Published in final edited form as:

Exp Eye Res. 2014 February ; 119: 77–87. doi:10.1016/j.exer.2013.12.009.

Novel Transgenic Mouse Models Develop Retinal Changes Associated with Early Diabetic Retinopathy Similar to Those Observed in Rats with Diabetes Mellitus

Changmei Guo¹, Zifeng Zhang¹, Peng Zhang¹, Jun Makita¹, Hiroyoshi Kawada¹, Karen Blessing¹, and Peter F. Kador^{1,2}

¹Department of Pharmaceutical Sciences, College of Pharmacy, University of Nebraska Medical Center, Omaha, NE

²Department of Ophthalmology, College of Medicine, University of Nebraska Medical Center, Omaha, NE

Abstract

Retinal capillary pericyte degeneration has been linked to aldose reductase (AR) activity in diabetic retinopathy (DR). Since the development of DR in mice and rats has been reported to differ and that this may be linked to differences in retinal sorbitol levels, we have established new murine models of early onset diabetes mellitus as tools for investigating the role of AR in DR. Transgenic diabetic mouse models were developed by crossbreeding diabetic C57BL/6-Ins2^{Akita}/J (AK) with transgenic C57BL mice expressing green fluorescent protein (GFP), human aldose reductase (hAR) or both in vascular tissues containing smooth muscle actin- α (SMAA). Changes in retinal sorbitol levels were determined by HPLC while changes of growth factors and signaling were investigated by Western Blots. Retinal vascular changes were quantitatively analyzed on elastase-digestion flat mounts.

Results show that sorbitol levels were higher in neural retinas of diabetic AK-SMAA-GFP-hAR compared to AK-SMAA-GFP mice. AK-SMAA-GFP-hAR mice showed induction of the retinal growth factors VEGF, IGF-1, bFGF and TGF β , as well as signaling changes in P-Akt, P-SAPK/JNK, and P-44/42 MAPK. Increased loss of nuclei per capillary length and a significant increase in the percentage of acellular capillaries presented in 18 week old AK-SMAA-GFP-hAR mice. These changes are similar to those observed in streptozotocin-induced diabetic rats. Retinal changes in both mice and rats were prevented by inhibition of AR. These studies confirm that the increased expression of AR in mice results in the development of retinal changes associated with the early stages of DR that are similar to those observed in rats.

Corresponding Author: Peter F. Kador, Ph.D., FARVO, College of Pharmacy, 986025 Nebraska Medical Center, Omaha, NE 68198-6025, Tel: 402-559-9261, pkador@unmc.edu.

4. Conflict of interest statement

The authors declare no conflict of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

diabetic retinopathy; transgenic diabetic mouse models; aldose reductase; green fluorescent proteins; diabetic rats; retinal vascular changes; growth factors VEGF, bFGF, TGF- β ; cell signaling P-ERK1/2, P-SAPK/JNK, P-Akt

1. Introduction

Diabetic retinopathy (DR) is a common microvascular complication in patients with type 1 and type 2 diabetes mellitus (DM). It is a major cause of blindness that affects approximately three-fourths of all diabetics within 15 years of the onset of DM (Stitt et al., 2013). Hyperglycemia is the central, underlying cause of DR and tight control of hyperglycemia reduces the progression of DR (D.C.C.T., 1995). However, the exact mechanism(s) through which hyperglycemia initiates both the vascular and neuronal alterations in retinopathy have not been completely defined.

While a number of microvascular modifications occur in the development of DR (Cai and Boulton, 2002), the distinguishing feature of retinal vascular changes in DR is the selective degeneration of pericytes (mural cells) from the retinal capillary vessels of diabetic humans (Beltramo and Porta, 2013; Cogan et al., 1961; Ejaz et al., 2008; Hammes et al., 2002) as well as diabetic and galactose-fed dogs (Kador et al., 1990; Kador et al., 2007). Pericytes encompass the endothelial cell tube and share a common basal membrane. Pericytes control retinal capillary blood flow through their contractile nature, i.e. presence of smooth muscle actin, and their loss is associated with decreased capillary tonicity, increased vascular permeability, the formation of microaneurysms, and vessel dilation. Pericytes also control neovascularization by suppressing the growth of endothelial cells (Armulik et al., 2005; Hall, 2006).

Experimentally, retinal pericyte destruction occurs under both hyperglycemic and galactosemic conditions and is linked to the excess formation of the sugar alcohols (polyols) sorbitol and galactitol by the enzyme aldose reductase (AR) (Miwa et al., 2003; Sato et al., 1999). AR has been observed by immunohistochemistry to be primarily present in pericytes of isolated retinal capillaries from human and dog (Akagi et al., 1983; Akagi et al., 1986) and *in vitro* cultured pericytes from dog, human, bovine and rat accumulate polyols (Kador et al., 2009; Miwa et al., 2003; Naruse et al., 2000; Takamura et al., 2008). This polyol accumulation has been linked to the induction of apoptosis that can be arrested by inhibition of AR (Dagher et al., 2004; Kador et al., 2009; Robison et al., 1991).

To date, several animal models of DM demonstrate a role for AR in the mechanism(s) initiating retinal lesions. These include streptozotocin-induced diabetic and galactose-fed rats (Robison, 2000), galactose-fed dogs (Kador et al., 1995), and transgenic and knock-out mice (Cheung et al., 2005; Grossniklaus et al., 2010; Ho et al., 2006). Moreover, these retinal lesions are reduced by the administration of structurally diverse aldose reductase inhibitors (ARIs) to rats and dogs (Neuenschwander et al., 1997; Obrosova et al., 2003; Robison et al., 1995). However, the polyol pathway for the treatment of DR has become a controversial target because clinical trials in contrast to the dog studies conducted at the

National Eye Institute have established only minor benefits in human diabetic retinopathy (1990) compared to the galactose-fed dog (Cusick et al., 2003; Kador et al., 1990; Neuenschwander et al., 1997; Takahashi et al., 1993). These differences are probably linked to the levels of AR inhibition achieved in these studies because the premise in ARI studies has been based entirely on the levels of sugar alcohol accumulation. Since galactitol is not further metabolized, it is well known that robust inhibition of AR is required to demonstrate efficacy in galactosemic animals. However, it has been assumed that less strenuous inhibition is required in diabetic animals because sorbitol levels are reduced not only by AR inhibition but by metabolism to fructose by sorbitol dehydrogenase (SDH). Since the focus has been on reducing sorbitol levels in target tissues while at the same time minimizing the potential safety concerns of ARIs, pharma development has only utilized “normalization” of sorbitol as the marker for adequate inhibition of AR with red cell sorbitol levels being the convenient marker for clinical inhibition (Peterson et al., 1986). Ignored was the 3 to 20-fold increase in fructose that still can occur *despite* the reduction of sorbitol to apparent “normal” levels in diabetic animals. Clinical dose selections for the Phase III trials for Tolrestat, Ponalrestat, Zopolrestat and Statil were extrapolated from sorbitol suppression endpoints obtained from these animal studies (Oates, 2002). No clinical efficacy was demonstrated with these ARIs despite sorbitol (but not elevated fructose) being reduced to normal levels in human target tissues. While these failures led many clinicians to conclude that the ARI concept does not warrant further studies, it has been recently determined that robust blockade of AR—i.e., inhibition of sorbitol so that excess fructose cannot be formed—is also required with diabetic animals (Oates, 2002). This premise has been confirmed with AR knockout mice (Chung and Chung, 2003). “Robust” AR inhibition can be interpreted to reduce both osmotic and oxidative stress. By blocking the first step of the Polyol Pathway flux, the entire pathway is blocked. As a result, the intracellular levels of NADPH and NAD nucleotides are preserved and normal intracellular levels of fructose and sorbitol (both of which contribute to intracellular osmolarity) maintained. This new experimental evidence, combined with the prevention of retinal changes in the AR knockout mouse (Cheung et al., 2005) and clinical findings in both type 1 and 2 patients that DR is linked to the presence of select AR alleles (Abhary et al., 2009; Demaine et al., 2000; dos Santos et al., 2006; Katakami et al., 2011; Kumaramanickavel et al., 2003; Olmos et al., 2000; Petrovic et al., 2005; Richeti et al., 2007; Szaflik et al., 2008; Uthra et al., 2010; Wang et al., 2003) merit further studies in this area.

