Increased Cytochrome Oxidase Activity in the Diabetic Rat Retinal Pigment Epithelium

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We have investigated the effects of diabetes on retinal oxidative metabolism. Since activity of the mitochondrial enzyme, cytochrome oxidase, has been demonstrated to be a reliable indicator of oxidative metabolism and physiological activity, we used cytochemical techniques to study the activity of this enzyme in spontaneously diabetic, streptozotocin-diabetic, and control rat retinas. Light microscope results showed an increase in staining for cytochrome oxidase activity in the diabetic RPE cell layer as compared with the control. Quantitative electron microscope analysis showed a significant increase in RPE cells with highly reactive mitochondria as compared with the controls. Mitochondrial staining within the diabetic photoreceptor and retinal vascular endothelial cells was normal. RPE cell volume and surface area, as well as number and volume of mitochondria, were unchanged. This increase in oxidative enzyme activity is further evidence of RPE cell alteration in diabetes. Invest Ophthalmol Vis Sci 30:591–599, 1989

Alterations in blood–retinal barrier functions may contribute to the development of diabetic retinopathy. Changes in the distribution of fluorescein across the blood–retinal barrier occur early in the disease in both humans and animals and are thought to be due to increased permeability of the retinal pigment epithelial (RPE) cell layer. Morphological studies in diabetic rats showing RPE lesions and abnormal RPE plasma membrane permeability suggest that paracellular permeability may increase through defects in the RPE cell layer. On the other hand, others report a similar incidence of apparent permeability alterations in normal retinas.

In the current study we have investigated the effects of diabetes on retinal metabolism using cytochemical techniques for the localization of the mitochondrial enzyme, cytochrome oxidase. Previous studies using cytochrome oxidase staining have demonstrated a close relationship between intensity of oxidative enzyme staining and physiological activity, metabolic activity, and relative amounts of immunolabeled mitochondrial cytochrome oxidase. In addition, alterations in cytochrome oxidase staining have been found in a variety of pathological conditions. We therefore hypothesized that if retinal physiology and metabolism were altered in diabetes, changes in cytochrome oxidase activity should be apparent in the affected cell layers. The results of these studies showed an increase in staining for cytochrome oxidase activity in the RPE of both drug-induced and spontaneously diabetic rats when compared with the controls. Activity within retinal vascular endothelial cells and photoreceptor cells was quantitatively normal. Morphometric analysis showed that the RPE cell alteration was not associated with any change in cell volume or surface area, or in the number or volume of mitochondria. This specific increase in RPE cell oxidative enzyme activity adds to the evidence that the RPE is one of the first cell types in the retina to be altered during diabetes, and suggests that RPE cell changes may contribute to some of the other alterations in diabetic retinopathy.

Materials and Methods

Animals

Spontaneously diabetic and diabetes-prone control BB/Wor-UTM rats, streptozotocin-induced diabetic
and 3-O-methyl glucose control Long-Evans rats were used in these studies. The diabetic rats were studied from 1–12 months after the onset of diabetes. The spontaneously diabetic rats received single daily injections of PZI insulin. Insulin dose was adjusted daily, based on urine sugar and ketone determination. At the time of the experiments, the animals ranged in age from 4–13 months. Diabetes was confirmed by glycosuria and elevated blood sugar (>300 mg/dl). Chemstrips uGK were used to measure glycosuria, and a reflectance photometer and chemstrips bG (Boehringer Mannheim, Indianapolis, IN) were used to measure blood glucose.

Animals were watered and fed ad libitum and maintained on a 12 hr light/12 hr dark cycle. Illumination in the cages was 2 lux. Experiments were conducted during the first 6 hr of the lights-on period. These investigations adhered to the ARVO Resolution on the Use of Animals in Research.

**Tissue Preparation**

Rats were anesthetized and then perfused transectially with 1% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer containing 4% sucrose, pH 7.3, at 37°C for 20 min. After perfusion, the cornea, lens and vitreous were removed, and the eyes were flushed with fixative for 2 min. Eyecups were then removed and stored in fixative at 4°C for an additional hour. Following fixation, eyecups were thoroughly rinsed in 0.1 M phosphate buffer, and 20–40 μm sections were prepared from the posterior 2 mm of the retina using a tissue chopper or vibratome. Sections were incubated in 15 mg cytochrome C (Type III, Sigma Chemical Company, St. Louis, MO), 25 mg diaminobenzidine, 45 ml 0.1 M phosphate buffer, pH 7.4, and 2 g sucrose for 2–5 hr in the dark at 37°C. Preliminary studies showed that substantial amounts of reaction product were present at 2 hr. At 4 hr reaction product had continued to increase, and had begun to diffuse into the surrounding tissue. Therefore, 2 hr incubations were used for electron microscopic analysis and for all quantitative studies.

