The occurrence of glutathione-insulin transhydrogenase in the retina*

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Insulin-degrading activity in the retina of cattle, rat, and rabbit has been studied. On the basis of activation by reduced glutathione, complete inhibition by N-ethylmaleimide, identification of one of the reaction products as the A chain of insulin, and reaction with antibody to purified beef pancreatic glutathione-insulin transhydrogenase, it is concluded that the insulin-degrading enzyme occurs in the retina. Preliminary studies showed that there is no difference in the levels of enzyme activity in retinal homogenates from cattle, rats, or rabbits and that the rat rod outer segments are probably devoid of the degrading activity. (INVEST OPHTHALMOL VIS SCI 22:715-719, 1982.)

Key words: glutathione-insulin transhydrogenase, insulin degradation, retina, diabetes mellitus, insulin receptors, rat, cattle, rabbit

Retinopathy, cataracts, and other clinical disorders of eye are commonly seen in diabetes mellitus. However, the exact relationship between eye abnormalities and various putative factors that might be involved in the pathogenesis and long-term complications of diabetes mellitus remains to be elucidated. The occurrence of insulin receptors in many tissues, including the retina, has been reported. Likewise, the insulin-degrading enzyme, glutathione-insulin transhydrogenase (GIT), occurs widely (see ref. 4 for a review). Nothing, however, is known about the metabolism of insulin in the retina. The purpose of this study was to determine whether the insulin-degrading enzyme, GIT, occurs within the retina.

Methods

All procedures utilized in this study have been previously described in detail. Briefly, retinas were homogenized with a Polytron or by sonication. Bovine eyes were collected at a local slaughterhouse and transported on ice in darkness to the laboratory. Retinas were immediately excised and stored at —15°C. Frozen retinas were thawed, minced, and mixed with 9 ml of 0.3M sucrose solution per 1 gm of retina. Homogenates were centrifuged at 600 x g for 15 min and the sediment was discarded. The supernatant was subjected to additional centrifugation at 15,000 x g for 20 min and 160,000 x g for 60 min. The pellets collected after each centrifugation and the high-speed supernatant (hereafter designated as retina extract) were assayed for insulin-degrading activity and protein content.

Sprague-Dawley albino rats or rats afflicted with hereditary retinal degeneration were bred and reared in the laboratory animal facility in either a cyclic light environment (5 ft.-cd. 12 hr/day) or in total darkness from the age of 7 days to 34 to 39 days as described. New Zealand white

Fig. 1. Insulin degradation as a function of the amount of bovine retina protein assayed. Excised bovine retinas were homogenized with a Polytron in 0.3M sucrose and subjected to centrifugation. A portion of the high-speed retina extract was dialyzed extensively (for 48 hr with seven changes). Both dialyzed and undialyzed retina extracts were then assayed for insulin-degrading activity as described in the text. A, Undialyzed extract and no GSH; B, dialyzed extract and no GSH; C, undialyzed extract plus GSH; D, dialyzed extract plus GSH; E, undialyzed extract plus 10 mM N-ethylmaleimide; F, dialyzed extract plus 10 mM N-ethylmaleimide.

rabbits were obtained commercially and were acclimated to the cyclic light environment before use. Retinal rod outer segments were prepared as described. All dissections were performed in dim red light.

Insulin-degrading activity was measured by the conversion of radioactivity of $^{[125]}$I-labeled insulin to a form soluble in 5% trichloroacetic acid. Each retina homogenate or extract was incubated for 5 min at 37° in 0.9 ml of 0.1M K-phosphate/5 mM EDTA buffer (pH 7.5) containing 1 μmol of glutathione (unless indicated otherwise). The reaction was started by the addition of 0.1 ml of a solution containing 1 nm of insulin (final concentration 1 μM), a tracer amount of $^{[125]}$I-labeled insulin (∼30,000 cpm), and 3 mg of bovine serum albumin. After 5 min the incubation was terminated by the addition of 1 ml of 10% trichloroacetic acid. The enzyme activities were measured using two to four concentrations of protein. All enzyme activities reported have been corrected for non-enzymatic insulin degradation. One unit of insulin-degrading activity is defined as the net degradation of 1% of the insulin present in 1 min.

