Effects of Insulin Insufficiency and High Glucose on Cell Survival of Dog Retinal Capillary Pericytes

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Abstract

The selective loss of capillary pericytes has been well established as a hallmark of diabetic retinopathy. In this study, using cultured dog retinal capillary pericytes, the effects of insulin and high glucose on the viability of pericytes have been investigated. Dog retinal capillary pericytes were cultured in DMEM medium supplemented with 20% fetal calf serum (FCS). Cell viability was evaluated in medium containing 1% FCS. The expression levels of the insulin receptor and bcl-2 were examined by RT-PCR. Mitogen-activated protein (MAP) kinase was assayed by Western blot using monoclonal antibody against phospho-Elk-1 (ser383). Cell viability of the cultured retinal pericytes was significantly higher when cultured in medium containing insulin for 7 days than when cultured without insulin. Cells treated with insulin increased the expressions of both the insulin receptor and bcl-2. Activation of MAP kinase was also detected. In contrast, cell viability and the expression levels of the insulin receptor and bcl-2 were all down-regulated when 50 mM glucose was added to the culture medium. The data suggest that insulin is of importance in maintaining dog pericytes. The data also suggest that, under hyperglycemic conditions, pericytes become more susceptible to the various insults that initiate the process of apoptosis.

Key words: *Retinal pericyte; dog; retinopathy; insulin; insulin receptors*

Introduction

The presence of retinal capillary occlusions is a characteristic feature of diabetic retinopathy. It has been well established that anoxia and/or hypoxia induced by capillary occlusions are associated with clinically-apparent retinal capillary changes that include the formation of microaneurysms, retinal haemorrhages, massive vitreous haemorrhages, and neovascularization. It is also well established that the loss of retinal capillary pericytes is one of the earliest morphological lesions of diabetic retinopathy.[1,2] Preventing pericyte degeneration may be a key for preventing the whole process of diabetic retinopathy.

In diabetes, insulin is either produced in insufficient quantities (insulin deficiency, Type 1) or fails to function (insulin resistance, Type 2). To initiate proper signaling, insulin requires cell surface receptors.[3] It has been reported that bovine retinal pericytes express insulin receptors and that both insulin and insulin-like growth factor stimulate the proliferation of bovine pericytes.[4]

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However, it has not been established whether an insufficient level of insulin contributes to the specific pericyte degeneration observed in diabetes. In contrast, the importance of chronic hyperglycaemia on the onset of various diabetic complications has been well established. Proposed mechanisms for initiating diabetic complications such as polyol accumulation, myoinositol depletion, non-enzymatic glycation, and NAD-redox imbalances are all based on increased levels of glucose.[5-7] Hyperglycaemia also antagonizes various actions of insulin and decreases secretion of insulin.[8] Moreover, the DCCT trial confirms that strict control of blood glucose delays the onset of various diabetic complications including retinopathy.[9-11]

The pericyte cell death in diabetic retinopathy has recently been linked to apoptosis.[12] Apoptosis of retinal microvascular cells is specifically increased in both diabetes and galactosaemia.[13] Nerve growth factor, which prevents apoptosis of Mueller cells and ganglion cells, also prevents the development of pericyte loss in diabetic rats.[14] Fluctuation of glucose levels has also been linked to apoptosis of pericytes.[15,16] Bovine retinal pericytes cultured in high glucose medium also undergo apoptosis that is prevented by aldose reductase inhibitors.[17] To understand the specific cause of pericyte cell death in diabetes, it is important to understand how diabetes affects the viability of pericytes. Here, the effects of insulin and high glucose on the survival of retinal capillary pericytes isolated from dogs, an animal model that develops retinal changes similar to human diabetic retinopathy, [18,19] have been investigated.

Materials and Methods

Materials

All chemicals utilized were of analytical grade. Collagenase was obtained from Worthington Biochemicals (Freehold, NJ). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Biofluids (Rockville, MD). Anti-rabbit smooth muscle actin, peroxidase conjugated rabbit anti-mouse IgG and DAB substrate kit were obtained from Zymed Laboratories (San Francisco, CA), ICN Pharmaceuticals (Aurora, Ohio) and VECTOR Laboratories (Burlingame, CA), respectively.

Beagle dog eyes were obtained from Marshall Farm USA (Rose, NY). All eyes were stored at 4°C and utilized within 24 hours of death.

