Polyol Effects on Growth Factors and MAPK Signaling in Rat Retinal Capillary Cells

Peng Zhang,¹ Zifeng Zhang,¹ and Peter F. Kador^{1,2}

Purpose: Recent studies report that growth factor and signaling changes in rat lenses do not directly result from the presence of diabetes or sorbitol/galactitol (polyol) formation/accumulation, but from secondary osmotic changes associated with the aldose reductase (AR) catalyzed polyol formation. AR is also present in rat retinal pericyte and endothelial cells; however, significant polyol formation only occurs in pericytes and this does not appear to be linked to osmotic changes. The purpose of this study was to determine whether polyol formation and AR activity are similarly linked to growth factor and signaling changes in the rat capillary cells despite the apparent absence of osmotic stress.

Methods: Conditionally immortalized rat retinal pericyte (TR-rPCT) and endothelial (TR-iBRB) cell lines were cultured on collagen type 1-coated dishes in the DMEM containing 5.5 mM glucose. After 24 h of initial culture, the medium was replaced with a serum-free medium containing 5.5, 25, or 50 mM glucose or galactose with/without the aldose reductase inhibitors (ARIs) AL1576 or tolrestat for periods of up to 48 h. Growth factors and transduction pathways were measured by Western blots using the antibodies against basic FGF, IGF-1, TGF- β , P-ERK1/2, P-SAPK/JNK, and P-Akt.

Results: Sorbitol accumulation was only observed in pericytes, while galactitol was present in both pericytes and endothelial cells. Pericytes cultured in high glucose showed increased expression of the growth factors basic FGF, IGF-1, TGF- β , and signaling in P-Akt, P-ERK1/2, and P-SAPK/JNK compared with those cultured in 5.5 mM glucose and these expressions were normalized by the presence of ARIs. Similar results were observed with galactose media. In contrast, endothelial cells cultured in high glucose media showed neither growth factor or signaling changes. In galactose media, endothelial cells showed increased expression of basic FGF, IGF-1, TGF- β , P-ERK1/2, and P-SAPK/JNK, which were only partially reduced by ARIs.

Conclusion: Growth factor and MAPK signaling expression in pericytes are linked to the presence of polyols. Pericytes, which readily accumulate sorbitol/galactitol that is inhibited by ARIs, show expression changes similar to those observed in rat lenses. In contrast, endothelial cells only show partial expression changes that are linked to galactitol accumulation.

Introduction

DIABETIC RETINOPATHY (DR) is primarily a vascular disease where hyperglycemia is the underlying cause in patients with either type 1 or type 2 diabetes mellitus. This premise is clinically supported by the Diabetes Control and Complications Trials (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) trials, which conclude that the development and progression of DR is directly related to the tight control of blood glucose levels.^{1,2} Microvascular lesions associated with DR also develop in animals fed a diet rich in galactose, indicating that the metabolism of either excess glucose or galactose results in the development of similar vascular lesions.^{3–5} The onset and progression of

these vascular lesions in both animals with either diabetes mellitus or galactosemia are reduced by the tight control of hyperglycemia or the reduction of galactosemia, respectively.^{6–8} In addition, the development of these vascular lesions in both diabetic and galactosemic animals is inhibited by the administration of adequate levels of aldose reductase inhibitors (ARIs). This indicates that the enzyme AR, which catalyzes the reduction of glucose and galactose to the polyols sorbitol and galactitol, respectively, plays a key role in the development of the vascular lesions associated with DR.

In the development of DR, vascular changes of the retinal capillary bed include pericyte loss, capillary basement membrane thickening, endothelial cell hypertrophy, and endothelial cell degeneration that lead to capillary nonperfusion

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

²Department of Ophthalmology, School of Medicine, University of Nebraska Medical Center, Omaha, Nebraska.

and hypoxia. Retinal capillaries are composed of endothelial cells and pericytes, which can influence and communicate with one another through soluble growth factors, gap junctions, and adhesion molecules. These communications are central to vessel assembly, growth control, and normal function.⁹ The selective loss of pericytes has been reported to be the hallmark of DR.^{10,11} AR is present in pericytes in cultured dog, human, bovine, and rat pericytes^{12–15} and experimentally, retinal pericyte destruction has been linked to the formation of polyols under both hyperglycemic and galactosemic conditions.^{13,16,17} This polyol accumulation has been shown to induce apoptosis that can be arrested by inhibition of AR.^{12,18,19}

