Loss of Insulin-Mediated Vasoprotection: Early Effect of Diabetes on Pericyte-Containing Microvessels of the Retina

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PURPOSE. Microvascular cell death is a prominent pathologic feature of the retinopathy associated with insulin-deficient diabetes. The aim of this study was to test the hypothesis that reduced insulin action may contribute to microvascular damage in the diabetic retina.

METHODS. Microvascular complexes were isolated from retinas of healthy rats and those made insulin deficient by streptozotocin. As a model of ischemia, freshly isolated microvessels were maintained in a glucose-free, low-oxygen solution. Cell viability in pericyte-containing retinal microvessels was assayed by trypan blue dye exclusion. Cleaved caspase-3 immunoreactivity and nuclear morphology were used to detect apoptotic cells.

RESULTS. Ischemia significantly increased apoptotic cell death in pericyte-containing microvessels of the normal rat retina. In a dose-dependent ($IC_{50} = 600$ pM) manner, physiological concentrations of insulin markedly decreased ischemic cell death in the retinal microvasculature. This insulin-mediated vasoprotection was prevented by inhibitors of phosphatidylinositol-3-OH kinase and extracellular signal–regulated kinase. Soon after the onset of diabetes, insulin failed to significantly diminish cell death in ischemic retinal microvessels, in contrast to the prosurvival effect of this hormone on nondiabetic microvessels.

CONCLUSIONS. Results of this study support the hypothesis that a physiological concentration of insulin exerts a prosurvival effect on ischemic retinal microvessels and that diabetes impairs the ability of insulin to protect against ischemia-induced microvascular cell death. The authors propose that the loss of insulin-mediated vasoprotection may be a previously unappreciated mechanism by which diabetic retinopathy progresses. (*Invest Ophthalmol Vis Sci.* 2007;48: 2350–2355) DOI:10.1167/iovs.06-1357

Dysfunction of pericyte-containing retinal microvessels and the resultant ischemia are well-known complications of diabetes.[1–4] In the past two decades, substantial evidence has accumulated that hyperglycemia-induced changes, such as increased oxidative stress, advanced glycation end-products, protein kinase C, and polyol flux,[3,5–8] are likely to play important roles in causing damage to the microvasculature in the diabetic retina. More recently, there has been a growing appreciation that in addition to the pathophysiological consequences of excess glucose, reduced insulin action may be an etiological factor contributing to the progression of diabetic retinopathy.[9] However, though pericyte-containing retinal microvessels express insulin receptors,[10–12] it is not well understood how deficiency in insulin or changes in insulin signaling result in damage to the microvasculature of the diabetic retina.

Based on the known ability of insulin to exert a prosurvival effect on a variety of nonvascular cells,[13–15] we postulated that this hormone may have a vasoprotective role in the retina. More specifically, we hypothesized that a function of insulin is to protect pericyte-containing retinal microvessels from ischemia-induced cell death. We further conjectured that because of diabetes-induced changes in insulin signaling,[16] the vasoprotective action of this hormone may be diminished in the diabetic retina.

We report that physiological concentrations of insulin markedly decreased ischemic cell death in pericyte-containing microvessels of the normal rat retina. In contrast, soon after the onset of streptozotocin-induced diabetes, the ability of insulin to be vasoprotective was profoundly attenuated. These observations support the idea that the loss of insulin-mediated vasoprotection is one mechanism by which diabetic retinopathy progresses.

METHODS

Animal use conformed to the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the University of Michigan Committee on the Use and Care of Animals. Long-Evans rats (Charles River Laboratory, Cambridge, MA) were maintained on a 12-hour alternating light/dark cycle and received food and water ad libitum.

Experimental Model of Diabetes

As in our previous studies,[17,18] 5-week-old rats were given intraperitoneal injections of streptozotocin (150 mg/kg) diluted in 0.8 mL citrate buffer. Immediately before retinal microvessels were harvested, the blood glucose level was 382 ± 18 mg/dL ($n = 12$).

