Accumulation of Topically Applied Porcine Insulin in the Retina and Optic Nerve in Normal and Diabetic Rats

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PURPOSE. To explore the pharmacokinetics of topical insulin administration in relation to retinal and optic nerve retention.

METHODS. Insulin eye drops (~15 μL: 0.75% porcine insulin + 0.5% permeation enhancer) were applied to the eyes of normal and diabetic rats. The rats were killed at various intervals up to 16 hours, and the retinas and optic nerves from both eyes were analyzed for the presence of insulin in an ELISA. The extent to which systemically absorbed insulin accounted for the findings of insulin in the retina was explored by examining the effects of intravenously injected insulin on retinal insulin levels and by examining the effects of eye drop administration in decapitated rats.

RESULTS. Insulin levels rose significantly and peaked in the retina of normal rats 20 minutes after eye drop application (0.7 pg/μg; P < 0.00001). Levels in diabetic retinas peaked at 60 minutes (0.66 pg/μg; P < 0.004) and remained elevated for a longer period than in normal rats. The contralateral retina showed delayed accumulation of lesser amounts of insulin in both normal and diabetic rats. Significant elevations also occurred in the optic nerves in normal and diabetic rats, with concentrations reaching 13 pg/μg in normal rats at 20 minutes and 26 pg/μg in diabetic rats at 5 hours. Topical insulin application resulted in a decrease in serum glucose concomitant with an increase in serum porcine insulin. It did not appear, however, that the systemic absorption of insulin contributed to the accumulation of insulin in the ipsilateral retinas, for two reasons: The intravenous injection of a high concentration of insulin did not appreciably influence retinal insulin levels, and the application of insulin eye drops to decapitated rats still resulted in the accumulation of insulin in the retina.

CONCLUSIONS. These results led to the conclusion that topically applied insulin accumulates in the retina and optic nerve in normal and diabetic rats, with levels remaining elevated longer in diabetic animals. It did not appear that systemically absorbed insulin, resulting from ocular drainage, contributed to this effect. (Invest Ophthalmol Vis Sci. 2002;43:797–804)

Several sight-threatening diseases of the posterior segment could benefit from the use of topical therapeutics. However, there are formidable physical obstacles, such as the corneal epithelium and stroma, that limit the delivery of topically applied agents to the back of the eye. Furthermore, nasolacrimal drainage significantly reduces ocular retention and contributes to the development of negative systemic side effects.

It was demonstrated sometime ago that the noncorneal absorption route also contributes to drug penetration into intraocular tissues. Specifically, it was demonstrated in rabbits that topical insulin (molecular weight [MW]: 5000) reaches intraocular tissues by way of the sclera if the corneal absorption route is blocked with a glass cylinder glued to the cornea. These investigators reported a significant concentration of insulin in the vitreous after the topical application of a 0.65% solution.

The revelation that a molecule with an MW of 5000 could accumulate in the vitreous after topical application led us to speculate that a molecule with only a somewhat larger size, insulin (MW: 5802), might similarly concentrate in the retina after topical application. It was theorized that the topical application of insulin to the eye may be potentially beneficial in preventing and possibly reversing diabetic retinopathy, even in the presence of sustained hyperglycemia, through its localized induction of nitric oxide and Na,K-adenosine triphosphatase (ATPase) production, among other mechanisms. Insulin receptors are nearly ubiquitous in the retina, being present not only on the retinal microvasculature but in nearly all cellular layers of the retina, suggesting an important role for insulin in retinal function and further supporting this hypothesis.

As a first step toward determining whether topical insulin administration is advantageous in the treatment of diabetic retinopathy, in this study we investigated the pharmacokinetics of topical insulin applied in Lewis rats, with specific attention given to retinal and optic nerve accumulation.

METHODS

Animals

Female Lewis rats, between 45 and 60 days of age, were obtained from colony 202B at Harlan Sprague-Dawley (Indianapolis, IN) and were housed and maintained in the New England College of Optometry’s animal facility in accordance with the Public Health Service’s Policy on Humane Care and Use of Laboratory Animals. Normal and diabetic rats were used in the study. Diabetes was induced by injection of 85 mg/kg streptozotocin (STZ; Sigma Chemical Co., St. Louis, MO) in citrate buffer (pH 4.0), and animals were considered diabetic if they had a sustained blood glucose level above 250 mg/dL. Animals that appeared moribund during the course of this study were killed. All experimental procedures were approved by the school’s Institutional Animal Care and Use Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Insulin Eye Drop Administration

Both normal and diabetic rats received insulin eye drops. The insulin used was porcine derived (Sigma Chemical Co.) and was reconstituted in an isotonic bicarbonate (0.75% solution) buffer containing 0.5% of the permeation enhancer, polyoxymethylene-20-stearyl ether (Brij-78; Sigma Chemical Co.). An insulin drop (approximately 15 μL) was applied to the left eye of unanesthetized rats and the lids spread apart.
and opened wide for 1 to 2 seconds before the eye was held closed for 30 seconds. This approach routinely resulted in some overflow (suggesting that a relatively uniform volume was retained in each animal), and was followed in an attempt to prevent the rapid drainage of fluid into the lacrimal drainage system.