Whereas AR is only present in retinal pericytes in the dog,¹⁶ it is present in both retinal capillary pericytes and endothelial cells in the rat. Using an immortalized rat cell line of retinal capillary pericytes (TR-rPCT) and endothelial (TR-iBRB) cells to gain insight into the role of AR activity in capillary cell destruction, we have reported that culture of these cells in 50 mM glucose or galactose results in significant polyol accumulation in pericytes, but not endothelial cells where little accumulation of galactitol and no accumulation of sorbitol were observed.¹² This suggests a difference in the levels and/or activity of AR in pericytes. TUNEL staining for apoptosis was also primarily limited to the pericytes, where significant levels of polyol accumulation occur. However, unlike in the lens epithelial cells where polyol-linked osmotic changes initiate endoplasmic (ER) stress that leads to the generation of reactive oxygen species through an unfolded protein response, neither pericytes or endothelial cells showed an induction of ER stress.²⁰ This indicates that the polyol-linked apoptosis of pericytes is not associated with the induction of ER stress.

The AR activity and polyol accumulation have also been linked to growth factor and signaling changes. Lenses from either diabetic rats or from glucose/galactose culture conditions show increased expressions of basic FGF, TGF- β , and increased signaling through P-Akt, P-ERK1/2, and P-SAPK/JNK. These changes are normalized by ARIs to the levels observed in nondiabetic controls or normal culture conditions. However, the increased growth factor expression or altered signaling is absent when rat lenses are cultured in osmotically compensated media containing 30 mM glucose or galactose. This indicates that neither the AR activity nor its resulting polyol formation specifically causes the growth factor/signaling changes in the rat lens. Instead, it is a biophysical response of the lens to osmotic stress that can result from the AR catalysed polyol accumulation that initiates the increased intralenticular production of basic FGF and TGF- β and the altered cytotoxic signaling. In our continuing studies with rat retinal capillary cell lines, we demonstrate that high glucose/galactose media and polyol formation also induce growth factors and signaling changes.

Methods

Chemicals

All other chemicals were of analytical grade. Electrophoretic materials all were obtained from Bio-Rad Laboratories (Hercules, CA). ARIs spiro-(2,7-difluoro-7H-fluorene-9,4'-imidazolidine)-2'-5'-dione (AL1576) was obtained from Alcon Laboratories (Fort Worth, TX) and tolrestat (N-[(5-

trifluoromethyl-6-methoxy-1-naphthalenyl)thioxomethyl]-N-methyl-glycine) was obtained from Wyeth-Ayerst Laboratories (Princeton, NJ). The bFGF rabbit monoclonal antibody, TGF- β rabbit monoclonal antibody, the phospho-Akt (Ser473) rabbit monoclonal antibody, phospho-ERK1/2 (phospho-44/42 MAPK) (Thr202/Tyr204) rabbit monoclonal antibody, phospho-SAPK/JNK monoclonal antibody, and GAPDH rabbit monoclonal antibody were obtained from Cell Signaling Technologies (Beverly, MA). The enhanced chemiluminescence system components, including the horseradish peroxidase (HRP)-conjugated anti-rabbit antibody and chemiluminescent reagent were obtained from Cell Signaling Technologies.

Retinal capillary pericytes

Frozen cell stocks of the TR-rPCT pericyte cells in cryotubes were thawed in a 37°C water bath and following repeated removal of DMSO with the DMEM, the cells were seeded to 75-cm² culture flasks coated with collagen type 1 and cultured in the DMEM containing glutamine, NaHCO₃, and streptomycin at 33°C in a humidified atmosphere of 5% CO₂ and 95% air until subconfluent (2–4 days). The cells were then passaged by treatment with trypsin-EDTA when the cells became 80–90% confluent. The cells were then plated at a density of 2×10^4 in 96-well plates or 1×10^5 in 24-well plates depending on the experiment. After an overnight incubation, the attached pericytes were washed twice and incubated with the serum-free DMEM containing specified concentrations of glucose for specific times. Only cells passaged less than 7 times were utilized.