Microvessel Isolation

With our tissue print procedure,[17,19–22] we isolated microvascular complexes from the adult rat retina. With increasing concentrations of carbon dioxide, 6- to 14-week-old nondiabetic and diabetic rats were killed. Retinas were rapidly removed and placed in solution A, which consisted of 140 mM NaCl, 3 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM Na-Hepes, 15 mM mannitol, and 5 mM glucose at pH 7.4 with osmolarity adjusted to 310 mOsm/L. Retinas were incubated at 30°C in 2.5 mL Earle balanced salt solution supplemented with 0.5 mM EDTA, 20 mM glucose, 6 U papain (Worthington Biochemicals, Freehold, NJ), and 2 mM cysteine for 25 to 30 minutes while 95% oxygen–5% carbon dioxide was bubbled into this solution to maintain pH and oxygenation. For each new lot of papain, the duration of incubation was empirically adjusted to optimize the yield of isolated microvessels.

After incubation in the papain-containing solution, the retinas were transferred to a 60-mm Petri dish containing 5 mL solution A. Adherent vitreous was carefully removed with fine forceps, and the retinas were cut into quadrants. Then, one by one, each retinal quadrant was positioned with its vitreal surface up in a glass-bottomed chamber that contained 1 mL solution A. Subsequently, each retinal quadrant was sandwiched between the bottom of the chamber and a 15-mm diameter glass coverslip (Warner Instrument Corp., Hamden, CT) that was gently applied onto the vitreal surface of the retina. After approximately 30 seconds of compression, the coverslip was carefully removed; it contained adherent microvascular complexes. Photomicrographs and time-lapse movies of freshly isolated retinal microvascular vessels are available in recent publications.[17,19,22-24] All experiments were performed at room temperature (22°-23°C).

Model of Ischemia

To simulate ischemia, the 5 mM glucose in solution A was replaced with 5 mM mannitol, and the solution was bubbled with 100% nitrogen for 15 minutes; the pH and osmolarity of this "ischemic solution" were 7.4 and 310 mOsm/L, respectively. Coverslips containing freshly isolated retinal microvessels were placed in 35-mm Petri dishes that contained 2-mL aliquots of the ischemic solution. Coverslip-containing Petri dishes were kept in a modular chamber (Billips-Rothberg, Del Mar, CA) flushed with 100% N_2 for 15 to 30 minutes before they were tightly sealed; as reported previously,[25] this flushing technique reduced the oxygen level in the chamber to less that 0.005%. A reservoir of water maintained a well-humidified environment within the chamber. For control groups, coverslips were bathed in solution A and kept in Billips–Rothberg chambers containing room air.

Cell Viability Assay

Microvascular cells that failed to exclude trypan blue dye were classified as dead. As previously detailed,[18,26,27] coverslips containing retinal microvessels were exposed for 15 minutes to 0.04% trypan blue in solution A. Microvessels were then promptly examined at 100× magnification with an inverted microscope equipped with bright-field optics. The percentage of cells stained with trypan blue was determined for at least 200 microvascular cells per coverslip; assessed microvessels were of at least 300 μm in length. After this initial count, the location on each coverslip of the assayed microvessels was documented to permit the subsequent reassessment of cell viability in the same microvessels. For experiments using insulin, microvessels were exposed to this hormone for 30 minutes before the onset of ischemia; insulin remained in the bathing solution throughout the experiment. Unless otherwise noted, the concentration of insulin was 1 nM. In experiments using enzyme inhibitors, microvessels were exposed to an inhibitor for 45 minutes before the onset of ischemia.