Animal studies also indicate that there are significant species differences in the development of vascular lesions (Wang and Hui, 2012). For example, the accumulation of retinal sorbitol and fructose in streptozotocin-induced diabetic rats is higher than in diabetic mice (Asnaghi et al., 2003; Obrosova et al., 2006). Compared to rats, diabetic mice also have lower AR activity in other tissues such as the lens and sciatic nerve (Chung and Chung, 2003; Gaynes and Watkins, 1989; Llewelyn et al., 1991; Markus et al., 1983). Since the lower AR activity in mice may contribute to differences in retinal changes observed between the mouse and rat, select transgenic mice expressing human aldose reductase (SMAA-hAR), green fluorescent protein (SMAA-GFP), or both (SMAA-GFP-hAR) under the control of the smooth muscle α -actin promoter have been established (Kador et al., 2012). DM was subsequently introduced into these transgenic mice by cross-breeding with naturally diabetic

C57BL/6-Ins2^{Akita}/J (AK) mice. This has resulted in naturally diabetic mice (AK-SMAA-GFP-hAR) that demonstrate increased retinal AR activity compared to similar mice not expressing hAR. Here, we report that the retinal growth factor and signaling changes as well as vascular lesion changes that we have demonstrated in AK-SMAA-GFP-hAR mice (Kador et al., 2012) are more similar to changes observed in diabetic rats than mice not expressing aldose reductase.

2. Materials and Methods

2.1 Reagents

All reagents and solvents were purchased from either Acros Organics, Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich Corporation (St. Louis, MO). All solvents were reagent or high-performance liquid chromatography (HPLC) grade. The aldose reductase inhibitor (ARI) AL1576 (2,4-difluorospirofluorene-9,5'-imidazolidine-2',4'-dione) was obtained from Alcon Laboratories (Ft. Worth, TX), Tolrestat (2-[[6-methoxy-5-(trifluoromethyl)naphthalene-1-carbothioyl]-methylamino]acetic acid) was obtained from Wyeth-Ayerst Laboratories (Princeton, NJ), Ranirestat (AS3201, (3R)-2'-(4-bromo-2-fluorobenzyl)-1'H, 2H,5H-spiro[pyrrolidine-3,4'-pyrrolo[1,2-a]pyrazine]-1',2,3',5(2'H)-tetrone) was obtained from Dainippon Sumitomo Pharma (Osaka, Japan). Statil ([3-(4-bromo-2-fluorobenzyl)-4-oxo-3H-phthalazin-1-yl]acetic acid) was obtained from ICI (Manchester, UK). Antibodies utilized were as follows: mouse VEGF mAb (ab1316; VEGF-A) and GAPDH rabbit Ab were obtained from Abcam Inc, Cambridge, MA; and IGF-1 rabbit mAb were obtained from Santa Cruz Biotech (Santa Cruz, CA); the phospho-ERK (phospho-44/42 MAPK) (Thr202/Tyr204) rabbit Ab, phospho-Akt (Ser473) rabbit Ab, phospho-SAPK/JNK (Thr183/try185) rabbit mAb, basic-FGF rabbit Ab, TGF- β rabbit Ab, horseradish peroxidase (HRP) conjugated anti-rabbit antibody, and HRP conjugated anti-biotin Ab were obtained from Cell Signaling Technology (Beverly, MA). Chemiluminescent reagent and peroxide, biotinylated protein ladder, prestained protein marker, and cell lysis buffer were from Cell Signaling Technology (Beverly, MA).

2.2 Experimental animals

All studies were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center and performed in strict accordance with the recommendation of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2.1 Transgenic diabetic mice and genotyping for SMAA-GFP, SMAA-hAR, and Ins2^{Akita}—The development of the AK-SMAA-GFP-hAR transgenic mice and their genotyping has previously been reported (Kador et al., 2012). In the AK-SMAA-GFP-hAR group initial blood glucose levels ranged from 247–600 mg/dL for females and 342–600 mg/dL for males and in the AK-SMAA-GFP group blood glucose levels ranged from 245–572 mg/dL for females and 332–600 mg/dL for males. Overall, the blood glucose levels in both the AK-SMAA-GFP-hAR and the AK-SMAA-GFP mice used were similarly elevated with average blood glucose levels (mean $\pm$ SEM) of 472 ± 7 mg/dL (86% male) and 471 ± 15 mg/dL (70% male), respectively. AK-SMAA-GFP-hAR of either sex were fed a

standard rodent diet containing either 0.02% AL1576 or 0.035% Ranirestat. Food consumption and body weights were monitored weekly. Based on these measurements, mice received an average dose of 11 mg/kg/day of AL1576 and 23 mg/kg/day of Ranirestat.

2.2.2 Diabetic rats—DM was induced in 80–100 g male Sprague Dawley rats by tail vein injection of 75 mg/kg of streptozotocin as previously described (Zhang et al., 2012). After 3 days, blood glucose levels were obtained and each rat with blood glucose levels <300 mg/dL were re-injected and again measured after 3 days. Twenty-four rats with blood glucose levels > 300 mg/dL were then equally divided into 3 groups. The first group consisted of untreated diabetic rats receiving standard rodent chow (Bio-Serve). The second group of diabetic rats received standard rodent chow containing 0.0125% AL1576 (Bio-Serve), while the third group of diabetic rats received rodent chow containing 0.015% Tolrestat (Bio-Serve) or 0.05% Statil (Bio-Serve). Experimental diets were initiated 10 days following initial Streptozotocin injections and continued for 10 weeks until the studies were terminated. A fourth group, consisting of non-diabetic age-matched rats, was added to the study as the non-diabetic control group. Food consumption and body weights, monitored weekly, indicated that the rats received an average dose of 20 mg/kg/day of AL1576, 24 mg/kg/day of Tolrestat or 85 mg/kg/day of Statil.

2.3 Sugar analysis

Retinal sorbitol levels were determined by high-performance liquid chromatography (HPLC) as previously described (Kador et al., 2012).

2.4 SDS-PAGE and western immunoblot analyses

The detection of the select proteins by western blot was conducted as follows (Kador et al., 2012; Zhang et al., 2012). The scanned images were subsequently photo enhanced using Corel Photo-Paint X-3. Immunoreactive band densities were measured using Image-Pro Plus software (Bethesda, MD) and the NIH ImageJ image analysis program (1.42q).

2.5 Preparation of retinal digests

To prepare retinal vasculature flat mounts, enucleated eyes were slit at the limbus and fixed at room temperature for at least 4 days in a solution of 4.0% w/v paraformaldehyde in 50 mmol/l sodium/potassium phosphate buffer with 6.0% sucrose at pH 7.2. The anterior segments were removed by microdissection at the ora serrata and the intact neural retina was carefully dissected from the posterior globe. Vascular whole mounts were prepared using elastase digestion (Kador et al., 2012). The isolated retinal vasculatures were flattened via a radial cut from the periphery of each retina to the optic nerve as needed to permit an even flattening of the preparation. The vascular preparation, mounted on gelatin-coated slides, were stained with a periodic acid Schiff (PAS) and counterstained with hematoxylin.

2.6 Analysis of retinal digests

Each retinal preparation was divided into 4 equal quadrants and the central areas in each quadrant mid-distance between the optic nerve and outer retinal edge were analyzed (Kador et al., 2012). At the central point in each of these quadrants, 4 adjacent 230 × 300 micron

areas were captured with an Olympus BX51 research microscope at 400x magnification and analyzed using PAX-it Image software (Chicago, IL). Approximately 1.1 mm² central areas of each mouse retina were analyzed. Retinas from 18 week old diabetic AK-SMAA-GFP-hAR and AK-SMAA-GFP-hAR mice fed diet containing AL1576 along with age-matched non-diabetic SMAA-GFP-hAR mice were analyzed. Similarly, retinal preparations from streptozotocin-diabetic rats and diabetic rats treated with AL1576 or Statil along with age-matched normal rats were analyzed.

2.7 Statistical analyses

Statistical analyses (ANOVA and 2-sample t-test) were conducted using OriginPro® software version 8.1 (OriginLab Corp., Northampton, MA) and ProStat ver. 5.01 (Pearl River, NY). Differences with a $p < 0.05$ were defined as significant.

