH/) [33] to impute autosomal genotypes that were present in HapMap Phase III data in the Mexican population from Los Angeles, California, but not genotyped in the Affymetrix 100 K SNP set. An average allele dosage was estimated with 100 iterations of the imputation algorithm conditional on a set of known haplotypes while simultaneously estimating the recombination map. The squared correlation (r^2) between imputed and true genotypes was estimated for each SNP to evaluate imputation performance [33]. The mask option within MACH was also used to hide 2% of genotypes from the haplotyping, and imputed genotypes at these locations

were compared with the actual genotypes to estimate the imputation error rate.

2.6. Statistical Analysis. χ^2 tests, Student T-tests, and logistic regressions were used to compare the basic characteristics between cases and controls using SAS/STAT system (SAS Institute Inc., Cary, NC). For single-marker case-control analyses, we did not initially filter any SNPs for quality-control reasons, but we labeled them with quality-control indicators for data interpretation. Logistic regression under an additive genetic model was performed for each directly genotyped SNP adjusting for the effects of age, gender, diabetes duration, and serum glycosylated hemoglobin level using PLINK [30]. Imputed markers with poor performance were filtered to obtain more reliable association results based on the per marker quality measures generated from MACH. A cutoff of 0.5 for r^2 between imputed and true genotypes was applied to remove about 90% of poorly imputed SNPs at a cost of 5% of good ones. To account for the probability distribution and the uncertainty of genotype imputation, logistic regression modeling adjusted for the same covariates was performed on these well-imputed markers using ProbABEL (http://mga.bionet.nsc.ru/~yurii/ABEL/) from the ABEL set of programs [34, 35], where the SNP effect was assessed by its average imputed allele dosage.

3. Results

Among 286 Mexican-Americans with type 2 diabetes from Starr County, Texas, 103 (36%) with severe nonproliferative or proliferative diabetic retinopathy were defined as cases. Compared to those without retinopathy or only with modest nonproliferative retinopathy, individuals with severe diabetic retinopathy had significantly longer duration of diabetes, higher glycohemoglobin levels, and higher systolic blood pressure measurements (all P-values of Student t-test <.005, Table 1) [36].

The average genotype call rate among study subjects was 93%, with 14% of the 112,666 autosomal SNPs having a call rate less than 90%, and 4% having P-values for χ^2 tests for genotype missingness between cases and controls less than .01. All polymorphic SNPs in this study ($n = 111,528$) remained in the single-marker analysis, and 5641 (5%) of them had minor allele frequency (MAF) less than 1%. The mean χ^2 statistics and the genomic inflation factor (λ) were 1.02 and 1.06, respectively, suggesting that any observed associations will unlikely be due to population stratification. However, there might be a slightly increased false positive rate since both values are greater than 1. There was no subpopulation identified from our study subjects using the IBS clustering analysis, and none of the permutation tests of IBS differences between cases and controls were significant (data not shown). The Q-Q plot in Supplementary Figure 1 also shows no strong evidence of population stratification, but with a deviation toward null possibly due to decreased power.

Figure 1 shows the P-value distribution for single-marker associations with severe retinopathy under an additive genetic model adjusted for age, gender, diabetes duration, and glycosylated hemoglobin level across the whole genome using the Affymetrix 100 K SNP set. All the top signals met all quality control criteria. Detailed information for the 10 strongest single marker associations is presented in Table 2. (Results for all SNPs are available as Supplementary Table 1) The best signals found in this study were rs2300782 ($P = 6.04 \times 10^{-5}$) in 5q21 and rs10519765 ($P = 6.21 \times 10^{-5}$) in 15q13, that locate in the intron of the calcium/calmodulin-dependent protein kinase IV (CAMK4) gene and formin 1 (FMN1) gene, respectively.

The 100 K SNP set alone is insufficient to cover most variants currently available, therefore, we extended the analysis to an imputation-based GWAS using HapMap data [37]. To improve imputation performance, one subject with an overall genotyping rate less than 90% and 8,094 SNPs not meeting quality control criteria were excluded from imputation. A total of 1,326,990 SNPs from the HapMap Phase III Mexican population in Los Angeles, California, were imputed on 102 cases and 183 controls of severe diabetic retinopathy based on 104,572 directly genotyped SNPs. Supplementary Table 2 summarizes the imputation performance of MACH [33] across the whole genome. More than 88% of the imputed SNPs had a minor allele frequency greater than 1%, and the MACH estimated per allele error rate after masking 2% of direct genotypes from imputation was 8.7%. For all imputed markers, the overall r^2 between imputed and true genotypes was 0.387, and the average posterior probability for the most likely genotypes was 0.783. The 421,010 (31.7%) markers considered well imputed with an $r^2 \geq 0.5$ were used for subsequent association analysis.

Figure 2 shows the genome-wide P-value distribution from the logistic regression models using the well-imputed SNPs. The genomic inflation factor (λ) and the mean χ^2 statistics were 1.03 and 0.47, respectively, implying that any observed associations are unlikely due to population stratification. Table 3 summarizes the genes nearest the top signals (P-value less than 10^{-4}) under logistic regression models adjusted for the effects of age, gender, diabetes duration, and glycosylated hemoglobin level. There were 32 SNPs associated with severe diabetic retinopathy at P-value less than 10^{-4}, with 7 of these top signals in chromosomal region 6p11-12, where tubulointerstitial nephritis antigen (TINAG) is encoded.

4. Discussion

Genome-wide association studies provide an additional tool, in conjunction with candidate gene and linkage studies to better understand the genetics of diabetic retinopathy. Our strongest signals from single-marker analysis implicated several genes as associated with severe diabetic retinopathy. SNP rs2300782 is located in an intronic region of CAMK4; its product has been reported within the Ca(2+)/calmodulin-dependent protein kinase subfamily and has been shown to increase transcriptional activity required for ATF-2 induced insulin gene expression [38]. The second best single-marker signal rs10519765 is located in FMN1, encoding a protein

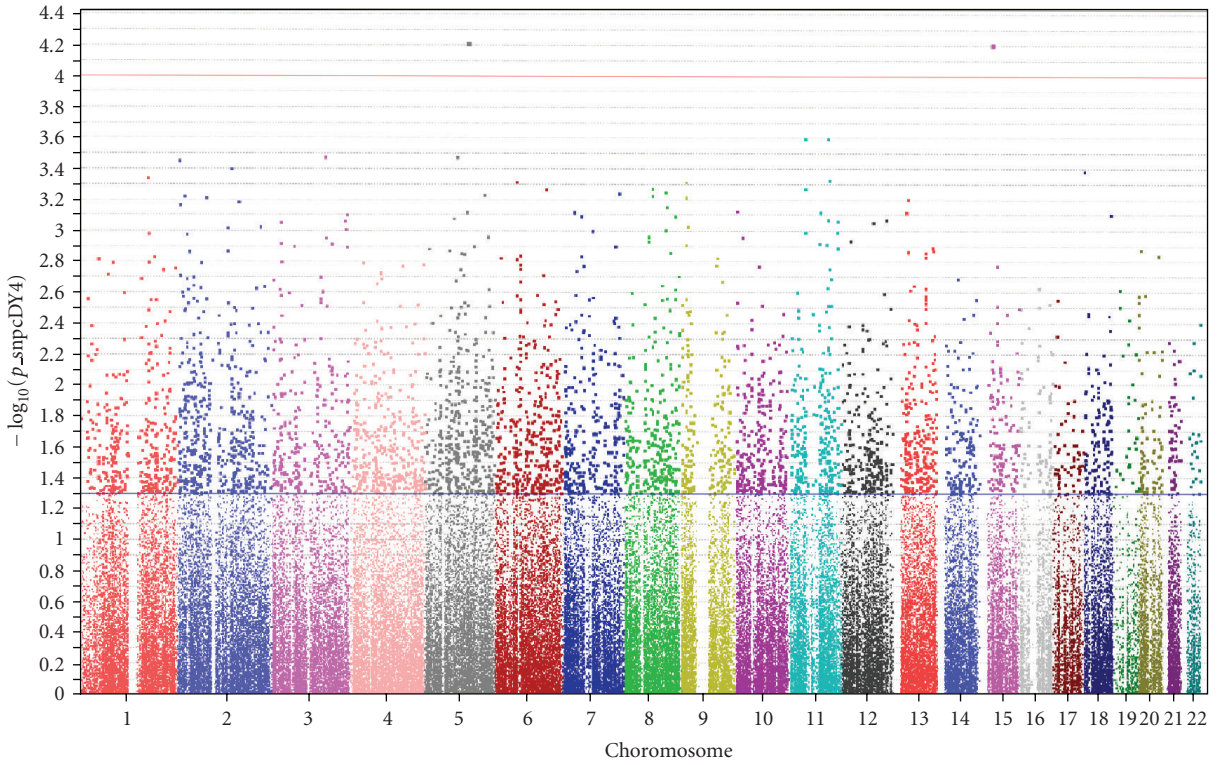

Figure 1: Summary of genome-wide associations between 111,528 SNPs and severe diabetic retinopathy under additive genetic model adjusted for age, gender, diabetes duration, and HbA1c level.

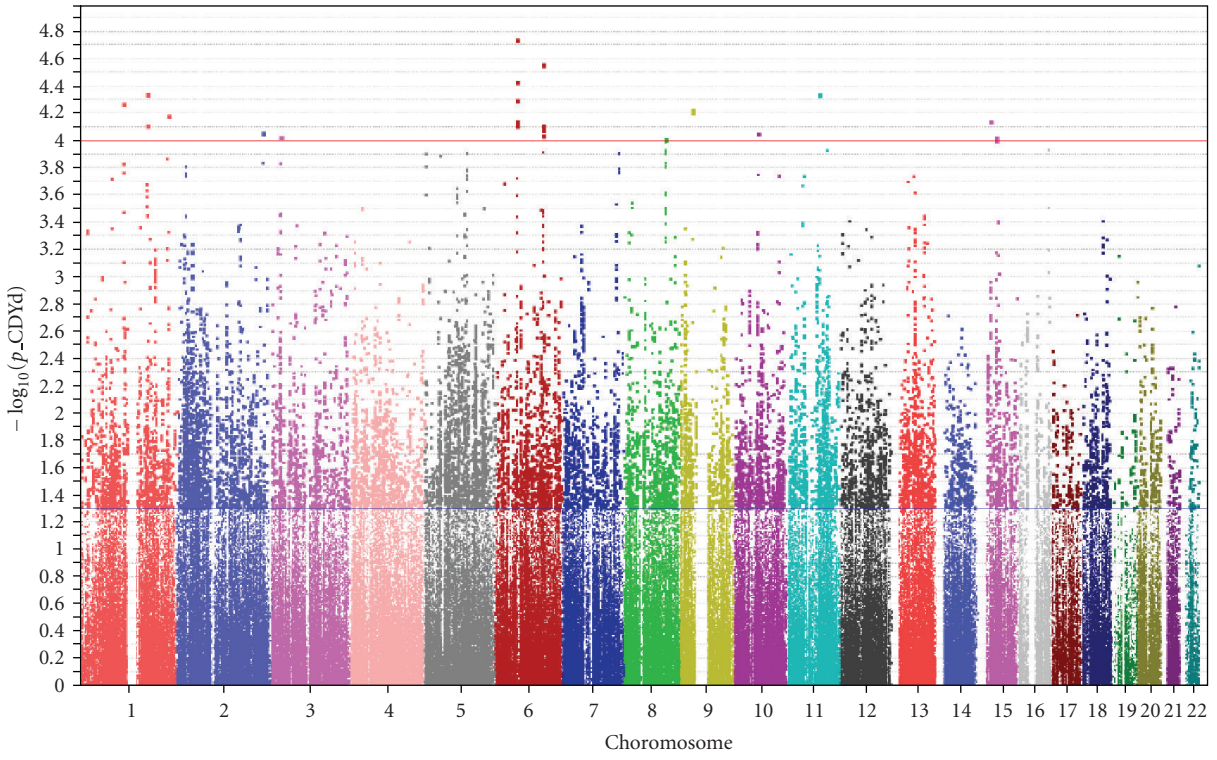

Figure 2: Summary of genome-wide associations between 421,010 well-imputed SNPs and severe diabetic retinopathy under additive genetic model adjusted for age, gender, diabetes duration, and HbA1c level.

Table 1: Basic characteristics of Mexican-Americans with type 2 diabetes from Starr County, Texas.

Variables		No DR + NPDR-E[#] (controls $n = 183$)	NPDR-S + PDR[#] (cases $n = 103$)	P-value[§]	OR	(95%CI)
Age	Mean ± SD	57.06 ± 11.54	58.67 ± 9.27	.1988	1.01	(0.99–1.04)
Gender	Male	66 (36.07%)	43 (41.75%)	.3422	1.00	—
	Female	117 (63.93%)	60 (58.25%)		0.79	(0.48–1.29)
Albumin	Negative	132 (74.58%)	57 (60.64%)	.0802	1.00	—
	20 mg/L	22 (12.43%)	14 (14.89%)		1.47	(0.70–3.08)
	50 mg/L	17 (9.60%)	17 (18.09%)		2.32	(1.10–4.86)*
	100 mg/L	6 (3.39%)	6 (6.38%)		2.32	(0.72–7.49)
Hypertension history	No	96 (54.24%)	59 (59.60%)	.3895	1.00	—
	Yes	81 (45.76%)	40 (40.40%)		0.80	(0.49–1.32)
Diabetes duration (yrs)	mean ± SD	12.02 ± 9.09	18.10 ± 8.22	<.0001	1.08	(1.05–1.11)***
HbA1C (%)	mean ± SD	10.87 ± 3.72	12.29 ± 3.90	.0028	1.10	(1.03–1.18)***
Fasting glucose (mg/dL)	mean ± SD	185.54 ± 68.71	201.63 ± 84.49	.0883	1.03	(0.99–1.06)
BMI (kg/m^2)	mean ± SD	31.82 ± 6.49	30.85 ± 5.93	.2175	0.98	(0.94–1.02)
Systolic BP (mmHg)	mean ± SD	126.79 ± 17.86	134.73 ± 23.26	.0037	1.21	(1.07–1.38)*** (every 10 unit)
Diastolic BP (mmHg)	mean ± SD	72.69 ± 10.03	73.32 ± 10.78	.6290	1.06	(0.84–1.35) (every 10 unit)
Total cholesterol (mg/dL)	mean ± SD	191.21 ± 41.81	199.06 ± 42.13	.1381	1.05	(0.99–1.11) (every 10 unit)
Triglycerides (mg/dL)	mean ± SD	184.79 ± 120.92	210.02 ± 123.59	.1031	1.02	(0.99–1.04) (every 10 unit)
HDL cholesterol (mg/dL)	mean ± SD	42.86 ± 11.73	40.62 ± 10.32	.1153	0.83	(0.66–1.05) (every 10 unit)
LDL cholesterol (mg/dL)	mean ± SD	111.49 ± 30.71	118.94 ± 34.84	.0790	1.08	(0.99–1.16)

[#]No DR: normal or nondiabetic retinopathy; NPDR-E: early nonproliferative diabetic retinopathy
[#]NPDR-S: moderate-to-severe nonproliferative diabetic retinopathy; PDR: proliferative diabetic retinopathy
[§]P-value of χ^2 test or Student t-test
*P-value < .05, **P-value < .01, ***P-value < .005.

involved in cell adhesion and morphogenesis by assembling radial action cables in epithelial cells [39, 40]. However, there is very limited evidence about genetic variation on CAMK4 or FMN1 and diabetes or its complications.

CNTN5 (contactin 5) at 11q22 is also among the nominally associated genes for severe diabetic retinopathy. The protein encoded by CNTN5 belongs to an immunoglobulin superfamily and may participate in the developing nervous system [41]. SNP variants in CNTN5 have been reported to be associated with atrial fibrillation and heart failure [42]. Another top single-marker signal was in COLEC12 (collectin subfamily member 12), encoding a protein of the C-lectin family, that possesses collagen-like sequences and carbohydrate recognition domains. This protein is a scavenger receptor recognizing oxidized phospholipids, so it may participate in removing oxidative damage [43], which is a potential etiological factor for diabetic retinopathy [44]. API5, EDIL3, BFSP2, HNMT, and SCYL1BP1 were also among the top signals from the single-marker analysis within their associated region, but there is no clear connection currently between these genes and diabetes or its complications.

Imputation of untyped SNP markers is as a useful tool to improve the coverage and power for genome-wide association studies without additional genotyping costs [45, 46]. A number of programs have been developed and routinely used to impute genotypes based on the observed haplotype structure from millions of SNPs in the HapMap project [37] or 1000 Genomes Project [47]. Given the uneven coverage of the genome by the Affymertrix 100 K SNP set, a more detailed SNP map was obtained by imputation conducted in MACH [32], since it consistently outperformed other algorithms with better accuracy and efficiency [48, 49]. The Mexican-American population in Starr County is relatively homogenous with 97.5% self-reporting as Hispanic. Genetically, this population is admixed with 68% European, 27% Asian, and 6% African ancestry [20]. Considering the importance of linkage disequilibrium pattern between the reference panel and study subjects, we used the HapMap III population with Mexican ancestry from Los Angles, California, who identified themselves as having at least three grandparents born in Mexico, as the reference set for imputation. The overall 8.7% estimate from MACH of overall allele error rate was comparable with the original MACH evaluation data (7.5%) [32]. Compared to all the autosomal SNP variants ($n = 1,387,466$) available in the HapMap III Mexican population, the genomic coverage in

Table 2: Top 10 single-marker associations with severe diabetic retinopathy among Mexican-Americans from Starr County, Texas.

dbSNP rs#	Chr	Position*	Minor/major allele	MAF in Cases	MAF in Controls	P-value§	OR+ (95%CI)	Nearest gene*	Distance/ relationship to gene
rs2300782	5	110816684	A/G	0.512	0.322	$6.04E-05$	2.64 (1.64–4.25)	CAMK4	intron/0
rs10519765	15	30992716	A/G	0.136	0.294	$6.21E-05$	0.30 (0.16–0.54)	FMN1	intron/0
rs899036	11	41639486	C/A	0.125	0.210	$2.52E-04$	0.32 (0.17–0.59)	API5	upstream/ 1650623
rs10501943	11	99452209	C/T	0.195	0.086	$2.53E-04$	3.04 (1.68–5.52)	CNTN5	intron/0
rs1445754	5	83611387	A/T	0.143	0.273	$3.35E-04$	0.37 (0.22–0.64)	EDIL3	intron/0
rs1197310	3	134610914	T/A	0.540	0.435	$3.35E-04$	2.25 (1.45–3.51)	BFSP2	intron/0
rs699549	2	4683138	T/C	0.115	0.043	$3.49E-04$	4.27 (1.93–9.47)	—	—
rs763970	2	138352603	A/C	0.330	0.206	$4.00E-04$	2.25 (1.44–3.52)	HNMT	upstream/ 85675
rs599019	18	284495	G/T	0.030	0.131	$4.06E-04$	0.15 (0.05–0.43)	COLEC12	downstream/ 24861
rs6427247	1	168647104	G/A	0.360	0.221	$4.56E-04$	2.17 (1.41–3.35)	SCYL1BP1	upstream/ 120790

*Affymetrix NetAffx annotation build 25, NCBI genome build 36.1.
§P-value of logistic regression under additive genetic model, adjusted for age, gender, diabetes duration, and HbA1c level.
+odds ratios of the minor allele.

Table 3: Regions with strongest imputation-inferred associations ($P < .0001$) using estimated allelic dosage under an adjusted additive genetic model.

Location	SNP with best P-value within this region	Position*	Best P-value§	# of top SNPs within this region	Nearest gene*
6p11-12	rs6909083	54290262	$1.80E-05$	7	TINAG
6q22	rs17083119	121443809	$2.76E-05$	4	C6orf170
1q23	rs1033465	171254353	$4.50E-05$	2	TNFSF18
1p13	rs11583330	109925036	$5.35E-05$	1	GNAI3
1q42	rs3014267	225619540	$6.58E-05$	1	CDC42BPA
15q13	rs11635920	30999949	$7.18E-05$	4	FMN1, GREM1
2q35-36	rs6726798	219009099	$8.66E-05$	1	VIL1
10q21	rs11812882	59712631	$8.85E-05$	1	ZCD1
2q35	rs1106412	219023301	$8.91E-05$	1	USP37
3p24	rs11927173	23200198	$9.39E-05$	2	UBE2E2
8q22	rs3098241	104494480	$9.72E-05$	2	SLC25A32

*Affymetrix NetAffx annotation build 25, NCBI genome build 36.1
§P-value of logistic regression under additive genetic model, adjusted for age, gender, diabetes duration, and HbA1C level.

the imputation analysis increases extensively with 30.3% ($n = 421,010$) of the variants tested here, whereas only 4.3% ($n = 60,283$) of them are directly covered in the Affymetrix 100 K SNP set.

Among all imputed markers associated with severe diabetic retinopathy at a P-value less than 10^{-4}, there are 7 SNPs located in 6p11-12 where TINAG (tubulointerstitial nephritis antigen) is encoded. TINAG is a glycoprotein originally identified as a target antigen involved in human antitubular basement membrane disease [50]. It has been recognized as an extracellular matrix protein and shows increased expression in the kidney of streptozotocin-diabetic rats [51], but there is yet no evidence how TINAG might be involved in human diabetes or its complications. Chromosomal region 15q13, where FMN1 (formin 1) and GREM1 (gremlin 1) map, also has 4 of the strongest imputation-based signals. FMN1 has one of the strongest associations from direct genotyping (rs10519765). Gremlin is located

~40 kb downstream of FMN1, a highly conserved protein involved in various disorders related to fibrotic changes in the kidney, lung, and liver [52]. This protein has been shown to have a potential role in diabetic nephropathy because of enhanced expression and interaction with TGF-β signaling pathways [53, 54]. Recent evidence of increased gremlin mRNA in bovine retinal perictyes [55] and immunohistochemistry data on the mouse retina [54] coupled with results reported here elevate interest in gremlin.

In addition to identifying novel candidate genes, we were interested in SNPs among genes that appear to be traditional candidates for diabetic retinopathy. A list of 208 retinopathy candidate genes has been generated [18, 56] (see Supplementary Table 3) to include genes involved in metabolic processes or clinical risk factors leading to retinopathy and those that have been previously reported to be associated with diabetic retinopathy. Among a total of 667 SNPs that are located in the coding region of these 208 candidate genes and also genotyped in the Affymetrix 100 K SNP set, 54 of them had nominal allelic association with diabetic retinopathy with *P*-values ranging from .001 to .05 (Supplementary Figure 2). The significance pattern found here is more than expected by chance alone.

Although we cannot completely exclude the possibility of undetected subpopulations affecting the results, we found no evidence of population stratification. Hayes et al. also used the genome-wide SNP data to estimate proportions of ancestry on the same population. Ancestry is unlikely to be a source of spurious association in this sample since the amount of African, Asian, and European ancestries were indistinguishable between cases and controls [20].

The primary objective of this study is searching for diabetic retinopathy genes without any assumption about the location of disease-associating variants. This initial genome-wide association analysis of severe diabetic retinopathy identified several unexpected loci that may contribute to the genetic susceptibility of diabetic retinopathy. The study was limited, however, in its ability to detect small effect sizes. A sample of 103 cases and 183 controls was only powerful enough to identify variants with odds ratios greater than 2.2 (assuming disease allele frequency of 0.5 and disease prevalence of 0.5 in an additive model, calculated by CaTS [57]) with a genome-wide significance level (*P*-value less than 5×10^{-8}). On the other hand, genetic association studies usually require replication in appropriate independent samples to validate any findings. No such comparable data among Mexican-Americans are available though we are expanding the sample and SNP coverage, even so, these results set some limits as to the magnitude of genetic effects for diabetic retinopathy.

In summary, we observed several SNPs and genes associated with severe diabetic retinopathy in this initial genome-wide analysis. None of these loci have been previously linked to diabetic retinopathy or diabetes itself. While the underlying mechanisms leading to diabetic retinopathy remain unresolved, these results implicate genetic regulation of oxidative stress and cell adhesion as possible players in the development of diabetic retinopathy.

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Clinical Study

Effect of Trandolapril on Regression of Retinopathy in Hypertensive Patients with Type 2 Diabetes: A Prespecified Analysis of the Benedict Trial

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Background. The effect of angiotensin converting enzyme inhibitors (ACEi) on regression of retinopathy in type 2 diabetics is still ill defined. *Methods*. We compared the incidence of retinopathy regression in 90 hypertensive type 2 diabetics randomized to at least 3-year blinded ACEi with trandolapril (2 mg/day) or non-ACEi therapy who had preproliferative or proliferative retinopathy at baseline. *Results*. Over a median (interquartile range) follow-up period of 35.8 (12.4–60.7) months, retinopathy regressed in 27 patients (30.0%). Regression occurred in 18 of 42 patients (42.9%) on ACEi and in 9 of 48 (18.8%) on non-ACEi therapy (adjusted for predefined baseline covariates HR (95% CI): 2.75 (1.18–6.42), $P = .0193$). Concomitant treatment with or without Non-Dihydropyridine Calcium Channel Blockers (ndCCBs) did not appreciably affect the incidence of retinopathy regression. *Conclusions*. Unlike ndCCB, ACEi therapy may have an additional effect to that of intensified BP and metabolic control in promoting regression of diabetic retinopathy.

1. Introduction

Despite the beneficial effects of photocoagulation, retinopathy remains the leading cause of blindness in people aged 30 to 69 years, that ultimately affects more than 60% of type 2 diabetics [1]. Duration of diabetes, poor metabolic control, and arterial hypertension have been associated with the development and progression of retinopathy, although their relative role appears to differ in different series and clinical conditions [1, 2]. Undoubtedly, strict metabolic control is essential for the prevention and treatment of retinopathy. Reducing Blood Pressure (BP), however, has been recognized as an additional, and probably even more effective, therapeutic intervention [2].

However, the specific effects on the retina of different medications used to control arterial hypertension in diabetic patients are still unclear. Studies suggest that inhibitors of the renin angiotensin system (RAS) may retard progression more effectively than other antihypertensive drugs. Still, their effect has been never formally compared with other agents such as non-dihydropyridine calcium channel blockers (ndCCBs). Moreover, no study primarily addressed whether regression of retinopathy can be achieved in those who already have retinal involvement.