**Quantifying Insulin Levels in the Retina and Optic Nerve**

At various time points after topical insulin administration, rats were killed by decapitation, blood collected, and serum prepared by centrifugation. The eyes (both the treated ipsilateral eye and the untreated contralateral eye) were removed using curved, serrated scissors in a manner that preserved approximately a 3- to 5-mm section of the optic nerve. The optic nerves were then sliced off at the posterior pole and pulled from surrounding tissue before being placed in 100 μL of buffer in 0.5 mL microfuge tubes (Kontes, Vineland, NJ) on ice. The posterior segments were then sliced away from the anterior segments with a pair of curved scissors, and the retinas scraped from the choroid and sclera with the aid of a dissecting scope. Confirmation that this technique yielded retinal tissue alone was assessed morphologically early in the study. The retinas were similarly placed in 100 μL of buffer in 0.5-mL microfuge tubes on ice.

The optic nerves and retinas were homogenized using a battery-powered, handheld homogenizer (Kontes) on ice and spun down in a microfuge (Beckman Instruments, Berkeley, CA), and the supernatants were transferred to separate microfuge tubes on ice. A porcine insulin ELISA (ALPCO, Windham, NH) was used to quantify porcine insulin levels. This assay has minimal cross-reactivity to rat insulin and does not cross-react with either insulin-like growth factor (IGF)-1 or -2 (Kim et al., 1991). This assay has minimal cross-reactivity to rat insulin, and was performed to determine to what extent, if any, systemic uptake contributed to our findings of porcine insulin in both the ipsilateral and contralateral retinas and optic nerves. In the first, 0.25 μg porcine insulin in 100 μL was injected intravenously into diabetic rats, some of which were then killed 3, 12, 20, 30, 45, and 60 minutes later. Serum and retinal porcine insulin levels were then assessed as described, and

Special care was taken to ensure that no cross-contamination of samples occurred during the isolation and homogenization process. This was accomplished by extensive and repeated washing of the instruments and dissecting microscope stage between samples. Eyes were not washed before isolation, however, because our early empiric measurements assured us that such a practice was not necessary.

**Experimental Design**

**Pharmacokinetics.** Baseline porcine insulin levels were measured and compared in normal and diabetic rats. (As expected, these levels were quite low and only registered a reading at all because of minimal cross-reactivity to rat insulin.) In normal rats, porcine retinal and optic nerve insulin levels were additionally assessed 10, 15, 20, 30, and 60 minutes after eye drop administration. In diabetic rats in which there were sustained elevations in retinal porcine insulin for longer periods, retinal and optic nerve insulin levels were assessed at 10 (except in contralateral nerves), 15, 20, 30, 60, 90, 180, 300, 480, and 960 minutes after porcine insulin eye drop administration. Porcine insulin levels in the aqueous humor of treated eyes only were also quantified, but only at 20 minutes after eye drop administration in diabetic rats.

**Effects of Topical Insulin Administration on Systemic Insulin and Glucose Levels.** A proportion of topically applied insulin enters the systemic circulation and can affect blood glucose levels. The degree to which this occurs was assessed in diabetic rats. Serum samples were obtained from these animals after decapitation, up to 210 minutes after topical insulin was applied, and were analyzed for their levels of porcine insulin using the porcine-insulin-specific ELISA; serum glucose was measured using a Beckman glucose analyzer.

**Role of Systemic Uptake in Accumulation of Porcine Insulin in the Retina and Optic Nerve.** Two experiments were performed to determine to what extent, if any, systemic uptake contributed to our findings of porcine insulin in both the ipsilateral and contralateral retinas and optic nerves. In the first, 0.25 μg porcine insulin in 100 μL was injected intravenously into diabetic rats, some of which were then killed 3, 12, 20, 30, 45, and 60 minutes later. Serum and retinal porcine insulin levels were then assessed as described, and
baseline samples were also obtained. In the second, the left eye of intact and decapitated diabetic rats was removed 20 minutes after the application of an insulin eye drop. Only the retinas were assessed in these animals.

Statistics

For each experiment, the differences between baseline measurements and the measurements taken at later time points were tested by a nonparametric statistical method, the Wilcoxon rank sum test. This test is nonparametric in the sense that no distributional assumptions regarding the data have to be made. Because of the small sample sizes, exact probabilities were computed using StatExact.11 A Bonferroni correction was applied to the test results because of the multiple within-experiment tests performed. The correction involves multiplying the probability ($P$) of the Wilcoxon test statistic by the number of time points tested for a particular experiment.