Detection of Apoptosis

Microvessels were assessed for cleaved capase-3 immunoreactivity or nuclear morphology after incubation for 8 or 14 hours, respectively. As detailed previously,[27] microvessels were fixed for 30 minutes with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature. Vessels to be assessed for cleaved capase-3 immunoreactivity were exposed to 0.3% hydrogen peroxide in PBS for 30 minutes (to block endogenous peroxidase activity), to 0.5% Triton X-100 for 60 minutes, and then to rabbit anti–cleaved caspase-3 (1:1000; Cell Signaling Technology, Beverly, MA) for 16 hours at 4°. Subsequently, the coverslips were incubated with biotin-conjugated goat anti–rabbit IgG (Vector Laboratories, Burlingame, CA) at 1:200 for 1 hour and subsequently with the avidin-biotin-peroxidase complex (ABC method; Vector Laboratories) at 1:100 for 30 minutes After development with diaminobenzidine, microvessels were examined by bright-field and phase-contrast optics to detect immunopositive and nonstained cells, respectively. Assessment of nuclear morphology was performed on microvessels that had been exposed to Hoechst dye 33258 (8 μg/mL in phosphate-buffered saline) for 15 minutes at room temperature. Nuclei

were examined at 400× through a UV filter of a microscope (Eclipse E800; Nikon, Tokyo, Japan). For anti–cleaved caspase-3 and nuclear morphology assays, we could not establish with certainty whether a positive cell was an endothelial cell or a pericyte; thus, subclassification of microvascular cells into these two types was not feasible.
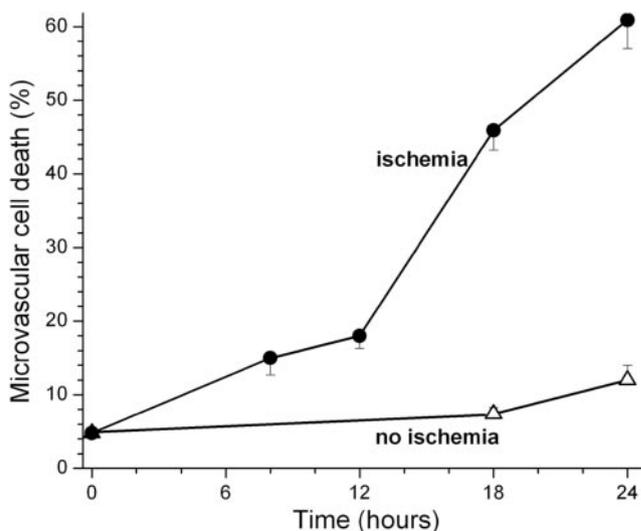
Chemicals

Insulin was derived from the bovine pancreas. DMSO, which was the solvent for the stock solutions of wortmannin, LY294002, PD98059, and SB203580, was present at 0.1% or less in the solutions that bathed the retinal microvessels. Unless noted otherwise, chemicals were obtained from Sigma (St. Louis, MO).

Statistical Analysis

Standard errors are presented in the text and shown in the figures. Unless noted otherwise, the two-tailed Student's *t*-test was used.

RESULTS

Cell viability was determined in pericyte-containing retinal microvessels isolated from adult rats and maintained under control or ischemic conditions for up to 24 hours (Fig. 1). Immediately after isolation from the retina, 4.8% ± 1% of the microvascular cells were unable to exclude the vital dye, trypan blue. After incubation for 18 hours in solution A (no ischemia), microvascular cell death was not significantly increased ($P = 0.063$; $n = 7$ coverslips assessed), although the rate of cell death increased to 12.0% ± 2% ($P = 0.002$; $n = 14$) after another 6 hours of incubation. In contrast to the modest amount of cell death observed under control conditions, microvessels maintained in the ischemic solution (no glucose, low oxygen; see Methods for details) showed a marked increase in nonviable cells. Specifically, a significant increase ($P = 0.004$) in cell death was detected within 8 hours of the onset of ischemia (Fig. 1). After 18 hours of ischemia, 45.9% ± 2.7% ($P < 0.001$; $n = 42$) of the microvascular cells failed to exclude trypan blue, and 60.9% ± 3.9% ($P < 0.001$; $n = 18$) of the microvascular cells were dead after 24 hours of ischemia;

FIGURE 1. Time course for cell death in isolated pericyte-containing retinal microvessels maintained under nonischemic (△) and ischemic conditions (●). For the zero time value, 290 vessel-containing coverslips were assessed; for other times, cell viability was quantified in 17 ± 5 vessel-containing coverslips. After 8 hours of ischemia, cell death increased significantly ($P = 0.004$). At 18 hours and 24 hours, cell death was markedly greater ($P < 0.001$) in ischemic than in nonischemic microvessels.

these levels of cell death were significantly greater ($P < 0.001$) than those observed in microvessels maintained under control conditions.