To formally explore these issues, we took advantage of a large cohort of hypertensive type 2 diabetics from the BErgamo Nephrologic Diabetes Complications Trial (BENEDICT) [3]. These patients were expected to have a high

prevalence of retinopathy at study entry because of the concomitance of two strong and possibly synergistic risk factors, arterial hypertension and type 2 diabetes. They were randomized to receive at least 3 years of treatment with the ACEi trandolapril, the nondihydropyridine CCB (ndCCB) verapamil, their combination (VeraTran), or placebo plus other antihypertensive drugs titrated to a systolic/diastolic BP goal of 120/80 mmHg or less. Data showed that patients on ACEi therapy (either as trandolapril alone or the combination VeraTran) compared to those on non-ACEi therapy (verapamil or placebo) had a significantly lower incidence of persistent microalbuminuria, which is an early marker of diabetic nephropathy and a major risk factor for cardiovascular disease in this population.

The primary aim of the present study was to evaluate whether, in type 2 diabetic patients, treatment with the ACEi trandolapril may promote regression of diabetic retinopathy more effectively than antihypertensive medications that do not directly interfere with angiotensin II production or activity, at comparable BP and metabolic control. Secondarily, we compared the effects of trandolapril and non-RAS inhibitor therapy on newly onset retinopathy in those patients without evidence of retinal involvement at study entry. The results of the analyses formed the basis of the present report.

2. Methods

2.1. Patients and Study Design. This study is a pre-specified analysis of data from the BENEDICT trial. Study design and patient characteristics have been described in detail elsewhere [3]. Briefly, BENEDICT was a prospective, randomized, double blind, parallel group study that evaluated the possibility of preventing the onset of persistent microalbuminuria in 1209 patients with type 2 diabetes (WHO criteria), arterial hypertension (systolic or diastolic BP more than 130 or 85 mmHg, or concomitant antihypertensive therapy), and normal Urinary Albumin Excretion (UAE) rate (UAE $< 20\,\mu$g/min in at least 2 of 3 consecutive overnight urine collections) randomly assigned to at least 3 years of treatment with one of the following study drugs: I, a ndCCB: verapamil SR, 240 mg/day; II, an ACEi: trandolapril 2 mg/day; III, the fixed-dose combination of verapamil SR, 180 mg/day plus trandolapril 2 mg/day: VeraTran; and IV, placebo. The target BP after randomization and throughout the whole study period was to be less than 120/80 mmHg for all the treatment groups. Other antihypertensive drugs (with the exception of RAS inhibitors and ndCCBs different from the study drugs) could be used to achieve and maintain target BP according to predefined guidelines.

The analysis was primarily aimed at evaluating the rate of regression of diabetic retinopathy in patients with retinal involvement at study entry considered as a whole and, then, according to their original randomization to RAS-inhibitor or non-RAS inhibitor therapy (Figure 1). Secondarily, the study compared the effect of RAS and non-RAS inhibitor therapy on newly onset retinopathy in those without evidence of retinal involvement at inclusion. Finally, for explorative purposes only, patients were considered according to their randomization to one of the four original treatment arms.

The study protocol was in accordance with the declaration of Helsinki and was approved by the institutional review board at each Center and by the Safety Committee of the BENEDICT study. All patients gave written informed consent.

2.2. Retinal Evaluation. Retinal evaluations by ophthalmoscopy and photography (in a subgroup) were scheduled at baseline, every year thereafter, and at final visit in all patients included in the BENEDICT trial who had been randomized at the Clinical Research Center (CRC) "Aldo and Cele Daccò" of The Mario Negri Institute and at the Unit of Diabetology of the Azienda Ospedaliera "Ospedali Riuniti di Bergamo". They were referred to the Unit of Ophthalmology of the Azienda Ospedaliera where they were evaluated independently by two ophthalmologists (I. I. AND M. F.) blinded to the clinical and laboratory data of the patients. The diagnoses were compared for consistency. Patients with an inconsistent diagnosis were evaluated by a third independent ophthalmologist (S.T.), and his diagnosis was recorded as final and considered for data analyses [5]. After mydriasis was induced, indirect binocular ophthalmoscopy was performed by a L-0185 slit-lamp biomicroscope (magnification 10x and 16x) and handheld lens (magnification 90x). Photographs of four standard 30° fields of each eye were taken through dilated pupils in stereo pairs (lateral to macula, macula, disc, and nasal) with Canon CF 60 UV fundus camera (Tokio, Japan) [4]. The pictures were printed on Kodak Ektachrome 100-colour slide film. Photographs were initially assessed for quality and adherence to the protocol. Inadequate photographs were discharged.

The eye with the most severe involvement was used for categorization of retinal involvement. Pre-proliferative retinopathy was defined by the presence of microaneurysms, hemorrhages, hard exudates, venous congestion, cotton wool spots, or intraretinal microvascular abnormalities. Proliferative retinopathy was diagnosed when new vessels, glial proliferation, preretinal hemorrhage, vitreous hemorrhage, scars of photocoagulation (known to have been directed at new vessels), and/or retinal detachment were found. Patients with none of these abnormalities were classified as not having retinopathy [5, 6]. Based on this simplified classification, regression of retinopathy was defined as a persistent (up to the final visit) change in the stage of retinal involvement from proliferative to pre-proliferative retinopathy, or from pre-proliferative retinopathy to no retinal involvement.

2.3. BP and Other Outcome Variables. Trough systolic and diastolic (Korotkoff phase I/V) BPs were measured in the morning before treatment administration by use of an appropriate cuff with a sphygmomanometer and with the patient in a sitting position after at least 5 minutes rest. Three measurements to the nearest 2 mmHg were obtained, two minutes apart at each time point, and the average of the three measurements was recorded for statistical analyses. Mean arterial pressure (MAP) was calculated as

diastolic BP plus one third of the pulse pressure. All the laboratory measurements were centralized at the Laboratory of the CRC. HbA1C was measured by ion exchange high-performance liquid chromatography and urinary albumin excretion rate by nephelometry.

Data were reported in dedicated case record forms and doubly entered in an ad hoc database that was eventually merged with the BENEDICT database. Before analyses, all data were monitored by the Monitoring Unit of the CRC.

2.4. Sample Size. Regression of retinopathy was the primary outcome variable of a substudy ancillary to BENEDICT phase A. At the time the present analyses were planned, no data were available on the regression of retinopathy in hypertensive patients with type 2 diabetes on intensified BP and metabolic control, as well as on a possible additional effect on disease regression of ACEi therapy. Thus, it was impossible to establish *a priori* the sample size required to provide the analyses with an adequate power to detect the hypothesized treatment effect on retinopathy. Actually, this was an explorative study performed in all consenting patients with available ophthalmologic evaluations.

2.5. Statistical Analyses. The analyses were performed by the Laboratory of Biostatistics of the CRC. Patients were eligible if they had a funduscopy evaluation at baseline. Main outcome variable was regression of retinal changes in patients with retinopathy at study entry. Secondary outcome variable was newly onset retinopathy in those with no retinal changes at study entry. In the primary outcome analyses, patients with retinopathy were considered as a whole regardless of the stage of retinal involvement. For outcome analyses, systolic and diastolic BP measurements were included separately in the model. Continuous variables were compared by unpaired t-test or Wilcoxon Rank Sum tests and categorical variables by χ^2 test or Fisher's Exact test. Regression of retinopathy was evaluated by means of Cox regression models in order to obtain the hazard ratio (HR) and its 95 percent confidence interval. Patients without funduscopy evaluation on follow up were conventionally classified as having one day of follow up and without the event of interest. Unless otherwise stated, statistical analyses were done according to the intention-to-treat principle and considered adjustments according to pre-defined baseline covariates (site, age, smoking status, diastolic BP, and log-transformed urinary albumin excretion). All the statistical analyses were performed using SAS version 9 (SAS Institute Inc, Cary, NC). A P-value of less than .05 was considered as statistically significant. No P-value adjustment was carried out for multiple comparisons. Data are expressed as mean ± standard deviation (SD) or median and interquartile (IQ) range or percentages.

3. Results

3.1. Baseline Characteristics. Of the 1209 patients randomized in the original BENEDICT cohort, 583 patients were referred to the two centers involved in the present study. Five-hundred-fifty patients had a baseline funduscopy evaluation (Figure 1). Patients with funduscopy evaluation, compared to those without, had a lower body mass index, poorer metabolic control and higher BP at baseline (Table 1). Four-hundred-sixty patients (83.6%) had no evidence of retinal involvement. Of the remaining 90 patients with funduscopy data, 82 had a pre-proliferative and 8 had a proliferative form of retinopathy. All had BP and HbA1C data at baseline and on follow-up and were therefore available for this analysis. Compared to patients without evidence of retinal involvement, those with retinopathy (either pre-proliferative or proliferative) at inclusion reported a significantly longer duration of diabetes, were more hypertensive and had significantly higher HbA1C, blood glucose levels, and urinary albumin excretion (Table 1). Age, gender distribution, smoking habit, serum creatinine, and lipid profile were similar between groups. A similar proportion of patients with retinopathy were on ACEL or non-ACEi therapy, and a similar proportion of patients were on ndCCB or non-ndCCB therapy (Table 1). Baseline characteristics of patients on ACEL or non-ACEi therapy, as well as of patients on ndCCB or non-ndCCB therapy were comparable, with the only exception of systolic BP that was lower in those on ndCCB compared to those on non-ndCCB therapy (Table 1). The proportion of patients on concomitant medications at baseline and on follow-up was also similar within each considered treatment group, with the only exception of the proportion of patients on fibrate therapy at baseline that was lower in the ndCCB than in the non-ndCCB treatment group (Table 2).

3.2. Regression of Diabetic Retinopathy According to ACEi, or Non-ACEi Therapy. Over a median (IQ range) follow-up period of 35.8 (12.4–60.7) months, retinal changes regressed in 27 of 90 patients (30.0%) who had retinopathy at study entry. Regression was observed in 18 of the 42 patients (42.9%) randomized to ACEi therapy and in 9 of the 48 patients (18.8%) randomized to non-ACEi therapy (Figure 2) (HR (95% CI): 2.62 (1.17–5.84), $P = .0188$, (unadjusted) and 2.75 (1.18–6.42), $P = .0193$ (adjusted for predefined baseline covariates)) (Figure 3(a)). Systolic and diastolic BP were similar in the two treatment groups at baseline (Table 1) and at different visits on follow-up. HbA1C was also similar between groups at baseline (Table 1) and on follow up. The regression rate of retinopathy was significantly different even after adjustment for baseline and follow-up systolic/diastolic BP and HbA1C and for systolic/diastolic BP and HbA1C changes versus baseline ($P < .05$ for all considered adjusted Hazard Ratios).

3.3. Regression of Diabetic Retinopathy According to ndCCB or Non-ndCCB Therapy. Regression of retinopathy was observed in 12 of the 50 patients (24.0%) randomized to ndCCB therapy and in 15 of the 40 patients (37.5%) randomized to non ndCCB therapy (HR (95% CI): 0.64 (0.30 to 1.37), $P = .25$ (unadjusted) and 0.56 (0.25 to 1.25), $P = .16$ (adjusted for predefined baseline covariates)) (Figure 3(b)). Systolic BP was lower in the ndCCB than in the non-ndCCB group at baseline (Table 1), but the difference progressively weaned on subsequent follow up visits, while

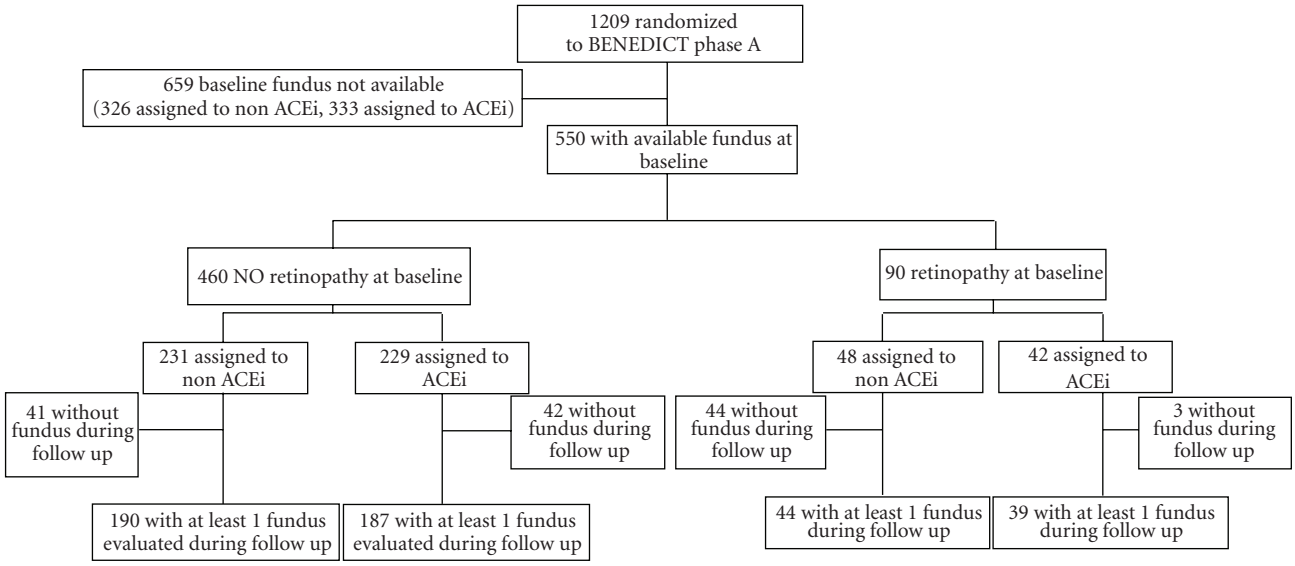
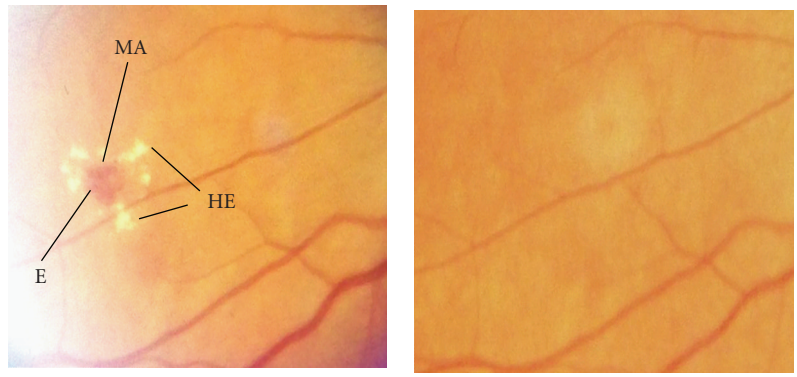


Figure 1: Study flow chart.



(a) (b)

Figure 2: Fundus photographs showing pre-proliferative changes (a) at baseline in a patient who had a regression of eye lesions after three years of trandolapril therapy (b). This picture provides a comprehensive example of three typical lesions, microaneurysms (MA), hemorrages (E), and hard exudates (HE, that may regress in type 2 diabetic patients on ACE inhibitor therapy combined to intensified metabolic and blood pressure control, as in the BENEDICT trial.

diastolic BP was similar in the two treatment groups at baseline (Table 1) as well as at different visits on follow-up. HbA1C was similar between groups at baseline (Table 1) and at different visits on follow up.

3.4. Regression of Diabetic Retinopathy According to the Original Treatment Arm. Regression of retinopathy was observed in 10 (52.6%), 8 (34.8%), 2 (7.4%), and 5 (23.8%) of the 19, 23, 27, and 21 patients randomized to trandolapril, VeraTran, verapamil, or placebo, respectively. The HR (95% CI) for trandolapril, VeraTran, or verapamil versus placebo was, respectively: 2.47 (0.84–7.23), $P = .10$; 1.72 (0.55–5.32), $P = .35$; 0.61 (0.16–2.27), $P = .46$ (unadjusted) and: 2.61 (0.84–8.13), $P = .10$; 1.89 (0.53–6.71), $P = .33$; and 0.91 (0.19–4.33), $P = .90$ (adjusted for predefined baseline covariates.) Systolic and diastolic BP and HbA1C were not significantly different between treatment groups both at baseline (Table 1) and on follow-up (data not shown).

3.5. Newly Onset Diabetic Retinopathy. Retinal changes developed in 61 of 460 patients (13.3%) who had no evidence of diabetic retinopathy at study entry. Newly onset retinopathy was observed in 33 of the 229 patients (14.4%) randomized to RAS inhibitor therapy and in 28 of the 231 patients (12.1%) randomized to non-RAS inhibitor therapy (unadjusted: HR (95% CI) 0.968 (0.582–1.610), $P = .90$; adjusted for predefined baseline covariates: HR (95% CI) 0.984 (0.588–1.646), $P = .95$). No significant difference between groups was detected even after adjustment for predefined baseline and follow up covariates, including baseline and follow up BP and HbA1 C (data not shown). No difference in new onset retinopathy was observed between

Table 1: Baseline characteristics of hypertensive patients with type 2 diabetes and normoalbuminuria according to availability of fundus evaluation YES/NO, evidence of retinopathy at study entry YES/NO, and to randomization to ACE inhibitor therapy YES/NO and ndCCB therapy YES/NO.

	Funduscopy Yes	Funduscopy No	Retinopathy Yes	Retinopathy No	With Retinopathy			
					ACEi Yes	ACEi No	ndCCB Yes	ndCCB No
Number of patients	550	659	90	460	42	48	50	40
Demography								
Age (yrs)	62.0 ± 7.8	62.6 ± 8.2	61.3 ± 7.9	62.1 ± 7.8	61.5 ± 7.7	61.1 ± 8.1	60.8 ± 8.4	62.0 ± 7.1
Males (*n*)	294 (53.5)	345 (52.3)	48 (53.3)	246 (53.5)	24 (57.1)	24 (50.0)	30 (60.0)	18 (45.0)
Clinics								
BMI (kg/m^2)	28.7 ± 4.5	29.4 ± 4.9*	28.3 ± 4.0	28.8 ± 4.6	28.0 ± 3.3	28.6 ± 4.5	28.4 ± 4.0	28.1 ± 4.0
Diabetes duration (yrs)	7.9 ± 6.6	7.4 ± 6.5	10.5 ± 7.2	7.4 ± 6.3°°°	11.2 ± 7.5	9.9 ± 7.1	10.9 ± 7.0	10.1 ± 7.6
Smokers								
Never	323 (58.7)	377 (57.2)	58 (64.4)	265 (57.6)	24 (57.1)	34 (70.8)	31 (62.0)	27 (67.5)
Former	169 (30.7)	194 (29.4)	24 (26.7)	145 (31.5)	14 (33.3)	10 (20.8)	15 (30.0)	9 (22.5)
Current	58 (10.5)	88 (13.3)	8 (8.9)	50 (10.9)	4 (9.5)	4 (8.3)	4 (8.0)	4 (10.0)
Laboratory								
HbA1c (%)	5.9 ± 1.5	5.7 ± 1.3**	6.5 ± 1.5	5.8 ± 1.4°°°	6.6 ± 1.4	6.5 ± 1.7	6.6 ± 1.7	6.4 ± 1.2
Systolic BP (mm Hg)	151.6 ± 14.3	149.3 ± 12.9*	158.4±16.5	150.3 ± 13.5°°°	161.2 ± 15.3	155.9±17.2	154.3±15.2	189.0 ± 55.6
Diastolic BP (mm Hg)	88.8 ± 8.3	86.4 ± 6.9***	90.7 ± 8.2	88.4 ± 8.3°	92.4 ± 7.9	89.1±8.3	89.9±8.5	91.6 ± 7.9
Albuminuria (μg/min)	6.8 ± 4.5	7.0 ± 4.6	8.1 ± 5.1	6.6 ± 4.4°°	8.0 ± 5.0	8.2 ± 5.2	8.3 ± 5.4	7.8 ± 4.6
Ser. creatinine (mg/dL)	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.2
Triglycerides (mg/dL)	143.5 ± 73.2	151.7 ± 83.4	146.3 ± 75.0	142.9 ± 72.9	146.7 ± 64.9	145.9 ± 83.6	139.7 ± 80.1	154.4 ± 68.2

Table 2: Concomitant medications in patients with type 2 diabetes and microalbuminuria at baseline and during follow-up according to treatment with ACE inhibitors YES or NO or with ndCCB YES or NO.

	Baseline				Follow-up			
	ACEi Yes	ACEi No	ndCCB Yes	ndCCB No	ACEi Yes	ACEi No	ndCCB Yes	ndCCB No
Number of patients	42	48	50	40	39	44	47	36
Concomitant medication	number (percent)				number (percent)			
Glucose-lowering regimen								
Diet alone	5 (11.9)	12 (25.0)	12 (24.0)	5 (12.5)	4 (10.3)	8 (18.2)	8 (17.0)	4 (11.1)
Oral hypoglycemic agent alone	29 (69.0)	21 (43.8)	26 (52.0)	24 (60.0)	24 (61.5)	21 (47.7)	26 (55.3)	19 (52.8)
Insulin and oral hypoglycemic agent	5 (11.9)	12 (25.0)	10 (20.0)	7 (17.5)	11 (28.2)	16 (36.4)	13 (27.7)	14 (38.9)
Insulin alone	3 (7.1)	3 (6.3)	2 (4.0)	4 (10.0)	3 (7.7)	3 (6.8)	3 (6.4)	3 (8.3)
Antihypertensive agents								
Any	22 (52.4)	26 (54.2)	23 (46.0)	25 (62.5)	32 (82.1)	38 (86.4)	37 (78.7)	33 (91.7)
Diuretic	5 (11.9)	11 (22.9)	9 (18.0)	7 (17.5)	10 (25.6)	14 (31.8)	16 (34.0)	8 (22.2)
Beta-blocker	6 (14.3)	2 (4.2)	3 (6.0)	5 (12.5)	4 (10.3)	3 (6.8)	4 (8.5)	3 (8.3)
Calcium-channel blocker (dihydropyridine)	11 (26.2)	15 (31.3)	12 (24.0)	14 (35.0)	14 (35.9)	16 (36.4)	14 (29.8)	16 (44.4)
Sympatholytic agent	7 (16.7)	8 (16.7)	9 (18.0)	6 (15.0)	28 (71.8)	32 (72.7)	29 (61.7)	31 (86.1)
Lipid-lowering agents								
Any	3 (7.1)	3 (6.3)	1 (2.0)	5 (12.5)	6 (15.4)	9 (20.5)	6 (12.8)	9 (25.0)
Statin alone	0	1 (2.1)	0	1 (2.5)	2 (5.1)	7 (15.9)	4 (8.5)	5 (13.9)
Fibrate alone	3 (7.1)	1 (2.1)	0	4 (10.0)*	2 (5.1)	0	1 (2.1)	1 (2.8)
Statin and fibrate	0	0	0	0	2 (5.1)	1 (2.3)	0	3 (8.3)

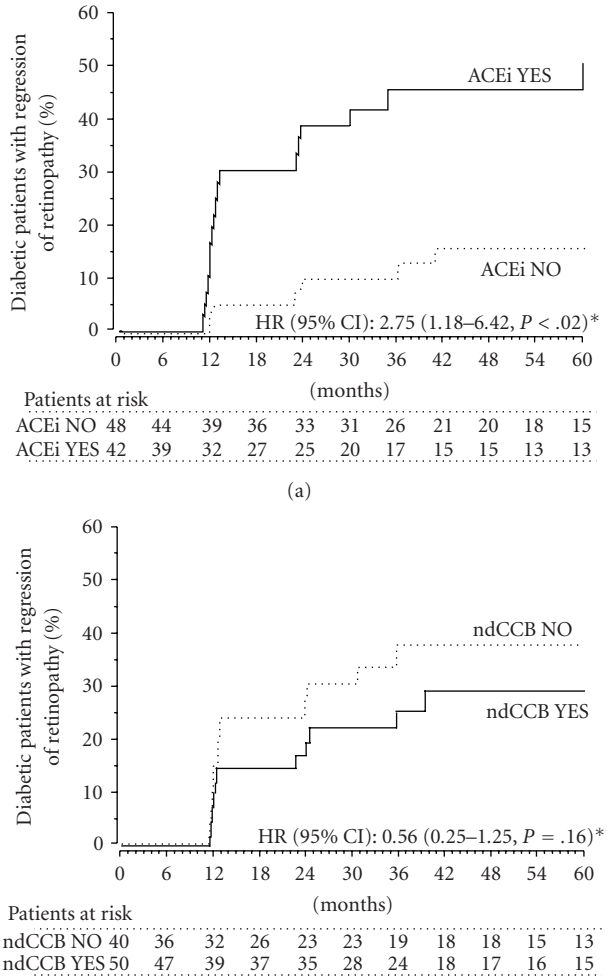


Figure 3: Cumulative incidence of patients with retinal involvement at baseline who achieved regression of diabetic retinopathy according to randomization to ACEi therapy YES or NO (a) or to ndCCB therapy YES or NO (b).

patients on ndCCB and non-ndCCB therapy (data not shown).

4. Discussion

Our study shows that regression of diabetic retinopathy is possible in a substantial proportion of hypertensive patients with type 2 diabetes and tight BP and metabolic control. Importantly, we found that therapy with antihypertensive drugs that directly interfere with the RAS, such as the ACEi trandolapril, is more effective in inducing regression than non-RAS inhibiting therapy, while treatment regimens including or not including the ndCCB verapamil have similar effects. Secondarily, data showed that the protective effect of trandolapril against diabetic retinopathy is not appreciably enhanced by combined therapy with verapamil, and the effect of verapamil is not different from that of placebo. On the other hand, trandolapril as well as verapamil had no specific protective effect against the development of retinopathy in patients with no evidence of retinal involvement at study entry.

These findings are in line with the recent DIRECT-Protect 2 trial showing that the angiotensin receptor blocker (ARB) candesartan increases regression of retinopathy by 34% over placebo in type 2 diabetic patients with mild to moderately severe retinal lesions but has no appreciable effect on progression of retinopathy in those patients without retinal involvement at inclusion [7].

Hypertension may increase the shear stress on the vascular wall, leading to hyperplasia of the vascular endothelium and to hypertrophia of its cytoskeleton [8]. This process may be magnified in the diabetic retina, since defective autoregulation may favor the transmission of high systemic BP down to the microcirculation, causing capillary hypertension and structural damage to the endothelium [9]. Thus, lowering BP might decrease the barotrauma to the vascular wall and therefore prevent or regress the microvascular changes of the diabetic retina.

Throughout the observation period the regression rate of diabetic retinopathy was more than double in patients on trandolapril than in controls. This finding was not explained by blood pressure control that was similar across different treatment groups and was consistent with a specific beneficial effect of trandolapril on the retina.