The nature of products of $^{[125]}$I-insulin degradation after incubation with bovine retina extracts was determined by Sephadex G-75 column chromatography with 50% acetic acid as eluent.

Double immunodiffusion experiments were carried out as previously described. The preparation of $^{[125]}$I-insulin and its purification, and pancreatic GIT and its antibodies have also been described.

Protein was determined by the method of Lowry et al., with bovine serum albumin as the standard.

Results and discussion

Assay of the insulin-degrading activity of bovine retina homogenates showed that, of the enzyme activity present in the 600 × g supernatant, 20% was present in the 15,000 × g sedimented material, 2% in the 160,000 × g precipitate, and 78% in the high-speed supernatant (i.e., retina extract). All further studies were therefore carried out on this retina extract. Previously we have demonstrated that in the liver GIT primarily occurs in the microsomal fraction and is readily solubilized or released into the supernatant by a Polytron treatment or sonication such as that used in the present studies.

The dependence of insulin degradation on the amount of protein in bovine retina homogenates is shown in Fig. 1. In the absence of reduced glutathione (GSH), the rate of insulin degradation is nonlinear (curve A, which begins at the origin point zero) and the nonlinearity is more pronounced with a dialyzed homogenate (curve B, which begins at the origin point zero). These curves, however, attain linearity (curves C and D) when 1 mM GSH is included in the assay buffer. The presence of 10 mM N-ethylmaleimide (curves E and F) completely abolished the insulin-degrading activity. These data sug-
Fig. 2. Radioactivity elution profiles on a Sephadex G-75 column of [125I]-insulin that was incubated with bovine retina extract at 37° for various time periods. Bovine retina extract (0.17 mg), insulin (1 μM), a tracer amount of [125I]-insulin and 1 mM GSH in 1 ml of 0.1M phosphate/5 mM EDTA, pH 7.5, were incubated. At the indicated time periods, the tubes received 10 mM N-ethylmaleimide (to terminate the reaction). The samples then received 1.44 gm of urea, 1 mg each of A chain and B chain as carriers, and 1 ml of glacial acetic acid, and the radioactivity products were separated on a 2 by 50 cm Sephadex column with 50% acetic acid as described previously.8 We have previously demonstrated8 that peak I represents a B-chain rich/A-chain aggregate; peak II, insulin; peak III, A chain; and peak IV, low-molecular weight fragments of insulin.

gest that the bovine retina probably contains a thiol-dependent insulin-degrading activity.

Examination of the products of insulin degradation by Sephadex G-75 chromatography after incubation of [125I]-insulin with retina extracts for various time intervals showed a radioactive product peak that eluted with the A chain of insulin (Fig. 2). The elution profiles also show that as the intact insulin peak decreased, the peak containing A chain increased, indicating that GIT is probably involved in the process of insulin degradation. In addition, no radioactive products were found at the position of low-molecular weight fragments, suggesting, as previously found with rat and mouse liver,7 that the insulin-degrading activity measured in the presence of GSH and EDTA is mainly that of GIT.

Double immunodiffusion of bovine retina extracts with antibody to purified bovine pancreas GIT showed a precipitin band in a similar position and continuous with that obtained with purified bovine pancreas GIT (Fig. 3).

Together, the above data (the effect of GSH and N-ethylmaleimide on the insulin-degrading activity, the formation of a radioactive product co-eluting with the A chain of
insulin, and the results of immunodiffusion clearly indicate that bovine retina contains GIT. The finding of GIT in retina is consistent with the ubiquitous nature of this enzyme.3

As can be seen from Table I, the insulin-degrading activity per milligram of protein in the retina is about the same in cattle, rat, or rabbit. This level of GIT activity is about one fifth to one third of that present in rat liver. GIT activity levels in retina and brain are similar, relative to liver activity. There was no difference in the enzyme activity in retinas of cyclic-light- or dark-reared rats, separately or together, from those of dark-reared dystrophic albino rats. In one set of experiments with purified rod outer segments from cyclic-light- or dark-reared rats we were unable to detect GIT activity. Taken together with the finding of similar enzymatic activity in the retinas of dystrophic rats, which have undergone extensive visual cell death, these data suggest that the enzymatic degradation of insulin may occur to a large degree in the inner retinal layers.