3. Results

3.1 Transgenic Mouse Models

New mouse models for investigating the pathogenesis of DR have been established where GFP or hAR has been introduced in all vascular tissues under the control of smooth muscle α -actin promoter (Kador et al., 2012). These were introduced to not only aid in the identification of capillary pericytes, but also to observe the effects of increasing levels of AR in the retinal capillary pericytes of mice where the retinal levels of AR are believed to be lower than in the rat (Obrosova et al., 2006). Diabetes was subsequently introduced into these colonies by cross-breeding with naturally diabetic C57BL/6-Ins^{2Akita}/J (AK) mice (Yoshioka et al., 1997) to produce a new colony of AK-SMAA-GFP and AK-SMAA-GFP-hAR mice. The traits in all offspring were confirmed by genotyping DNA isolated from tail snips or ear punches with polymerase chain reaction (PCR).

In these transgenic mice, the presence of GFP in vascular tissues can easily be detected by fluorescent microscopy not only in the retina, but also in the kidney, aorta, heart, brain, lung and mammary glands (Fig. 1 and 2). Higher magnification of retinal vessels confirmed the presence of GFP in the finger-like cytoplasmic projections of the pericytes that encompass the vascular endothelial cells (Fig. 2B and C). These observations confirm that the selective presence of GFP in pericytes may be a useful tool for differentiating pericytes from endothelial cells in retinal capillaries by fluorescent confocal microscopy.

3.2 Retinal Sorbitol Levels

The cross-bred AK-SMAA-GFP-hAR and the AK-SMAA-GFP mice naturally develop DM by 8 weeks after birth with blood glucose levels higher in males than females. Overall, the blood glucose levels in both the AK-SMAA-GFP-hAR and the AK-SMAA-GFP mice groups used were similarly elevated with average blood glucose levels (mean $\pm$ SEM) of 472 ± 7 mg/dL (86% male) and 471 ± 15 mg/dL (70% male), respectively. While blood glucose levels were similar, sorbitol levels of the isolated neural retinas from the diabetic AK-SMAA-GFP-hAR mice were significantly higher than those from the diabetic AK-SMAA-GFP mice (Fig. 3A). Sorbitol levels in non-diabetic SMAA-GFP-hAR mice were also significantly higher than those from non-diabetic SMAA-GFP mice. This is attributed to the

increased presence of hAR in the neural retina of the SMAA-GFP-hAR mice. In rats, induction of DM also resulted in a time-dependent increase in retinal sorbitol levels (Fig. 3B).

3.3 Vascular Changes

To determine whether the observed increase in sorbitol levels is associated with retinal vascular changes, histological studies were conducted on isolated retinal capillaries from 18 week old diabetic AK-SMAA-GFP-hAR treated with or without the ARI AL1576 along with age-matched non-diabetic SMAA-GFP-hAR mice. The central areas of each of 4 equal quadrants located mid-distance between the optic nerve and outer retinal edge of each isolated intact retinal vasculature was analyzed by light microscopy (Fig. 4).

The similar appearance of pericyte and endothelial cell nuclei in the hematoxylin and eosin-stained retinal vasculature makes differentiation of these cells difficult; therefore, all nuclei were counted and the results were expressed as capillary nuclei/100 μ m of counted capillary length. With this approach, a small (5.2%) decrease in the number of nuclei per capillary length was observed in the diabetic AK-SMAA-GFP-hAR mice compared to non-diabetic SMAA-GFP-hAR mice (Fig. 5G). A similar decrease was not observed in diabetic AK-SMAA-GFP-hAR mice treated with the ARI AL1576. Similar analyses of the number of nuclei/100 μ m of counted capillary lengths of isolated retinal capillaries from 10-week diabetic rats revealed a significant, 10.1 % reduction compared to either age-matched non-diabetic rats or diabetic rats treated with either the ARIs AL1576 or Statil (Fig. 5G).

Similar analyses of the presence of acellular capillaries in both mice and rats revealed significant increases in the untreated diabetic animals which were not observed in similar animals treated with ARI. There was a small but significant increase in the percent acellular vessels present in diabetic AK-SMAA-GFP-hAR mice compared to non-diabetic SMAA-GFP-hAR ($p = 0.03$) mice or diabetic AK-SMAA-GFP-hAR mice treated with AL1576 ($p = 0.007$) (Fig. 5B and H). In rats, a similar significant increase in the presence of acellular capillaries ($p < 0.05$) was observed (Fig. 5E, H). This increase was not observed in similar diabetic rats treated with either AL1576 or Statil. This shows that ARIs play an important role in protecting retinal capillaries from hyperglycemia and sorbitol accumulation.

3.4 Growth Factor Changes

Capillary changes in diabetic retinopathy are associated with increased VEGF expression (Hattori et al., 2010; Obrosova et al., 2003) that is reduced by treatment with ARIs (Amano et al., 2002; Frank et al., 1997; Hattori et al., 2010; Obrosova et al., 2003). Induction of diabetes resulted in an increase in VEGF-A levels that were slightly higher in the AK-SMAA-GFP-hAR mice compared to the AK-SMAA-GFP or non-diabetic SMAA-GFP and SMAA-GFP-hAR mice (Fig. 6A). This induction was not observed in AK-SMAA-GFP-hAR mice treated with the ARI AL1576 but was observed with the ARI Ranirestat. This induction was also observed in a time-dependent manner in diabetic rats and concomitant treatment with the ARIs AL1576 and Tolrestat reduced the expression of VEGF (Fig. 6B). Other growth factors such as TGF- β , IGF-1, and b-FGF have also been linked to the development of DR (Paques et al., 1997; Van Geest et al., 2010). Compared to 18 week old

AK-SMAA-GFP mice, the retinal expression of TGF- β , IGF-1, and b-FGF increased in the neural retinas of AK-SMAA-GFP-hAR mice and the expression of these growth factors was reduced in similar AK-SMAA-GFP-hAR mice treated with the structurally diverse ARIs AL1576 or Ranirestat (Fig. 7A, C and E). Similarly, the neural retinas from the 10 week diabetic rats showed an increase in the retinal expression of TGF- β , IGF-1, and b-FGF that was reduced by the ARIs AL1576 and Tolrestat (Fig. 7B, D and F).

3.5 Cellular Signaling Changes

In the neural retina, growth factor expression changes are accompanied by cellular signaling changes. The expression levels of the pathway signals P-Akt, P-SAPK/JNK, and P-Erk1/2 (P-44/42 MAPK) were altered in both the neural retinas from 18 week diabetic AK-SMAA-GFP-hAR mice and 10 week diabetic rats. Compared to AK-SMAA-GFP mice, increased signaling in P-Akt, P-SAPK/JNK, and P-ERK1/2 were observed in the AK-SMAA-GFP-hAR mice and these were reduced by the administration of the ARIs AL1576 or Ranirestat (Fig. 8A, C and E). Similarly, changes in signaling expression levels in the diabetic rats were normalized to levels seen in non-diabetic rats by the ARIs AL1576 or Tolrestat (Fig 8B, D and F).

4. Discussion

Animal models serve as indispensable tools not only for elucidating the onset and development of diabetic complications but also for evaluating drugs for the management of diabetes and its complications. For DR, dogs are the animal model of choice because they demonstrate both clinical and histological retinal changes that mirror those observed in human DR. In galactose-fed dogs, pericyte dropout is the first prominent histological finding and retinal changes progress from the initial background to the advanced proliferative stage. More importantly, these changes can all be clinically observed with basic funduscopy as well as fluorescein angiography (Cusick et al., 2003). Research with dogs, however, is expensive and retinal changes require up to 6 years to develop. Rats are the most commonly used animal model for investigating DR. In contrast to dogs, initial pericyte degeneration in rats is not as prominent as capillary basement membrane thickening and rats do not progress to the advanced proliferative stage. The clinical appearance of microaneurysms and other subsequent vascular changes are also not readily observed by funduscope. Nevertheless, this relatively inexpensive animal model is well-suited for evaluating drug effects on the initial biochemical stages of DR. In contrast to rats, mice have not been widely used for evaluating drugs for the treatment of DR. The ability to modify specific biochemical parameters in mice through gene manipulation makes them versatile animal models for investigating the biochemical mechanisms of DR (Robinson et al., 2012).