We also observed that microvascular cells with spheroid nuclei or with cleaved caspase-3 immunoreactivity were significantly ($P < 0.001$) more plentiful in ischemic microvessels than in nonischemic microvessels (Table 1). These findings, though not excluding the presence of necrosis, indicated that apoptosis of microvascular cells was triggered in our experimental model of ischemia.

Based on reports that insulin exerts a prosurvival effect on a variety of cell types[13-15,28] and that insulin receptors are expressed in the retinal vasculature,[10-12] we tested the hypothesis that insulin protects retinal microvessels from ischemia-induced cell death. In agreement with insulin having a vasoprotective role, Figure 2 shows that this hormone significantly diminished ($P < 0.001$) the microvascular cell death induced by 18 hours of ischemia; this was a concentration-dependent action of insulin. The half-maximally effective concentration for the vasoprotective effect of insulin was approximately 600 pM, which is close to the insulin concentration in the plasma of healthy rats (670 ± 150 pM).[29] Of note, we observed that insulin at a concentration of 100 pM, which is approximately twice that detected in the plasma of rats with streptozotocin-induced diabetes,[29] did not significantly ($P = 0.6$) protect retinal microvessels from ischemic cell death (Fig. 2). A maximal vasoprotective effect of insulin was achieved at a concentration of approximately 1 nM ($P < 0.001$; $n = 10$); at this concentration, insulin does not effectively activate IGF-1 receptors. Over the concentration range of 1 nM to 10 nM, insulin was equally ($P = 0.3$) effective in protecting retinal microvessels from ischemic cell death (Fig. 2). On the other hand, for reasons that remain to be elucidated, at the supraphysiological concentrations of 100 nM and 1 μM, insulin failed to protect retinal microvessels from ischemia-induced cell death. In another series of experiments, we found that in addition to providing vasoprotection during 18 hour-exposure to ischemic conditions, 1 nM insulin decreased by 35% ± 9% ($P = 0.008$; $n = 12$) the cell death that occurred during 24 hours of ischemia; insulin-induced decreases in ischemic cell death at 18 hours and 24 hours were not significantly different ($P = 0.08$).

Consistent with insulin exerting an antiapoptotic effect, Table 1 shows that exposure of ischemic retinal microvessels to 1 nM insulin significantly decreased the percentage of cells that were positive for the apoptotic markers, cleaved caspase-3 ($P = 0.009$; Fisher exact test) and spheroid nuclei ($P = 0.047$; Fisher exact test). These data are consistent with the protective
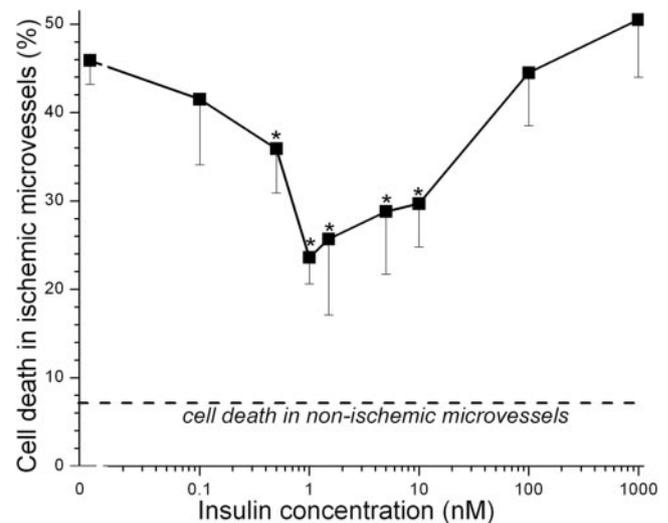

FIGURE 2. Dose–response relationship for the protective effect of insulin on cell death in isolated retinal microvessels maintained under ischemic conditions for 18 hours. For ease of comparison, the *dashed line* shows the percentage of trypan blue cells (7.4% ± 0.1%; $n = 7$) observed in microvessels maintained under nonischemic conditions for 18 hours. *$P < 0.017$. For experimental groups incubated in the absence of insulin, 42 vessel-containing coverslips were assessed; for the other groups, 8.6 ± 0.3 coverslips were examined.

effect of insulin being mediated, at least in part, by an inhibition of ischemia-induced apoptosis.