In a preliminary study we have also found that the bovine retina binds [125I]-insulin, which can be displaced by unlabeled insulin (data not shown). These results indicate that, as reported by others,2 insulin receptors occur in retina. These observations are in keeping with the occurrence of insulin receptors and the insulin-degrading enzyme (GIT) in close proximity in other tissues.4 However, the physiologic function of insulin receptors and insulin-degrading enzymes in the retina (and in the brain) is unclear, since these tissues are insensitive to insulin in the classic sense of requiring the hormone for glucose entry into cells. The following three possibilities exist: (1) Since insulin produces a variety of biological effects, in the retina and brain it performs functions other than simply facilitating glucose transport. Insulin receptors subserve these other functions. (2) GIT utilizes, besides insulin, other disulfide-containing proteins as substrates (see ref. 11 for a review). In the brain and retina GIT metabolizes primarily other polypeptides, particularly those molecularly similar to insulin, e.g., somatomedins. By its action on polypeptide substrates, the function of GIT may be to simply adjust the sulfhydryl-disulfide status in these tissues (see ref. 12 for a review). (3) Alternatively, insulin receptors and insulin-degrading enzymes might not have any physiologic function in the retina and brain.

### Table I. Insulin-degrading activity content of bovine, rabbit, and rat retinas

<table>
<thead>
<tr>
<th>Animal</th>
<th>No. of retinas</th>
<th>Insulin-degrading activity (U/mg of protein)</th>
</tr>
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<tbody>
<tr>
<td>Bovine retina</td>
<td>10</td>
<td>4.21 ± 0.32</td>
</tr>
<tr>
<td>Rabbit retina</td>
<td>2</td>
<td>4.46</td>
</tr>
<tr>
<td>Rat retina</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclic-light reared</td>
<td>4</td>
<td>4.38 ± 0.72</td>
</tr>
<tr>
<td>Dark reared</td>
<td>4</td>
<td>4.78 ± 0.98</td>
</tr>
<tr>
<td>Dark reared dystrophic</td>
<td>4</td>
<td>3.84 ± 0.61</td>
</tr>
<tr>
<td>Rod outer segments</td>
<td>2</td>
<td>Undetectable</td>
</tr>
<tr>
<td>Rat liver</td>
<td>16 - 20</td>
<td></td>
</tr>
</tbody>
</table>

*All excised retinas were sonicated in 0.1M K-phosphate/5 mM EDTA buffer (pH 7.5) and were then assayed for insulin-degrading activity in the presence of 1 mM GSH. Values are mean ± S.D.

†A range of values based on several experiments for rat liver is given for comparison.
brain, but may simply be atavistic remnants. Work is in progress in this laboratory to shed light on these possibilities.

Vascular abnormalities and increased vascular permeability in the retina are nearly always observed in human diabetes and in diabetic animal models. In alloxan-diabetic dogs and diabetic rats, normalization of blood sugar with insulin reduces microvascular abnormalities and reverses vascular permeability. The re-introduction of insulin, via islet-cell isografts, in streptozotocin-induced diabetic rats resulted in a decrease in the accumulation of ocular fluorescein typical of their diabetic counterparts. These authors concluded that the reduction in ocular vascular permeability could be more closely correlated to the increased insulin levels rather than the reduced blood glucose levels. The finding of similar levels of GIT activity in the poorly vascularized (rabbit) and fully vascularized (bovine and rat) retinas and brain raises the possibility that the hormone effects may occur in conjunction with insulin metabolism. Whether retinal vascular abnormalities, insulin receptors, and the ability to enzymatically degrade insulin in the retina are related remains to be determined. Clearly, further studies will be required to correlate insulin metabolism and vascular permeability changes in the retina of diabetic animals.

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REFERENCES