The selective degeneration of pericytes is a hallmark of DR with the pericyte to endothelial cell ratio observed to increase from a normal ratio of 1:1 up to 1:18 in diabetics (Beltramo and Porta, 2013; Cogan et al., 1961; Kuwabara et al., 1961). A number of *in vitro* and *in vivo* studies in rats and dogs have linked this selective degeneration to AR activity (Kador et al., 2009; Murata et al., 2002; Neuenschwander et al., 1997; Robison et al., 1991; Takamura et al., 2008). Cheung, using a knockout mouse model with deletion of AR (AR^{-/-}), has

reported that AR is responsible for the early events in the pathogenesis of DR which lead to a cascade of retinal lesions, including blood-retinal barrier breakdown, loss of pericytes, neuro-retinal apoptosis, glial reactivation, and neovascularization (Cheung et al., 2005). Similarly, deletion of AR prevents diabetes-induced defects in visual function (Lee et al., 2013). Obrosova, comparing diabetic mice with rats, however, reported that mouse retinas are deficient in AR and fail to demonstrate sorbitol accumulation and oxidative stress (Obrosova et al., 2006). Based on our studies on the importance of pericyte degeneration on the progression of DR in dogs, we have focused on the induction of hAR into the mouse retinal capillary pericytes. In the present studies we have utilized transgenic mice expressing either GFP, hAR or both under the control of smooth muscle α -actin promoter that also develop diabetes by crossbreeding with the naturally diabetic C57BL/6-Ins2^{Akita}/J (AK) mice (Kador et al., 2012). As in the nondiabetic rat, sorbitol levels in the transgenic SMAA-GFP mice were negligible but crossbreeding these mice with the naturally diabetic C57BL/6-Ins2^{Akita}/J (AK) mouse resulted in neural retinal sorbitol levels in their offspring (AK-SMAA-GFP) that were similar to the baseline sorbitol levels in nondiabetic transgenic mice expressing hAR (SMAA-hAR). However, similar introduction of diabetes in the hAR expressing mouse resulted in AK-SMAA-hAR mice possessing significantly increased sorbitol levels (Fig. 3A). These AK-SMAA-hAR mice also develop retinal vascular changes that are similar to those observed in rats (Fig. 5). Moreover, these vascular lesions are prevented by ARI treatment.

Retinal vascular changes in DR are also linked to growth factor imbalances (Frank, 2009; Obrosova and Kador, 2011). Among these, the role of VEGF in retinal neovascularization is well-established, with numerous early clinical studies with anti-VEGF agents suggesting that they may have an important potential role in the management of DR (Nicholson and Schachat, 2010). Other growth factors such as TGF- β , IGF-1, and b-FGF have also been linked to the development of DR (Paques et al., 1997; Van Geest et al., 2010). bFGF is a pro-angiogenic growth factor whose expression precedes the expression of VEGF (Song et al., 2003). TGF-beta is involved in the control of endothelial cell proliferation, adhesion and deposition of extracellular matrix, and it has been suggested that TGF-beta signaling plays a role in vascular basement membrane thickening in preclinical diabetic retinopathy (Gacka and Adamiec, 2006; Van Geest et al., 2010). The growth factor IGF-1 enhances ischemia-induced VEGF expression (Chantelau et al., 2008). Its pro-angiogenic effects are supported by the observation that normoglycemic transgenic mice overexpressing IGF-1 in the retina develop vascular changes that include venule dilatation, intraretinal microvascular abnormalities, and neovascularization of the retina and vitreous cavity that are associated with DM (Ruberte et al., 2004). In the present studies, the expressions of these pro-angiogenic growth factors were increased in both diabetic rats and transgenic AK-SMAA-hAR mice that developed retinal vascular changes (Fig. 6 and 7). Moreover, with the exception of Ranirestat on VEGF levels in diabetic mice, these expression levels were reduced to nondiabetic levels in rats by the ARIs AL1576 and Tolrestat and in the diabetic AK-SMAA-hAR mice to levels in non-hAR expressing diabetic AK-SMAA-GFP mice by the ARIs AL1576 and Ranirestat (Fig. 6 and 7). The failure of Ranirestat to normalize VEGF expression may be due to the levels of inhibitor employed (0.035% in diet) because a

slightly higher 0.05% Ranirestat diet in rats while inhibiting cataract formation only partially reduced sorbitol formation in the lens (Matsumoto et al., 2008).

Similar to growth factor expressions, the expression levels of the pathway signals P-Akt, P-SAPK/JNK, and P-Erk1/2 (P-44/42 MAPK) were increased in the neural retinas of both diabetic AK-SMAA-GFP-hAR mice and diabetic rats (Fig. 8A, B). These signaling changes were also reduced to levels observed in nondiabetic rats by the ARIs AL1576 and Tolrestat and in the diabetic AK-SMAA-GFP-hAR mice to levels observed by diabetic AK-SMAA-GFP mice by the ARIs AL1576 and to a lesser extent by Ranirestat. The importance of signaling changes as well as growth factor induction in the development of DR has been previously discussed in detail (Frey and Antonetti, 2011; Khan and Chakrabarti, 2007).

These observations, combined with the fact that both vascular and expression changes in growth factors and signaling are normalized by concomitant treatment with ARIs, strongly suggest that AR contributes to early retinal changes associated with DR in both the mouse and the rat. These studies also suggest that AK-SMAA-GFP-hAR mice can be substituted for rats in studies of early DR development and the evaluation of drugs for the treatment and prevention of early DR. Not answered by these studies is the mechanism(s) by which either sorbitol levels, AR activity, or diabetes itself are linked to the growth factor and signaling changes. Retinal sorbitol levels in nondiabetic SMAA-GFP mice are virtually absent and sorbitol levels in diabetic AK-SMAA-GFP mice only reach sorbitol levels that are similar to those of the nondiabetic SMAA-hAR mice (Fig. 3A). Moreover, no difference in the expression levels of growth factors or signaling was observed among these three mice (Fig. 6 and unpublished results). That is why in Fig 7A and 8A expression changes only in diabetic AK-SMAA-GFP versus AK-SMAA-GFP-hAR mice treated with or without ARI treatment are compared. This suggests that DM by itself does not initiate these expression changes and that there is a threshold of either cellular sorbitol levels or AR activity that is required to initiate the cascade of biochemical events that result in these downstream expression changes. This premise is also supported by the observation that expression levels in the AK-SMAA-hAR mice treated with ARIs were not statistically decreased to levels below those of nondiabetic SMAA-hAR mice. In the rat lens, *in vitro* studies show that the initiating factor is osmotic stress resulting from specific levels of intracellular sorbitol accumulation rather than specific sorbitol or galactitol formation or increased AR activity by themselves (Zhang et al., 2012). This osmotic stress induces endoplasmic reticulum (ER) stress and the subsequent generation of ROS in the lens and other cells (Dihazi et al., 2011; Mulhern et al., 2006). However, similar growth factor and signaling expression changes have been observed with cultured rat retinal pericyte (TR-rPCT) and endothelial (TR-iBRB) cells where apparent osmotic changes and ER stress are not induced by sorbitol or galactitol accumulation (Makita et al., 2011; Zhang et al., 2013). This suggests that in these cells the induction of growth factor and signaling changes appears to be directly linked to AR activity rather than to osmotic changes. We hypothesize that the induction of the present growth factors and signaling pathway changes in response to the adverse effects of AR-linked activity and excess sorbitol accumulation in the neural retina represent an “initial survival factor” response which upon their prolonged induction could eventually lead to pathological cellular changes. Future studies comparing diabetic and galactose-fed SMAA-hAR mice may help clarify the complex relationship(s) between polyol formation, accumulation, AR

activity, changes in growth factor and signaling, and the development of vascular changes observed in these mice.