To begin to clarify the mechanism by which insulin exerts its vasoprotective effect, we used pharmacologic inhibitors of putative insulin-signaling pathways. In one series of experiments, we tested inhibitors of phosphatidylinositol-3-OH kinase (PI3K), which is known to play a role in the antiapoptotic effect of insulin in certain nonvascular cells.[15,30] As shown in Figure 3, when ischemic retinal microvessels were exposed to the PI3K inhibitors wortmannin (100 nM) or LY294002 (10 μM), insulin did not reduce the occurrence of cell death. In other experiments, we used an inhibitor of extracellular signal-regulated kinase (ERK), which is part of a prosurvival signaling cascade in various types of cells.[31-34] As illustrated in Figure 3, exposure of retinal microvessels to the ERK inhibitor PD98059 (5 μM), prevented insulin from exerting a significant ($P = 0.4$) protective effect on ischemic retinal microvessels. Taken together, these pharmacologic experiments suggest that PI3K

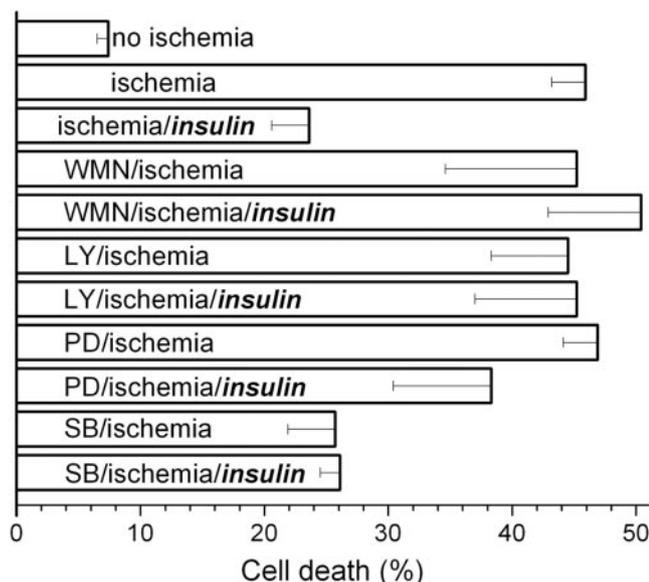**TABLE 1.** Apoptosis in Retinal Microvessels

Apoptosis Assay	Positive Cells (no.)	Negative Cells (no.)	Apoptotic Cells (%)
Cleaved caspase-3			
No ischemia	12	920	1.3
Ischemia	143	966	12.9*
Ischemia/insulin	78	780	9.1†
Spheroid nuclei			
No ischemia	23	1016	2.2
Ischemia	121	2357	4.9*
Ischemia/insulin	75	1985	3.6‡