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933592/)

**Figure 2.** (A) Insulin levels in the ipsilateral optic nerve after topical administration in normal rats. All levels except that at 60 minutes were significantly above baseline ($P < 0.003$ or lower). Sample sizes were 26, 4, 5, 11, and 4 for the baseline, 10-, 15-, 20-, and 60-minute time points, respectively. (B) Insulin levels in the contralateral optic nerve after topical administration in normal rats. All levels were significantly above baseline ($P < 0.003$ or lower). Sample sizes were 29, 4, 4, 12, and 3 for the baseline and 10-, 15-, 20-, and 60-minute points, respectively.
RESULTS

Effects of Topical Insulin on Retinal, Optic Nerve, and Aqueous Humor (Diabetic Only) Insulin Concentrations

Normal Rats. Porcine insulin accumulated in the retina after topical application, with peak levels observed at 20 minutes after application. Insulin in the ipsilateral retina reached levels of more than 0.7 pg/μg protein, whereas in the contralateral control retina levels reached slightly less than 0.1 pg/μg (Fig. 1). The concentrations of insulin isolated from the optic nerves after topical application were markedly higher than retinal concentrations. Insulin levels in the ipsilateral optic nerve peaked significantly at 20 minutes after application at 13 pg/μg (Fig. 2A), whereas the levels in the contralateral eye peaked significantly at 10 minutes, at nearly 18 pg/μg (Fig. 2B).

Diabetic Rats. When topical insulin was applied to the eyes of diabetic rats, insulin was extractable from the ipsilateral and contralateral retinas for several hours (Fig. 3). Levels peaked at 0.66 pg/μg in the ipsilateral retina at 60 minutes, whereas concentrations in the contralateral retina reached a high point of 0.25 pg/μg at 90 minutes. Insulin levels in the optic nerve of treated eyes peaked 360 minutes after application at nearly 26 pg/μg (Fig. 4A), whereas levels in the contralateral optic nerve reached a pinnacle of 19.9 pg/μg at 15 minutes (Fig. 4B).

A significant concentration of porcine insulin was measurable in the aqueous humor of diabetic rats 20 minutes after topical application. Specific values (mean ± SEM) and sample sizes (n) were as follows: Baseline: 0.05 ± 0.01 ng/mL (n = 3). 20 minutes: 4.4 ± 0.6 ng/mL (n = 8; P < 0.01).

Effects of Topical Insulin on Serum Insulin and Glucose Levels

Serum glucose levels decreased by 30 minutes after eye drop administration, and remained approximately 20% suppressed for 120 minutes (Fig. 5). Because baseline glucose levels varied somewhat between animals, these data were expressed as the percentage of baseline levels. The decline in serum glucose corresponded with an increase in serum insulin (Fig. 6), with levels reaching the initial peak of 0.52 pg/μg at 20 minutes and remaining elevated for 90 minutes.

Role of Systemic Uptake in Accumulation of Porcine Insulin in the Retina and Optic Nerve

When 0.25 μg porcine insulin was injected intravenously into diabetic rats, serum insulin levels peaked at 3 minutes and returned to baseline by 30 minutes (Fig. 7). Simultaneously, retinal insulin levels were also assessed and revealed a slight increase (levels were not more than 0.1 pg/μg) but significant increase at 3 and 20 minutes after injection.

As another measure of the role that systemic insulin levels play in ocular uptake, the levels of insulin in the retina of decapitated diabetic rats were analyzed 20 minutes after topical insulin application and were compared with the levels found in control rats (Fig. 8). Results showed that the retinas of decapitated rats accumulated significant amounts of insulin that were comparable to the levels in the retinas of animals that received eye drops while they were still alive.

DISCUSSION

The results reported herein represent the first step in determining whether diabetic retinopathy can be treated by the administration of topical therapeutic drugs. Recent evidence that an eye drop form of an extracellular protease inhibitor can prevent retinal neovascularization in an animal model supports this approach. We chose to study insulin, because we theorized that it might reverse the harmful effects of hyperglycemia in the retina through its action on such compounds as nitric oxide, phospholipase C-β, and platelet-activating factor, among other mechanisms of action. Insulin receptors are ubiquitous in the retina, being present not only on the retinal microvasculature, but in nearly all cellular layers of the retina.