In isolated retinal preparations from rodents, the appearance of nuclei from capillary pericytes versus endothelial cells can be difficult to differentiate. Similarly, pericyte ghost are difficult to identify. Since retinal pericytes contain smooth muscle α -actin (Kador et al., 2009), GFP has been introduced into the retinal vasculature through a smooth muscle α -actin promoter to aid in the identification of pericytes by confocal microscopy. GFP can clearly be seen in the finger-like cytoplasmic projections of pericytes that encompass the vascular endothelial cells pericytes (Fig. 2). This selective presence of GFP in pericytes of SMAA-GFP mice used to establish our colony has previously been documented (Yokota et al., 2006). However, the presence of GFP is not limited to retinal capillaries, since many other tissues express smooth muscle α -actin. For example, GFP can easily be detected by fluorescent microscopy in the vasculature of other tissues, including the heart, kidney, brain, lung and mammary gland (Fig. 1). This makes this animal model attractive for investigating diabetic vascular changes in other tissues.

The tissue distribution of hAR and GFP is assumed to be identical since the same smooth muscle α -actin promoter was used to introduce both GFP and hAR into these transgenic mice. Retinal capillary pericytes are only a small component of the neural retina; therefore, the increase in sorbitol observed in the mouse neural retinas expressing hAR (Fig. 3) appears to be too large to be contributed from the pericytes alone. The retinal distribution of smooth muscle α -actin is not limited to retinal pericytes since retinal cone bipolar cells (Hickman et al., 2002), rod inner segments (Drenckhahn and Groschel-Stewart, 1977), Müller cells (Joussen et al., 2009) and retinal pigment epithelia (RPE) (Kivela and Uusitalo, 1998) have also been reported to contain smooth muscle α -actin. Therefore, one can conclude that these other tissues can contribute to the neural retinal sorbitol levels observed. However, it is certain that the sorbitol levels observed were not contributed by the RPE or choroid, since the neural retinas were separated from the posterior segment in the present studies. In addition to pericytes, AR is also present in Müller cells, some ganglion and cone cells, and in the axons in the optic nerve of human eyes (Akagi et al., 1983; Akagi et al., 1984). Similarly, in db/db mice, AR has been reported to be present in Müller cells and neuronal cells in both the retinal ganglion cell and the inner nuclear layer (INL) as well as in capillary pericytes (Cheung et al., 2005). AR is also present in RPE cells (Henry et al., 2000). Ongoing immunohistochemical studies are clarifying the cellular source(s) of increased expression levels in the SMAA-hAR mice in all retinal tissues. Moreover, further studies comparing retinal AR levels between the rat, mouse, and human neural retinas are planned.

By demonstrating that similar vascular, growth factor and signaling expression changes occur in both the present diabetic transgenic mice and rats, the present studies suggest that these novel diabetic AK-SMAA-GFP-hAR mice may be useful not only for investigating early changes in the pathogenesis of DR, but also for evaluating the effects of various drugs on the onset and progression of early DR. Moreover, the presence of GFP along with increased expression levels of hAR in other select tissues possessing smooth muscle α -actin makes this an attractive animal model for investigating other diabetic complications.

Acknowledgments

This work was supported by the National Institutes of Health grant (EY016730).

References

- Abhary S, Hewitt AW, Burdon KP, Craig JE. A systematic meta-analysis of genetic association studies for diabetic retinopathy. *Diabetes*. 2009; 58:2137–2147. [PubMed: 19587357]
- Akagi Y, Kador PF, Kuwabara T, Kinoshita JH. Aldose reductase localization in human retinal mural cells. *Invest Ophthalmol Vis Sci*. 1983; 24:1516–1519. [PubMed: 6417042]
- Akagi Y, Terubayashi H, Millen J, Kador PF, Kinoshita JH. Aldose reductase localization in dog retinal mural cells. *Curr Eye Res*. 1986; 5:883–886. [PubMed: 3096638]
- Akagi Y, Yajima Y, Kador PF, Kuwabara T, Kinoshita JH. Localization of aldose reductase in the human eye. *Diabetes*. 1984; 33:562–566. [PubMed: 6427040]
- Amano S, Yamagishi S, Kato N, Inagaki Y, Okamoto T, Makino M, Taniko K, Hirooka H, Jomori T, Takeuchi M. Sorbitol dehydrogenase overexpression potentiates glucose toxicity to cultured retinal pericytes. *Biochem Biophys Res Commun*. 2002; 299:183–188. [PubMed: 12437967]
- Armulik A, Abramsson A, Betsholtz C. Endothelial/pericyte interactions. *Circ Res*. 2005; 97:512–523. [PubMed: 16166562]
- Asnaghi V, Gerhardinger C, Hoehn T, Adeboje A, Lorenzi M. A role for the polyol pathway in the early neuroretinal apoptosis and glial changes induced by diabetes in the rat. *Diabetes*. 2003; 52:506–511. [PubMed: 12540628]
- Beltramo E, Porta M. Pericyte loss in diabetic retinopathy: mechanisms and consequences. *Curr Med Chem*. 2013; 20:3218–3225. [PubMed: 23745544]
- Cai J, Boulton M. The pathogenesis of diabetic retinopathy: old concepts and new questions. *Eye (Lond)*. 2002; 16:242–260. [PubMed: 12032713]
- Chantelau E, Kimmeler R, Meyer-Schwickerath R. Insulin, insulin analogues and diabetic retinopathy. *Arch Physiol Biochem*. 2008; 114:54–62. [PubMed: 18465359]
- Cheung AK, Fung MK, Lo AC, Lam TT, So KF, Chung SS, Chung SK. Aldose reductase deficiency prevents diabetes-induced blood-retinal barrier breakdown, apoptosis, and glial reactivation in the retina of db/db mice. *Diabetes*. 2005; 54:3119–3125. [PubMed: 16249434]
- Chung SS, Chung SK. Genetic analysis of aldose reductase in diabetic complications. *Curr Med Chem*. 2003; 10:1375–1387. [PubMed: 12871135]
- Cogan DG, Toussaint D, Kuwabara T. Retinal vascular patterns. IV. Diabetic retinopathy. *Arch Ophthalmol*. 1961; 66:366–378. [PubMed: 13694291]
- Cusick M, Chew EY, Ferris F 3rd, Cox TA, Chan CC, Kador PF. Effects of aldose reductase inhibitors and galactose withdrawal on fluorescein angiographic lesions in galactose-fed dogs. *Arch Ophthalmol*. 2003; 121:1745–1751. [PubMed: 14662595]
- DCCT. Progression of retinopathy with intensive versus conventional treatment in the Diabetes Control and Complications Trial. Diabetes Control and Complications Trial Research Group. *Ophthalmology*. 1995; 102:647–661. [PubMed: 7724182]
- Dagher Z, Park YS, Asnaghi V, Hoehn T, Gerhardinger C, Lorenzi M. Studies of rat and human retinas predict a role for the polyol pathway in human diabetic retinopathy. *Diabetes*. 2004; 53:2404–2411. [PubMed: 15331552]
- Demaine A, Cross D, Millward A. Polymorphisms of the aldose reductase gene and susceptibility to retinopathy in type 1 diabetes mellitus. *Invest Ophthalmol Vis Sci*. 2000; 41:4064–4068. [PubMed: 11095596]
- Dihazi H, Dihazi GH, Mueller C, Lahrichi L, Asif AR, Bibi A, Eltoweissy M, Vasko R, Mueller GA. Proteomics characterization of cell model with renal fibrosis phenotype: osmotic stress as fibrosis triggering factor. *J Proteomics*. 2011; 74:304–318. [PubMed: 21118732]
- dos Santos KG, Canani LH, Gross JL, Tschiedel B, Souto KE, Roisenberg I. The –106CC genotype of the aldose reductase gene is associated with an increased risk of proliferative diabetic retinopathy in Caucasian-Brazilians with type 2 diabetes. *Mol Genet Metab*. 2006; 88:280–284. [PubMed: 16545977]