Retinal capillary endothelial cells

Frozen cell stocks of the TR-iBRB endothelial cells in cryotubes were thawed in a 37°C water bath and following removal of DMSO with the DMEM, the cells were seeded to 75-cm² culture flasks coated with collagen type 1. The cells were then cultured in the DMEM containing glutamine, ECGF, NaHCO₃, and streptomycin at 33°C in a humidified atmosphere of 5% CO₂ and 95% air until subconfluent (2–4 days). The cells were passaged by treatment with trypsin-EDTA when the cells became 80–90% confluent. The cells were then plated at a density of 2×10^4 in 96-well plates, 1×10^5 in 24-well plates, or 2×10^6 in 100-mm dishes depending on the experiment. After an overnight incubation, the attached endothelial cells were washed twice and incubated with the serum-free DMEM containing specified concentrations of glucose for specific times. Only cells passaged less than 7 times were utilized.

Western blot analysis

Cells were washed three times with phosphate-buffered saline, harvested, and lysed in a buffer (1% NP-40, 0.5% deoxycholate, 1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH=8) supplemented with a mixture of protease inhibitors (Cell Signaling Technologies). Cells were disrupted in a syringe fitted with a 21-gauge needle, with cell debris removed by centrifugation at 13,000 rpm for 15 min at 4°C. Protein samples were prepared in the SDS-PAGE sample buffer and equal amounts of protein (50–200 μ g) were loaded onto polyacrylamide gels. Protein levels were quantified using the colorimetric DC Protein Assay (Bio-Rad Laboratories) and bovine serum albumin (BSA) protein standards. After

FIG. 1. Comparison of growth factor expression levels normalized to GAPDH of rat retinal pericytes (TR-rPCT) and endothelial (TR-iBRB) cells cultured for 48 h in media containing up to 50 mM glucose with/without the presence of 10 μ M of the aldose reductase inhibitors (ARIs) AL1576 or Tolrestat. *Panels (A, C, E)* show changes in pericytes of bFGF, IGF-1, and TGF- β levels, respectively, while *(B, D, F)* show changes in endothelial cell levels of bFGF, IGF-1, and TGF- β , respectively. No expression changes were observed in endothelial cells, while pericytes showed expression changes in high glucose that were normalized by the presence of ARIs. Mean $\pm$ standard error of mean (SEM); $n=3-5$. *Significantly different ($P \leq 0.05$) compared with 5 mM glucose control.

electrophoresis, the separated proteins were blotted onto the nitrocellulose membrane. The membranes were then separately incubated overnight at 4°C with antibodies against bFGF, TGF- β , Phospho-Akt, phospho-ERK1/2, and phospho-SAPK/JNK in accordance with the manufacturer's instructions. After final washings with 0.05% TBS-Tween, membrane bound antibody complexes were visualized by applying the HRP-conjugated anti-rabbit antibody to the membrane for 1 h at room temperature. The blots were again washed with TBS and processed for chemiluminescence detection of the immunoreactive proteins after incubation for 5 min at room temperature. The visualized protein levels were quantified using the NIH ImageJ program (<http://rsbweb.nih.gov/ij/>)

and Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

Statistical analysis

Analyses were conducted with the ANOVA software on ProStat ver. 5.01 (Pearl River, NY) and Origin Pro 8.1 (OriginLab Corporation, Northampton, MA). All results with a $P \leq 0.05\%$ were considered to be significant.

Results

Rat retinal capillary pericytes (TR-rPCT) and endothelial cells (TR-iBRB) established from a transgenic rat harboring

FIG. 2. Comparison of expression levels normalized to GAPDH of rat retinal pericytes (TR-rPCT) and endothelial (TR-iBRB) cells cultured for 48 h in media containing up to 50 mM glucose with/without the presence of 10 μ M of the ARIs AL1576 or Tolrestat. Panels (A, C, E) show signaling changes in pericytes of Akt, Erk1/2 (44/42 MAPK)m or JNK (SAPK/JNK) levels, respectively, while (B, D, F) show changes in endothelial cell levels of Akt, Erk1/2, or JNK, respectively. No expression changes in response to glucose were observed in endothelial cells. Mean $\pm$ SEM; $n=3-5$. *Significantly different ($P \leq 0.05$) compared with 5 mM glucose control.