Activation of the RAS has been involved in defective autoregulation of the retinal microvasculature, and inhibition of the RAS may therefore explain the improved perfusion observed during ACE inhibition therapy. Moreover, the hemodynamic effects of RAS inhibition have been suggested to contribute to the partial regression of retinal changes observed during ACEi therapy in hypertensive rats with streptozotocin induced diabetes [10].

ACE inhibitors, however, may also interfere with a series of nonhemodynamic effects mediated by the RAS. The RAS is involved in growth factor expression, in particular of vascular endothelial growth factor (VEGF) and tumor growth factor (TGF)-beta. Increased VEGF expression in the diabetic rat is normalized by ACE inhibition, which eventually translates into an amelioration of retinal injury. In the presence of high glucose, angiotensin II stimulates TGF-beta secretion, which increases matrix accumulation by activating the synthesis of collagen I and fibronectin and by decreasing matrix degradation [11]. Consistently, blockade of angiotensin II synthesis by ACEi decreases the expression of TGF-beta, reduces the accumulation of matrix protein synthesis, and accelerates its degradation.

Finally, ACEi may interfere with RAS-independent metabolic pathways. By inhibiting bradykinin degradation, they may increase the bioavailability of nitric oxide and prostacyclin which, through an increased bioavailability and activity of Na^+, K^+-ATPase in the vasculature of the diabetic retina, might contribute to the functional improvement detected by electroretinography [12] during ACEi therapy.

Finding that trandolapril was not protective against the development of retinopathy confirms and extends data from

DIRECT-Protect 2 trial that the angiotensin II receptor blocker candesartan had no appreciable effect on progression of retinopathy in type 2 diabetic patients without retinal involvement at inclusion [7]. A possible explanation is that all patients were on intensified BP and metabolic control, which substantially decreased the overall incidence of events, reducing the statistical power of comparative analyses between treatment groups. Indeed, while optimized BP and metabolic control increased the number of regressions in those with retinal involvement at baseline, which increased the power of comparative analyses between treatment groups, in those without retinal disease optimized treatment decreased the incidence of newly onset retinopathy, which decreased the statistical power of between-group comparisons. An alternative or complementary explanation would be that mechanisms sustaining progression of retinopathy may differ from those at the basis of disease regression, which might translate into different response to ACEi therapy of patients with or without retinal changes to start with.

On the other hand, present data on the retina, combined with the results of the BENEDICT Phase 1 study showing that, in patients with type 2 diabetes and normal urinary albumin excretion, verapamil therapy failed to prevent microalbuminuria [3], confirm that this drug has no specific protective effects against microvascular disease of type 2 diabetes.

4.1. Limitations. We graded the severity of retinal involvement by a simplified score that has been validated in previous studies by our [5] and other [6, 13–15] groups and has been recently used in other large-scale, prospective, and randomized clinical trials [16]. Compared to a more complex score implemented to grade retinal involvement in people with diabetes [17], this approach discriminates only two stages (pre-proliferative and proliferative) of retinopathy. Thus changes in one or more grades within the same pre-proliferative or proliferative stage could not be captured by this approach. This reduced the sensitivity and precision of the assessment and, secondarily, the power of the analyses but did not introduce a systematic bias since the same limitation was applied to the same extent to each considered patient group. On the other hand, compared to more complex approaches, the criteria we used to grade retinal involvement in our present study more closely reflect the criteria normally used in every-day clinical practice, which enhances the generalizability of our present findings to the average population of patients with type 2 diabetes.

Regression of retinopathy was the main outcome variable of a substudy ancillary to BENEDICT phase A. Thus, the substudy was not powered *a priori* on the basis of an expected treatment effect on the regression of retinal involvement but rather aimed at including all BENEDICT patients with available funduscopy evaluation at baseline. However, a posteriori evaluations showed that, due to the strong treatment effect of trandolapril, the probability of a false positive finding was less than two percent.

Finally, analysis was restricted to patients referred to two of the nine Centers involved in the BENEDICT trial. This was because of the logistic possibility for these Centers to refer randomized patients to the Unit of Ophthalmology where funduscopy evaluations were performed (these three Institutions were in the same urban area). However, since randomization to different treatment arms was balanced within each Center, this did not introduce any appreciable bias. This is consistent with the evidence that patient distribution was balanced, and baseline characteristics were similar among different treatment groups.

5. Conclusions

The present study, along with the recently published DIRECT-Protect 2 trial, provided the evidence that diabetic retinopathy can regress. This may have important clinical implications since regression of retinal damage may limit the risk of visual loss in the long term. Moreover, microvascular complications of diabetes, such as nephropathy and retinopathy, may reflect coronary ischemic disease and predict cardiovascular morbidity and mortality [18]. Conceivably, as already reported for regression of renal disease, regression of retinopathy might also predict reduced cardiovascular risk in the long-term.

Our present data showed that RAS inhibition with the ACEi trandolapril, unlike calcium channel blockade by verapamil, had a beneficial effect that exceeded the benefit expected from the reduction in arterial BP and blood glucose observed during the study. Importantly, the ACEi trandolapril, compared to the ARB candesartan tested in the DIRECT-Protect 2 trial, has the advantage of remarkably lower treatment costs (US$ 1.10 versus 2.60, $ 0.24 versus 0.58, or Euro 0.49 versus 0.94 for one day therapy with trandolapril 2 mg or candesartan 32 mg, resp.). Costs can be further reduced by using the generic compound that is currently available in most countries. Improving cost-effectiveness of intervention programs at population level would have major implications, as diabetic retinopathy is a leading cause of visual impairment worldwide [1], and its incidence is expected to further increase along with the forecasted epidemic of diabetes, in particular in developing countries [19].

Considering the tremendous burden of diabetes and of its chronic complications, these findings may have important clinical and social implications for patients, physicians, and other health care providers.

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Review Article

Involvement of TAGE-RAGE System in the Pathogenesis of Diabetic Retinopathy

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Diabetic complications are a leading cause of acquired blindness, end-stage renal failure, and accelerated atherosclerosis, which are associated with the disabilities and high mortality rates seen in diabetic patients. Continuous hyperglycemia is involved in the pathogenesis of diabetic micro- and macrovascular complications *via* various metabolic pathways, and numerous hyperglycemia-induced metabolic and hemodynamic conditions exist, including increased generation of various types of advanced glycation end-products (AGEs). Recently, we demonstrated that glyceraldehyde-derived AGEs, the predominant structure of toxic AGEs (TAGE), play an important role in the pathogenesis of angiopathy in diabetic patients. Moreover, recent evidence suggests that the interaction of TAGE with the receptor for AGEs (RAGE) elicits oxidative stress generation in numerous types of cells, all of which may contribute to the pathological changes observed in diabetic complications. In this paper, we discuss the pathophysiological role of the TAGE-RAGE system in the development and progression of diabetic retinopathy.

1. Introduction

Diabetic complications are a leading cause of end-stage renal failure, acquired blindness, and cardiovascular disease (CVD) and are involved in the disabilities and high mortality rates observed in patients with type 1 or type 2 diabetes [1]. Although various hyperglycemia-induced metabolic and hemodynamic conditions are proposed to contribute to complications in diabetes [2, 3], recent clinical studies have suggested the concept of "hyperglycemic memory" in the pathogenesis of vascular injury in diabetes [4–6]. Indeed, the Diabetes Control and Complications Trial-Epidemiology of Diabetes Interventions and Complications (DCCT-EDIC) Study demonstrated that the reduction in the risk of progressive retinopathy and nephropathy brought about by intensive therapy in patients with type 1 diabetes persisted for at least eight years, despite increasing hyperglycemia [4, 5]. The intensive therapy administered during the DCCT resulted in decreased progression of intima media thickness (IMT) and had reduced the risk of nonfatal myocardial infarction, stroke, or death from CVD by 57% by 11 years after the end of the trial [6].

Furthermore, a recent follow-up study, the United Kingdom Prospective Diabetes Study (UKPDS), has also shown that the benefits of intensive therapy in patients with type 2 diabetes were sustained after the cessation of the trial [7]. In this study, despite the early loss of glycemic differences between intensive and conventional therapy, the reductions in microvascular risk and emergent risk reductions for myocardial infarction and death from any cause were maintained during 10 years of posttrial follow-up [7]. These observations indicate that intensive therapy to control blood glucose has long-term beneficial effects on the risk of diabetic retinopathy, nephropathy, CVD, and death in patients with type 1 or type 2 diabetes, strongly suggesting that so-called "metabolic memory" causes chronic damage

in diabetic vessels that is not easily reversed, even by subsequent, relatively good control of blood glucose. Among the various pathways activated under diabetes, as described above, the biochemical nature of advanced glycation end-products (AGEs) and their mode of action are the most compatible with the theory of "hyperglycemic memory" [8, 9].

There is a growing body of evidence to suggest that continuous hyperglycemia under diabetic conditions enhances the formation of AGEs, senescent macroprotein derivatives, through nonenzymatic glycation (called the "Maillard reaction"). There is also accumulating evidence that the binding of the receptor for AGEs (RAGE) with AGEs elicits oxidative stress generation and subsequently evokes inflammatory and/or thrombogenic responses in various types of cells, thus participating in the development and progression of diabetic angiopathies [10–18]. Recently, we demonstrated that glyceraldehyde-derived AGEs (Glycer-AGEs), the predominant structure of toxic AGEs (TAGE), play an important role in the pathogenesis of angiopathy in diabetic patients [10, 19, 20]. Furthermore, there is a growing body of evidence to suggest that the interaction of TAGE with the RAGE alters intracellular signaling, gene expression, and the release of proinflammatory molecules and elicits oxidative stress generation in numerous types of cells, all of which may contribute to the pathological changes seen in diabetic complications. Therefore, the inhibition of TAGE formation, blockade of TAGE-RAGE interactions, and the suppression of RAGE expression or its downstream pathways are promising targets for therapeutic intervention against diabetic complications.

In this paper, we discuss the pathophysiological role of the TAGE-RAGE-oxidative stress system in the development and progression of diabetic retinopathy and related therapeutic interventions.

2. Alternative Routes for the Formation of AGEs In Vivo

AGEs are formed by the Maillard process, a nonenzymatic reaction between aldehyde or ketone group of the reducing sugars (such as glucose, fructose, and trioses etc.) and the amino groups of proteins that contribute to the aging of proteins and to the pathological complications of diabetes [10–13, 19–24]. In the hyperglycemia elicited by diabetes, this process begins with the conversion of reversible Schiff base adducts to more stable, covalently bound Amadori rearrangement products. Over the course of days to weeks, these Amadori products undergo further rearrangement reactions to form irreversibly bound moieties known as AGEs. AGEs were originally characterized by their yellow-brown fluorescent color and their ability to form cross-links with and between amino groups, but the term is now used for a broad range of advanced products of the glycation process, including *N*-(carboxymethyl)lysine (CML) and pyrraline, which show neither color nor fluorescence and are not cross-linked proteins [8, 21–25]. The formation of AGEs in vivo is dependent on the turnover of the chemically modified target, the time available, and the sugar concentration. The structures of the various cross-linked AGEs that are generated in vivo have not yet been completely determined. Due to their heterogeneity and the complexity of the chemical reactions involved, only some AGEs have been structurally characterized in vivo. The structural identities of AGEs with cytotoxic properties therefore remain unknown.

Recent studies have suggested that AGEs can arise not only from reducing sugars, but also from carbonyl compounds derived from the autoxidation of sugars and other metabolic pathways [26–28]. Indeed, we have recently demonstrated that glucose, α-hydroxyaldehydes (glyceraldehyde and glycolaldehyde), and dicarbonyl compounds (methylglyoxal; MGO, glyoxal; GO, and 3-deoxyglucosone, 3-DG) are actively involved in the protein glycation process [21, 29–31]. Six immunochemically distinct classes of AGEs (glucose-derived AGEs; Glc-AGEs, glyceraldehyde-derived AGEs; Glycer-AGEs, glycolaldehyde-derived AGEs; Glycol-AGEs, MGO-derived AGEs; MGO-AGEs, GO-derived AGEs; GO-AGEs, and 3-DG-derived AGEs; 3-DG-AGEs) are found in the sera of type 2 diabetic patients during hemodialysis [21, 29–31]. Based on these data, we proposed a pathway for the in vivo formation of distinct AGEs involving the Maillard reaction, sugar autoxidation, and sugar metabolic pathways, as shown in Figure 1.

3. Receptors for AGEs

Such receptors may play a critical role in AGEs-related biology and the pathology associated with diabetic complications and aging disorders. Several types of AGEs binding proteins and/or receptors for AGEs such as RAGE [32–36]; oligosaccharyl transferase-48 (AGE-R1) [37]; galectin-3 (AGE-R3) [38]; CD36 [39]; macrophage scavenger receptors types 1 and 2 (MSRs-1 & -2) [40]; and fasciclin EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptors 1 and 2 (FEELs-1 & -2) [41] have been reported. The relative pathogenic contributions of these receptors to diabetic complications are poorly defined, although RAGE is by far the best characterized, and mechanistic in vitro and in vivo studies on AGEs and their regulatory fragments such as soluble RAGE (sRAGE) have indicated that they play important roles in pathobiology [36, 42]. RAGE is normally expressed in a variety of cells, including endothelial cells (EC), pericytes, neurons, and microglia, [32–34]. We have recently found that glyceraldehyde rapidly reacts with the amino groups of proteins to form Glycer-AGEs both in vitro and in vivo [19, 21, 30]. Furthermore, Glycer-AGEs have the strongest binding affinity for RAGE and subsequently elicit oxidative stress generation and vascular inflammation and are therefore implicated in accelerated atherosclerosis in diabetes [43, 44]. Recently, we also demonstrated that Glycer-AGEs, the predominant structure of toxic AGEs (TAGE), play an important role in the pathogenesis of angiopathy in diabetic patients [19, 20]. Moreover, there is a growing body of evidence to suggest that the interaction of TAGE with RAGE elicits oxidative stress generation in numerous types of cells, all of which may contribute to the pathological changes observed in diabetic complications [10, 19, 20].

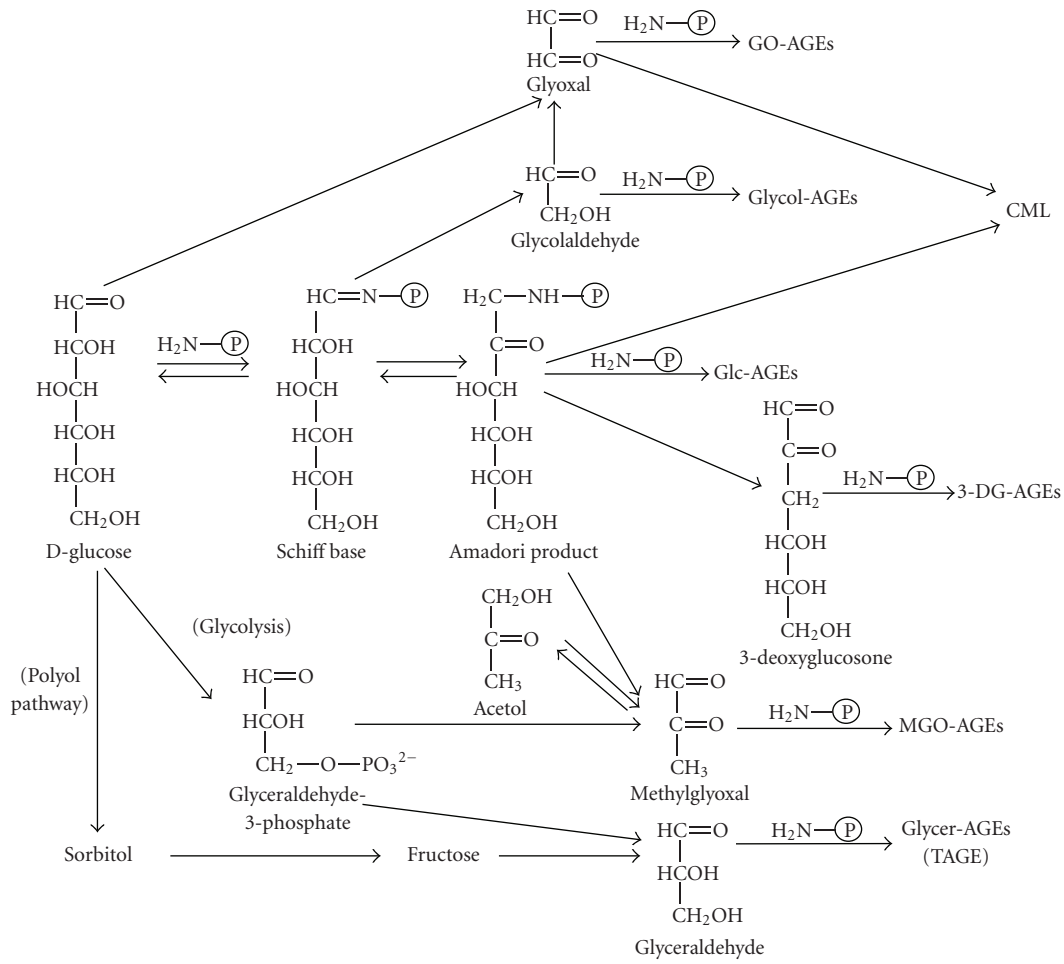

Figure 1: *Alternative routes for the formation of various distinct AGEs in vivo*. Glc-AGEs; glucose-derived AGEs, Glycer-AGEs; glyceraldehyde-derived AGEs, Glycol-AGEs; glycolaldehyde-derived AGEs, MGO-AGEs; methylglyoxal (MGO)-derived AGEs, GO-AGEs; glyoxal (GO)-derived AGEs, and 3-DG-AGEs, 3-deoxyglucosone (3-DG)-derived AGEs, CML; *N*-(carboxymethyl)lysine, and P-NH_2; free amino residue of protein.

4. Pathway of Glycer-AGEs (TAGE) Formation In Vivo

Glyceraldehyde is derived from two distinct pathways in vivo, (1) the glycolytic pathway and (2) the fructose metabolism pathway [19, 20, 45]. (1) The glycolytic intermediate glyceraldehyde-3-phosphate (G-3-P) is normally catabolized by the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). With a decline in GAPDH activity, G-3-P accumulates intracellularly. G-3-P metabolism then shifts to another route, and the amount of glyceraldehyde is increased, which leads to an increase in the formation of TAGE. This suggests a positive feedback mechanism; that is, the TAGE-induced GAPDH suppression further stimulates the generation of TAGE. (2) Under hyperglycemic conditions, the increased intracellular glucose concentration stimulates the polyol pathway to generate fructose in insulin-independent tissues such as the lens, kidney, nerve tissue, brain, and red blood cells [46–48]. Furthermore, fructose, a component of table sugar and high-fructose corn syrup, is also obtained from the diet [49]. Fructose is phosphorylated to fructose-1-phosphate (F-1-P) and then catabolized to glyceraldehyde and dihydroxyacetone-phosphate by aldolase B [48–51]. The newly synthesized glyceraldehyde is then transported or leaks passively across the plasma membrane. Glyceraldehyde promotes the formaion of TAGE both intracellularly and extracellularly (Figure 2).

5. Diabetic Retinopathy

Diabetic retinopathy is one of the most important microvascular complications in diabetes and is a leading cause of acquired blindness among people of occupational age [52]. Hyperglycemia damages retinal microvascular cells and causes various changes in retinal tissues such as enhanced vascular permeability due to pericyte loss, which is followed by microvascular occlusion in the retina [53, 54]. Pericytes are elongated cells of mesodermal origin, which wrap around and along the EC of small vessels [55]. As pericytes contain contractile muscle filaments on their EC side, they are regarded as microvascular counterparts of

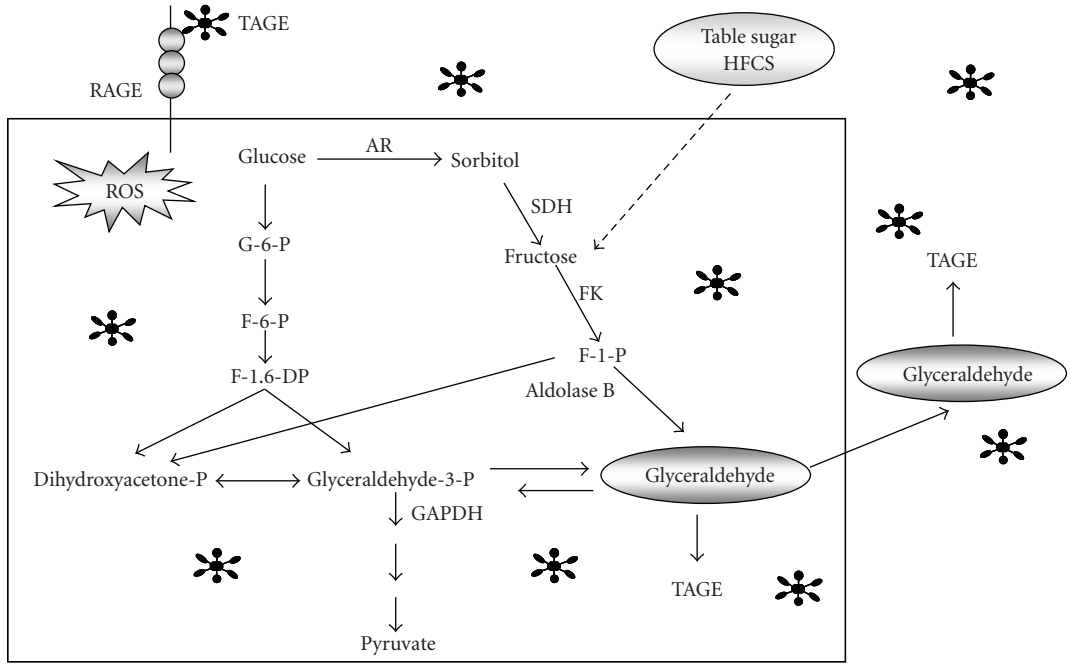

Figure 2: *Production routes of glyceraldehyde-derived AGEs (Glycer-AGEs) in vivo*. TAGE; toxic AGEs (glyceraldehyde-derived AGEs), RAGE; receptor for AGEs, ROS; reactive oxygen species, HFCS; high-fructose corn syrup, AR; aldose reductase, SDH; sorbitol dehydrogenase, FK; fructokinase, GAPDH; glyceraldehyde-3-phosphate dehydrogenase, $*$; TAGE.

smooth muscle cells and are considered to be involved in the maintenance of capillary tone [56, 57]. AGEs have been postulated to play a role in the development and progression of microvascular disease in diabetes. Vascular endothelial growth factor (VEGF) is a specific mitogen to EC, which is also known as vascular permeability factor and is generally thought to be involved in the pathogenesis of proliferative diabetic retinopathy. Indeed, clinical observations have demonstrated that the VEGF level in ocular fluid is positively correlated with the amount of neovascularization in diabetic retinopathy [58, 59].

Retinal pericytes accumulate AGEs during diabetes [60], which is expected to have a detrimental influence on pericyte survival and function [61]. We have found that TAGE causes the apoptosis of retinal pericytes and induces the expression of VEGF by interacting with RAGE, indicating the involvement of TAGE in the pathogenesis of diabetic retinopathy, especially in the early stage [62–64]. TAGE also induces VEGF expression, DNA synthesis, and angiogenesis in EC. These changes are the hallmark of proliferative diabetic retinopathy [65, 66]. These findings suggest that the TAGE-RAGE interaction facilitates angiogenesis by two distinct mechanisms, by relieving the restriction on EC growth due to the apoptotic cell death of pericytes and by autocrine and paracrine induction of VEGF proteins by vascular wall cells. Although the molecular mechanisms of the VEGF overexpression elicited by TAGE are not fully understood, our recent investigation suggested that the TAGE-RAGE interaction increases VEGF gene transcription in EC by NADPH oxidase-mediated reactive oxygen species (ROS) generation and the subsequent activation of nuclear factor κB (NF-κB) *via* the Ras-mitogen activated protein kinase pathway [65, 66]. There has been increasing interest in the role of inflammatory reaction in diabetic retinopathy [67]. AGEs have recently been shown to increase leukocyte adhesion to cultured retinal microvascular EC by inducing intracellular cell adhesion molecule-1 (ICAM-1) expression [68]. Furthermore, TAGE also induces monocyte chemoattractant protein-1 (MCP-1) expression in EC through intracellular ROS generation [69].

6. Postprandial Hyperglycemia is Associated with Increased Risk of Diabetic Retinopathy

While it is well known that postchallenge and postprandial hyperglycemia are related to the development and progression of diabetic macrovascular disease [70, 71], there are limited data on the relationship between postprandial hyperglycemia and microvascular complications. A recent observational prospective study from Japan demonstrated that postprandial hyperglycemia is a better predictor of diabetic retinopathy that glycated hemoglobin A_{1c} (HbA_{1c}) [72]. Shiraiwa et al. performed a cross-sectional study of 232 people with type 2 diabetes who were not being treated with insulin injections. Multiple regression analysis revealed that postprandial hyperglycemia was independently correlated with the incidence of diabetic retinopathy and neuropathy. Additionally, postprandial hyperglycemia was also found to be associated, although not independently, with the incidence of diabetic nephropathy.

We have previously shown that glyceraldehyde reacts rapidly with the amino groups of proteins to form TAGE

in vivo, which evokes vascular inflammation and oxidative stress generation, thereby implicating them in accelerated atherosclerosis in diabetes [10, 19, 20]. More recently, we investigated the effects of nateglinide, which has been known to improve postprandial hyperglycemia, on HbA_{1c}, Glc-AGE, and TAGE levels in Goto-Kakizaki (GK) rats, one of the rat models of type 2 diabetes, fed twice a day [73]. After 6 weeks, nateglinide treatment was found to not only prevent postprandial hyperglycemia, but also to reduce TAGE levels in GK rats. However, it did not cause a significant difference in HbA_{1c} or Glc-AGE levels [73]. This study suggests that TAGE is formed more rapidly than HbA_{1c}, a precursor of Glc-AGEs, under postprandial hyperglycemic states and shows potential as novel markers of cumulative postprandial hyperglycemia. In this study, although we did not clarify the exact molecular mechanism by which TAGE is formed under postprandial hyperglycemic conditions, hyperglycemia-induced oxidative stress-mediated inhibition of GAPDH may lead to the elevation of glyceraldehyde levels and subsequently enhance the formation of TAGE during the postprandial period [74]. The relative contribution of postprandial glucose decreased progressively from the lowest to the highest quintile of HbA_{1c}; whereas, the relative contribution of fasting glucose increased gradually with increasing levels of HbA_{1c} [75]. These observations suggest that a decrease in HbA_{1c} levels does not necessarily reflect a reduction in postprandial hyperglycemia, especially in poorly controlled diabetic patients.