Dog Retinal Capillary Pericytes

Dog retinal capillary pericytes were cultured from dog eyes as described by Capetandes et al.[20] The neural retina was carefully removed from dog eyes under a dissecting microscope and was homogenized in phosphate buffered saline (PBS) in a Dounce tissue homogenizer. The homogenate was filtered through two stainless steel sieves (210 μm and 86 μm sieves, Tetko, Elmsford, NY) to remove large vessels. Tissues retained on the 86 μm sieve were digested with PBS containing 0.066% collagenase for 30 min at 37^0C. After the digestion, the microvessel fragments were collected, suspended in DMEM medium containing 20 % FCS, and cultured in plastic culture flasks. The identity of pericytes was confirmed by demonstrating the presence of α-smooth muscle actin.[21] Unless otherwise stated, the experiments in this study were conducted on confluent cultures at the third passage.

Table 1: Primers utilized for RT-PCR amplification

Insulin receptor (378 bp)	
Sense:	5'-GAAGATTTCCGAGACCTCAG -3'
Antisense:	5'-GGCAGTTGGTCTTGCCCTT -3'
Bcl-2 (229 bp)	
Sense:	5'-GTGGAGAGCGTCAACCGG-3'
Antisense:	5'-AGGCACCCAGGGTGATGC -3'
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 253 bp)	
Sense:	5'-CATCACCATCTTCCAGGAGC-3'
Antisense:	5'-TAAGCAGTTGGTGGTGCAGG-3'

The numbers closed by parentheses are the predicted lengths of amplified cDNA products with these primers.

Cell Viability Assay

Cells suspended in DMEM medium supplemented with 20% FCS were plated at sparse densities (50,000 cells/well) onto multiwell dishes (2.1 cm^2/well). After incubating overnight to allow the cells to attach, the medium was replaced with DMEM medium containing 1% FCS and either 1 μg/ml insulin or 50mM glucose. The medium was replenished with fresh medium every 3 days. After culture for 7 days, the cells were rinsed with PBS, harvested with trypsin, and the cell number was counted in a hemocytometer. Cell viability was expressed as the cell number per well after 7-day culture. Each assay was repeated at least five times.

RNA Preparation

mRNA was isolated using the quick prep micro mRNA purification kit (Pharmacia, Piscataway, NJ). Briefly, the cells were extracted in a buffered solution containing a high concentration of guanidinium thiocyanate (GTC). The extracts were diluted 3-fold with 10 mM Tris Buffer, pH 7.5 containing 1 mM EDTA and transferred to microcentrifuge tubes containing oligo (dT)-cellulose. After the oligo (dT)-cellulose was washed 5 times with 10 mM Tris buffer, pH 7.5 containing 1 mM EDTA and 0.5 M NaCl, the cellulose was washed 3 times with the same Tris buffer containing 0.1 M NaCl. The polyadenylated material (mRNA) was then eluted with 10 mM Tris buffer, and pH 7.5 containing 1 mM EDTA. The obtained mRNA was precipitated by the addition of 95% ethanol at –20°C for 30 min, collected by centrifugation, dried, and suspended in deionized water treated with diethylpyrocarbonate (0.1%).

Reverse Transcription - Polymerase Chain Reaction (RT - PCR)

Cell mRNA was reversely transcribed to cDNA using oligo (dT) as a primer from 100 ng of cell mRNA. Samples were incubated at 40°C for 60 min. The reaction was stopped by incubating at 95°C for 2 min and cooled at 4°C. One microliter of the cDNA reaction was then amplified using PCR.

The primers utilized are listed on Table 1. All primers were designed in the highly conserved region (at least 85% of homology) between human and rat. The amplification reactions contained 50 mM KCl, 10 mM Tris-HCl, pH 8.0, 1.5 mM $MgCl_2$, 0.2 mM of dATP, dTTP, dGTP and dCTP, 2 units of amplitaq DNA polymerase and 0.4 mM of 5' and 3' primers. Amplifications were carried out for 30 cycles of 94°C for 1 min