the temperature-sensitive simian virus 40 (SV 40) large T-antigen gene (Tg rat) were grown at 33°C on rat tail type I collagen-coated plates with the DMEM containing 10% FBS as previously described.¹² The cells were passaged by treatment with trypsin-EDTA when 80–90% confluent and then plated at a density of 1×10^5 cells onto 24-well plates, or 2×10^6 cells onto 100-mm dishes. After an overnight incubation, the attached pericytes were washed twice and incubated with the serum-free DMEM containing 5, 25, or 50 mM of glucose or galactose for up to 48 h. This resulted in polyol accumulation in cells cultured in media containing 25 or 50 mM of sugar, with maximum polyols generated when cells were cultured for 48 h with 50 mM of sugar. A forty-eight-hour incubation of the pericytes in 50 mM glucose or galactose resulted in sorbitol concentrations of up to 0.96 ± 0.04 mmol/mg protein and galactitol concentrations of 3.84 ± 0.27 mmol/mg protein (mean $\pm$ SD). Both sorbitol and

galactitol were inhibited by the presence of 10 μ M of the ARIs AL1576 or tolrestat. In contrast, sorbitol formation was not observed when endothelial cells were similarly cultured with 50 mM glucose. However, 0.75 ± 0.11 mmol/mg protein of galactitol was observed when endothelial cells were cultured with 50 mM galactose and formation was also inhibited by 10 μ M of the ARIs AL1576 or tolrestat.

Since growth factor and signaling changes are induced in rat lenses accumulating sorbitol or galactitol,²¹ we investigated whether similar changes occur in rat retinal capillary pericytes and endothelial cells (TR-rPCT and TR-iBRB) accumulating polyols. As shown in Fig. 1, culturing the retinal cells in 25 or 50 mM glucose media resulted in a significant induction of the growth factors bFGF, IGF-1, and TGF- β in the retinal pericytes, but not endothelial cells. This induction was reduced to levels observed when pericytes were cultured in 5 mM glucose by treatment with 10 μ M of the ARIs

FIG. 3. Comparison of growth factor expression levels normalized to GAPDH of rat retinal pericytes (TR-rPCT) and endothelial (TR-iBRB) cells cultured for 48 h in media containing up to 50 mM galactose with/without the presence of 10 μ M of the ARIs AL1576 or Tolrestat. Panels (A, C, E) show changes in pericytes of bFGF, IGF-1, and TGF- β levels, respectively, while (B, D, F) show changes in endothelial cell levels of bFGF, IGF-1, and TGF- β , respectively. Similar expression changes in response to high galactose were observed in both pericyte and endothelial cells, and these changes were reduced by the presence of ARIs. Mean $\pm$ SEM; $n=3-5$. *Significantly different ($P \leq 0.05$) compared with 5 mM galactose control.

AL1576 or Tolrestat. Since sorbitol formation in pericytes is reduced by ARIs and endothelial cells do not accumulate sorbitol, this suggests that the observed induction of bFGF, IGF-1, and TGF- β is linked to sorbitol accumulation. A similar sorbitol-linked induction of cellular signaling changes in Akt, Erk1/2 (44/42 MAPK), or JNK (SAPK/JNK) was observed in retinal pericytes, but not endothelial cells (Fig. 2).

In contrast to glucose, galactose is reduced to galactitol by AR in both pericytes and, to a lesser extent, in endothelial cells. As shown in Fig. 3, culture of both retinal cells in 25 or 50 mM galactose media showed a similar induction of bFGF, IGF-1, and TGF- β growth factors. This induction was also reduced by ARI treatment in both retinal capillary cells. In the pericytes, culture in galactose media resulted in increased

cellular signaling in the Akt, Erk1/2, and SAPK/JNK pathways similar to that observed with glucose (Fig. 4). This increased signaling was also reduced by treatment with ARIs. However, in the galactose cultured endothelial cells, increased signaling was observed in the Erk1/2 and SAPK/JNK pathways, but not the Akt pathway.