7. Serum Levels of TAGE in Diabetes

The above-discussed effect of TAGE strongly suggests a pathological role for these senescent macroproteins in diabetic complications. Furthermore, Glc-AGEs and TAGE are present in human serum, and the level of both AGEs is elevated in type 1 and type 2 diabetes [76–79]. These AGEs, especially TAGE-epitopes, elicit angiogenesis at the concentrations present in the sera of diabetic patients. These results suggest the involvement of TAGE-epitopes in pathologic angiogenesis in vivo. Recently, we demonstrated that the vitreous levels of both TAGE and VEGF were significantly higher in diabetic patients than in control subjects and that these levels were elevated in association with the severity of neovascularization in diabetic retinopathy. In addition, there was a significant correlation between vitreous TAGE and VEGF levels [80, 81]. Furthermore, we have recently found that serum TAGE levels are positively correlated with thrombogenic markers in humans. Plasminogen activator inhibitor-1 and fibrinogen levels are positively associated with serum TAGE levels [82].

While many of the reported studies measured a range of ill-defined AGEs moieties, others evaluated defined adducts such as CML, pentosidine, and crossline in association with diabetic retinopathy [83, 84]. In addition, other studies have reported no correlation between AGE levels and retinopathy in diabetic patients [83, 85], although the apparent disparity between the findings of various studies may be related to variations in patient populations and/or the nonuniform assays used for plasma AGEs-quantification. Our studies suggest that an elevated TAGE level in diabetic patients is an important factor for the initiation and progression of retinopathy. Therefore, the inhibition of TAGE formation and the blockade of TAGE-RAGE interactions are potential therapeutic strategies for the prevention of diabetic retinopathy.

8. Serum Levels of Soluble RAGE in Diabetes

The administration of a recombinant soluble form of RAGE (sRAGE) consisting of its extracellular ligand-binding domain has recently been shown to not only suppress the development of atherosclerosis but also to stabilize established atherosclerosis in diabetic apolipoprotein E-null mice [86, 87]. The blockade of the AGEs-RAGE axis by the administration of sRAGE also ameliorates neuronal dysfunction and reduces the development of acellular capillaries and pericyte ghosts in hyperglycemic and hyperlipidemic mice [88]. Furthermore, Kaji et al. have also shown that attenuation of the RAGE axis with sRAGE inhibits retinal leukostasis and blood-retinal barrier breakdown in diabetic C57/BJ6 and RAGE-transgenic mice, which are accompanied by decreased expression of VEGF and ICAM-1 in the retina [89]. These observations suggest that exogenously administered sRAGE captures and eliminates circulating AGEs, thus protecting against AGEs-elicited tissue damage by acting as a decoy.

Recently, endogenous sRAGE has been identified in humans [42]. Endogenous sRAGE may be generated from the cleavage of cell surface full-length RAGE or novel splice variants of RAGE (the C-truncated splice isoform of secretory RAGE; esRAGE) [42]. Endogenous total sRAGE levels are elevated in patients with type 1 or 2 diabetes [90–93]. Furthermore, we, along with others, have recently demonstrated that serum total sRAGE levels are positively, rather than inversely, associated with TAGE levels in both nondiabetic and diabetic subjects [93, 94]. Age-, sex-, and body mass index-adjusted TAGE levels are also significantly increased in proportion to the increasing levels of sRAGE in nondiabetic subjects [93, 94]. These findings suggest that the sRAGE pool is not able to efficiently capture and eliminate circulating TAGE in vivo by working as a decoy receptor. Since TAGE is a positive regulator of the cell expression of RAGE, circulating sRAGE levels may reflect tissue RAGE expression and be elevated in parallel with serum TAGE levels as a counter system against TAGE-elicited tissue damage [95–98].

The serum levels of esRAGE are also correlated with the levels of circulating AGEs such as CML and pentosidine in type 1 diabetes [99]. However, in contrast to the case for total sRAGE, circulating esRAGE levels are decreased, rather than increased, in both type 1 and 2 diabetes. Katakami et al. reported in Japanese that the serum levels of esRAGE were significantly decreased in patients with type 1 diabetes compared with nondiabetic subjects [100], and esRAGE levels were found to be significantly lower in type 1 diabetic patients with retinopathy than in those

without retinopathy [100, 101]. Decreased esRAGE levels were also found to be an independent risk factor for carotid atherosclerosis [102]. Indeed, Koyama et al. reported that esRAGE levels were decreased in Japanese type 2 diabetic patients compared with nondiabetic subjects and that low levels of esRAGE were associated with the components of metabolic syndrome and carotid atherosclerosis [102]. These observations were contrary to the finding of previous reports that total sRAGE levels were associated with conventional coronary risk factors including inflammatory markers and were independent determinants of coronary artery disease in diabetes [91, 95, 96]. Therefore, the kinetics and role of sRAGE and esRAGE in diabetes may differ [97]. Decreased levels of esRAGE may be associated with comorbidities such as diabetic retinopathy and atherosclerosis *via* mechanisms other than its role as a decoy because esRAGE levels are approximately 3~4-fold lower than total sRAGE levels and may not be sufficient to efficiently eliminate circulating AGEs in humans. Furthermore, sRAGE, but not esRAGE, was recently found to be independently correlated with albuminuria in type 2 diabetic patients [103].

9. Agents That Could Potentially Suppress TAGE-RAGE Interaction

9.1. Inhibitors of the Renin-Angiotensin System (RAS). The interaction of the RAS and TAGE-RAGE systems has also been proposed. We have found that angiotensin II potentiates the deleterious effects of TAGE in pericytes by inducing RAGE protein expression [64]. In vivo, TAGE-injection stimulated RAGE expression in the eyes of spontaneously hypertensive rats, which was blocked by telmisartan. In vitro, angiotensin II-type 1 receptor-mediated ROS generation elicited RAGE gene expression in retinal pericytes through NF-κB activation. Furthermore, angiotensin II augmented TAGE-induced pericyte apoptosis, the earliest hallmark of diabetic retinopathy. Telmisartan also blocks angiotensin II-induced RAGE expression in EC [104].

There is an increasing interest in the role of inflammatory reactions and immune phenomena in the pathogenesis of diabetic complications [105–107]. Indeed, leukocyte adhesion to diabetic retinal vasculature is considered to be a critical early event in diabetic retinopathy, the development of which is mainly mediated by VEGF, ICAM-1, and MCP-1 expression [105–107]. ICAM-1 and MCP-1 are essential chemokines that mediate the recruitment of leukocytes to mesangial lesions [108, 109]. The selective targeting of ICAM-1 or MCP-1 was also shown to markedly decrease albuminuria and renal injury in experimental diabetic nephropathy [108, 109]. Furthermore, several experimental studies have supported the pathological role of VEGF in diabetic nephropathy: antibodies raised against VEGF have been reported to improve hyperfiltration and albuminuria in diabetic rats [110, 111]. In addition, atherosclerosis is also an inflammatory-proliferative disease [112], and the administration of VEGF is reported to enhance atherosclerotic plaque progression in animals [113]. We have recently found that treatment with telmisartan or olmesartan inhibits the TAGE-evoked inflammatory responses in EC *via* downregulation of RAGE expression [114–118]. These observations suggest that the blockade of the TAGE-RAGE signaling pathways by RAS inhibitors may be clinically relevant to the prevention of diabetic complications.

9.2. Pigment-Epithelium-Derived Factor (PEDF). PEDF is a glycoprotein that belongs to a superfamily of serine protease inhibitors with complex neurotrophic, neuroprotective, antiangiogenic, antioxidative, and anti-inflammatory properties, any of which could potentially be exploited as a therapeutic option for the treatment of vascular complications in diabetes [119, 120]. PEDF inhibits TAGE-induced ROS generation and subsequently prevents apoptotic cell death in pericytes by restoring downregulation of the gene expression of the antiapoptotic factor bcl-2 [121]. Furthermore, PEDF also inhibits TAGE-induced ICAM-1, VEGF, and MCP-1 upregulation as well as NO suppression in EC by blocking NADPH oxidase-mediated ROS generation [69, 122–126]. In vivo, the administration of PEDF or pyridoxal phosphate, an AGEs inhibitor, decreased the retinal levels of 8-hydroxydeoxyguanosine (8-OHdG), an oxidative stress marker, and subsequently suppressed ICAM-1 gene expression and retinal leukostasis in diabetic rats [127]. Moreover, intravenous administration of TAGE to normal rats increased ICAM-1 gene expression and retinal leukostasis, which were blocked by PEDF [127]. PEDF inhibited diabetes- or TAGE-induced RAGE gene expression by blocking superoxide-mediated NF-κB activation [128]. In addition, we have recently found that intravenous administration of TAGE to normal rats not only increases retinal vascular permeability by stimulating VEGF expression, but also decreases retinal PEDF levels [129]. Simultaneous treatment with PEDF inhibited TAGE-elicited VEGF-mediated permeability by downregulating the mRNA levels of $p22^{phox}$ and $gp91^{phox}$, membrane components of NADPH oxidase, and subsequently decreasing retinal levels of the oxidative stress marker, 8-OHdG. PEDF also inhibited TAGE-induced vascular hyperpermeability (as measured by transendothelial electrical resistance) by suppressing VEGF expression. PEDF decreased ROS generation in TAGE-exposed EC by suppressing NADPH oxidase activity *via* downregulation of the mRNA levels of $p22^{phox}$ and $gp91^{phox}$. This led to blockade of TAGE-elicited Ras activation and NF-κB-dependent VEGF gene induction in EC. These results indicate that the central mechanism of PEDF inhibition of the TAGE-signaling related to vascular permeability is the suppression of NADPH oxidase-mediated ROS generation and subsequent VEGF expression [129].

The PEDF levels in the aqueous humor and vitreous are decreased in diabetic patients, especially in those with proliferative retinopathy, suggesting that loss of PEDF in the eye contributes to the pathogenesis of proliferative diabetic retinopathy [130, 131]. We have also found that the vitreous levels of TAGE and VEGF are significantly higher in diabetic patients than in control subjects [81] and detected a significant correlation between vitreous TAGE and VEGF levels. Total antioxidant status was also decreased in the vitreous in patients with diabetes compared with that in

the controls. Furthermore, both the TAGE and VEGF levels (inversely) and those of PEDF (positively) were associated with the total antioxidant status of the vitreous [132, 133]. These observations further support the concept that PEDF is an endogenous anti-inflammatory and antioxidative agent that blocks the TAGE-VEGF axis, thereby protecting against the progression of diabetic retinopathy.

9.3. Statins and Bisphosphonates. We have found that protein prenylation is crucial for TAGE-RAGE signaling in EC [65, 66]. Cerivastain completely prevented TAGE-induced increases in NF-κB activity and VEGF expression and the resultant increase in DNA synthesis as well as tube formation in microvascular EC [65]. Since mevalonate blocked the growth-inhibitory effects of cerivastatin on TAGE-exposed EC and that FTI-276, an inhibitor of farnesyltransferase, mimicked the effects of cerivastatin, cerivastatin may block the TAGE-RAGE signaling involved in vascular hyperpermeability and angiogenesis *via* the suppression of protein prenylation. Furthermore, we have recently found that atorvastatin dose-dependently inhibited TAGE-induced ROS generation in Hep3B cells [134]. Atorvastatin as well as the antioxidant N-acetylcysteine (NAC) was found to suppress C-reactive protein (CRP) expression in TAGE-exposed Hep3B cells at both the mRNA and protein levels [134]. These results demonstrate that atorvastatin is able to block the TAGE-signaling involved in CRP expression through its antioxidative action. Taken together, these observations suggest that statins have vasculoprotective effects by inhibiting the deleterious effects of TAGE *via* the suppression of their downstream signaling.

Bisphosphonates are potent inhibitors of bone resorption and are widely used for the treatment of osteoporosis, osteolytic bone metastasis, and tumor-associated hypercalcemia [135–137]. These compounds have a high affinity for calcium ions and therefore target bone mineral, where they are internalized by bone-resorbing osteoclasts and inhibit osteoclast function. Recently, farnesyl pyrophosphate synthase has been shown to be a molecular target of nitrogen-containing bisphosphonates such as incardronate disodium and minodronate, and the inhibition of the posttranslational prenylation of small molecular weight G proteins including Ras and Rac-1 is probably involved in their antiresorptive activity in osteoclasts [135–137]. Since the protein prenylation of GTP-binding proteins is associated with various cellular functions such as cell growth and differentiation [135–137], nitrogen-containing bisphosphonates may have pleitrophic effects by blocking the synthesis of isoprenoid intermediates. Indeed, incardronate disodium was found to inhibit TAGE-induced increases in NF-κB activity and VEGF expression as well as the proliferation and tube formation of EC [66]. Furthermore, we have recently found that minodronate inhibits TAGE–induced NF-κB activation and subsequently suppresses VCAM-1 gene expression by reducing ROS generation in EC [135]. Geranylgeranyl pyrophosphate reversed the antioxidative properties of minodronate in TAGE-exposed EC [135]. Taken together, these findings suggest that nitrogen-containing bisphosphonates are able to inhibit TAGE-elicited inflammatory-proliferative changes in EC by suppressing NADPH oxidase-derived ROS generation, probably *via* the inhibition of the geranylgeranylation of Rac-1, a component of endothelial NADPH oxidase [136, 137].

10. Conclusion

There is accumulating evidence that the TAGE-RAGE-oxidative stress system is actively involved in the pathogenesis of diabetic complications, especially diabetic retinopathy. We have reviewed the inhibitors of the TAGE-RAGE axis and their potential therapeutic implications in these devastating disorders.

Expert Opinion. Two recent large prospective clinical studies, DCCT and UKPDS, have shown that intensive blood glucose control effectively reduces the incidence of vascular complications among patients with diabetes [138, 139]. However, strict control of hyperglycemia is often very difficult to maintain and may increase the risk of severe hypoglycemia in diabetic patients. Inhibition of TAGE formation, blockade of TAGE-RAGE interactions, and the suppression of RAGE expression or its downstream pathways by the agents discussed here are promising novel therapeutic strategies for the treatment of patients with diabetic retinopathy. Further clinical studies are needed to clarify whether the use of these agents is able to reduce the risk of diabetic retinopathy beyond blood glucose-, blood pressure- or cholesterol-lowering effects.

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Review Article

ROCK as a Therapeutic Target of Diabetic Retinopathy

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The increasing global prevalence of diabetes is a critical problem for public health. In particular, diabetic retinopathy, a prevalent ocular complication of diabetes mellitus, causes severe vision loss in working population. A better understanding of the pathogenesis and the development of new pharmacologic treatments are needed. This paper describes the relevance between Rho/ROCK pathway and the pathogenesis of diabetic retinopathy from its early to late stages. Moreover, the therapeutic potential of ROCK inhibitor in the total management of diabetic retinopathy is discussed.

1. Introduction

The growing prevalence of diabetic retinopathy (DR), the common ocular complication of diabetes mellitus, is a critical problem for global public health [1, 2]. Early nonproliferative stages of DR are characterized by blot hemorrhages and vascular abnormality such as retinal vascular microaneurysms or hyperpermeability which could cause diabetic macular edema (DME). Proliferative diabetic retinopathy (PDR), later stage of the diseased state, causes neovascularization, vitreous hemorrhages, preretinal fibrovascular proliferation, and tractional retinal detachment. While visual acuity is not always affected in early stages, progression of the disease leads to severe vision loss.

Panretinal photocoagulation (PRP) and vitreoretinal surgery remain the primary therapeutic strategies for progressed DR. However, PRP is destructive to the retina and accompanied by adverse effects such as decreased visual acuity, increased risk of macular edema, and pain [3, 4]. Moreover, recent advances in vitreous surgery cannot always achieve a satisfying visual acuity [5]. Now it is at a point where new alternative and adjunctive agents from the earlier stages are urgently required because of overwhelming patient's physical and economic burdens of these treatments.

The various clinical findings in earlier DR stages are related to endothelial damage secondary to increased leukocyte adhesion mediated through adhesion molecules, intercellular adhesion molecule-1 (ICAM-1), and leukocyte β_2-integrins (CD18/CD11a and CD18/CD11b) [6–8]. In addition, the critical mechanism of this leukocyte-induced endothelial damage is the interaction of endothelial Fas with Fas ligand, expressed on adherent leukocytes in diabetic rats [9] and DR patients [10].

Recent accumulating evidences indicate that vascular endothelial growth factor (VEGF) plays a critical role in pathogenesis of both DME and neovascularization in PDR [11, 12]. Clinical studies with anti-VEGF antibodies are potentially useful strategy and improve outcome for treating DR [13]. However, the adaptation is only for progressed states and there is a possibility of systemic adverse complications such as hypertension, cerebrovascular accidents, and myocardial infarcts by anti-VEGF therapy [14].

Meanwhile, neovascularization and proliferative vitreoretinopathy (PVR) are hallmark of the later DR stages. VEGF-induced endothelial migration and proliferation is essential process for angiogenesis. ERK1/2 signaling promotes the proliferative activities of endothelial cells in angiogenic processes [15, 16]. Moreover, endothelial migration is mediated by Rho-kinase (ROCK) pathway which activates remodeling of endothelial F-actin cytoskeleton [17].

Our recent findings indicated that hyalocytes, a known resident macrophage in the cortical vitreous under physiological conditions, appeared to be involved in the pathogenesis of PVR associated with a cicatricial contraction of

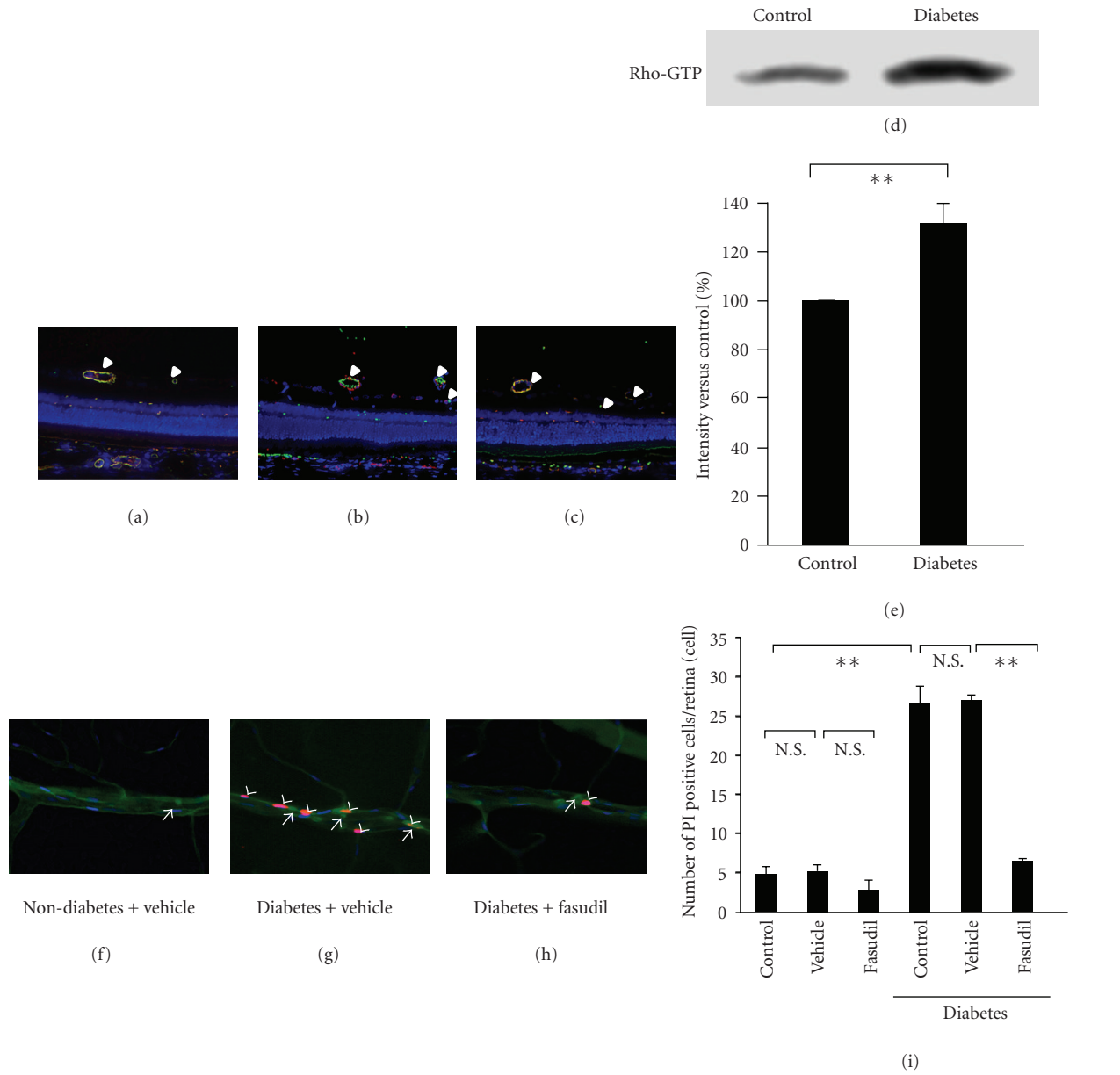
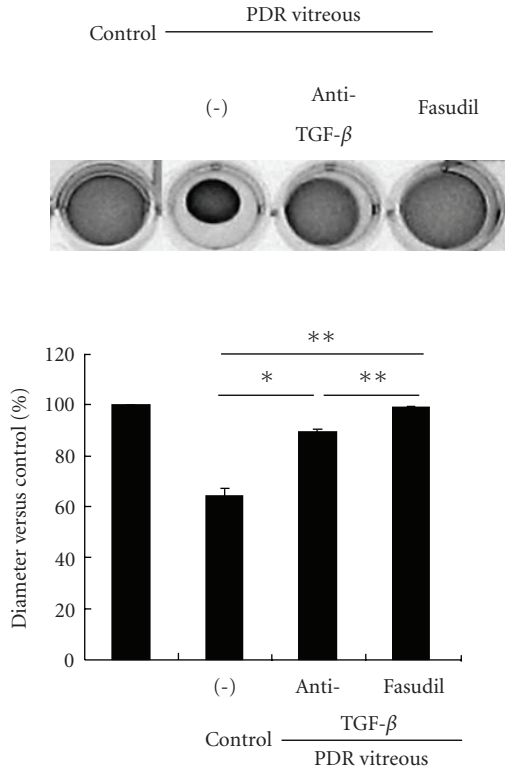


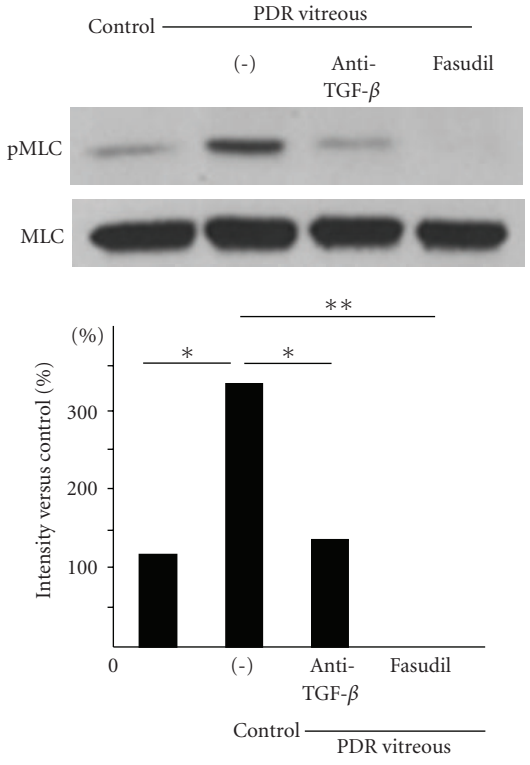
Figure 1: Rho/ROCK activation in retinal vessels. In immunohistochemical analysis, RhoA (a), ROCK1 (b) and ROCK2 (c) were detected in retinal vessels. Yellow (white arrowhead) indicates double-stained vasculature (magnification: ×400). (d and e) The levels of Rho-GTP were significantly higher in streptozotocin induced-diabetic rat bretinas, compared with those in nondiabetic control, detected by Rho pull-down assay. Average signal intensities are quantified and expressed as percentage of the ratio of control ($^{**}P < .01$, $n = 5$ each). Prevention of leukocyte-induced retinal endothelial damage by fasudil. (f–i) *In vivo* visualization of adhering leukocytes (green, concanavalin A lectin) and injured endothelial cells (red, propidium iodide (PI)) and endothelial nuclei (blue, DAPI) in rat retinas. PI positive cells (white arrowhead) widely coincided with adherent leukocytes (white arrow). The number of PI positive cells per retina was significantly higher in the diabetic animals, compared with the nondiabetic controls. Fasudil caused a significant reduction in the number of PI positive cells in the retinas of the diabetic animals, compared with the vehicle-treated controls ($^{**}P < .01$, N.S., not significant, $n = 5$ each).

proliferative membranes in PDR [18–20]. The expression of α-smooth muscle actin (α-SMA) and phosphorylation of myosin light chain (MLC) in hyalocytes, which is associated with stress-fiber formation and contractile rings, are facilitating cell contraction [21, 22]. We demonstrated that vitreous from patients with PDR significantly promoted expression of α-SMA and phosphorylation of MLC, and enhanced contraction of hyalocyte-containing collagen gels, compared with vitreous from patients with nondiabetic controls [23].

In this paper, we first place the Rho/ROCK pathway plays a critical role in diabetic retinal microvasculopathy, neovascularization, and tractional retinal detachment associated with a cicatricial contraction of proliferative membranes. We then describe our current knowledge about ROCK inhibition



(a)



(b)

Figure 2: Continued.