Cleaved caspase-3 immunoreactivity and nuclear morphology were assessed in retinal microvessels after incubation under various experimental conditions for 8 hours and 14 hours, respectively. Ischemia significantly (* $P < 0.001$; Fisher exact test) increased the percentage of microvascular cells with cleaved caspase-3 immunoreactivity and spheroid nuclei; insulin decreased the percentage of cells in ischemic microvessels with cleaved caspase-3 immunoreactivity († $P = 0.009$; Fisher exact test) and spheroid nuclei (‡ $P = 0.047$; Fisher exact test).
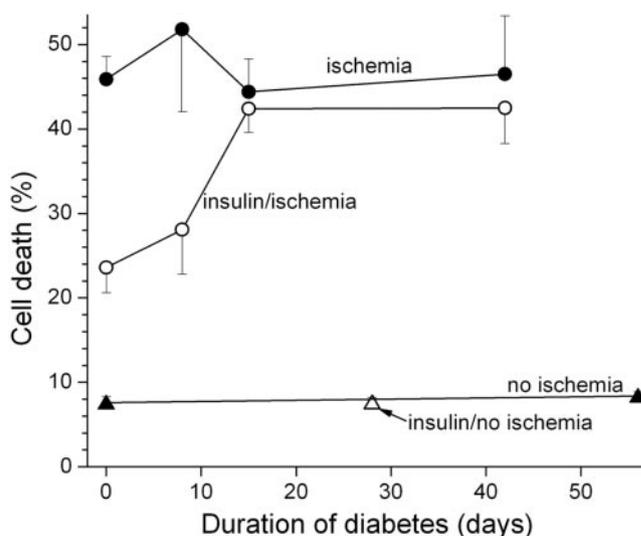
and ERK are critically involved in the insulin-mediated prevention of cell death in ischemic retinal microvessels.

We also considered the possibility that the mitogen-activated protein kinase p38 plays a role in the cell death that occurs in retinal microvessels maintained in an ischemic environment. Although not previously studied in retinal microvessels, this kinase was of interest because its activation is known to be associated with ischemia-induced death of various types of cells.[35,36] Consistent with p38 playing a role in ischemic death in retinal microvessels, the experiments summarized in Figure 3 show that the p38 inhibitor SB203580 (10 μM) had a significant ($P < 0.001$) prosurvival effect, suggesting that SB203580 and insulin may share a common mechanism of action (i.e., the inhibition of p38-mediated death and the protective effects of SB203580 and insulin on ischemic cell death were not additive).

Because the ability of insulin to activate PI3K is markedly diminished in diabetic vessels of streptozotocin-injected rats,[16] we hypothesized that diabetes may adversely affect the ability of insulin to mediate vasoprotection. As shown in Figure 4, although 1 nM insulin continued to significantly decrease ($P = 0.046$) ischemic cell death for 8 ± 1 days in retinal microvessels isolated from diabetic rats, this hormone no longer effectively reduced ($P = 0.7$) cell death in ischemic retinal microvessels of rats that were diabetic for 15 ± 1 days or longer. We ruled out the possibility that this increase in cell death was caused by a vasotoxic effect of insulin because it did not affect the viability of cells in diabetic microvessels maintained under nonischemic conditions (Fig. 4). Taken together, our experiments indicated that the ability of insulin to protect pericyte-containing retinal microvessels from ischemic cell death is lost early in the course of streptozotocin-induced diabetes.

FIGURE 4. Effect of the duration of streptozotocin-induced diabetes on the ability of insulin to protect retinal microvessels from ischemia-induced cell death. Cell viability was assessed after microvessels were maintained in solution A (no ischemia, ▲), in solution A plus 1 nM insulin (insulin/no ischemia, △), in solution A without glucose and with minimal oxygen (ischemia, ●), and in the ischemic solution plus 1 nM insulin (insulin/ischemia, ○). For the no ischemia (▲) group, 6 ± 1 coverslips were assessed per point. For the insulin/no ischemia group (△, $n = 4$) and for the insulin/ischemia group (○), 11 ± 2 coverslips were assessed per point. For the ischemia group (●), the number of assessed vessel-containing coverslips was 42 at 0 days, 4 at 8 ± 1 days, 6 at 15 ± 1 days and 15 at 44 ± 2 days after streptozotocin injection. Error bars under the conditions of "no ischemia" and "insulin/no ischemia" were relatively small and are obscured by the symbols ▲ and △. Means for cell death after 0, 8, 12, and 42 hours of ischemia (●) are not significantly ($P > 0.5$) different.