Discussion

Diabetic and galactosemic animal studies show that pericyte degeneration is associated with localized endothelial cell proliferation and the formation of microaneurysms, while endothelial cell degeneration leads to the formation of acellular capillaries and areas of vascular nonperfusion.^{14,17,22,23}

FIG. 4. Comparison of expression levels normalized to GAPDH of rat retinal pericytes (TR-rPCT) and endothelial (TR-iBRB) cells cultured for 48 h in media containing up to 50 mM galactose with/without the presence of 10 μ M of the ARIs AL1576 or Tolrestat. Panels (A, C, E) show signaling changes in pericytes of Akt, Erk1/2 (44/42 MAPK), or JNK (SAPK/JNK) levels, respectively, while (B, D, F) show changes in endothelial cell levels of Akt, Erk1/2, or JNK, respectively. Mean $\pm$ SEM; $n = 3-5$. *Significantly different ($P \leq 0.05$) compared with 5 mM galactose control.

Administration of ARIs to these animals reduces the development of these retinal vascular changes.^{5,24,25} The development of these vascular changes is preceded by the formation of cataracts that are also linked with the AR catalyzed polyol formation and inhibited by ARI administration.^{6,26-28}

Rats have been primarily used as animal models for investigating the onset and progression of DR. However, the specific physiological and biochemical changes induced by diabetes/hyperglycemia on rat retinal capillary pericytes and endothelial cells during the development of DR have not been adequately defined. This may be due, in part, to the difficulty of obtaining adequate amounts of retinal vascular tissue from the rat eye for such studies. The availability of rat TR-rPCT pericytes and TR-iBRB endothelial cell lines may facilitate such studies.¹²

The present studies show that the growth factors bFGF, IGF-1, and TGF- β and the cellular signaling of Akt, Erk1/2

(44/42 MAPK), and JNK (SAPK/JNK) are induced when pericyte cells are cultured in high glucose or galactose media. It has been documented that the ocular changes associated with diabetes (elevated blood glucose levels) and galactosemia (elevated galactose levels) are linked to the AR activity, which reduces both glucose and galactose to their respective sugar alcohols. However, while both hexoses are substrates for AR, galactose is a better substrate for AR than glucose and galactitol accumulation is more rapid compared with sorbitol because galactitol is not further metabolized by sorbitol dehydrogenase. The prominence of growth factor induction is contrasted to that of endothelial cells, where similar induction of growth factors and signaling only occurs when the cells are cultured in the galactose medium. Support for a central role of AR in the induction of growth factor and signaling in both pericytes and endothelial cells comes from the observation that this induction is reduced in both

pericytes and endothelial cells by ARIs. Although both rat pericytes and endothelial cells express AR, only pericytes accumulate sorbitol, suggesting that AR levels in endothelial cells are too low for significant sorbitol production to occur. This may explain why the induction is only observed in endothelial cells cultured in galactose. This is also consistent with the observation that pericytes cultured for 24 h in serum-free media containing ≥ 25 mM glucose demonstrate apoptosis that is linked to sorbitol accumulation, while similar culture of endothelial cells does not result in either sorbitol formation or apoptosis.¹²

Whereas sugar cataracts in diabetic and galactosemic rats are directly linked to the AR catalyzed accumulation of sugar alcohols, recent lens culture studies in both glucose of galactose media indicate that growth factors and signaling changes do not directly result from either the AR activity or the accumulation of sugar alcohols.²¹ Instead, they are due to osmotic stress resulting from the abnormal accumulation of sorbitol or galactitol.^{29,30} Osmotic stress also induces ER stress in the lens and other cells.^{31,32} Since GRP78 is not induced in either the rat pericytes or endothelial cell lines present, this suggests that the induction of growth factor and signaling changes appears to be directly linked to the AR activity rather than to osmotic changes. However, the absence of Akt induction in galactose-cultured endothelial cells and inhibition by ARIs suggest that the relationship between the AR activity and signaling changes may be more complex. We hypothesize that the induction of the present growth factors and signaling pathway changes in response to the adverse effects of AR linked activity/sugar alcohol accumulation represent the cells' initial survival factor response. However, their prolonged induction could eventually lead to pathological cellular changes. Further studies are required to elucidate the exact relationship between the AR activity, sugar alcohol accumulation, and the induction of these growth factors and signaling pathways.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Dr. Peter F. Kador
Department of Pharmaceutical Sciences
College of Pharmacy
University of Nebraska Medical Center
986025 Nebraska Medical Center
Omaha, NE 68198-6025

E-mail: pkador@unmc.edu