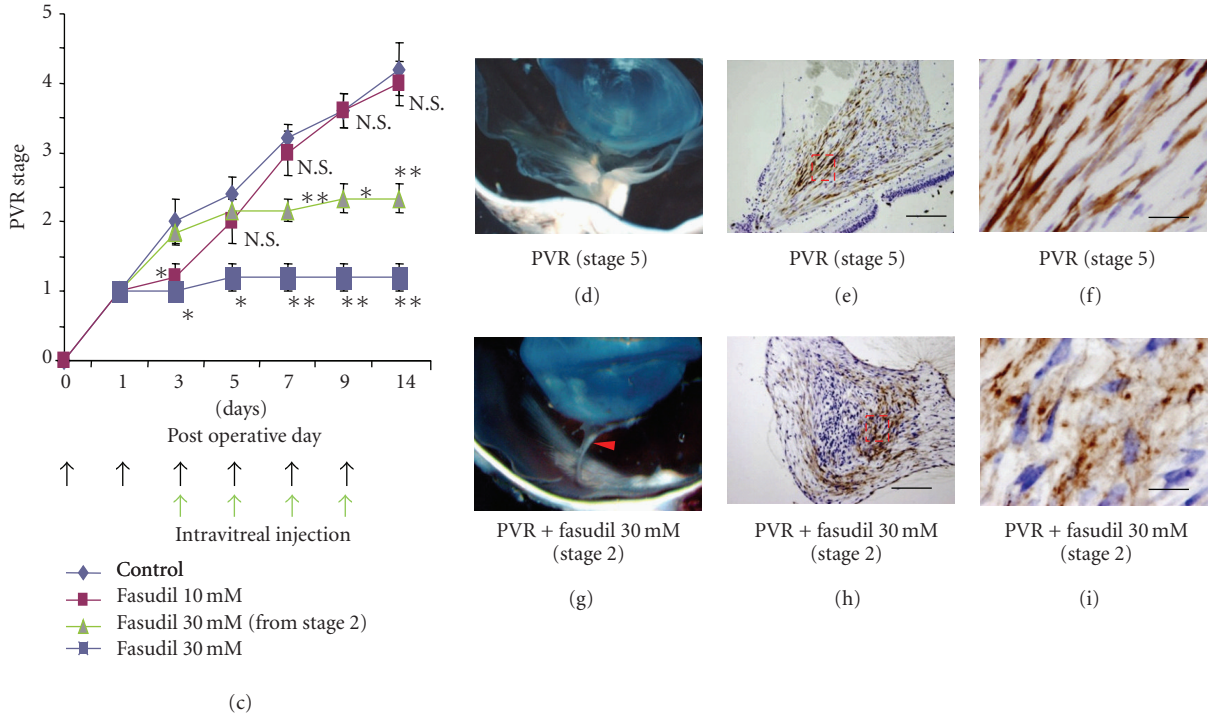


FIGURE 2: Impact of fasudil on PDR vitreous-induced collagen gel contraction and MLC phosphorylation. After pretreatment with or without anti-TGF-β mAb or fasudil, hyalocytes were stimulated with vitreous with PDR. (a) In hyalocyte-containing collagen gels, fasudil almost completely suppressed the contraction of collagen gels treated with PDR vitreous. The diameter of the gels was measured and statistically analyzed ($^*P < .05$; $^{**}P < .01$; NS, not significant, $n = 3$ each). (b) Western blot analysis was performed to detect phosphorylated MLC (pMLC). Fasudil abolished MLC phosphorylation, induced by PDR vitreous. Lane-loading differences were normalized by MLC. Signal intensities were quantified and expressed as percentages of the pMLC/MLC ratio compared with control ($^*P < .05$; $^{**}P < .01$, $n = 3$ each). Experimental PVR in rabbit eyes. (c) Therapeutic potential of fasudil in reducing the progression of experimental PVR. PVR was classified into six stages (0–5). Rhombus, vehicle ($n = 5$); purple square, fasudil 10 μM ($n = 5$); trigone, fasudil 30 μM from stage 2 ($n = 6$); blue square, fasudil 30 μM ($n = 5$) ($^*P < .05$, $^{**}P < .01$, not significant versus vehicle). (d) Tractional retinal detachment because of formation and cicatricial contraction of preretinal proliferative membrane was observed by stereomicroscopy in vehicle-treated eyes (stage 5 PVR). (g) In contrast, intravitreal membranes adhered to the retina without causing retinal detachment (arrowhead) in 30 μM fasudil-treated eyes with stage 2 PVR. Micrographs depict α-SMA expression (brown) in preretinal proliferative membrane with stage 5 PVR (e) and stage 2 PVR (h) by immunohistochemical analysis. (Scale bar, 200 μm). (f and i) Magnified images of (e) and (h), respectively, (Scale bar, 10 μm).

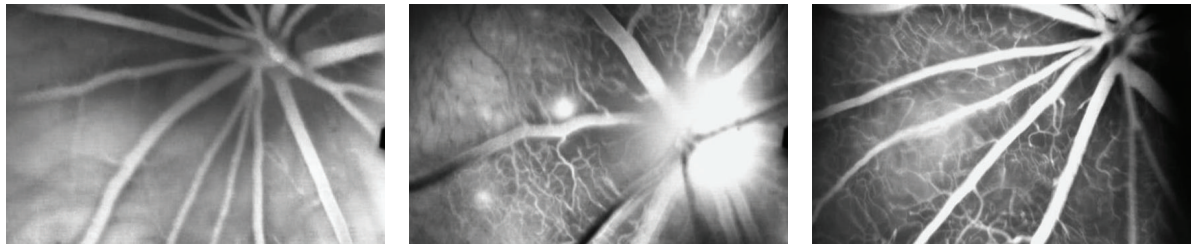
as a new strategy in the total management of DR from its early to late stages.

2. Involvement of Rho/ROCK Pathway in the Pathogenesis of Diabetic Retinopathy

Recent studies have revealed that small GTP-binding protein Rho and its target protein ROCK are implicated in the important physiological roles such as cell adhesion and migration mediated through MLC phosphorylation [19, 24]. Rho activity is also increased in bovine aortic endothelial cells treated with high glucose [25], and involved in the pathogenesis of renal and aortic complications during diabetic states [26, 27]. Furthermore, we revealed that Rho/ROCK pathway is activated in retinal microvessels during diabetes (Figures 1(a)–1(e)).

Rho/ROCK pathway promotes leukocyte adhesion to the microvasculature by affecting the expression and function of adhesion molecules, including ICAM-1 [28, 29] and integrins [30]. Moreover, ROCK causes firm adhesion through the activation of ezrin, radixin, and moesin in endothelial cells, which jointly form the anchoring structures for leukocytes' integrins [31, 32]. These findings suggest that elevated activity of the Rho/ROCK pathway is involved in the pathogenesis of diabetic microvasculopathy mediated through leukocyte adhesion.

Rho/ROCK signaling is also involved in the pathogenesis of VEGF-induced angiogenesis. In endothelial cells ROCK pathway plays a critical role in VEGF-induced endothelial migration by regulating stress fiber formation associated with MLC phosphorylation [17, 33]. Moreover, recent *in vivo* studies have also demonstrated that the ROCK pathway plays a critical role in angiogenesis [34, 35].



(a) (b) (c)

Figure 3: Contractile impacts of ROCK activation in rat retinal vasculature. Intravitreal injections ofllysophosphatidic acid (LPA), Rho activator, were performed into rat's eyes over a period of 1 minute with a 33-gauge needle. The final intraocular concentration of LPA was 20 μM. We monitored the retinal fluorescein with a scanning laser ophthalmoscope ((a) no injection, (b) 5 minutes after injection, (c) 10 minutes after injection). Intravitreal injection of induced sever retinal vessel contraction.

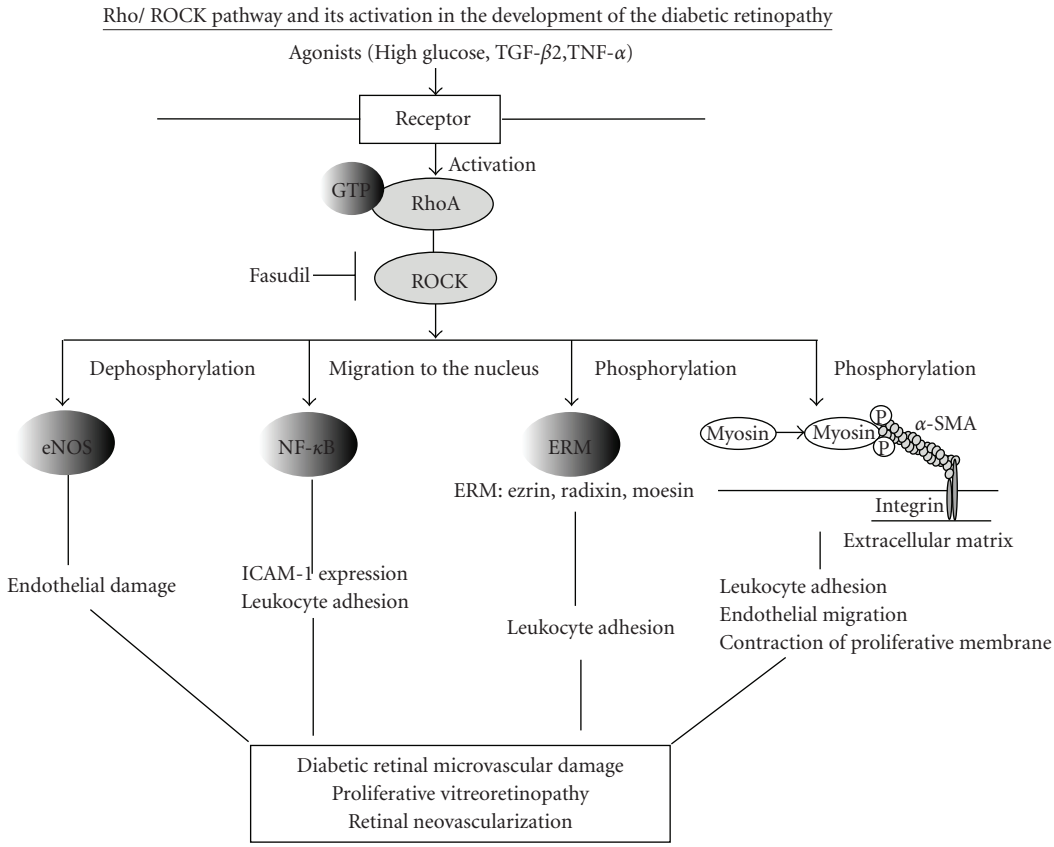


Figure 4: Rho/ROCK pathway and its activation in the development of diabetic retinopathy. RhoA is a small GTP-binding protein, and Rho-kinase (ROCK) is its target protein. Glucose, TNF-α, and TGF-β, elevated in diabetic serum or vitreous, activate Rho/ROCK pathway in endothelial cells or hyalocytes. ROCK activation induces endothelial damage mediated through inactivation of endothelial nitric oxide synthase (eNOS), which has endothelial protective potential. Moreover, ROCK causes firm leukocyte adhesion through the increase of ICAM-1 expression and activation of ezrin, radixin, and moesin (ERM) in endothelial cells. ROCK also has important roles such as leukocyte adhesion, endothelial migration, and contraction of proliferative membrane mediated through myosin light chain (MLC) phosphorylation in diabetic retinopathy. These findings suggest that elevated activity of the Rho/ROCK pathway is involved in the pathogenesis of diabetic microvascular damage, proliferative vitreoretinopathy, and retinal neovascularization.

Previously we showed TGF-β2 contributes to transdifferentiation of hyalocytes into α-SMA positive myofibroblast-like cells that causes hyalocyte-containing collagen gel contraction [19]. Moreover, we revealed that TGF-β2, overexpressed in the vitreous and contractile membranes of PDR patients, activates ROCK pathway and forms stress fibers and contractions mediated through ROCK activation [20, 23]. These results suggest the central role of ROCK in the cicatricial contraction of proliferative membrane in PDR patients.

3. Therapeutic Strategy in Diabetic Retinopathy by ROCK Inhibition

3.1. ROCK Inhibition Ameliorates Diabetes-Induced Microvascular Damage. Fasudil, a potent and selective ROCK inhibitor, is relatively safe and effective in the treatment of cardiovascular disease including cerebral and coronary vasospasm, angina, hypertension, and heart failure with no serious adverse side effect in fasudil-treated patients [36]. In our animal experiments, intravitreal injection of fasudil did not cause apparent electrophysiological or morphological changes in retinal tissues at least within its effective concentrations [23]. However, several adverse effects such as hepatic function abnormal, intracranial hemorrhage, and hypotension have been reported [37, 38]. We thus need further examination regarding the safety and adverse effects of ROCK inhibitor before its clinical use in the field of intraocular diseases.

Nevertheless, we recently could reveal the therapeutic potential of fasudil in the management of earlier stages of DR. Treatment with intravitreal injection of fasudil significantly decreased retinal leukocyte adhesion in diabetic rat mediated through reduction of ROCK activation. Moreover, fasudil effectively suppressed endothelial damage, even when leukocytes firmly adhered to the endothelium (Figures 1(f)–1(i)). This suggests that fasudil directly causes endothelial protection in addition to its impact on leukocyte adhesion. Rho/ROCK inactivates endothelial nitric oxide synthase (eNOS) in human umbilical venous cells [39]. eNOS generates physiological levels of nitric oxide (NO), a potent vasodilator [40] and antiapoptotic factor [41, 42]. Fasudil treatment almost completely reversed the decreased eNOS activity in diabetic rat retinas. In addition, the protective effect of fasudil on microvascular endothelial cells was significantly blocked by NOS inhibition with L-NAME, without apparent effect on leukocyte adhesion *in vitro*. These findings suggest that fasudil has a direct endothelial protective potential through induction of physiological levels of NO, synthesized by eNOS.

3.2. Antiangiogenic Properties of ROCK Inhibitor. We demonstrated that ROCK inhibitor could inhibit VEGF-elicited bovine retinal capillary endothelial cell (BREC) migration and proliferation *in vitro* and corneal neovascularization *in vivo*. A ROCK inhibitor fasudil had inhibitory effect on BREC migration with a scratch-wound assay. Moreover, fasudil could inhibit VEGF-induced BRECs [^{3}H]-thymidine incorporation and ERK1/2 phosphorylation, whose activity indicates the proliferative activities of endothelial cells in angiogenic processes [15]. *In vivo*, fasudil strongly attenuated VEGF-induced corneal neovascularization in a corneal pocket assay [43].

3.3. ROCK Inhibition Suppresses Critical Contraction of Proliferative Membrane. We could also demonstrate the therapeutic potential of ROCK inhibitor fasudil in the management of later stages of DR. In hyalocyte-containing collagen gels assay, fasudil almost completely abolished the PDR vitreous-induced collagen gel contraction mediated through the suppression of MLC phosphorylation (Figures 2(a) and 2(b)). In experimental PVR rabbit model, fasudil also effectively disrupted α-SMA organization and blocked contraction of proliferative membrane (Figures 2(c)–2(i)).

Statins, inhibitors of the 3-hydroxy-3-methyl-glutaryl-(HMG-) CoA reductase, are widely used to reduce endogenous cholesterol synthesis and improve hypercholesterolemia [44]. By inhibiting HMG-CoA reductase, statins also block ROCK activation mediated through the mevalonate pathway [45].

We demonstrated that simvastatin almost completely inhibited vitreous-induced contraction of the collagen gels in *exvivo* and proliferative membrane in experimental PVR model mediated through ROCK inhibition [46]. Our results indicate that ROCK inhibition suppresses PVR progression in later DR stages.

3.4. Other Beneficial Effects of Fasudil on Retinal Tissue during Diabetes

3.4.1. Vasodilatory Property and Improvement of Hemodynamics in the Retinal Vessels. Development of chronic retinal ischemic state aggravates diabetic retinopathy. Rho/ROCK activation plays an important role in the pathogenesis of vasoconstriction, such as cerebral and coronary spasm [47] or hypertension [48], by NO-dependent mechanisms. We could show that intravitreal injection of lysophosphatidic acid, a potential Rho activator, induced severe retinal vessel constriction (Figures 3(a)–3(c)). Recent studies suggested that ROCK inhibitor fasudil improved hemodynamic states in human [49], and also dilated rat retinal vessels, and increases blood flow [48]. These results suggest that fasudil has preventable benefit on retinal ischemia during diabetes through improvement of hemodynamics in the retinal vessels.

3.4.2. Retinal Neuroprotective Effect of Fasudil. Retinal ischemia secondary to DR causes functional and irreversible damage not only in retina vasculature but also in retinal neuronal cells. Chronic loss of neuronal cells from the inner retina by increasing the frequency of apoptosis reduces the thickness of the nerve fiber layer in diabetic retina [50]. Impaired retinal electrophysiology and neurodegeneration have been shown in diabetic patients [51, 52]. Recent studies revealed that Rho/ROCK pathway also seems to be associated with the pathogenesis of this neuronal damage. Abnormal activation of the Rho/ROCK pathway is important in the

pathogenesis of several neurological diseases [53]. In rat retina, Rho/ROCK pathway is also involved in N-methyl-D-aspartat-induced neurotoxicity in the rat retina. [54]. These studies suggest that ROCK inhibitor would protect against neuronal damage by acting directly on neurons. In fact, the ROCK inhibitor Y-27632 increases regeneration of retinal ganglion cell in the rat optic nerve crush model [55]. Moreover, ROCK inhibition attenuates ischemia-induced retinal neuronal cell death by inhibiting leukocytes extravasation and release of proinflammatory cytokines such as TNF-α or IL-6 *in vitro/vivo* [56, 57]. These data suggest that inhibition of Rho/ROCK pathway leads to neuroprotective effect and promote retinal cell survival during diabetes.

4. Conclusion

Rho/ROCK pathway is involved throughout the pathogenesis of DR, particularly in diabetic retinal microvasculopathy, neovascularization, and tractional retinal detachment associated with cicatricial contraction of preretinal proliferative membranes (Figure 4). Since we must consider frequent intravitreal injections as administration method due to a short biological half-life time of the compound in the vitreous cavity, we are considering intravitreal implantation of a slowly releasing drug-delivery system. For preventable benefit on progressing retinal microvascular damage and keeping good visual acuity, timing of intravitreal fasudil implantation prefers when early clinical DR findings such as microaneurysm begin to appear. In addition, pre- and postoperative intravitreal implantations for active PDR patients with proliferative membrane are also considered to be effective for prevention of PVR and tractional retinal detachment. Whereas further basic and clinical studies to reveal the effectiveness and safety of ROCK inhibitor are needed for clinical use in the field of eye diseases, ROCK inhibition might become a novel therapeutic strategy in the total management of DR from its early to late stages.

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Research Article

ERK5 Contributes to VEGF Alteration in Diabetic Retinopathy

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Diabetic retinopathy is one of the most common causes of blindness in North America. Several signaling mechanisms are activated secondary to hyperglycemia in diabetes, leading to activation of vasoactive factors. We investigated a novel pathway, namely extracellular signal regulated kinase 5 (ERK5) mediated signaling, in modulating glucose-induced vascular endothelial growth factor (VEGF) expression. Human microvascular endothelial cells (HMVEC) were exposed to glucose. In parallel, retinal tissues from streptozotocin-induced diabetic rats were examined after 4 months of follow-up. In HMVECs, glucose caused initial activation followed by deactivation of ERK5 and its downstream mediators myocyte enhancing factor 2C (MEF2C) and Kruppel-like factor 2 (KLF2) mRNA expression. ERK5 inactivation further led to augmented VEGF mRNA expression. Furthermore, siRNA mediated ERK5 gene knockdown suppressed MEF2C and KLF2 expression and increased VEGF expression and angiogenesis. On the other hand, constitutively active MEK5, an activator of ERK5, increased ERK5 activation and ERK5 and KLF2 mRNA expression and attenuated basal- and glucose-induced VEGF mRNA expression. In the retina of diabetic rats, depletion of ERK5, KLF2 and upregulation of VEGF mRNA were demonstrated. These results indicated that ERK5 depletion contributes to glucose induced increased VEGF production and angiogenesis. Hence, ERK5 may be a putative therapeutic target to modulate VEGF expression in diabetic retinopathy.

1. Introduction

Diabetic retinopathy (DR) is a devastating complication of diabetes, manifesting primarily as vascular structural and functional changes in the retina, eventually leading to vision loss. DR is the most common cause of blindness in North America in the 25–74-years age group [1]. Glucose-induced increased production of vasoactive factors are characteristics of all chronic diabetic complications including DR. Vascular endothelial growth factor (VEGF) is a key vasoactive factor, which is upregulated in the retina in DR. VEGF is an important mediator of increased vascular permeability in early DR and a major contributor of retinal neovascularization in proliferative DR [1–4]. In human, VEGF mRNA expression is increased in the neovascular membranes from diabetic patients obtained by vitrectomy compared to those removed from the nondiabetic individuals [4]. Augmented VEGF protein production was further observed in human retina in diabetes using immunohistochemistry [2, 3]. We and others have demonstrated increased VEGF expression in the retina of streptozotocin-induced diabetic rat model causing increased microvascular permeability [5, 6]. In a murine model of ischemic retinopathy, inhibition of VEGF has also been shown to suppress retinal neovascularization [7]. Furthermore exposure of endothelial cells (ECs) to high glucose causes increased VEGF expression [8, 9]. VEGF mRNA and protein expression are stimulated by long-term high glucose treatment in bovine microvascular retinal ECs [10]. Previous studies in our lab have demonstrated that VEGF interacts with other vasoactive factors such as endothelin-1 (ET-1) in mediating glucose-induced increased permeability in the ECs [5, 8]. These data indicate that VEGF plays an important role in causing increased vascular permeability and angiogenesis in DR [2, 3]. Several glucose induced signaling mechanisms such as protein kinase C (PKC) activation, nonenzymatic glycation and mitogen-activated protein kinase (MAPK) activation are instrumental in causing glucose induced alteration of vasoactive factors in diabetes [11].

ERK5, also known as big MAPK1 (BMK1), was identified as a member of the MAPK family in 1995 [12, 13]. ERK5 is a protein of 816 amino acid residues with a large COOH

terminal. BMK1 is different from other MAPK as it has a transcriptional activation domain. MAPK/ERK kinase 5 (MEK5) is the specific MAPK kinase for ERK5. ERK5 is highly expressed in the ECs [14]. Studies on ERK5 knockout mice have shown that the ERK5 pathway is critical for endothelial function and for maintaining blood vessel integrity [15]. In addition, ERK5 signaling mediates stress response in the ECs [14, 16]. More recently, studies have shown that ERK5 signaling controls migration and morphology of the ECs [17].

It has been demonstrated that in the nondiabetic conditions, ERK5 have a regulatory role on VEGF expression [18–20]. In mouse, mutant ERK5 gene (homozygous knockout) increases expression of VEGF mRNA and protein [19, 20]. Moreover, ERK5 represses VEGF expression in bovine lung microvascular ECS [18]. Investigations have shown that overexpression of Krupple-like factor 2 (KLF2), a target gene of ERK5, inhibited VEGF receptor promoter activation [21]. KLF2 overexpression also counteracts VEGF-mediated inflammatory responses in the ECs [22]. In addition, microarray studies have revealed that KLF2 overexpression decreases mRNA expression of human VEGF receptor 2 [23, 24]. Hence it is potentially possible that ERK5 through KLF2 may also have a regulatory role on the production of VEGF in DR. However such possibilities have not been investigated. Here, we examined the mechanisms of glucose-induced ERK5-mediated regulation of VEGF in the ECs and in the retina of diabetic rats. We further explored the significance of such changes in DR.

2. Materials and Methods

2.1. Cell Culture. A dermal-derived human microvascular endothelial cell (HMVEC) was obtained from Lonza, Inc. (Walkersville, MD). HMVECs were grown in endothelial cell basal medium 2 (EBM-2, Lonza, Walkersville, MD) containing human epidermal growth factor (hEGF), 1‰; Hydrocortisone, 0.4‰; gentamycin, 1‰; fetal bovine serum (FBS), 10‰; vascular endothelial growth factor (VEGF), 1‰; human fibroblast growth factor-basic (hFGF-B), 4‰; long R3 insulin-like growth factor (R3-IGF-1), 1‰; Ascorbic Acid, 1‰. In EBM2, the glucose concentration was 5 mmol/l. Cells were grown in 25 cm^2 tissue culture flasks and maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Cells at 80% confluence were growth arrested by incubation in serum-free medium overnight prior to incubation with high glucose (25 mmol/l D-glucose) or osmotic control (L-glucose) of the same concentration.

2.2. Gain and Loss of Function Studies. For gain of function study, constitutively active human recombinant MEK5 (CAMEK5, 20MOI) adenovirus (Cell Biolabs, San Diego, CA) was used to activate ERK5. Cells were seeded in 6-well plate, cultured overnight and infected with adenovirus for 48 hrs. A nonspecific GFP adenovirus with the same multiplicity of infection (20MOI) was used as a negative control. For loss of function study, ERK5 siRNA (siERK5) was used to knock down ERK5 expression in endothelial cells. Endothelial cells were transfected with ERK5 siRNAs (ON-TARGET*plus* siRNA, 100 nmol/l; Dharmacon Inc. Lafayette, CO) for 48 hrs using siRNA transfection reagent (DharmaFECT 4; Dharmacon Inc. Lafayette, CO) as described before in [25]. A nontargeting siRNA (siGENOME NonTargeting Pool; Dharmacon Inc. Lafayette, CO) with the same concentration of ERK5 siRNA was used as a negative control (control siRNA). siRNA knock down efficiency was determined by real-time RT-PCR.

Table 1: Oligonucleotide sequences for real-time PCR.

Gene	Sequence 5′ → 3′
ERK5 (human)	CTGGCTGTCCAGATGTGAA
	ATGGCACCATCTTTCTTTGG
MEF2C (human)	TACAACGAGCCGCATGAGAG
	CCTGTGTTACCTGCACTTGG
KLF2 (human)	GCACGCACACAGGTGAGAAG
	ACCAGTCACAGTTTGGGAGGG
VEGF (human)	GGCCTCCGAAACCATGAACTTTCTGCT
	GCATGCCCTCCTGCCCGGCTCACCGC
VEGF (rat)	CTGCTGTCTTGGGTGCATTGG
	CACCGCCTTGGCTTGTCACAT
β-actin (human and rat)	CCTCTATGCCAACACAGTGC
	CATCGTACTCCTGCTTGCTG
18S (human and rat)	GTAACCCGTTGAACCCCATT
	CCATCCAACGGTAGTAGCG

2.3. RNA Isolation and cDNA Synthesis. TRIzol reagent (Invitrogen, Burlington, ON, Canada) was used to isolate RNA as previously described in [26]. RNA was extracted with chloroform followed by centrifugation to separate the sample into aqueous and organic phases. RNA was recovered from the aqueous phase by isopropyl alcohol precipitation and suspended in diethylpyrocarbonate-treated water. Total RNA (2–4 μg) was used for cDNA synthesis with High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA). The resulting cDNA products were stored at −20°C.