DISCUSSION

This study shows that physiological concentrations of insulin significantly decrease ischemic cell death in pericyte-containing microvessels isolated from the retinas of healthy rats. However, in retinal microvessels assessed after 2 weeks of streptozotocin-induced diabetes, insulin is dramatically less effective in exerting a vasoprotective effect. These observations suggest that with a loss of pancreatic beta cells, the vulnerability of the retinal microvasculature to ischemia is increased because of the limited prosurvival action of subphysiological levels of insulin and also because of diabetes-induced impairment of the signaling pathway that mediates insulin-induced vasoprotection.

It seems likely that the loss of insulin's vasoprotective effect would exacerbate the effects of ischemia on the retinal microvasculature. This may be a particularly important pathophysiological mechanism at advanced stages of diabetic retinopathy, when capillary perfusion can be significantly compromised. Whether a failure of insulin-induced vasoprotection also plays a role in the early stages of diabetic retinopathy remains uncertain. Although the dysregulation of blood flow and the formation of leukocyte plugs result in suboptimal delivery of oxygen and nutrients well before the onset of histologic and clinical signs of retinopathy,[4,37,38] there is little documentation in humans that overt retinal ischemia occurs early in the course of diabetes. On the other hand, we postulate that later in the course of diabetes, the loss of insulin-mediated vasoprotection may be an important factor contributing to the progression of vascular damage and the retinal changes that lead to the production of angiogenic signals.

FIGURE 3. Microvascular cell death under a variety of experimental conditions. Cell viability was quantified after the retinal microvessels were maintained under the various conditions for 18 hours. When present, insulin was at a concentration 1 nM. WMN, wortmannin (100 nM); LY, LY294002 (10 μM); PD, PD98059 (5 μM); SB, SB203580 (10 μM). In the presence of these inhibitors, insulin did not have a significant effect ($P > 0.4$) on cell death in ischemic retinal microvessels. For ischemic microvessels incubated in the absence of insulin, 42 vessel-containing coverslips were assessed; for the other groups, 5.0 ± 0.7 coverslips were examined.

Our use of pharmacological inhibitors indicates that the insulin-mediated protection of retinal microvessels from ischemic cell death is dependent on PI3K and ERK pathways, both of which are implicated in the prosurvival action of insulin on other cell types.[15,30,39] At present, the mechanism by which diabetes minimizes the vasoprotective effect of insulin remains to be elucidated. However, the observation by Kondo and Kahn[16] that the ability of insulin to activate PI3K is markedly impaired in the retinal vasculature of streptozotocin-injected rats raises the possibility that this change in insulin signaling accounts for the inability of this hormone to prevent cell death in ischemic microvessels of the diabetic retina. Thus, our experimental findings support the emerging concept that in addition to damage caused by hyperglycemia, impaired insulin signaling may contribute to the development of diabetic complications in the retina.[9]

Our findings concerning the vasoprotective role of insulin are based on experiments using pericyte-containing microvessels freshly isolated from the rat retina. Benefits of studying microvessels in isolation include the elimination of potentially confounding effects of insulin on nonvascular retinal cells and the ability to control the duration of exposure to specific concentrations of insulin. On the other hand, isolated microvessels cannot be used to test hypotheses concerning the effects of diabetes on retinal neurons and glia. In addition, our observation that insulin protects against ischemic cell death in isolated retinal microvessels remains to be demonstrated as a significant pathophysiological process in vivo. In addition, the possibility of species differences warrants caution in extrapolating findings with rodent microvessels to the pathophysiology of diabetic retinopathy in the human retina. Despite these caveats, freshly isolated microvessels have proven useful in generating and testing hypotheses concerning novel mechanisms by which microvascular complications may develop in the diabetic retina.[18,27,40,41]

In conclusion, we propose that the loss of insulin-mediated vasoprotection may be an important mechanism contributing to microvascular damage in the diabetic retina. Based on the results of this study, it appears that insulin-deficient diabetes markedly diminishes the ability of insulin to protect retinal microvessels from ischemia. Similarly, although type 2 diabetes was not assessed in this study, future investigations may disclose that the insulin resistance characteristic of this form of diabetes may interfere with insulin-mediated vasoprotection.

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