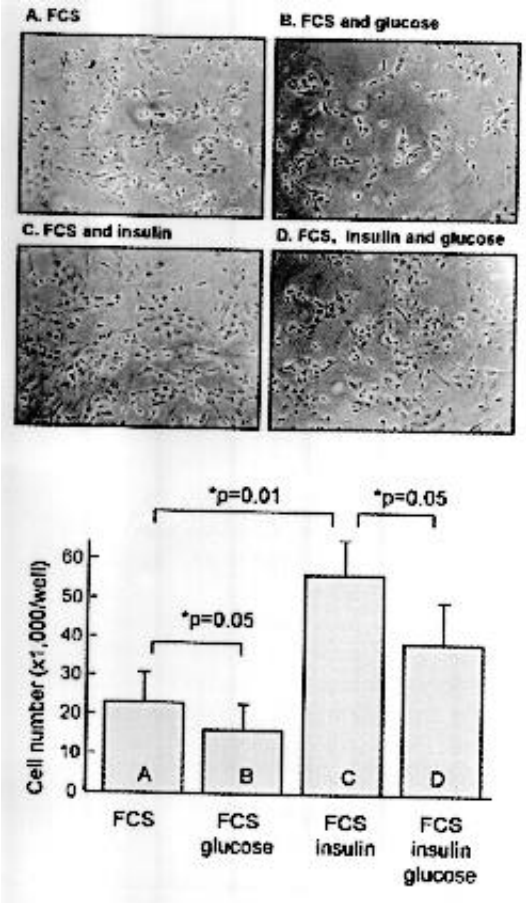

Figure 1. Cell viability of dog retinal capillary pericytes cultured for 7 days in either medium containing 1% FCS (A), medium containing 1% FCS and 50 mM glucose (B), medium containing 1% FCS and 1 µg/ml insulin (C), or medium containing 1% FCS, 1 µg/ml insulin and 50 mM glucose (D). The bar graph illustrates pericyte cell number in one well of multiwell dish (2.1 cm^2). mean ± S.D. (n=5).

(denaturing), 60°C for 1 min (annealing), 72°C for 1 min (extension) with a final extension at 72°C for 10 min. The PCR products were visualized by electrophoresing the reaction in 2% agarose gels containing 0.5 µg/ml ethidium bromide.

p44/42 MAP Kinase Activity Assay

Mitogen-activated protein (MAP) kinase activity was assayed using the p42/44 MAPK assay system (New England Biolabs, MA).

Dog pericytes (10^6 cells) were treated with either insulin or high glucose for 3 days as described by Adachi et al.[22] and Chao.[23] After treatment, the cells were incubated with 0.5 ml cell lysis buffer, sonicated on ice, and then microcentrifuged. A monoclonal phospho-antibody to p44/42 MAP kinase (15 µl) was used to selectively immunoprecipitate active MAP kinase from cell lysates. The resulting immunoprecipitate was incubated with an Elk-1 fusion protein in the presence of 1 µl ATP and 50 µl kinase buffer. This allows immunoprecipitated active MAP kinase to phosphorylate Elk-1. Phosphorylation of Elk-1 at Ser383 was detected by Western blot using a phospho-Elk-1 (Ser383) antibody (1:1000 dilution).

Statistical Analysis

Significance of results was determined using Statview software (BrainPower, CA). Cell number in each assay was compared using the Kruskal-Wallis test and Post-hoc test (Scheffe's F). P values less than 0.05 were considered to be significant.

Results

Dog retinal capillary pericytes continuously grow in DMEM medium supplemented with 20% FCS through at least 5 to 6 passages. However, when FCS is removed or reduced to an extremely low concentration, pericytes

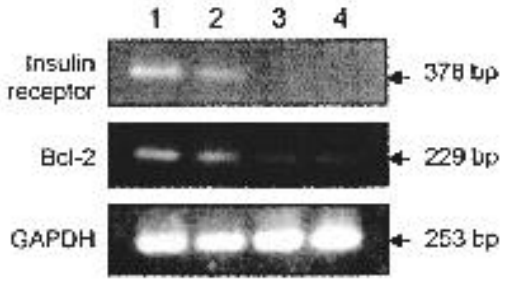

Figure 2. RT-PCR of insulin receptors and bcl-2 of dog retinal capillary pericytes cultured for 3 days in either medium containing 1% FCS and 1 μg/ml insulin (lane 1), medium containing 1% FCS, 1 μg/ml insulin and 50 mM glucose (lane 2), medium containing only 1% FSC (lane 3), or medium containing 1% FCS and 50 mM glucose (lane 4).

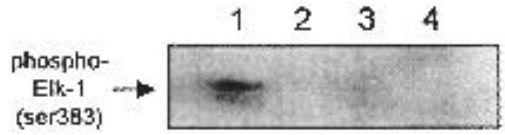

Figure 3. MAP kinase activation in dog retinal capillary pericytes cultured for 3 days in either medium containing 1% FCS and 1 μg/ml insulin (lane 1), medium containing 1% FCS, 1 μg/ml insulin and 50 mM glucose (lane 2), medium containing only 1% FSC (lane 3), or medium containing 1% FCS and 50 mM glucose (lane 4). The activation of MAP kinase was detected using monoclonal antibody against phospho-Elk-1 (ser383).

are no longer able to survive and many of these cells are gradually detached from the culture plate. Insulin appears to protect these cells from FCS starvation (Figure 1). When dog pericytes are cultured in DMEM medium containing 1% FCS, the number of live cells attached to the plate was 23,000 ± 7,800/ well (n=5) compared to 56,400 ± 8,700/well (n=5) when 1 μg/ml insulin was added to the medium. In contrast, high glucose reduced the viability of pericytes cultured in 1% FCS medium. The cell numbers after culture in the presence of 50 mM glucose for 7 days decreased to 16,200 ± 6,500 without insulin and 38,600 ±10,900/well (n=5) with insulin, respectively.