2.4. Real-Time RT-PCR. Real-time RT-PCR was performed using the LightCycler (Roche Diagnostics Canada, Laval, PQ, Canada) as previously described in [27]. For a final reaction volume of 20 μl, the following reagents were added: 10 μl SYBR Advantage qPCR Premix (Clontech, Mountain View, CA), 1 μL of each forward and reverse 10 μM primers (Table 1), 7 μL H_2O, and 1 μL cDNA template. Messenger RNA (mRNA) levels were quantified using the standard curve method. Standard curves were constructed by using serially diluted standard template. The data were normalized to 18S ribosomal RNA or β-actin RNA to account for differences in reverse transcription efficiencies and the amount of template in the reaction mixtures.

2.5. Western Blot Analysis. Total proteins from endothelial cells were isolated by homogenizing cells in lysis buffer (contains 25 mmol/l Tris·HCl, pH 7.5, 150 mmol/l NaCl, 5 mmol/l $MgCl_2$, 1% NP-40, 1 mmol/l DTT and 5% glycerol) and protease inhibitor (complete Mini tablet, Roche) and phosphatase inhibitor cocktail 1 and 2 (Sigma-Aldrich, Saint Louis, MO). Protein concentrations were determined by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). 30 μg of protein was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (BIO-RAD, Hercules, CA). The membrane was then incubated with the rabbit antiphospho-ERK5 antibody (1 : 1000; Cell Signaling Technology, MA). Horseradish peroxidase-conjugated antirabbit antibody (1 : 10000; Upstate Biotechnology, Charlottesville, VA) was used for detection. The signals from the western blots were visualized with an ECL plus chemiluminescence detection kit (Amersham Pharmacia Biotechnology, Buckinghamshire, UK). Blots were stripped with ReBlot Plus Strong Antibody Stripping Solution (Millipore Corporation, Billerica, MA) and reprobed with ERK5 antibody (1 : 1000; Cell Signaling Technology, MA). Blots were then stripped again and reprobed with β-actin antibody (1 : 1000, Santa Cruz Biotechnology, CA) as a control for protein loading. Blots were quantified by densitometry using Mocha software (SPSS, Chicago, IL) and the data expressed as a ratio of phosphor-Erk5 to β-actin.

2.6. In Vitro Angiogenesis Assay. The angiogenic responses to high glucose (25 mmol/l) and ERK5 siRNA transfection were assessed using an in vitro Matrigel analysis. Following appropriate treatment, HMVECs were seeded in 96-well culture plates precoated with ECMatrix (In Vitro Angiogenesis Assay Kit, Milipore, Billerica, MA) at 1×10^4 cells/well. Cells were maintained in serum-free medium at 37°C for 6 hrs. The tube-like structures were visualized by a Leica inverted light microscope. Images were captured with Infinity Capture software Version 3.5.1 at ×10 magnification after 6 hrs incubation. To quantify the image of tube formation, branch points were counted in several random microscopic fields (3–5) per sample and the values averaged. At least 3 different cultures were counted per experimental group. The data were expressed as number of branch points per100× field.

2.7. Animal Experiments. Male Sprague-Dawley rats (Charles River) weighing between 200 and 250 g were used. Diabetes was induced by a single intravenous injection of streptozotocin (65 mg/kg, in citrate buffer, pH 5.6). Age-and sex-matched rats were used as controls and given equal volume of citrate buffer [28]. The animals were monitored for glucosuria and ketonuria (Uriscan Gluketo; Yeong Dong, Seoul, South Korea). All diabetic rats were implanted with slow release insulin implants to prevent ketosis (approximately 2 U/day) (LinShin, Scarborough, ON, Canada). They were sacrificed after 4 month of diabetes. We have previously demonstrated that they develop diabetes induced tissue damage in the retina and kidney at this time [29]. The eyes were immediately enucleated, lens and vitreous removed. The retinas of the right eye were gently peeled off, snap-frozen in liquid nitrogen, and stored at −70°C. The left retinas were fixed in 4% paraformaldehyde (PFA), as described before in [5]. All animals were cared for according to the Association for Research in Vision and Ophthalmology's *Guiding Principles in the Care and Use of Animals*. All experiments were approved by the University of Western Ontario Council on Animal Care Committee.

2.8. Immunohistochemistry. Formalin fixed retinal tissues were embedded in paraffin, sectioned at 4 μM thickness, and placed on positively charged slides for phosphor-ERK5 (pERK5) immunohistochemical staining. Briefly, the sections were incubated with rabbit anti-pERK5 antibody (Invitrogen, Carlsbad, CA, USA) at 1 : 200 dilutions overnight at 4°C, followed by incubation with labeled polymer-HRP anti-rabbit antibody (Dako North America, Carpinteria, CA, USA) for 30 minutes at room temperature. Visualization was conducted using diaminobenzidine (DAB, Dako North America, Carpinteria, CA, USA) as HRP substrate. Slides were counterstained with Hematoxylin. Staining with nonimmune rabbit serum instead of primary antibodies was used as negative controls. Images were recorded by an Olympus BX51 microscope (Olympus Canada Inc, ON, Canada) with Northern Eclipse software (Empix Inc, ON, Canada).

2.9. Statistical Analysis. Data were presented as the mean ± standard error. Statistical significance of difference between groups was tested using Student's t-test or if there were more than two groups, using one way analysis of variance (ANOVA) followed by posthoc analysis. A P value of .05 or less was considered to be significant. All calculations were performed using SPSS version 15.0.

3. Results

3.1. Glucose Caused ERK5 Alteration and VEGF Upregulation. We initially established whether in the ECs, glucose causes any alteration of ERK5 signaling. No change in the mRNA expression of ERK5 and its downstream molecules MEF2C, KLF2, or VEGF were seen after exposure of the cells to 25 mmol/l glucose (HG) for 1 hour compared to 5 mmol/l glucose (LG) (Figures 1(c)–1(f)). L-glucose was used as an osmotic control (OC). Following 24 hrs of glucose exposure, ERK5 signaling was significantly activated as evidenced by increased phosphorylation (Figures 1(a) and 1(b)) and mRNA expression of ERK5 (Figure 1(c)) and augmented mRNA expression of MEF2C (Figure 1(d)) and KLF2 (Figure 1(e)), which are downstream mediators of ERK5 signaling. Interestingly, at this time point there were no increases of VEGF mRNA expression (Figure 1(f)). However, with increased duration of high-glucose treatment (48 hrs), ERK5 phosphoryation and mRNA expression (Figures 1(a)–1(c)) as well as MEF2C and KLF2 mRNA expression (Figures 1(d) and 1(e)) were decreased, while VEGF mRNA expression was increased (Figure 1(f)). Similar results were also observed after 72 hrs of HG treatment (data not shown).

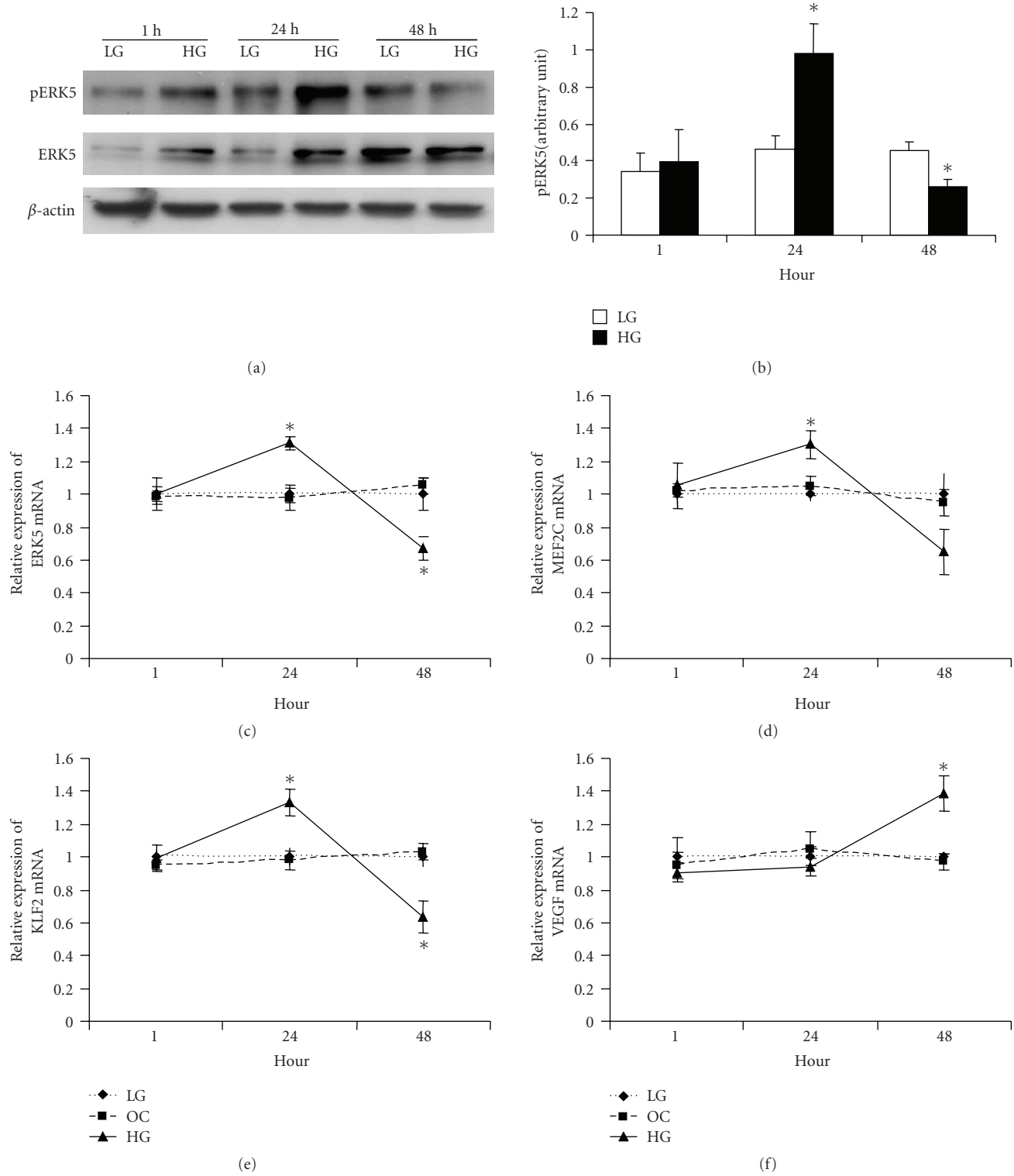

Figure 1: (a) Representative Western Blot showing increased pERK5 and total ERK5 after 24 hrs HG treatment which subsequently decreased after 48 hrs. (b): Densitometric quantification of pERK5 expression. (c–f): Real-time PCR showed that following incubation in HG, ERK5, MEF2C and KLF2 mRNA expression were increased after 24 hrs and then were decreased after 48 hrs. On the other hand, VEGF mRNA expression did not increase until after 48 hrs of HG treatment. LG : 5 mmol/l D-glucose; OC: osmotic control, 25 mmol/l L-glucose; HG: 25 mmol/l D-glucose. [Data in "B" are expressed as a ratio to β-actin. mRNAs are expressed as a ratio to 18S, normalized to controls, * significantly different from LG, $n = 3 - 6$/group.]

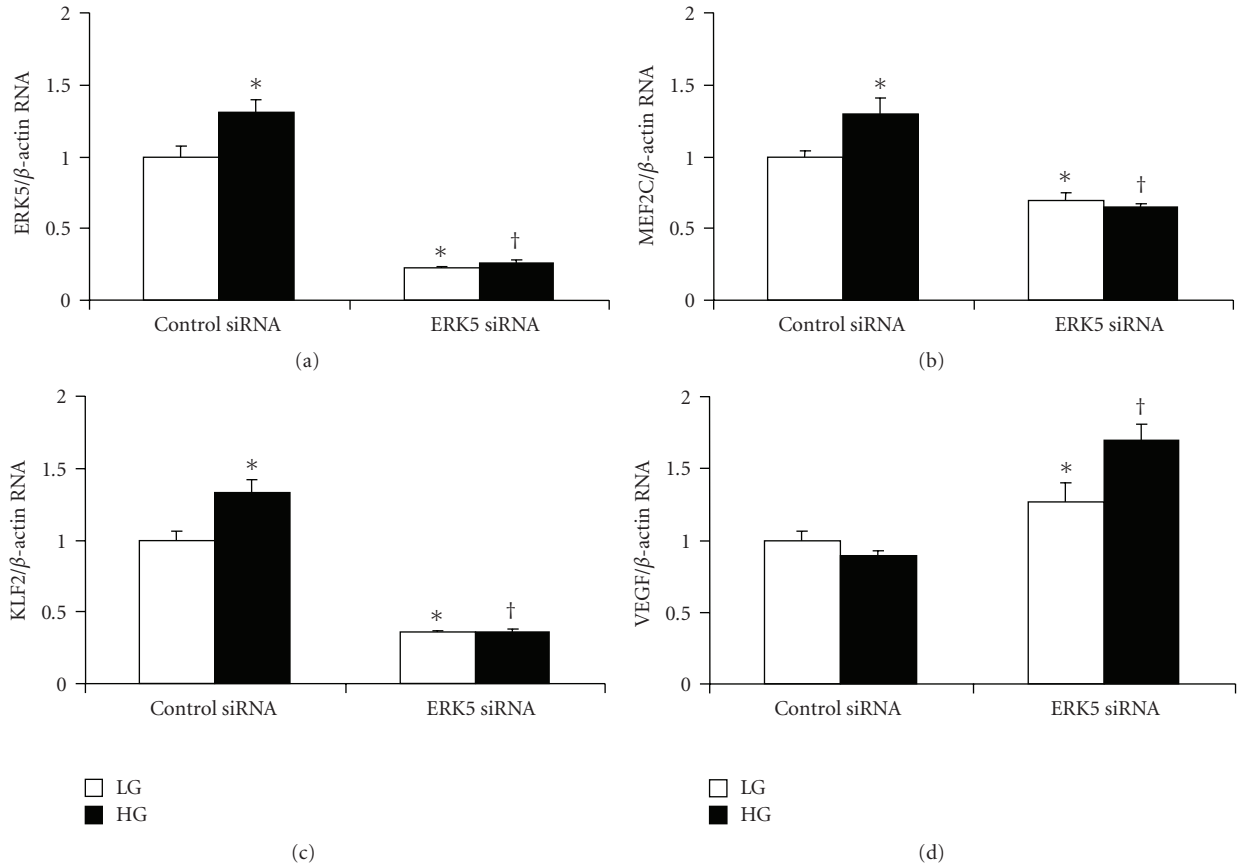

Figure 2: ERK5 siRNA transfection reduced basal (5 mmol/l, LG) and glucose induced (25 mmol/l, HG) mRNA expression of ERK5 (a), MEF2C (b), KLF2 (c) in endothelial cells after 24 hrs of HG treatment. On the other hand, such transfection augmented VEGF mRNA expression (d). [Data are expressed as a ratio to β-actin, normalized to controls, *: significantly different from control siRNA in LG, **: significant difference from control siRNA in HG, $n = 6$/group].

Since expression of total ERK5 was also changed after glucose treatment, western blot of pERK5 was normalized to β-actin. To further delineate the mechanistic role and significance of glucose-induced ERK5 activation, especially with its regulatory effects on VEGF expression, gain and loss of function studies were performed.

3.2. ERK5 Downregulation Led to Increased VEGF Expression. Our initial investigation indicated that VEGF mRNA expression was inversely related with ERK5 activation following high-glucose treatment. To further explore the interaction of ERK5 signaling and VEGF expression, loss of function study was performed in the HMVECs using ERK5 siRNA. ERK5 siRNA was transfected to endothelial cells to knock down ERK5 gene. Real-time PCR analyses demonstrated that such transfection led to >70% reduction of ERK5 mRNA expression (Figure 2(a)). ERK5's downstream substrates, MEF2C and KLF2, were also significantly reduced (Figures 2(b) and 2(c)). As expected, VEGF mRNA expression was increased following ERK5 siRNA transfection measured by real-time PCR (Figure 2(d)). To further study the effect of ERK5 knockdown on glucose induced VEGF upregulation, HMVECs were transfected with ERK5 siRNA and then treated with high glucose for short period (24 hrs). Results showed that ERK5 siRNA transfection abolished high-glucose-induced increase of ERK5, MEF2C, KLF2 mRNA (Figures 2(a)–2(c)), whereas promoted upregulation of VEGF mRNA (Figure 2(d)).

3.3. Functional Significance of Glucose-Induced–ERK5 Mediated VEGF Upregulation. We further expanded the investigations to examine functional significance of glucose-induced ERK5 mediated VEGF expression. As glucose-induced VEGF upregulation plays an important role in neovascularization in proliferative DR, we examined whether alteration of ERK5 and subsequent change in VEGF has any effects on endothelial tube formation using and an *in vitro* angiogenesis assay. High-glucose treatment (48 hrs) stimulated branching and tube formation in endothelial cells transfected with control siRNA (Figures 3(a) and 3(b)), which was similar as our finding in endothelial cells without transfection (data not shown). Glucose induced tube formation paralleled increased VEGF mRNA expression measured by real-time PCR (Figure 1(e)). Endothelial cells transfected with ERK5 siRNA rapidly formed capillary-like tube structures (Figure 3(c)). High-glucose treatment further

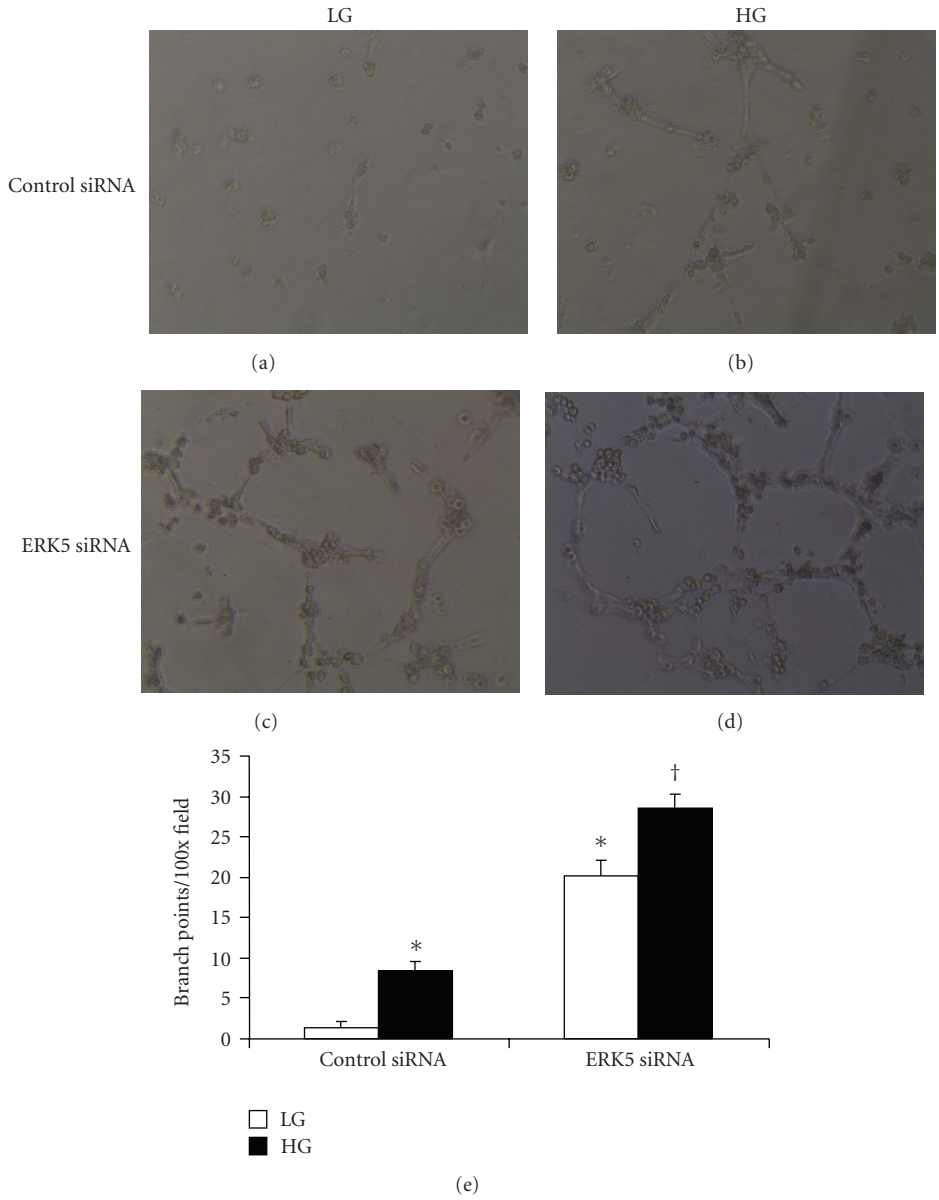

Figure 3: ERK5 siRNA enhanced tube formation in ECs under normal and high glucose conditions. (a–d): Representative phase-contrast photographs of in vitro angiogenesis assay showing tube formation in ECs. Such tube formations were pronounced following ERK5 siRNA transfection ((c) and (d)), compared with control siRNA transfection ((a) and (b)), both in 5 mmol/l (LG) or 25 mmol/l (HG) glucose. Original magnification at 100x. (e): Quantification of tube formation by counting branch points of tube-like structures confirmed stimulatory effect of ERK5 siRNA on tube formation. [Data are expressed as number of branch points per 100× field, * significantly different from control siRNA in LG, †: significant difference from control siRNA in HG, $n = 3$/group].

augmented the number and size of tube-like structure formation (Figure 3(d)). Quantification of tube formation is shown in Figure 3(e). Such increase in tube formation was associated with pronounced VEGF mRNA expression (Figure 2(d)). These results suggest that decreased ERK5 stimulates angiogenesis by increasing VEGF expression.

3.4. ERK5 Upregulation Inhibited VEGF Expression in Endothelial Cells. We then investigated whether ERK5 upregulation can protect endothelial cells in pathological conditions mediated by glucose. As glucose-induced tissue damage is mediated by vasoactive factors such as VEGF, we proceeded to examine whether glucose induced upregulation of VEGF mRNA can be prevented by constitutively active MEK5 (CAMEK5).

MEK5 is a specific MAPK kinase for ERK5 [13, 30, 31]. Hence, use of CAMEK5 adenovirus to upregulate ERK5 signaling is a rational approach. CAMEK5 not only activated ERK5 phosphorylation, but also augmented ERK5 transcription (Figures 4(a) and 4(b)). Western Blot confirmed increased pERK5 after CAMEK5 infection in HMVECs (Figure 4(a)). Real-time PCR showed that ERK5 mRNA level

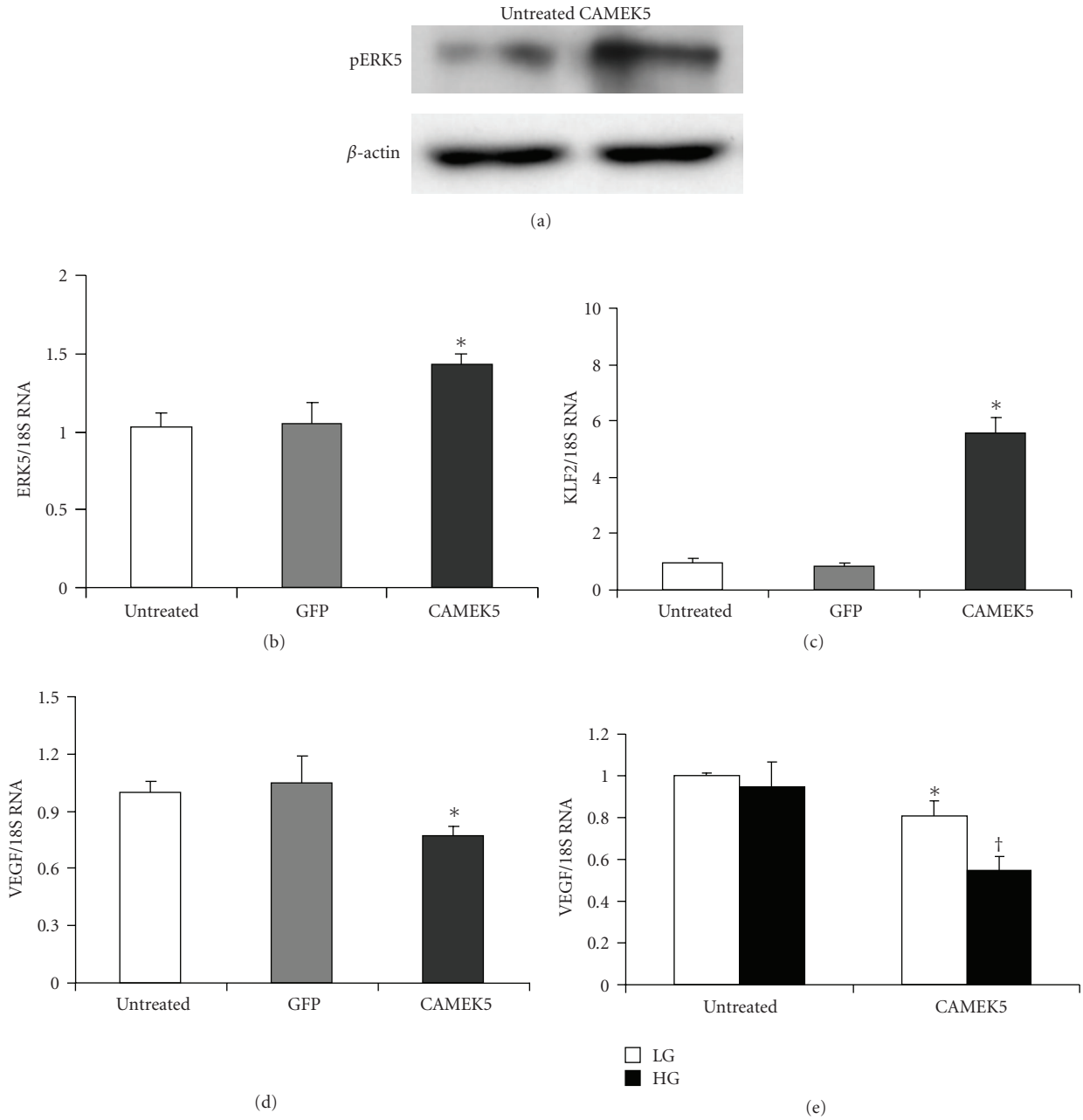

Figure 4: In ECs, constitutionally active MEK5 (CAMEK5) caused increased ERK5 phosphorylation as indicated by (a) Western blot using phospho ERK5 antibody. CAMEK5 also caused mRNA upregulation of ERK5 (b), KLF2 (c) and downregulation of VEGF (d). Twenty-four hours exposure of 25 mmol/l of glucose (HG) significantly decreased VEGF mRNA expression after CAMEK5 infection (e). [mRNAs are expressed as a ratio to 18S, normalized to control, $n = 5$/group, *: significantly different from untreated or GFP controls, †: significantly different from other groups].

after CAMEK5 infection was significantly higher than that of GFP control and untreated control (Figure 4(b)). ERK5 activation caused upregulation of KLF2 mRNA expression (Figure 4(c)) and downregulation of VEGF mRNA expression. (Figure 4(d)).