In initial experiments (one diabetic and one control animal) catalase (200 μg/ml) was used to control for the presence of endogenous H2O2. Since no difference in either mitochondrial or background staining pattern was apparent in these experiments, catalase was not used in subsequent experiments. As a control for the specificity of the cytochemical reaction, tissue from one diabetic and one control rat was processed in incubation medium with 0.01 M KCN. In order to control for possible variability in cytochemical reactions between experiments, sections from diabetic and control animals were always incubated under the same conditions at the same time. Tissue for light microscope analysis was mounted on glass slides, dehydrated, and coverslipped. Tissue for ultrastructural analysis was postfixed in osmium, embedded in plastic, cut into superficial thin sections, and studied in the electron microscope.

**Quantitative Analysis**

Retinas from six spontaneously diabetic and five age-matched diabetes prone, normoglycemic control rats were studied quantitatively. These tissues were prepared in three replications and were processed under identical conditions. Superficial thin sections of the retina prepared from four blocks each from each animal were photographed at a magnification of ×10,000. In each block, randomly selected photoreceptor inner segments and four to eight RPE cell profiles were photographed. Photographs were coded and enlarged to a final magnification of ×28,000. Two independent observers counted the mitochondria in each RPE cell or inner segment profile and classified each mitochondrion according to its level of reactivity. Vascular endothelial cells were analyzed directly in the electron microscope. Coded tissue sections from the same 11 animals and 44 tissue blocks used for the inner segment and RPE analysis were studied, and all mitochondria in all retinal vessels of each section were classified as described above. Differences in mitochondrial staining intensity between diabetic and control animals were evaluated in each cell type using a multivariate t-test (Wilks' Lambda).

To investigate possible changes in the number or size of the RPE cells or mitochondria, a computer assisted digitizing tablet (Hewlett-Packard, Sunnyvale, CA) was used. The following parameters were determined: the area of each RPE cell profile, the area of each cell occupied by mitochondria, and the number of mitochondria within each RPE cell profile. The data were compared using a t-test.

The relationship between possible changes in basal membrane surface area and cytochrome oxidase activity in the diabetic RPE was evaluated using stereological and correlational techniques. For this analysis, the extent of basal infolding for each RPE cell profile was estimated as described previously. A test grid of parallel lines spaced 1 cm apart was placed over each micrograph, and the number of intersections of the basal membrane with the grid lines was counted. Counts for a given field were averaged from counts made with the lines oriented at 45° and 135° to the plane of the cell base to compensate for the anisotropic orientation of the basal infoldings. The boundary length of the cell membrane (Lb) was calculated from

\[
L_b = \frac{\pi}{2} ND
\]

(1)
where $N$ is the average number of intersections, and $D$ is the distance between test lines. The resulting value was then expressed as a length of basal cell membrane per unit length of Bruch's membrane ($L_c / L_b$). The values obtained for the two groups were compared using a t-test. To evaluate possible relationships between basal membrane surface area and cytochrome oxidase activity, the estimate of basal membrane infolding for each RPE cell was correlated with the intensity of staining for cytochrome oxidase activity within that cell.

**Results**

**Light Microscopy**

In tangential light microscope sections through the RPE and photoreceptor layers of the neural retina, a laminar pattern of oxidative enzyme staining was apparent (Fig. 1). The cytoplasm of the hexagonally shaped RPE cells was diffusely stained, outlining the unstained nuclei. Photoreceptor outer segments were unstained, but staining was intense within the inner segment zone. Oxidative enzyme staining was consistently more intense in the diabetic RPE (Fig. 1A) than in the RPE of control retinas prepared simultaneously (Fig. 1B). Staining differences were not apparent in the other retinal layers.

**Electron Microscopy: RPE**

Electron microscopic analysis showed that the cytochemical reaction product for oxidative enzyme activity was present on the outer surface of the inner mitochondrial membranes and within the intracisternal space (Fig. 2). In all animals, enzyme activity within individual mitochondria of a given cell was somewhat variable, but, within each cell, the majority of mitochondria exhibited a similar pattern of activity. Three categories of mitochondrial oxidative enzyme activity were distinguishable: dark, moderate and light. In darkly reactive mitochondria, reaction product filled the intracisternal space and the space between the outer and inner mitochondrial membranes (Fig. 2A). In moderately reactive mitochondria, reaction product was confined to the intracisternal space (Fig. 2B), whereas in lightly reactive mitochondria, little or no reaction product was apparent (Fig. 2C). In sections incubated in medium to which KCN was added, all mitochondria were free of reaction product (data not shown). The amount or distribution of reaction product was not affected by the addition of catalase to the incubation medium.