- Drenckhahn D, Groschel-Stewart U. Localization of myosin and actin in ocular nonmuscle cells. Immunofluorescence-microscopic, biochemical, and electron-microscopic studies. *Cell Tissue Res.* 1977; 181:493–503. [PubMed: 328160]
- Ejaz S, Chekarova I, Ejaz A, Sohail A, Lim CW. Importance of pericytes and mechanisms of pericyte loss during diabetes retinopathy. *Diabetes Obes Metab.* 2008; 10:53–63. [PubMed: 17941874]
- Frank RN. Treating diabetic retinopathy by inhibiting growth factor pathways. *Current opinion in investigational drugs.* 2009; 10:327–335. [PubMed: 19337953]
- Frank RN, Amin R, Kennedy A, Hohman TC. An aldose reductase inhibitor and aminoguanidine prevent vascular endothelial growth factor expression in rats with long-term galactosemia. *Arch Ophthalmol.* 1997; 115:1036–1047. [PubMed: 9258227]
- Frey T, Antonetti DA. Alterations to the blood-retinal barrier in diabetes: cytokines and reactive oxygen species. *Antioxid Redox Signal.* 2011; 15:1271–1284. [PubMed: 21294655]
- Gacka M, Adamiec J. The role of transforming growth factor-beta in the pathogenesis of diabetic retinopathy. *Przegl Lek.* 2006; 63:296–298. [PubMed: 17036509]
- Gaynes BI, Watkins JB 3rd. Comparison of glucose, sorbitol and fructose accumulation in lens and liver of diabetic and insulin-treated rats and mice. *Comp Biochem Physiol B.* 1989; 92:685–690. [PubMed: 2498032]
- Grossniklaus HE, Kang SJ, Berglin L. Animal models of choroidal and retinal neovascularization. *Prog Retin Eye Res.* 2010; 29:500–519. [PubMed: 20488255]
- Hall AP. Review of the pericyte during angiogenesis and its role in cancer and diabetic retinopathy. *Toxicol Pathol.* 2006; 34:763–775. [PubMed: 17162534]
- Hammes HP, Lin J, Renner O, Shani M, Lundqvist A, Betsholtz C, Brownlee M, Deutsch U. Pericytes and the pathogenesis of diabetic retinopathy. *Diabetes.* 2002; 51:3107–3112. [PubMed: 12351455]
- Hattori T, Matsubara A, Taniguchi K, Ogura Y. Aldose reductase inhibitor fidarestat attenuates leukocyte-endothelial interactions in experimental diabetic rat retina in vivo. *Curr Eye Res.* 2010; 35:146–154. [PubMed: 20136425]
- Henry DN, Frank RN, Hootman SR, Rood SE, Heilig CW, Busik JV. Glucose-specific regulation of aldose reductase in human retinal pigment epithelial cells in vitro. *Invest Ophthalmol Vis Sci.* 2000; 41:1554–1560. [PubMed: 10798676]
- Hickman F, Fariss R, Pagan-Mercado, Yamamoto T, Wawrousek E, Yabe-Nishimura C, Tsai J. Characterization Of Retinal Neurons Over-expressing Human Aldose Reductase In Smaa-har Mice. *Invest Ophthalmol Vis Sci.* 2002; 43:742.
- Ho EC, Lam KS, Chen YS, Yip JC, Arvindakshan M, Yamagishi S, Yagihashi S, Oates PJ, Ellery CA, Chung SS, Chung SK. Aldose Reductase-Deficient Mice Are Protected From Delayed Motor Nerve Conduction Velocity, Increased c-Jun NH2-Terminal Kinase Activation, Depletion of Reduced Glutathione, Increased Superoxide Accumulation, and DNA Damage. *Diabetes.* 2006; 55:1946–1953. [PubMed: 16804062]
- Joussen AM, Doehmen S, Le ML, Koizumi K, Radetzky S, Krohne TU, Poulaki V, Semkova I, Kociok N. TNF-alpha mediated apoptosis plays an important role in the development of early diabetic retinopathy and long-term histopathological alterations. *Mol Vis.* 2009; 15:1418–1428. [PubMed: 19641635]
- Kador PF, Akagi Y, Takahashi Y, Ikebe H, Wyman M, Kinoshita JH. Prevention of retinal vessel changes associated with diabetic retinopathy in galactose-fed dogs by aldose reductase inhibitors. *Arch Ophthalmol.* 1990; 108:1301–1309. [PubMed: 2119169]
- Kador PF, Randazzo J, Blessing K, Makita J, Zhang P, Yu K, Hosoya K, Terasaki T. Polyol formation in cell lines of rat retinal capillary pericytes and endothelial cells (TR-rPCT and TR-iBRB). *J Ocul Pharmacol Ther.* 2009; 25:299–308. [PubMed: 19450153]
- Kador PF, Takahashi Y, Akagi Y, Blessing K, Randazzo J, Wyman M. Age-dependent retinal capillary pericyte degeneration in galactose-fed dogs. *J Ocul Pharmacol Ther.* 2007; 23:63–69. [PubMed: 17341153]
- Kador PF, Takahashi Y, Wyman M, Ferris F 3rd. Diabeteslike proliferative retinal changes in galactose-fed dogs. *Arch Ophthalmol.* 1995; 113:352–354. [PubMed: 7887849]

- Kador PF, Zhang P, Makita J, Zhang Z, Guo C, Randazzo J, Kawada H, Haider N, Blessing K. Novel diabetic mouse models as tools for investigating diabetic retinopathy. *PLoS One*. 2012; 7:e49422. [PubMed: 23251343]
- Katakami N, Kaneto H, Takahara M, Matsuoka TA, Imamura K, Ishibashi F, Kanda T, Kawai K, Osonoi T, Matsuhisa M, Kashiwagi A, Kawamori R, Shimomura I, Yamasaki Y. Aldose reductase C-106T gene polymorphism is associated with diabetic retinopathy in Japanese patients with type 2 diabetes. *Diabetes Res Clin Pract*. 2011; 92:e57–60. [PubMed: 21420193]
- Khan ZA, Chakrabarti S. Cellular signaling and potential new treatment targets in diabetic retinopathy. *Experimental Diabetes Research*. 2007;31867. [PubMed: 18288248]
- Kivela T, Uusitalo M. Structure, development and function of cytoskeletal elements in non-neuronal cells of the human eye. *Prog Retin Eye Res*. 1998; 17:385–428. [PubMed: 9695798]
- Kumaramanickavel G, Sripriya S, Ramprasad VL, Upadyay NK, Paul PG, Sharma T. Z-2 aldose reductase allele and diabetic retinopathy in India. *Ophthalmic Genet*. 2003; 24:41–48. [PubMed: 12660865]
- Kuwabara T, Carroll JM, Cogan DG. Retinal vascular patterns. III. Age, hypertension, absolute glaucoma, injury. *Arch Ophthalmol*. 1961; 65:708–716. [PubMed: 13755463]
- Lee CA, Li G, Patel MD, Petrash JM, Benetz BA, Veenstra A, Amengual J, Von Lintig J, Burant C, Tang J, Kern TS. Diabetes-induced impairment in visual function in mice: contributions of p38 MAPK, RAGE, leukocytes, and aldose reductase. *Invest Ophthalmol Vis Sci*. 2013;13–11659. Epub ahead of print.
- Llewelyn JG, Thomas PK, Mirrlees DJ. Aldose reductase activity and myo-inositol levels in sciatic nerve and dorsal root ganglia of the diabetic mutant mouse [C57/BL/Ks (db/db)]. *Metabolism*. 1991; 40:1084–1087. [PubMed: 1943734]
- Makita J, Hosoya K, Zhang P, Kador PF. Response of rat retinal capillary pericytes and endothelial cells to glucose. *J Ocul Pharmacol Ther*. 2011; 27:7–15. [PubMed: 21091050]
- Markus HB, Raducha M, Harris H. Tissue distribution of mammalian aldose reductase and related enzymes. *Biochem Med*. 1983; 29:31–45. [PubMed: 6404249]
- Matsumoto T, Ono Y, Kuromiya A, Toyosawa K, Ueda Y, Bril V. Long-term treatment with ranirestat (AS-3201), a potent aldose reductase inhibitor, suppresses diabetic neuropathy and cataract formation in rats. *J Pharmacol Sci*. 2008; 107:340–348. [PubMed: 18612195]
- Miwa K, Nakamura J, Hamada Y, Naruse K, Nakashima E, Kato K, Kasuya Y, Yasuda Y, Kamiya H, Hotta N. The role of polyol pathway in glucose-induced apoptosis of cultured retinal pericytes. *Diabetes Res Clin Pract*. 2003; 60:1–9. [PubMed: 12639759]
- Mulhern ML, Madson CJ, Danford A, Ikesugi K, Kador PF, Shinohara T. The unfolded protein response in lens epithelial cells from galactosemic rat lenses. *Invest Ophthalmol Vis Sci*. 2006; 47:3951–3959. [PubMed: 16936110]
- Murata M, Ohta N, Fujisawa S, Tsai JY, Sato S, Akagi Y, Takahashi Y, Neuenschwander H, Kador PF. Selective pericyte degeneration in the retinal capillaries of galactose-fed dogs results from apoptosis linked to aldose reductase-catalyzed galactitol accumulation. *J Diabetes Complications*. 2002; 16:363–370. [PubMed: 12200082]
- Naruse K, Nakamura J, Hamada Y, Nakayama M, Chaya S, Komori T, Kato K, Kasuya Y, Miwa K, Hotta N. Aldose reductase inhibition prevents glucose-induced apoptosis in cultured bovine retinal microvascular pericytes. *Exp Eye Res*. 2000; 71:309–315. [PubMed: 10973739]
- Neuenschwander H, Takahashi Y, Kador PF. Dose-dependent reduction of retinal vessel changes associated with diabetic retinopathy in galactose-fed dogs by the aldose reductase inhibitor M79175. *J Ocul Pharmacol Ther*. 1997; 13:517–528. [PubMed: 9436155]
- Nicholson BP, Schachat AP. A review of clinical trials of anti-VEGF agents for diabetic retinopathy. *Graefes Arch Clin Exp Ophthalmol*. 2010; 248:915–930. [PubMed: 20174816]
- Oates PJ. Polyol pathway and diabetic peripheral neuropathy. *Int Rev Neurobiol*. 2002; 50:325–392. [PubMed: 12198816]
- Obrosova IG, Drel VR, Kumagai AK, Szabo C, Pacher P, Stevens MJ. Early diabetes-induced biochemical changes in the retina: comparison of rat and mouse models. *Diabetologia*. 2006; 49:2525–2533. [PubMed: 16896942]