Our data showed that the topical application of a high concentration of porcine insulin to the rat eye resulted in its accumulation in the retina. In both normal and diabetic rats, it took insulin approximately 20 minutes to reach the retina. The route taken by insulin to the retina has not yet been thoroughly explored. In experiments in rabbits in which the corneal route...
was blocked, topical timolol and insulin were shown to penetrate the sclera to enter the intraocular tissues. This occurred as a result of entry of the drug into the conjunctiva, followed by its diffusion into the sclera.6,23 It has been reported that the conjunctival epithelium, because of its higher membrane permeability and larger absorptive and intercellular space, was the most viable route for the ocular delivery of peptides and oligonucleotides.24 However, our data showing high levels of insulin in the aqueous humor at the 20-minute time point, support a role, in part, for transcorneal diffusion.

A more prolonged accumulation of insulin was demonstrated in the retina and optic nerve in diabetic than in normal rats. The most characteristic functional alteration in the diabetic vasculature, whether human or rat, is an increased vascular permeability resulting in the development of microangiopathy.25–30 At first thought, it seemed reasonable to speculate

**Figure 4.** (A) Insulin levels in the ipsilateral optic nerve after topical administration in diabetic rats. All levels except at the 960-minute point were significantly above baseline ($P < 0.006$ or lower). Sample sizes were 26, 3, 3, 3, 3, 3, 3, and 3 for the baseline and 10-, 15-, 20-, 60-, 90-, 180-, 300-, 480-, and 960-minute time points, respectively. (B) Insulin levels in the contralateral optic nerve after topical administration in diabetic rats. All levels except at the 960-minute point were significantly above baseline ($P < 0.003$ or lower). Sample sizes were 33, 3, 3, 3, 3, 3, 3, and 3 for the baseline and 15-, 20-, 60-, 90-, 180-, 300-, 480-, and 960-minute time points, respectively.
that increased vascular permeability, and perhaps increased blood flow, may have resulted in the prolonged deposition of insulin in the back of the eye. However, it is not immediately clear how these vascular changes could affect retinal insulin levels. Furthermore, in our decapitation study, we were able to show that an intact vasculature was not necessary for insulin absorption to occur in the retina. An alternate possibility is that the insulin was derived from the aqueous humor, which served as a temporary sink for the peptide, or from conjunctival or scleral areas that similarly concentrated the insulin and through which it leached across connective tissue and epithelial barriers into the retina. However, these still do not explain the differences between diabetic and nondiabetic animals.

Because the RPE barrier is thought to restrict only molecules with an MW of 20,000 or more, it is reasonable to speculate that a transscleral route of entry may play a role in retinal insulin accumulation in both normal and diabetic rats. In support of this route of entry are data that show that large molecules, such as IgG (MW, 150,000), diffuse across the sclera and into the retina. However, these still do not explain the differences between diabetic and nondiabetic animals.

As expected, the topical application of insulin resulted in its distribution throughout the retina, with levels comparable to those reported in this study (data not shown). This is consistent with data from other sources.35,38 The ability of topical insulin to be absorbed into the blood has led others to the exploration of this approach as an alternative to systemic administration.35,38 It has been reported that the long-term application of insulin to the eyes of both humans and animals was well tolerated, and, in the rabbit, which was used as a model for the human eye, resulted in no adverse corneal effects for up to 3 months of use.38

Although it is clear that insulin applied topically enters the blood, it did not appear that systemic absorption of porcine insulin contributed significantly to levels in the retina. This conclusion was supported by the data showing that porcine insulin levels were present in the retina of rats that received topical porcine insulin after they were decapitated, as well as by the finding that the intravenous injection of a high concentration of insulin did not appreciably elevate retinal insulin levels. Although not explicitly stated previously, both the normal and diabetic rat retinas contain a small amount of rat insulin (data not shown). This is comparable to the levels present in the retinas of rats that received topical porcine insulin after they were decapitated, as well as by the finding that the intravenous injection of a high concentration of insulin did not appreciably elevate retinal insulin levels.
It is not immediately clear whether the accumulation of insulin in the contralateral retina and optic nerve was due to systemic uptake or to some diffusional mechanism from the treated eye. Our data show that the intravenous injection of a dose of insulin in diabetic rats that was roughly comparable to the amount contained within an eye drop resulted in a very slight increase in retinal insulin levels that was similar to the increase seen in the contralateral retina of eye drop-treated animals. However, we did not examine the optic nerves in these animals. That both optic nerves showed very high levels of insulin after eye drop administration to the left eye, suggests that, at the very least, nonvascular mechanisms may play a role in the accumulation of insulin in the contralateral optic nerve, if not in the retina.

In conclusion, these data support the hypothesis that topically applied insulin can accumulate in the retina and optic nerve. It appears that this effect was not influenced by systemic insulin levels. The route taken by insulin to the back of the eye remains to be investigated, although high levels in the aqueous humor after application suggest at least a partial role for transcorneal migration.

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References