RPE cells from both spontaneously diabetic rats (Fig. 3A) and streptozotocin diabetic rats (data not shown) were structurally similar to those in retinas of
normoglycemic diabetes prone (Fig. 3B) and 3-O-methyl glucose control rats. Abnormally flattened or proliferated basal membranes and degenerating RPE cells were not observed. However, the number of darkly reactive mitochondria was substantially greater in the diabetic RPE than in the control RPE (Compare Fig. 3A and 3B). Quantitative analysis confirmed this, showing a significant increase in the number of darkly reactive mitochondria in the spontaneously diabetic as compared with the non-diabetic control retinas ($P < 0.05$, Fig. 4).

To determine whether morphological changes in RPE cells or mitochondria correlated with the increase in cytochrome oxidase activity observed in the diabetic RPE cells, we calculated the cytoplasmic area of the RPE cell profiles analyzed, the proportion of this area occupied by mitochondria, the number of mitochondria per RPE cell profile, and the surface area of the RPE basal infoldings. As shown in Table 1, diabetic and control RPE cell profiles were similar in all measures. Furthermore, a low correlation between the number of darkly reactive mitochondria within each cell and the basal membrane surface area in both control ($r = 0.04$) and diabetic RPE ($r = 0.16$) suggested that variations in oxidative enzyme activity were not related to variations in RPE basal mem-

Fig. 2. Criteria used for classifying levels of cytochrome oxidase staining. (A) In darkly reactive mitochondria reaction product fills the intracristate space and the area between the inner and outer mitochondrial membranes. (B) In moderately reactive mitochondria reaction product occupies the intracristate space but appears absent from the mitochondrial envelope (arrow). (C) In lightly reactive mitochondria reaction product is sparse and is confined to the intracristate space (arrow) ($\times 38,000$).
Fig. 3. Electron micrographs showing cytochrome oxidase staining in RPE cells from diabetic (A) and normoglycemic control (B) rats (BB-WorUt). Most mitochondria in the diabetic RPE are darkly stained. The majority of mitochondria in the control RPE are sparsely stained (arrows). Bruch's membrane (BM) is at the top (×20,000).
Fig. 4. Bar chart showing percent darkly reactive mitochondria (mean ± standard error) in the RPE, photoreceptor inner (I) segments and retinal vascular endothelium of diabetic and control rats (BB-WorUtm). Data are based on evaluation of 7080 mitochondria in 11 animals.

brane surface area. Stereological measures from control retinas fixed by immersion were comparable with those from perfusion-fixed retinas (data not shown).

Electron Microscopy: Neural Retina

To see whether oxidative activity was altered in other metabolically active cells of the retina, we also analyzed mitochondrial cytochrome oxidase activity within the retinal vascular endothelial cells and within the photoreceptor inner segments. In endothelial cells in both diabetic (Fig. 5) and control retinas numerous darkly reactive mitochondria were present. Quantitative analysis showed a small increase in the number of darkly reactive mitochondria in diabetic animals, but the difference between the two groups was not statistically significant (Fig. 4). In the inner segment region, numerous reactive mitochondria were present in both diabetic and control retinas (Fig. 6). Quantitative analysis of randomly selected inner segments showed similar levels of activity in diabetic and control retinas (Fig. 4).

Table 1. Morphometric analysis of RPE cell profiles and mitochondria (mean ± standard error)

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<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetic</th>
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<tr>
<td>Cytoplasmic area of RPE cell profiles (µm²)</td>
<td>31.88 ± 1.01</td>
<td>32.00 ± 0.52</td>
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<tr>
<td>Percent RPE cytoplasm occupied by mitochondria</td>
<td>9.06 ± 0.52</td>
<td>9.60 ± 0.48</td>
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<td>Number mitochondria per RPE cell profile</td>
<td>14.96 ± 0.90</td>
<td>15.41 ± 0.72</td>
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<tr>
<td>Surface area RPE basal infoldings (LC/LB µm)</td>
<td>10.16 ± 1.85</td>
<td>10.43 ± 2.31</td>
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Fig. 5. Electron micrograph showing cytochrome oxidase staining in vascular endothelial cells from a diabetic rat (BB-WorUtm). Most mitochondria are darkly stained (x11,200).

Discussion

This study shows an increase in cytochrome oxidase activity in RPE cells of diabetic rats. Because the change occurred in cells which appeared otherwise normal, it seems to reflect a primary metabolic alteration rather than an increased demand for energy due to increased cell volume or surface area. The results of this study are also unlikely to be due to ketoacidosis. First, the diabetic rats were rarely ketotic, and second, previous studies of cerebral metabolites in alloxan-treated diabetic mice suggest that cerebral activity is depressed rather than increased during diabetic acidosis. Finally, the observation of normal levels of cytochrome oxidase activity within the retinal photoreceptor inner segments and the vascular endothelium suggests that the alteration is specific to RPE cells and is not due to an overall increase in retinal oxidative metabolism.