- Obrosova IG, Kador PF. Aldose reductase/polyol inhibitors for diabetic retinopathy. *Curr Pharm Biotechnol.* 2011; 12:373–385. [PubMed: 20939801]
- Obrosova IG, Minchenko AG, Vasupuram R, White L, Abatan OI, Kumagai AK, Frank RN, Stevens MJ. Aldose reductase inhibitor fidarestat prevents retinal oxidative stress and vascular endothelial growth factor overexpression in streptozotocin-diabetic rats. *Diabetes.* 2003; 52:864–871. [PubMed: 12606532]
- Olmos P, Futers S, Acosta AM, Siegel S, Maiz A, Schiaffino R, Morales P, Diaz R, Arriagada P, Claro JC, Vega R, Vollrath V, Velasco S, Emmerich M. (AC)23 [Z-2] polymorphism of the aldose reductase gene and fast progression of retinopathy in Chilean type 2 diabetics. *Diabetes Res Clin Pract.* 2000; 47:169–176. [PubMed: 10741565]
- Paques M, Massin P, Gaudric A. Growth factors and diabetic retinopathy. *Diabetes Metab.* 1997; 23:125–130. [PubMed: 9137900]
- Peterson MJ, Page MG, Just LJ, Aldinger CE, Malone JI. Applicability of red blood cell sorbitol measurements to monitor the clinical activity of sorbinil. *Metabolism.* 1986; 35:93–95. [PubMed: 3515121]
- Petrovic MG, Peterlin B, Hawlina M, Petrovic D. Aldose reductase (AC)n gene polymorphism and susceptibility to diabetic retinopathy in Type 2 diabetes in Caucasians. *J Diabetes Complications.* 2005; 19:70–73. [PubMed: 15745835]
- Richeti F, Noronha RM, Waetge RT, de Vasconcellos JP, de Souza OF, Kneipp B, Assis N, Rocha MN, Calliari LE, Longui CA, Monte O, de Melo MB. Evaluation of AC(n) and C(–106)T polymorphisms of the aldose reductase gene in Brazilian patients with DM1 and susceptibility to diabetic retinopathy. *Mol Vis.* 2007; 13:740–745. [PubMed: 17563730]
- Robinson R, Barathi VA, Chaurasia SS, Wong TY, Kern TS. Update on animal models of diabetic retinopathy: from molecular approaches to mice and higher mammals. *Dis Model Mech.* 2012; 5:444–456. [PubMed: 22730475]
- Robison, WG. *Galactosemic Animal Models.* Harwood Academic Publishers; Amsterdam: 2000.
- Robison WG Jr, McCaleb ML, Feld LG, Michaelis OEt, Laver N, Mercandetti M. Degenerated intramural pericytes ('ghost cells') in the retinal capillaries of diabetic rats. *Curr Eye Res.* 1991; 10:339–350. [PubMed: 1829996]
- Robison, WG.; Laver, NM.; Lou, MF. The Role of Aldose Reductase in Diabetic Retinopathy: Prevention and Intervention Studies. In: Osborne, NN.; Chader, GJ., editors. *Progress in Retinal and Eye Research.* Pergamon; Oxford: 1995. p. 593-639.
- Ruberte J, Ayuso E, Navarro M, Carretero A, Nacher V, Haurigot V, George M, Llombart C, Casellas A, Costa C, Bosch A, Bosch F. Increased ocular levels of IGF-1 in transgenic mice lead to diabetes-like eye disease. *J Clin Invest.* 2004; 113:1149–1157. [PubMed: 15085194]
- SRTRG. A randomized trial of sorbinil, an aldose reductase inhibitor, in diabetic retinopathy. Sorbinil Retinopathy Trial Research Group. *Arch Ophthalmol.* 1990; 108:1234–1244. [PubMed: 2119168]
- Sato S, Secchi EF, Lizak MJ, Fukase S, Ohta N, Murata M, Tsai JY, Kador PF. Polyol formation and NADPH-dependent reductases in dog retinal capillary pericytes and endothelial cells. *Invest Ophthalmol Vis Sci.* 1999; 40:697–704. [PubMed: 10067973]
- Song E, Dong Y, Sui DM, Zhao XZ, Wu JX. Effects of vascular endothelial growth factor and basic fibroblast growth factor in early stage diabetic retinopathy and their molecular pathological mechanism. *Zhonghua Yi Xue Za Zhi.* 2003; 83:996–999. [PubMed: 12899804]
- Stitt AW, Lois N, Medina RJ, Adamson P, Curtis TM. Advances in our understanding of diabetic retinopathy. *Clin Sci (Lond).* 2013; 125:1–17. [PubMed: 23485060]
- Szaflik JP, Majsterek I, Kowalski M, Rusin P, Sobczuk A, Borucka AI, Szaflik J, Blasiak J. Association between sorbitol dehydrogenase gene polymorphisms and type 2 diabetic retinopathy. *Exp Eye Res.* 2008; 86:647–652. [PubMed: 18289528]
- Takahashi Y, Augustin W, Wyman M, Kador PF. Quantitative analysis of retinal vessel changes in galactose-fed dogs. *J Ocul Pharmacol.* 1993; 9:257–269. [PubMed: 8228533]
- Takamura Y, Tomomatsu T, Kubo E, Tsuzuki S, Akagi Y. Role of the polyol pathway in high glucose-induced apoptosis of retinal pericytes and proliferation of endothelial cells. *Invest Ophthalmol Vis Sci.* 2008; 49:3216–3223. [PubMed: 18362110]