To study the effect of CAMEK5 on glucose-induced VEGF expression, HMVECs was infected with CAMEK5 and then treated with high glucose for 24 hrs. VEGF mRNA was slightly decreased by CAMEK5-induced activation of ERK5 in LG groups, while constitutive activation of ERK5 by CAMEK5 infection led to a significant decrease of VEGF after HG treatment (Figure 4(e)).

3.5. Reduced ERK5 Activation Is Associated with Increased VEGF mRNA Expression in Retinas of Diabetic Rats. From the perspective of DR, it is important to examine whether the alterations demonstrated in the endothelial cells are indeed important in a clinically relevant model of diabetic retinal microangiopathy. Hence, we investigated retinas from

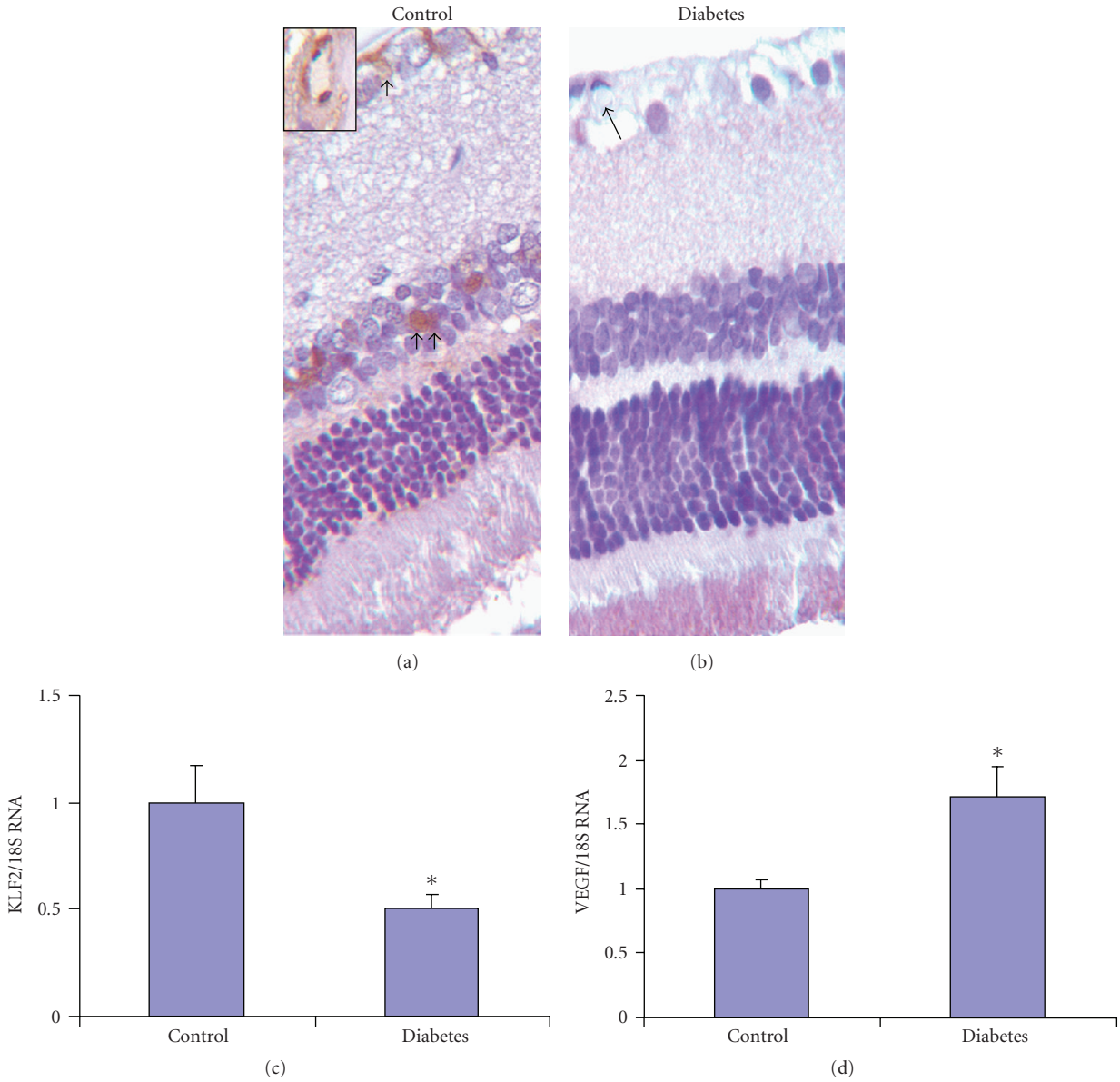

Figure 5: Immunohistochemical staining of pERK5 in the retina showing reduced pERK5 protein expression in the retina of diabetic rats (b) compared to the control animals (a). Positive pERK5 staining was localized in the ganglion cell and inner nuclear layers (arrows). Inset is an enlarged view of a microvessel showing positive pERK5 staining in the endothelial cells. Real-time PCR analysis showed reduced KLF2 (c) and augmented VEGF (d) mRNA expression in the retina of diabetic rats compared to the controls. [Original magnification at 100x. mRNAs are expressed as a ratio to 18S, normalized to control, $n = 5$/group, * significantly different from controls.]

a well-established model of diabetic retinal microangiopathy. Phospho-ERK5 immunohistochemical staining was performed in the retinal tissues of STZ-induced diabetic rats after 4 month of followup. Diabetic animals showed increased blood glucose levels, reduced body weight gain, glucosuria, and occasional ketonuria (data not shown). Positive pERK5 staining was localized in the ganglion cell layer and in the inner nuclear layer. Microscopic examination further revealed that such positivity was in the microvasculature as well as in other cells. The number of positive cells and the intensity of staining were reduced in the retina of diabetic animals compared with that of controls (Figures 5(a) and 5(b)). We also examined the mRNA expression of ERK5, KLF2, and VEGF in retinal tissues of 4-month diabetic and control rats. Real-time PCR showed that ERK5 mRNA expression in diabetic group was too low to be detected (data not shown). In parallel, mRNA expression of KLF2, a downstream molecule of ERK5 signaling, in diabetic group was markedly lower than that of control group (Figure 5(c)). In keeping with our previous data from our lab and others, real-time PCR analyses demonstrated a significant increase of VEGF mRNA expression in the retina of diabetic rats (Figure 5(d)).

4. Discussion

Here we have demonstrated a novel mechanism of glucose mediated VEGF gene upregulation in diabetes. We have shown that in the ECs glucose causes transient activation of ERK5 followed by deactivation. Reduced activity of ERK5 was associated with upregulation of VEGF mRNA and angiogenesis. We have confirmed such negative regulation of ERK5 on VEGF using ERK5 silencing and overexpression. Furthermore, we have found similar alteration of ERK5 and VEGF in the retina of diabetic animals.

A substantial body of evidence indicates that VEGF is a major angiogenic factor involved in DR [32, 33]. MAPK signaling pathways play essential roles in modulating expression of VEGF [34]. Constitutive activation of ERK1/2 elevated expression of VEGF mRNA [35]. Overexpression of p38 and JNK activation increased halflife of VEGF mRNA [36]. In addition, knockout animal study showed that JNK regulated VEGF expression at the transcriptional level in hypoxia induced retinal VEGF production [37]. A recent publication revealed that Wnt signaling is activated in DR and upregulates VEGF expression [38]. On the other hand ERK5, as demonstrated in this paper, is the only protective signaling that is activated by high glucose. In this study, we examined the effect of ERK5 on high-glucose-induced VEGF expression and demonstrated a novel pathway that potentially contributes to VEGF expression and subsequent angiogenesis in DR.

ERK5 is different from other MAPKs because of its unique C-terminal, which contains transcriptional activation domain. Transcriptional activation can enhance the effect of ERK5 signaling [30]. Hence, we examined ERK5 transcription in our study which paralleled its downstream effects. Expression of this transcription domain is sufficient to drive MEF2 and regulate MEF2-dependent gene expression [39]. The importance of transcriptional activation ERK5 have also been previously demonstrated by its effects on inhibition of ERK5 SUMOylation and prevention of diabetes-mediated left ventricular dysfunction [40]. However, ERK5 also activates signaling using traditional phosphorylation [41]. Studies have shown that the activated kinase activity of ERK5 undergoes autophosphorylation on its most C-terminal region, which is required for the C-terminal-half to enhance the ERK5 activity [30]. In this study we have demonstrated both attenuated ERK5 transcription and phosphorylation in high-glucose treated endothelial cells. Our data indicate that both mechanisms may be operating in glucose-induced VEGF upregulation.

It has been previously reported that MEF2-KLF2 counteracted VEGF-mediated inflammatory responses in endothelial cells [22]. In addition, KLF2, a downstream molecule of ERK5, is a transcriptional regulator of angiogenesis, and overexpression of KLF2 counteracts VEGF-mediated angiogenesis due to a potent inhibition of VEGFR2 expression and promoter activity [21]. Our study found that reduced ERK5, MEF2C, and KLF2 expression were along with increased VEGF level after 48 hrs glucose exposure (Figure 1). On the other hand, after 24 hrs, although ERK5 was activated, we failed to observe VEGF upregulation. Exact reason for such findings is not clear. Possible explanation may include other regulatory factors and cell specific factors, which need further characterization. Nevertheless, ERK5 siRNA transfection significantly reduced MEF2C and KLF2 mRNA expression (Figure 4), suggesting that ERK5 negatively regulates VEGF through MEF2C and KLF2 upon high glucose treatment. Under hypoxic conditions, ERK5 inhibits VEGF via hypoxia inducible factor 1α (HIF1α) in endothelial cells [18]. In addition, it has been shown that KLF2 inhibits HIF1a and hypoxia-mediated angiogenesis [42]. High glucose induced a state of pseudo-hypoxia in diabetic complications [43, 44]. It is therefore possible that depletion of ERK5/KLF2 signaling may promote high glucose-induced angiogenesis via HIF1a. However, such notion has to be further established by specific experiments.

In keeping with our finding it has been demonstrated that ERK5 activation is induced transiently by high glucose in endothelial cells, which ultimately decreased after long-term treatment [45]. As following long-term glucose exposure in ECs and in the retina of chronically diabetic animals, similar pattern of ERK5 and VEGF were seen, it is possible that in long-term diabetes inhibitory effects of ERK5 is lost, leading to VEGF upregulation. In this study we have seen VEGF upregulation after 48 hrs. This is in keeping with studies in endothelial cells from other sources [9, 46]. Some studies have demonstrated VEGF upregulation following a short period of glucose exposure. Various sources of cells and culture conditions may in part be responsible for such discrepancy. In addition simultaneously other mechanisms may also be responsible for glucose induced VEGF upregulation. Both PKC and ERK1/2 activation have been demonstrated to regulate VEGF in glomerular podocytes [47]. In this study we observed that after 24 hrs glucose treatment, although there was significant activation of ERK5, there were no significant downregulation of VEGF in this system. This suggests that other mechanisms mentioned above may also modulate glucose-induced VEGF, further investigation is required to delineate the relationship of ERK5 and other signaling pathways.

It is well established that oxidant and shear stress can regulate ERK5 alteration. Exact mechanism of glucose induced ERK5 alteration is still not known, however it is possible that glucose-induced oxidative stress is a key player in ERK5 change. Growth factors such as epidermal growth factor (EGF) and nerve growth factor (NGF) activate ERK5 [48, 49]. Hence several additional mechanisms may potentially regulate glucose induced ERK5 alteration. We, however, understand some of the limitations of this study as the in vitro experiments were performed in the microvascular cells of nonretinal origin. We tried to address some of these problems with simultaneous experiments at levels of complexities, that is, retinal tissues. However, additional future studies are needed in various cell types to further characterize these changes.

In summary, this is the first study to show that ERK5 may potentially regulate VEGF upon high-glucose treatment in the ECs and in the retina of diabetic rats. Although this study was done in the context of diabetic retinopathy, it is possible that such process is of importance in other diabetic

complications involving VEGF signaling. ERK5 may also provide an attractive target for drug development in DR and other diabetic vascular complications.

Acknowledgment

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Research Article

NADPH Oxidase *versus* Mitochondria-Derived ROS in Glucose-Induced Apoptosis of Pericytes in Early Diabetic Retinopathy

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Objectives. Using apocynin (inhibitor of NADPH oxidase), and Mitoquinol 10 nitrate (MitoQ; mitochondrial-targeted antioxidant), we addressed the importance of mitochondria versus NADPH oxidase-derived ROS in glucose-induced apoptosis of pericytes. *Methods*. NADPH oxidase was localised using Western blot analysis and cytochrome C reduction assay. Apoptosis was detected by measuring caspase-3 activity. Intracellular glucose concentration, ROS formation and Nε-(carboxymethyl) lysine (CML) content were measured using Amplex Red assay kit, dihydroethidium (DHE), and competitive immunoabsorbant enzyme-linked assay (ELISA), respectively. *Results*. NADPH oxidase was localised in the cytoplasm of pericytes suggesting ROS production within intracellular compartments. High glucose (25 mM) significantly increased apoptosis, intracellular glucose concentration, and CML content. Apoptosis was associated with increased gp91phox expression, activity of NADPH oxidase, and intracellular ROS production. Apocynin and not MitoQ significantly blunted the generation of ROS, formation of intracellular CML and apoptosis. *Conclusions*. NADPH oxidase and not mitochondria-derived ROS is responsible for the accelerated apoptosis of pericytes in diabetic retinopathy.

1. Introduction

Diabetic retinopathy is a leading cause of blindness that is characterized by vascular changes of the retinal capillary bed [1]. One of earliest changes is the accelerated apoptosis of retinal microvascular cells and the formation of acellular capillaries [2]. Although, the frequency of pericytes and endothelial cell apoptosis is thought to predict the development of the histological lesions in retinopathy [3], the underlying cause is not fully understood. Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) have confirmed that hyperglycaemia is the major factor in the development of diabetic retinopathy [4, 5]. In addition to the prevailing biochemical mechanisms [1] on how glucose leads to pathological changes in retinopathy, recent evidence suggests a key role for oxidative stress, a state in which excess reactive oxygen species (ROS) overwhelm endogenous antioxidant systems [6]. ROS can be produced from the mitochondrial transport chain and a number of enzymes that are localised in the plasma membrane, and the cytoplasm of cells [7].

The elegant study carried out by Brownlee and colleagues [8] suggests that glucose-induced mitochondria production of ROS stimulates several of the biochemical mechanism thought to be involved in hyperglycaemia-mediated complications of diabetes, including retinopathy. The authors proposed that the causal link between glucose and vascular damage in diabetes is the increased production of superoxide

by the mitochondrial electron transport chain [8]. However, increasing evidence suggests that NADPH oxidase is the most important source of cellular ROS in blood vessels [9]. NADPH oxidase complex in neutrophils and, most probably, endothelial cells and other cell types, involves four essential subunits. The subunits gp91phox and p22phox reside in the plasma membrane [9]. These subunits bind the components of the electron transport chain heme and FAD, forming cytochrome b_{558}. The cytosolic NADPH oxidase subunits p47phox and p67phox are involved in the activation of the enzyme complex. Unlike the phagocytic type, the NADPH oxidases present in blood vessels are constitutively active, producing relatively low levels of ROS under basal conditions, and generating higher levels of oxidants in response to cytokines [9]. Among the nonphagocytic cells examined so far, endothelial NADPH oxidase has been investigated more extensively [9].

By using apocynin and Mitoquinol 10 nitrate (MitoQ) we addressed the importance of mitochondria versus NADPH oxidase-derived ROS in glucose-induced apoptosis of cultured retinal capillary pericytes. Apocynin is a methoxy-substituted catechol that does not act as a ROS scavenger, but inhibits NADPH oxidase by impeding the assembly of p47phox and p67phox subunits within the membrane NADPH oxidase complex [10]. MitoQ which has an antioxidant ubiquinol moiety attached to a triphenylphosphonium cation by an aliphatic carbon chain is recently developed mitochondria-targeted antioxidants that selectively block mitochondrial oxidative damage [11]. The selective accumulation of MitoQ prevents mitochondrial oxidative damage far more effectively than untargeted antioxidants. It is not only accumulated by the mitochondria but also can be regenerated in its reduced form by mitochondrial respiratory chain [11]. Here we show for the first time, that it is ROS derived from NADPH oxidase and not the mitochondria, which is involved in apoptosis of pericytes induced by chronic exposure to high glucose.

2. Materials and Methods

2.1. Culture of Bovine Retinal Capillary Pericytes. Bovine retinal capillary pericytes (BRPs) were established from bovine retinas dissected from eyes of freshly slaughtered cattle as described previously in [12]. Briefly, the isolated retinas were homogenised in serum-free minimal essential medium (MEM; Sigma, UK), and filtered through 80 μm nylon mesh. The trapped micro vessels were digested with collagenase-dispase (1 mg/ml) for 30 min at 37°C, filtered through a 45 μm nylon mesh and then plated in tissue culture flasks and maintained in MEM supplemented with 10% foetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The cells were characterized as described previously [12] and used at passage 2-3.

For experiments, confluent cultures of BRP were exposed to continuous normal (5.6 mM) glucose and high (25 mM) glucose for 4 days. In some experiments, 500 μM apocynin and 1 μM of the mitochondrial targeted antioxidant [11], MitoQ (quinol attached to triphenylphosphonium; kindly provided by Dr Murphy, MRC-Dunn Human Nutrition Unit, Cambridge, UK) was added to normal and high-glucose medium.

2.2. Caspase-3-Like Activity. Cellular caspase-3 activity was determined using the colorimetric protease assay. This assay detects p-nitroanilide (p-NA) photometrically at 405 nm after its cleavage from the colorimetric substrate, N-acetyl-Asp-Glu-Val p-nitroanilide (Ac-DEVD-pNA) by the caspase-3 [13].

2.3. DNA Fragmentation. DNA fragmentation was quantified using the Cell Death Detection ELISA plus (1774425; Roche, Hertfordshire, UK). The assay was carried out according to manufacturer's instructions, which measures mono-, and oligonucleosomes in the cytoplasmic fraction of cell lysate. The assay was based on a quantitive sandwich enzyme-immunoassay directed against cytoplasmic histone-associated DNA fragments.

2.4. Western Blot Analysis. Antibodies directed against NADPH oxidase subunits, Gp91phox, and p47phox were from Upstate (UK). After treatment, cells were lysed on ice in the following lysis buffer (20 mM Tris-HCL, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 1 mM PMSF, 1 μg/ml aprotinin, 10 μg/ml leupeptin). Equal amounts of protein were separated on 10% polyacrylamide gels and transferred to Hybond-ECL membranes (Amersham, UK). Membranes were blocked with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST). After immunodetection of p47phox, and gp91phox, the membranes were stripped in a buffer containing 50 mM Tris, 2% SDS, and 100 mM mercaptoethanol at 55°C for 30 min, washed and immunoblotted with tubulin antibody (Chemicon, Hampshire, UK). Immunoreactive bands were quantified by scanning densitometrically and calculating the density of individual bands using Image J software (National Institute of Health, Bethesda, Maryland, http://rsb.info.nih.gov/ij/). The levels of NADPH oxidase subunits were expressed as a ratio of intensity of p47phox, and gp91phox immunoreactive bands/intensity of tubulin immunoreactive bands.

2.5. Measurement of Intracellular ROS Production. The cell permeant dihydroethidium (DHE; Molecular Probes) was used to assess real-time formation of superoxide (O_2^-) in BRP exposed to normal (5.8 mM) and high (25 mM) glucose. DHE enters the cells and is oxidized by superoxide to form ethidium (ETH), which binds to DNA to produce the fluorescent ETH-DNA that displays red fluorescence [14]. DHE was prepared as 2 mM stock solution in DMSO and stored at −20°C. The cells were loaded with 10 μM for the final 2 h of incubation. The cells were washed with PBS, lysed with 50 μl lysis buffer (20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 50 mM NaF and 0.2 mM sodium orthovanadate) on ice. The lysates were then transferred into black 96-well plates (Fisher, Loughborough,

UK) and the fluorescence was measured using spectrofluorometer (Plate Chameleon; Hidex, Baringstoke, UK). ETH-DNA red fluorescence was measured with excitation at 530 nm and emission at 616 nm. Background fluorescence intensity was subtracted from the results and the level of ROS was expressed as fluorescence intensity/μg protein.

2.6. Measurement of NADPH Oxidase Activity. Measurement of O_2^- was based on the capacity to reduce ferricytochrome *c* in ferrocytochrome at pH 7.8 [15]. Total cell lysate (50 μg protein/experiment), cytochrome c (250 μg/l final concentration), and NADPH (100 μM) were incubated at 37°C for 120 min, either in the presence or absence of diphenyleneiodonium (DPI, 100 μM). The reduction of cytochrome c was measured by reading absorbance at 550 nm. O_2^- production in nmol/mg protein was calculated from the difference between absorbance of samples at 0 and 120 min and the extinction coefficient 21 mmol/l/cm.

2.7. Measurement of Intracellular CML. The intracellular formation of N^{ε}-(carboxymethyl) lysine (CML) in pericytes was quantified using competitive imuunoabsorbant enzyme-linked assay (ELISA) as previously described in [16]. CML-BSA, used as a standard, was dissolved in 0.05 M carbonate buffer, pH 9.6, to a concentration of 0.5 μg/ml. A 50 μl aliquot was added to each well of a 96-well microtitre ELISA plate (Nunc, Loughborough, UK). After incubation at 4°C overnight, the coating solution was discarded and the wells washed three times with 400 μl of PBS containing 0.05% Tween-20 (PBST). The wells were then filled with 100 μl of 2% normal goat serum (NGS) containing 0.1% BSA for blocking and left for 1 h at room temperature. After washing with three times with 400 μl PBST, 50 μl of the diluted standards and samples were added in triplicate wells followed by 50 μl of the 1 : 1000 diluted CML-antibody (Kindly provided by Dr D Ruggiero, INSA, Lyon, France). After incubation for 2 h at room temperature, the wells were washed three times with 400 μl PBST, and developed with goat antirabbit peroxidase-conjugated IgG (diluted 1 : 10,000 in PBST) and o-phenylenediamine (Sigma, Dorset, UK). The absorbance of the samples was read at 490 nm and the levels of CML determined using the standard curve prepared using various concentrations of CML-BSA.

2.8. Measurement of Intracellular Glucose Concentration. After treatment, cells were washed and medium was removed and cells lysed. Intracellular glucose concentration was then determined using Amplex Red Glucose Assay Kit (A 22189; Molecular Probes, Paisley, UK) according to the manufacturer's instructions. Briefly, 50 μl of the reaction solution (10 mM Amplex Red, 10 U/ml HRP, 100 U/ml glucose oxidase, 50 mM sodium phosphate buffer, pH 7.4) was added to 50 μl of cell lysate in 96-well microtitre plate and incubated in the dark for 45 min at room temperature. The absorbance was then measured at 560 nm using a SpectraMax 190 microplate reader. Intracellular glucose concentration was determined from a standard curve generated using various concentrations of glucose.

2.9. Measurement of Protein Kinase Cβ1/2 Activity. Cellular PKCβ1/2 activity was determined using the TruLight assay kit (Calbiochem, Nottingham, UK) according to the manufacturer's instructions. The activity was based on fluorescence superquenching and the assay was carried out in a white 96-well microtitre plates (Nunc, Loughborough, UK). Fluorescence was measured using microplate reader (Plate Chameleon, Hidex, Basingstoke, UK) with excitation at 450 nm and emission at 535 nm. PKCβ1/2 activity was calculated from the generated phosphopeptide calibrator curve and the results expressed PKCβ1/2-dependent phosphorylation/mg protein.

2.10. Protein Measurement. Total protein was measured using the BCA protein assay kit (Pierce, UK).

2.11. Statistical Analysis. The statistical software Graph Pad Prism version 3.0 was used. A two-tailed Student's *t*-test was used to test the significance of paired data. Data are expressed as means ± S.E.M of measurements in the different experiments. Differences between groups were considered statistically significant at $P < .05$.

3. Results

3.1. Retinal Capillary Pericytes Express NADPH Oxidase. NADPH oxidase is a membrane-bound enzyme complex that is a source of cytosolic O_2^- and is composed of at least four subunits, including two important membrane-bound subunits p22phox and gp91phox and two cytosolic subunits p47phox and p67phox. On a Western blot, the antibody directed against the N-terminal peptide labelled specifically only one broad band at approximately 80 kDa in lysates of BRP (Figure 1(a)). This immunoreactive band was detected using the antibody directed against the peptide corresponding to amino acids 548–560 of human gp91phox. The antibody also detected similar molecular weight band in cultured bovine retinal capillary endothelial cells (BREC), human umbilical endothelial cells (HUVEC), and human leukocytes (U937 cells) (Figure 1(a)). The anti-gp91phox antibody used in our study is specific to human gp91phox (Nox2) and corresponds to amino acids 548–560 of human Nox2.

Specific anti-p47phox also confirmed the presence of p47phox NADPH oxidase subunit with approximate molecular weight of 55 kDa in BRP. An immunoreactive band of similar weight was also localized in BREC, and HUVEC (Figure 1(a)). However, in U937 cells the p47phox immunoreactive band had a lower molecular weight of approximately 45 kDa. Thus, two major components of a functional NADPH oxidase are present in BRP.

Immunoblot analysis showed that 48 h exposure to high glucose significantly increased gp91phox protein expression (Figures 1(b) and 1(c)) to 249 ± 38% of normal glucose ($n = 5$, $P < .05$). In contrast, high glucose failed to cause a significant change in the expression of p47phox (Figures 1(b) and 1(d)).