The energy demands of the RPE are relatively high as indicated by the presence of numerous mitochondria and of oxidative and glycolytic enzymes (reviewed in refs. 27, 28). Histochemical results indicate that the activity of some oxidative enzymes is higher in the RPE than in any other retinal region with the possible exception of the photoreceptor inner segments. Our finding of substantially increased cytochrome oxidase staining in diabetic RPE cells when compared with the controls suggests that ox-
Fig. 6. Electron micrograph showing cytochrome oxidase staining in photoreceptor inner segments (IS) from a control rat (BB-WorUttn). Most mitochondria are moderately or darkly stained. Outer segments (OS) are at the top (×20,000).

dative metabolism within the RPE is enhanced during diabetes.

Little is known about the effects of diabetes on RPE cell metabolism. Studies in acutely diabetic rabbits two weeks after alloxan treatment suggest that metabolism is depressed in both the RPE and neural retina. Quantitative histochemical analysis of pure samples of RPE and individual retinal layers showed increases in glucose and sorbitol. RPE and retinal myo-inositol levels were decreased. Measurable Na-K-ATPase activity was lost in the diabetic RPE, and activity appeared reduced in photoreceptor inner segments. Our finding of an increase in oxidative metabolism specific to the diabetic RPE is unexpected in view of the above data. However, this apparent discrepancy could be due to the difference between the acute and chronic effects of diabetes. Our animals were diabetic for 1 to 12 months compared with less than 2 weeks in the alloxan study. Differences between the animal models could also contribute to the contradictory experimental findings. The avascular rabbit retina may respond differently to diabetes than the vascular rat retina. Moreover, diabetic acidosis following alloxan treatment may have contributed to metabolic depression in the previous investigation.

A variety of energy requiring processes occur in the RPE (reviewed in refs. 27, 28). These include chemical reactions of the vitamin A cycle, phagocytosis and digestion of discarded photoreceptor outer segment membranes, transport of small molecules and ions against concentration gradients, energy dependent fluid transport (reviewed in ref. 32), autophagic degradation of worn out cellular components, and protein synthesis for the renewal of RPE membranes and organelles and of collagen, glycosaminoglycans, and other proteins secreted by the RPE into Bruch's membrane and the subretinal space. Alterations in phagocytosis and the vitamin A cycle are unlikely to contribute to increased metabolic activity in the diabetic RPE, since changes in either process would also be likely to affect morphology and/or visual function. It is possible that oxidative metabolism in the diabetic RPE increases to support an increase in active transport of fluid which reaches the subretinal space due to blood-retinal barrier alterations. However, our studies in dystrophic retinas in which breakdown of the blood-retinal barrier is known to occur show...
normal levels of oxidative enzyme staining (unpublished).

Protein synthesis is one RPE metabolic function whose increase seems likely to contribute to an increase in metabolic activity. As pointed out by Young and Bok, this function requires more energy than any other RPE cell activity. Little is known about the proteins synthesized by the RPE or about the effects of diabetes on their synthesis. However, previously observed alterations in the membrane infoldings and increases in freeze-fracture intramembrane particles within the RPE basal membrane of diabetic rats,0 suggest that synthesis and turnover of membrane proteins may accelerate in diabetes. Proteins secreted by the RPE may also increase. Collagen synthesis increases in many cell types and probably also increases in the RPE, since the RPE basal lamina becomes thickened in diabetes.38 Finally, cultured RPE cells have been found to secrete other proteins, which may include vascular modulating peptides (reviewed in refs. 40, 41) and which could be altered in diabetes.

Increased cytochrome oxidase activity has been observed previously in surviving hypertrophic RPE cells which surround areas of focal damage following continuous exposure to low levels of light. RPE cell lesions and areas of apparent attempted repair have also been reported in diabetic retinas. Although RPE lesions were not observed in the present study, the acceleration of oxidative metabolism in the diabetic RPE may reflect a response to physiological stress similar to the metabolic hypertrophy occurring in light damaged cells.

In conclusion, our data add to the growing evidence that the RPE is one of the first cell types in the eye to be affected in diabetes. Alterations in the RPE cell layer may contribute to some of the other retinal complications in diabetes. Additional work is needed to determine the physiological basis for the RPE cell change.

Key words: cytochrome oxidase, retinal pigment epithelium, diabetes, rats, retina

Acknowledgments

The authors are grateful to Dr. A. Y. Chang, the Upjohn Company, Kalamazoo, Michigan for providing the streptozotocin used in this study and to Dr. S. S. Solomon and Dr. J. Hessler (Animal Resources Division of the University of Tennessee, The Health Science Center, Memphis, Tennessee) for supplying the spontaneously diabetic and control BB/Wor-UTM rats. Excellent technical assistance was provided by D. Lum.

References