- Uthra S, Raman R, Mukesh BN, Rajkumar SA, Kumari P, Lakshmipathy P, Gnanamoorthy P, Sharma T, McCarty CA, Kumaramanickavel G. Diabetic retinopathy: Validation study of ALR2, RAGE, iNOS and TNFB gene variants in a south Indian cohort. *Ophthalmic Genet.* 2010; 31:244–251. [PubMed: 21067489]
- Van Geest RJ, Klaassen I, Vogels IM, Van Noorden CJ, Schlingemann RO. Differential TGF- β signaling in retinal vascular cells: a role in diabetic retinopathy? *Invest Ophthalmol Vis Sci.* 2010; 51:1857–1865. [PubMed: 19959647]
- Wang Y, Ng MC, Lee SC, So WY, Tong PC, Cockram CS, Critchley JA, Chan JC. Phenotypic heterogeneity and associations of two aldose reductase gene polymorphisms with nephropathy and retinopathy in type 2 diabetes. *Diabetes Care.* 2003; 26:2410–2415. [PubMed: 12882871]
- Wang YL, Hui YN. The problems that should be noticed in choosing animal models with diabetic retinopathy. *Zhonghua Yan Ke Za Zhi.* 2012; 48:587–590. [PubMed: 22943861]
- Wilson GL, Leiter EH. Streptozotocin interactions with pancreatic beta cells and the induction of insulin-dependent diabetes. *Curr Top Microbiol Immunol.* 1990; 156:27–54. [PubMed: 2143132]
- Yokota T, Kawakami Y, Nagai Y, Ma JX, Tsai JY, Kincade PW, Sato S. Bone marrow lacks a transplantable progenitor for smooth muscle type alpha-actin-expressing cells. *Stem cells.* 2006; 24:13–22. [PubMed: 16099999]
- Yoshioka M, Kayo T, Ikeda T, Koizumi A. A novel locus, Mody4, distal to D7Mit189 on chromosome 7 determines early-onset NIDDM in nonobese C57BL/6 (Akita) mutant mice. *Diabetes.* 1997; 46:887–894. [PubMed: 9133560]
- Zhang P, Xing K, Randazzo J, Blessing K, Lou MF, Kador PF. Osmotic stress, not aldose reductase activity, directly induces growth factors and MAPK signaling changes during sugar cataract formation. *Exp Eye Res.* 2012; 101:36–43. [PubMed: 22710095]
- Zhang P, Zhang Z, Kador PF. Polyol Effects on Growth Factors and MAPK Signaling in Rat Retinal Capillary Cells. *J Ocul Pharmacol and Therapeutics.* 2013 in press.

Highlights

- Diabetic AR transgenic mice show retinal changes similar to diabetic rats
- These include increased sorbitol, growth factor, signaling and vascular changes
- AR inhibitors prevent these retinal changes of both diabetic transgenic mice and rats
- Aldose reductase activity is linked to retinal changes in the early stages of DR

Fig. 1.

GFP expression in tissues containing smooth muscle α -actin in SMAA-GFP transgenic mice can be observed by fluorescent microscopy. Illustrated is the presence of GFP in the brain (A), heart (B), kidney (C), lung (D) and mammary glands (E).

Fig. 2. GFP expression in the retinal capillaries from SMAA-GFP transgenic mice. The presence of GFP is visible by fluorescent microscopy at both low (A) and higher magnification (B) and in greater detail by confocal microscopy (C). The white arrows show the location of capillary pericyte nuclei.

Fig. 3.

Sorbitol levels in the isolated neural retinas of diabetic transgenic mice and diabetic rats. (A) Sorbitol levels are significantly increased in both non-diabetic SMAA-GFP-hAR mice and diabetic AK-SMAA-GFP and AK-SMAA-GFP-hAR mice compared to non-diabetic SMAA-GFP mice. The highest retinal sorbitol levels are present in AK-SMAA-GFP-hAR mice and this is significantly higher (25%) than that of either the diabetic AK-SMAA-GFP mice or the non-diabetic SMAA-GFP-hAR mice. (B) Sorbitol levels were also significantly increased in the isolated neural retinas of diabetic rats in a time-dependent manner. This increase was normalized to the levels of control rats by treatment with ARI AL1576 or Statil. Mouse data in A has been previously reported (Kador et al., 2012) $n = 4-6$; mean $\pm$ S.E.M. * *vs* non-diabetic control or SMAA-GFP, $p < 0.05$. ** *vs* SMAA-GFP-hAR mice and AK-SMAA-GFP mice. $p < 0.05$.

Fig. 4.

Schematic illustrating the approximate location of capillaries evaluated in the isolated retinal digests from (A) mouse and (B) rat. Each retinal preparation was divided into 4 equal quadrants and the central areas in each quadrant mid-distance from the optic nerve and outer retinal edge were analyzed.

Fig. 5.

Appearance and changes of isolated retinal capillaries from 18 week non-diabetic SMAA-GFP-hAR mice and diabetic AK-SMAA-GFP-hAR mice treated with or without ARI and 10 week diabetic rats treated with or without ARI. Representative appearances of retinal vessels from SMAA-GFP-hAR mice (**A**), AK-SMAA-GFP-hAR mice (**B**) and AK-SMAA-GFP-hAR mice treated with AL1576 (**C**) are shown. The red arrow indicates acellular vessels. The capillary cell density expressed as capillary nuclei per 100 μ m of capillary length for the mice and rats is summarized in (**G**) showing a small decrease in the number of nuclei per capillary length in the diabetic AK-SMAA-GFP-hAR mice compared to either the SMAA-GFP-hAR mice or AK-SMAA-GFP-hAR mice treated with AL1576. In the 10 week diabetic rats, the untreated diabetic rats showed a significant reduction in retinal capillary nuclei compared to either non-diabetic controls or diabetic rats treated with ARIs AL1576 or Statil. The percent of acellular capillaries present in the examined neural retinal capillaries is summarized in (**H**). A small, but significant increase in the percent acellular vessels present in the diabetic AK-SMAA-GFP-hAR mice compared to either AK-SMAA-GFP-hAR mice treated with AL1576 ($p = 0.007$) or non-diabetic SMAA-GFP-hAR ($p = 0.03$) mice. The percent of acellular capillaries present was also significantly increased in diabetic rats (**E**) comparing to either normal rats (**D**) or diabetic rats treated with ARIs (**F**). Mouse data in **G** and **H** has been previously reported (Kador et al., 2012) $n = 5-7$; mean $\pm$ S.E.M. * $p < 0.05$ compared to SMAA-GFP-hAR. ** $p < 0.05$ compared to non-diabetic control. Bar=50 μ m.

Fig. 6.

A: Induction of VEGF in neural retinas from 18 week old non-diabetic SMAA-GFP and SMAA-GFP-hAR mice and diabetic AK-SMAA-GFP and AK-SMAA-GFP-hAR mice. A small increase of VEGF expression can be seen in diabetic AK-SMAA-GFP mice and AK-SMAA-GFP-hAR compared to SMAA-GFP mice. Highest VEGF induction was observed in AK-SMAA-GFP-hAR mice and this induction was reduced in similar mice treated with the ARI AL1576 but not Ranirestat. **B:** Time-dependent increase in VEGF expression in the neural retinas from diabetic rats. This induction was decreased by treatment with the ARIs AL1576 and Tolrestat. Mouse data in **A** has been previously reported (Kador et al., 2012). Bars represent the mean $\pm$ S.E.M of a minimum of 3 separate analyses. * $p < 0.05$ compared to non-diabetic control. The gel illustrates the representative appearance of one analysis.

Fig. 7. Comparison of (A) b-FGF, (C) IGF-1, and (E) TGF-β growth factor changes in AK-SMAA-GFP versus AK-SMAA-GFP-hAR mice treated with or without the ARIs AL1576 or Ranirestat and (B) b-FGF, (D) IGF-1, and (F) TGF-β growth factor changes in normal versus diabetic rats treated with or without the ARIs AL1576 or Tolrestat. With the exception of Ranirestat, mouse data *in A, C and E* has been previously reported (Kador et al., 2012). Bars represent the mean $\pm$ S.E.M of a minimum of 3 separate analyses. The gel illustrates the representative appearance of one analysis. * $p < 0.05$.

Fig. 8.

Induction of (A) P-Akt (C) P-SAPK/JNK and (E) P-ERK1/2 signaling changes in AK-SMAA-GFP versus AK-SMAA-GFP-hAR mice treated with or without the ARIs AL1576 or Ranirestat and (B) P-Akt, (D) P-SAPK/JNK, and (F) ERK1/2 signaling changes in normal versus diabetic rats treated with or without the ARIs AL1576 or Tolrestat. With the exception of Ranirestat, mouse data in A, C and E has been previously reported (Kador et al., 2012). Bars represent the mean $\pm$ S.E.M of a minimum of 3 separate analyses. The gel illustrates the representative appearance of one analysis. * $p < 0.05$.