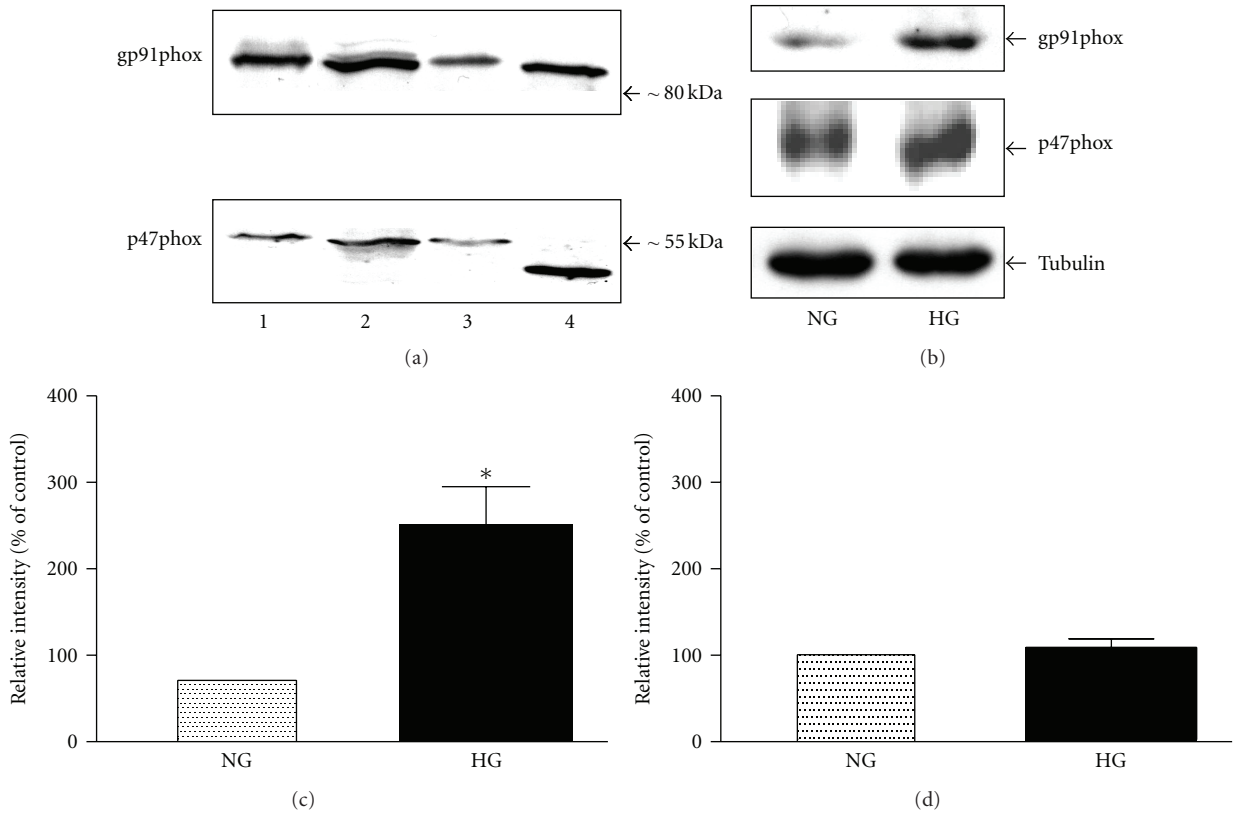

Figure 1: *Retinal capillary pericytes express NADPH oxidase*. Aliquots of total cellular protein were separated by SDS-PAGE, electrophoretically transferred onto Hybond-ECL membranes. Blots were probed with (a) anti-gp91phox antibody and anti-p47phox antibody. Protein antibody complexes were detected using secondary antibody conjugated with horseradish peroxidase and the ECL detection system. Lane 1: BRP, lane 2: BREC (bovine retinal capillary endothelial cells), Lane 3: HUVEC (human umbilical vein endothelial cells), and lane 4: U937 cells (human leukocytes). (b) Representative Western blots of gp91phox, p47phox and tubulin. (c) Expression of gp91phox protein expression in BRP exposed to high glucose (HG, 25 mM) for 48 h. Densitometric ratio (intensity gp91phox immunoreactive band/intensity of the tubulin immunoreactive band) is expressed as % of normal glucose (NG, 5.6 mM). (d) Expression of p47phox protein expression in BRP exposed to high glucose (HG, 25 mM) for 48 h. Densitometric ratio (intensity of p47phox immunoreactive band/intensity of the tubulin immunoreactive band) is expressed as % of normal glucose (NG, 5.6 mM). Data are represented as Means ± SEM of 3-4 separate experiments. $^{*}P < .05$ versus control.

3.2. Glucose Increases NADPH Oxidase Activity. To provide further and more direct evidence for the effect of high glucose on NADPH oxidase, cellular activity was measured using the cytochrome c reduction assay. BRP expresses active NADPH oxidase (Figure 2(a)). High glucose increased the NADPH-induced O_2^- generation by almost 1.65-fold compared to normal glucose (Figure 2(b)). The addition of apocynin (500 μM) significantly inhibited glucose-induced NADPH oxidase activity (115 ± 22% of normal glucose, $n = 4$, $P = .013$).

3.3. Glucose Stimulates ROS in Pericytes. Exposure to high glucose significantly increased the intracellular O_2^- production as detected by ETH-DNA fluorescence (Figure 3). Oxidant generation increased 126 ± 9% of normal glucose ($n = 6$, $P < .05$) and was significantly reversed by treatment with 500 μM apocynin (101 ± 7% of normal glucose versus 126 ± 9% of normal glucose, $n = 6$, $P < .05$). MitoQ slightly reversed glucose-induced ROS production (122 ± 4% of normal glucose, $n = 6$, $P = .05$).

3.4. Apocynin and Not MitoQ Reverse Glucose-Induced Apoptosis. Figure 4 compares the effect of high glucose on apoptosis determined by measuring cellular caspase-3 activity (A) and the level of DNA fragmentation (B) using the Cell Death Detection ELISA Plus (Roche, Hertfordshire, UK) which measures mono- and oligonucleosomes in the cell lysate. Both assay confirmed high glucose-induced apoptosis of BRP after 4 days.

As shown in Figure 5, incubation for 4 days in continuous high glucose caused a significant increase in apoptosis of pericytes compared to normal glucose (127 ± 9% of normal glucose, $n = 7$, $P < .001$). This glucose-induced caspase-3 activity was significantly reversed (Figure 5) by 500 μM apocynin (91 ± 7% of normal glucose versus 127 ± 9% of normal glucose, $n = 6-13$, $P < .05$). In contrast, the addition

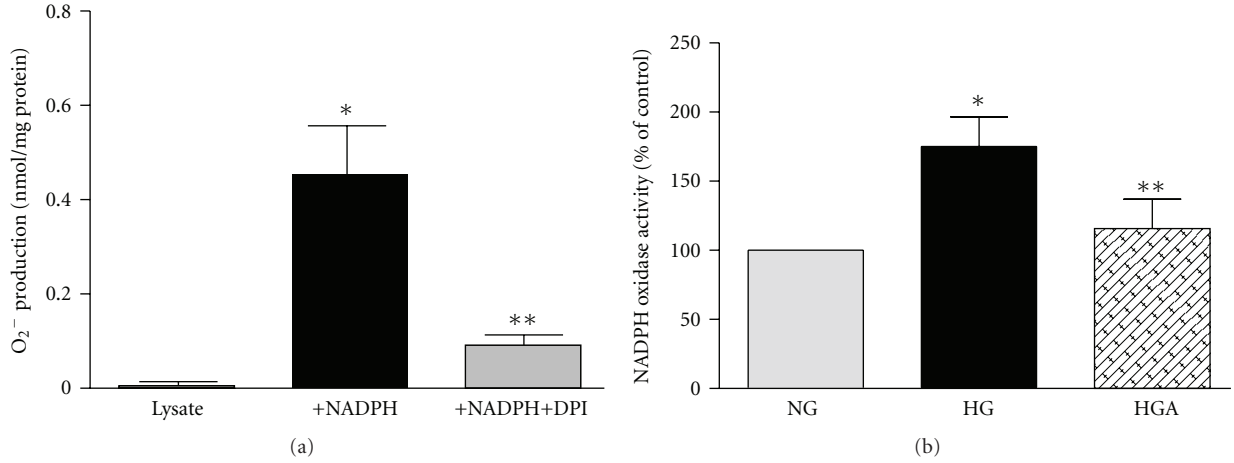

Figure 2: *Apocynin reverses glucose-induced NADPH oxidase activity*. (a) Cultured bovine retinal capillary pericytes express active NADPH oxidase. The activity was determined by cytochrome c reduction. Lysate (25 μg protein) was added to 250 μM cytochrome c in a 96-well multiwell plate and incubated for 120 min, either in the presence or absence of NADPH (100 μM) or NADPH oxidase inhibitor or flavoprotein inhibitor, diphenyleneiodonium (DPI, 100 μM). The reduction of cytochrome c was measured by reading absorbance at 550 nm (SpectraMax 190). Superoxide (O_2^-) production in nmol/mg protein was calculated from absorbance of samples and the extinction coefficient for change of ferricytochrome c to ferrocytochrome c of 21 mmol/l/cm. Data are presented as mean ± SEM of 4–6 separate experiments. $^*P < .001$ versus lysate alone, $^{**}P < .01$ versus lysate with NADPH. (b) Exposure to high glucose for 4 days increases NADPH oxidase which is significantly reversed by the addition of apocynin. Confluent cultures of BRP in 30 mm^2 dishes were exposed to normal glucose (NG, 5.6 mM) and high glucose (HG, 25 mM) with and without 500 μM apocynin (HGA) for 4 days. After incubation, the cells were washed twice with ice-cold PBS; lysed, and NADPH-dependent O_2^- production was measured by DPI-inhibitable cytochrome c reduction assay. Data are presented as mean ± SEM of 4 separate experiments. $^*P < .05$ versus HG. $^{**}P < .013$ versus HG.

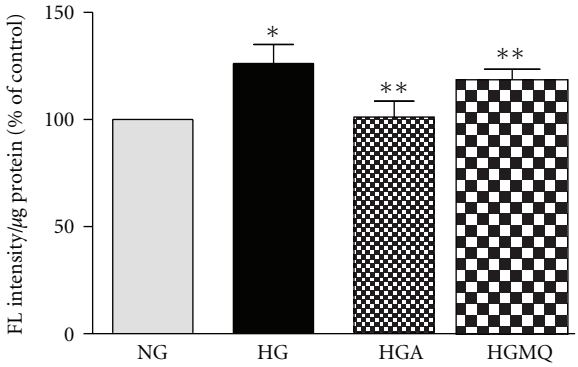

Figure 3: *Apocynin reverses continuous high glucose-induced ROS production in BRP*. Subconfluent cells in 24-well plate were exposed to normal glucose (NG, 5.6 mM) and continuous high glucose (HG, 25 mM) in the absence and presence of 500 μM apocynin (HGA) or 1 μM MitoQ (HGMQ) for 3 days. Approximately, 2 h before the end of incubation, DHE (10 μM) was added. After the treatment, the cells were washed twice with PBS, lysed and fluorescence (FL) intensity was measured. Data are presented as mean ± SEM of 6 separate experiments. $^*P < .05$ versus NG, $^{**}P < .05$ versus HG.

of MitoQ failed to prevent glucose-induced activation of caspase-3 (124 ± 17% of normal glucose, $n = 4$).

3.5. Intracellular CML Content after Exposure to High Glucose. Intracellular levels of CML were quantified by competitive enzyme-linked immunoabsorbant assay (ELISA) using specific CML antibody. Standard curve was established using various concentration of CML-BSA. As shown in Figure 6, exposure to continuous high glucose for 4 days significantly increased the intracellular CML content by almost 2.8-fold compared to normal glucose (16.3 ± 1.9 μg/mg protein versus 5.9 ± 0.9 μg/mg protein in normal glucose, $n = 10$–12, $P < .05$). The addition of apocynin at 500 μM, a concentration that reverses glucose-induced apoptosis, significantly prevented the accumulation of CML in pericytes exposed to high glucose (10.7 ± 1.4 μg/mg protein versus 16.3 ± 1.9 μg/mg protein in high glucose, $n = 8$–12, $P < .05$). In contrast, MitoQ, (1 μM) had no significant effect on the CML content in pericytes exposed to high glucose (16.9 ± 5.1 μg/mg protein, $n = 8$).

3.6. Glucose-Induced PKC-β Activity. There was no significant increase in PKC-β1/β2 activity measured using fluorescence superquenching-based assay. After 4 days exposure, the activity expressed as PKC-β1/β2-dependent phosphorylation/mg protein was found to be 6.9 ± 3.5 ($n = 6$) in HG compared to 6.8 ± 3.5 ($n = 6$).

3.7. Increased Accumulation of Intracellular Glucose after Exposure to High Glucose. Continuous exposure to high glucose (25 mM) for 4 days increased the intracellular glucose concentration by almost 6-fold compared to BRP exposed to normal glucose (92.6 ± 6.0 nmol/mg protein versus 15.2 ± 3.0 nmol/mg protein in normal glucose, $n = 13$-14, $P < .001$) (Figure 7). At 500 μM, apocynin failed to prevent the accumulation of intracellular glucose (87.8 ± 22.3 nmol/mg protein, $n = 5$). Interestingly, although

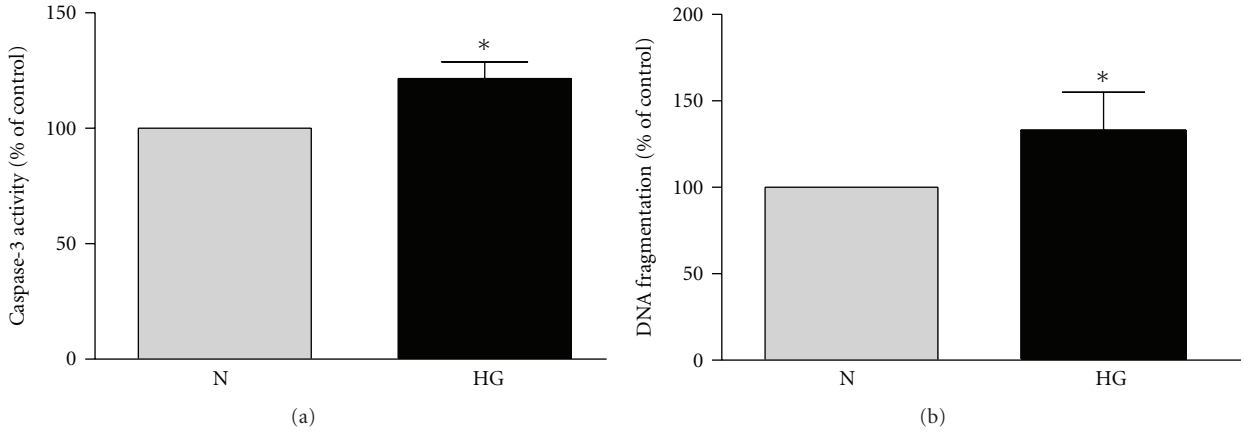

Figure 4: *Continuous high (25 mM) glucose induces apoptosis of BRP.* Subconfluent cells in 30 mm^2 dishes were exposed to normal (N, 5.6 mM) and high (CG, 25 mM) glucose for 4 days. (a) High glucose activates caspase-3. After 4 days, the cells were washed twice with ice-cold PBS, lysed and apoptosis was measured using colorimetric caspase activity assay. Data are represented as Means ± SEM of 6 separate experiments. $^*P = .0315$ versus N. (b) High glucose induces DNA fragmentation. After 4 days, DNA fragmentation was measured using Cell Death Detection ELISA plus (Roche, Hertfordshire, UK). Data are represented as Means ± SEM of 5 separate experiments. $P = .043$ versus N.

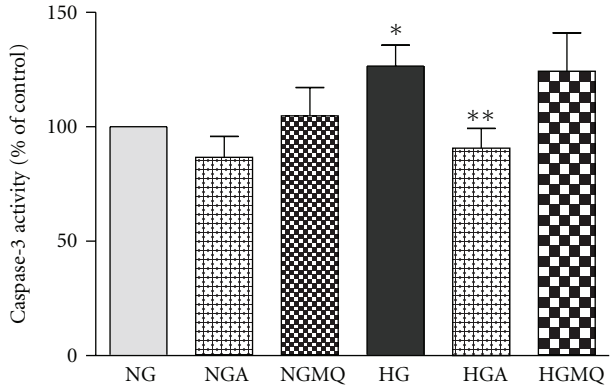

Figure 5: *Chronic exposure to high glucose induces apoptosis in BRP.* Subconfluent cells in 3 cm dishes were exposed to normal (NG, 5.6 mM) and high (HG, 25 mM) glucose in the absence (NG, HG) and presence of 500 μM apocynin (NGA, HGA) or 1 μM MitoQ (NGMQ and HGMQ). After 4 days, the cells were washed twice with ice-cold PBS, lysed and apoptosis was measured using colorimetric caspase-3 activity. Data are represented as Means ± SEM of 3–6 separate experiments. $^*P < .05$ versus NG, $^{**}P < .05$ versus HG.

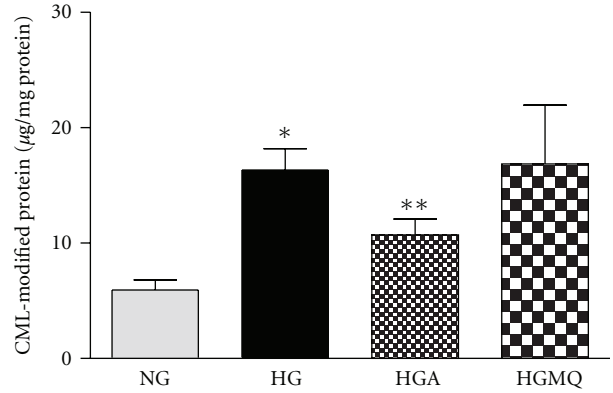

Figure 6: *Apocynin reverses glucose-induced CML formation in BRP.* Subconfluent cells in 24-well plate were exposed to normal glucose (NG, 5.6 mM) and continuous high glucose (HG, 25 mM) and in the presence of 500 μM apocynin (HGA) or 1 μM MitoQ (HGMQ) for 4 days. After incubation, the cells were washed twice with ice-cold PBS, lysed and CML were detected using competitive ELISA. Data are presented as Mean ± SEM of 8–13 samples. $^*P < .05$ versus NG, $^{**}P < .05$ versus HG.

MitoQ (1 μM) failed to prevent the activation of caspase-3, it significantly lowered the level of intracellular glucose concentration in BRP exposed to high glucose (57.9 ± 8.6 nmol/mg protein versus 92.6 ± 6.0 nmol/mg protein in high glucose, $n = 5$–13, $P < .05$). However, apocynin and MitoQ did not alter the intracellular glucose concentration in BRP exposed to normal glucose.

4. Discussion

There is now growing evidence that oxidative stress plays an important role in the pathogenesis of chronic complications of diabetes [6], but the exact source, and cellular location of the glucose-induced ROS is still unclear. Brownlee and co-workers proposed that in cultured macrovascular bovine aortic endothelial cells (BAECs), the production of ROS by the mitochondria *via* the respiratory chain is the most important causal link between high glucose and the main pathways responsible for hyperglycaemic damage [8]. Besides mitochondria, NADPH oxidase also generates a significant amount of ROS and is a major source of superoxide in vascular cells [9]. In the present study, we used apocynin, an inhibitor of NADPH oxidase [10] and MitoQ, a mitochondria-targeted antioxidant [11, 12], to explore the importance of mitochondria versus NADPH oxidase

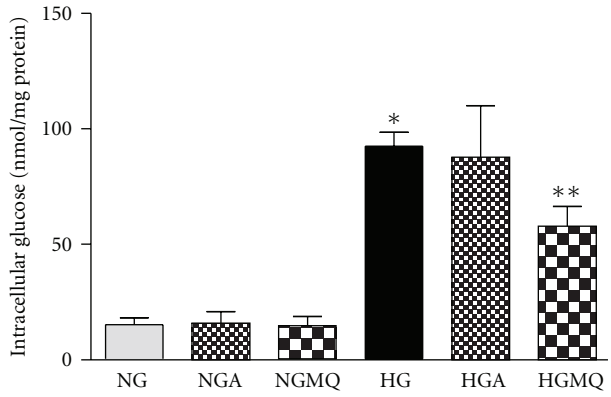

Figure 7: *Exposure to high glucose increases intracellular glucose concentration.* Subconfluent cells in 24-well plate were exposed to normal glucose (NG, 5.6 mM) and high glucose (HG, 25 mM) and in the presence of 500 μM apocynin (NGA, HGA) or 1 μM MitoQ (NGMQ, HGMQ) for 4 days. The cells were washed twice with ice-cold PBS, lysed and intracellular glucose concentrations were measured using the Amplex Red glucose assay kit (Molecular Probe). Data are Means ± SEM of 5–14 separate experiments. $^{*}P <$ .001 versus NG; $^{**}P <$ .05 versus HG.

derived ROS in glucose-induced apoptosis of cultured retinal capillary pericytes.

Our observations of glucose-induced apoptosis of pericytes is consistent with previous *in vitro* studies [17–20] and the activation of caspase-3 in the retina of diabetic animals and humans [21, 22]. Consistent with recent reports [23, 24], retinal capillary pericytes express NADPH oxidase as indicated by the immunoblotting of Nox2 and p47phox, major membrane and cytosolic subunits [9, 10], respectively. In contrast, Manea et al. [24] using reverse transcriptase-polymerase chain reaction (RT-PCR) detected Nox1 and Nox2 in pericytes isolated from rat adipose tissue microvasculature. Since Nox2 and Nox1 shows only 56% of homology [25], we safely assume that our antibody does not cross-react with Nox1. NADPH oxidase, as in other cell types [26, 27] could be mostly present in the cytoplasm of pericytes, suggesting that ROS is produced within the intracellular compartments. Although, glucose increased NADPH oxidase *via* Nox2 expression, the exact mechanism of control is at present unclear. In support of our observations, increased mRNA and/or protein levels of gp91phox have been reported in blood vessels from animals [28, 29] and patients with diabetes [30]. As reported in other cell types [22, 31, 32], exposure to high glucose increased NADPH oxidase activity and ROS production in pericytes. In addition to changes in the expression of NADPH oxidase subunits [33, 34], high glucose could stimulate ROS production through protein kinase C (PKC)-dependent phosphorylation of the p47phox subunit [32]. The β isoform of PKC has been implicated in the phosphorylation of p47phox [33], but in our study, we failed to demonstrate activation of PKCβ1/2 in pericytes exposed to high glucose.

Our findings of increased oxidative stress in response to high glucose is in line with some previous studies [20, 23], but it appears to argue against a recent report suggesting that pericytes are resistant to glucose-induced oxidative stress [35]. The reason for this inconsistency is at present unclear. Our results show that ROS derived from NADPH oxidase and not the mitochondria, is involved in caspase-3-mediated apoptosis of pericytes induced by high glucose. This possibility is supported by recent studies [36] demonstrating the potential of apocynin to block glucose-induced ROS in BAEC. Other workers have demonstrated the role of NADPH oxidase in diabetic retinopathy [36–39], and complications of diabetes [40, 41] including the loss of podocytes in diabetic nephropathy [42]. Results with MitoQ suggested that some ROS is produced from the mitochondria, but it does not play a significant role in glucose-induced apoptosis. The mitochondria is known to play a key role in activating apoptosis through enhanced cytochrome (cyt c) release resulting in the activation of caspases and subsequent cell death [43]. Apoptotic cell death is mediated by stimulated caspase-3 activity, and the accumulation of p53, a known signalling molecule that acts upstream of caspase-3. Although, there is no direct evidence available to show that ROS derived from NADPH oxidase interact with mitochondria, we suggest that there is some interaction in pericytes exposed to high glucose.

In bovine aortic endothelial cells (BAECs) the mitochondria-derived ROS was also involved in glucose-induced intracellular AGE formation [6]. However, our observation that apocynin reverses glucose-induced N^{ε}-(carboxymethyl) lysine (CML) production, suggests a role of NADPH oxidase-derived ROS in the formation of intracellular CML-modified proteins in pericytes exposed to high glucose. In support of this, there is evidence that phagocytic NADPH oxidase plays an important role in CML formation *in vivo* [44]. Since CML is a biomarker of cellular oxidative stress [45], it may explain our previous failure to observe intracellular formation of AGEs, as detected using AGE-antibody [16] in pericytes exposed to high glucose. As reported previously [16, 46, 47] intracellular glucose levels were significantly increased in pericytes exposed to high glucose. During the initial step of auto-oxidative glycation, ROS fragments glucose to generate glyoxal with potential to react and form CML on lysine residues of intracellular and extracellular proteins [48]. The formation of CML from intermediates of the Mailard reaction, such as Schiff's base and the Amadori product that are raised in pericytes exposed to high glucose [16], requires ROS-mediated oxidative cleavage of the carbon backbone [49]. Although, ROS controls the formation of intracellular CML, its role in glucose-induced apoptosis is unclear, but may be involved in the observed reduced proliferation and increased necrosis of pericytes in high glucose. This notion is supported by a recent study showing that CML-modification of histones is associated with decreased proliferation of keratinocytes exposed to glyoxal [50]. Intracellular formation of CML in pericytes could well be important since increased levels of CML are present in the retina of rats with experimental diabetes [51] and levels in lymphocytes are associated with the pathogenesis of diabetic retinopathy [52].

In summary, we have demonstrated for the first time that in contrast to BAEC, ROS from NADPH oxidase and not the mitochondria plays a key role in glucose-induced intracellular formation of CML and apoptosis of retinal capillary pericytes. The results support the recent reports linking NADPH oxidase and diabetic retinopathy [39] and the beneficial effect of antioxidants to prevent oxidative stress and caspase-3-dependent apoptosis of pericytes [19]. Specific pharmacological NADPH oxidase inhibitors might also be of potential use during the treatment of early diabetic retinopathy.

Nonstandard Abbreviations

ROS:	Reactive oxygen species
BRP:	Bovine retinal capillary pericytes
BAEC:	Bovine aortic endothelial cells
HUVEC:	Human umbilical vein endothelial cells
Ac-DEVD-pNA:	N-acetyl-Asp-Glu-Val p-nitroanilide
p-NA:	p-nitroaniline
FCS:	Foetal calf serum
PBS:	Phosphate buffered saline
OH:	Hydroxyl radical
H_2O_2:	Hydrogen peroxide
O_2^-:	superoxide radical
BSA:	Bovine serum albumin
DPI:	diphenyleneiodonium
SOD:	Superoxide dismutase
DHE:	Dihydroethidium hydroethidine
ETH:	Ethidium
SDS-PAGE:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
ELISA:	Enzyme linked immunoabsorbant assay
S.E.M:	Standard error of mean
FAD:	Flavin adenine dinucleotide
MitoQ:	10-(6′-ubiquinonyl) decyltriphenylphosphonium
AGE:	Advanced glycation product.

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