To verify the cell viability results, the expression of insulin receptor was examined (Figure 2). With RT-PCR, the expression of insulin receptors was clearly observed in pericytes cultured in the medium containing insulin. The cells cultured in the presence of 50 mM glucose also expressed the insulin receptors as far as the medium contained insulin. However, the level of expression was slightly lower in the presence of 50 mM glucose. In the absence of insulin, insulin receptor expression was not detected.

To further confirm the effects of insulin and glucose on cell viability, the expression of bcl-2, an apoptosis regulatory gene, was investigated (Figure 2). Although the expression of bcl-2 was detected in pericytes cultured in the medium containing only 1% FCS, this expression clearly increased when these cells were cultured in medium containing insulin. When the cells were cultured in high glucose medium, the expression level of bcl-2 appeared to be decreased.

When dog pericytes were treated with insulin, activation of MAP kinase was also clearly detected (Figure 3). However, in the presence of 50 mM glucose, MAP kinase activation by insulin was significantly decreased, and the immunostaining for phosphorylated Elk-1 was almost undetectable. The MAP kinase activity was also below the detectable levels when the cells were cultured in media containing only 1% FCS or 1% FCS and 50 mM glucose.

Discussion

In the 1960s, Kuwabara and Cogan[1,2] first described the selective loss of retinal capillary pericytes in the human as the hallmark of diabetic retinopathy. Similar

pericyte degeneration has been observed with various diabetic animal models such as the rat[24] and dog.[25,26] Interestingly, when fed with galactose diet for a long time, both rats and dogs also develop the retinal capillary changes similar to diabetic retinopathy in the human with the apparent pericyte loss.[24,27,28] Since insulin levels are not affected by galactose, these animal models clearly indicate that elevated sugar levels can initiate the degeneration of capillary pericytes. The present data, however, provides another perspective on pericyte degeneration. Both insulin deficiency and hyperglycaemia decrease the viability of retinal capillary pericytes and this, in turn, may affect their susceptibility to various insults such as osmotic imbalance induced by sorbitol accumulation.

When dog pericytes are cultured in the medium containing insulin, the cell survival is significantly increased. This was confirmed by the observation that insulin receptor expression also increased when the cells were treated with insulin. This is consistent with a previous study with bovine capillary pericytes.[4] Insulin stimulates the proliferation of bovine pericytes with an increase in receptor expression. Since insulin promotes pericyte cell viability from two different species - dog and cow, insulin may also be important in maintaining the cell viability of retinal pericytes in other species including humans.

While the present study does not answer the question of how insulin promotes pericyte survival, the present data does indicate that the level of bcl-2 substantially increases when the cells are stimulated by insulin. It is well known that this gene protects cells from apoptosis induced by various insults. This appears to be consistent with evidence that insulin protects dog pericytes from FCS starvation. Signal transduction is also important. Insulin action is mediated through tyrosine phosphorylation of the insulin receptor itself and various signaling molecules. [29] MAP kinase is a serine threonine kinase that is mainly a part of the Ras pathway. This signaling pathway is also well known as the major pathway for cell proliferation and differentiation by various growth factors or lymphokines. [30,31] In the present study, MAP kinase, assayed by detecting the phosphorylation of a transcription factor Elk-1, was clearly activated when dog pericytes were stimulated by insulin. This is again consistent with the evidence that insulin promotes the viability of pericytes.

It has been suggested that high glucose causes a replicative delay between DNA synthesis and mitosis in human endothelial cells.[32] In adipocytes, high glucose depletes intracellular glucose transporters.[33] Insulin receptor tyrosine kinase is also modulated by glucose.[34] The present data also demonstrated that, when dog pericytes were exposed to high glucose environment, insulin mediated cell survival, receptor expression and MAP kinase activation were all negatively modulated. Because the glucose concentration (50 mM) utilized in this study is extremely high compared to physiological blood glucose levels, the evidence of this study cannot be simply applied to human diabetics. However, combining all of these evidences may suggest that hyperglycaemia can deteriorate insulin action on cell viability of retinal pericytes. This may also suggest that the severity of diabetes and poor control of blood glucose not only increase the insults that initiate the cell death of pericytes but also increase the susceptibility of these cells to various insults associated with hyperglycaemia and diabetes.

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