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ORNITHINE DECARBOXYLASE ACTIVITY IN RABBIT RETINA FOLLOWING TREATMENT WITH ALPHA-DIFLUOROMETHYLORNITHINE

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Abstract—The rabbit retina has been utilized as a model for the study of abnormal cellular proliferation on the retinal surface and into the vitreous, a process commonly initiated by trauma and generally leading to retinal detachment. This study characterizes the ability of alpha-difluoromethylornithine (alpha-DFMO), a suicide inactivator of L-ornithine decarboxylase (EC 4.1.1.17) to inactivate normal retinal ornithine decarboxylase (ODC) activity in the crude supernatant fraction after incubation with different concentrations of alpha-DFMO and at various times after intraocular administration. Partial inactivation of ODC activity occurred following preincubation of crude retinal supernatant fraction with 10^{-5} M alpha-DFMO (N = 3; 34 ± 6.9% of control), whereas preincubation with 10^{-8} M alpha-DFMO did not alter ODC activity significantly (N = 3; 94 ± 2% of control). Different concentrations of alpha-DFMO administered intraocularly inactivated retinal ODC activity to varying degrees with different rates of recovery. No gross toxicity occurred with ocular tissues following intravitreal administration of alpha-DFMO as determined by electrophysiologic measurements, by indirect examination of the retina, and by measurement of intraocular pressure. These results suggest that alpha-DFMO may be a useful tool in which to define the physiologic role of ODC and polyamines in intraocular cellular proliferative diseases.

L-Ornithine decarboxylase (EC 4.1.1.17), the first and rate-limiting enzyme in mammalian polyamine biosynthesis, responds rapidly to hormones, growth factors, drugs, and proliferative stimuli [1]. The activity of ornithine decarboxylase (ODC) also changes dramatically during the developmental process, in which it plays an essential role in early mammalian embryogenesis [2]. Research on the role of polyamines in cell growth and differentiation has advanced rapidly in recent years due to the development of a potent mechanism-based irreversible ODC inactivator, DL-alpha-difluoromethylornithine (alpha-DFMO) [3–6]. Utilization of [^{14}C]-alpha-DFMO has demonstrated that changes in the activity of ODC apparently are due to changes in the number of active enzyme molecules [7].

Stimulated by either trauma or separation of the neural retina from the underlying pigment epithelium [8, 9], intraocular proliferation of cells occurs on both the retina and the posterior surface of the detached vitreous gel [10]. Following migration of these cells into the vitreous, cellular contraction of such cells, which are of non-neuronal origin, produces tractional forces between the retina and vitreous leading to traction retinal detachment and subsequent severe visual loss [11]. Recent studies in our laboratory have demonstrated that formation of intravitreal membranes in a rabbit model is accompanied by a 2-fold increase in retinal-associated ODC activity [12]. Whether increased ODC activity followed by increased tissue polyamines plays an essential physiologic role in such retinal-associated proliferative processes is not known. Past studies also have shown that cell proliferation rate and ODC activity are elevated concomitantly in the developing chick embryo retina [13], 30-day-old chickens [14], and in rat embryo retina [15].

If induction of ODC activity is a requirement for cellular proliferation on the retina and in the vitreous, then specific inactivators of ODC such as alpha-DFMO (or related analogues) may be of use in determining the importance of both ODC and polyamines in pathological intraocular proliferative processes and in its prevention. Therefore, this study will explore the conditions required for alpha-DFMO to inactivate retinal ODC both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

L-[1-^{14}C]Ornithine (57 Ci/mol) was purchased from both Amersham-Searle, Arlington Heights, IL, and ICN Biochemicals, Irvine, CA, whereas [^{14}C]-labelled $NaHCO_3$ (52.0 mCi/mmol) was obtained from New England Nuclear, Boston, MA; alpha-DFMO was a gift from Dr. Peter McCann of Merrell Dow, Cincinnati, OH. All other reagents were obtained from either the Sigma Chemical Co., St. Louis, MO, or from the Aldrich Chemical Co., Milwaukee, WI.

Methods

Both adult female and male pigmented rabbits

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weighing between 3 and 4 kg were obtained from ABC Rabbitry. The animals were maintained on a normal 12-hr light/dark lighting cycle and fed standard laboratory chow and water *ad lib*. Animals were housed in animal care facilities fully accredited by the American Association of Laboratory Animal Science. The rabbits were anesthetized intramuscularly with a combination of xylazine and ketamine hydrochloride prior to enucleation. Immediately after enucleation the eyes were cut in half, and the resulting posterior eyecup was placed in a chilled Petri dish filled with Ringer's solution and kept on ice. After cutting the eyecup into two pieces through the optic nerve, the half retinas were gently brushed off in ice-cold Ringer's solution with a fine sable brush. The retinas were immediately homogenized and centrifuged at 4°. ODC activity of the resulting crude supernatant fraction was assayed as described below.

Measurement of ODC activity

Retinal ODC activity in the crude supernatant fraction was measured by ascertaining the amount of $^{14}CO_2$ released in 60 min at 37° using a modification of the method of Lapointe and Cohen [16]. Enzyme activity is given in pmol of $^{14}CO_2$ formed per mg protein per hr. Protein was determined by the method of Bradford [17] utilizing Bio-Rad dye reagents and bovine serum albumin as standard. Using a Brinkmann Polytron homogenizer, individual retinas were homogenized in glass tubes containing 500 μl of a buffer consisting of 1 mM EDTA, 5 mM dithiothreitol, 50 mM Tris/HCl, pH 7.5, set in an ice-water bath. After centrifugation at 4° for 20 min at 10,000 *g*, the resulting supernatant fraction was assayed immediately for ODC activity. 200 μl of the retinal supernatant containing 48 μM pyridoxal phosphate was added to the well of a spot plate (Corning Glass), which was set in a water bath at 37°, containing 50 μl of both 0.29 mM L-ornithine and 1.25 μCi L-[1-^{14}C]ornithine. A glass filter disk (Whatman GF/A, 2.4 cm) wetted with 175 μl of a 2% solution of barium hydroxide was placed immediately on top of the well to trap the $^{14}CO_2$. After 1 hr, the filter disk was lifted, and 50 μl of 2 N sulfuric acid was added quickly to stop the reaction. The disk was put back in place for another 30 min to trap any remaining $^{14}CO_2$. The radioactive $^{14}CO_2$ absorbed on the disks was counted in a Beckman LS 9000 scintillation counter utilizing Altex Ready-Solv scintillation mixture for aqueous samples. Statistical analysis was accomplished by Student's two-tailed *t*-test.

To check the efficiency of absorption of $^{14}CO_2$ by the barium hydroxide-saturated disks, the following procedure was performed. Radiolabeled [^{14}C] $NaHCO_3$ was diluted with unlabeled Na_2CO_3 to provide a stock solution of 1 mM (2 mCi/mol). After the appropriate dilutions with Na_2CO_3, 10-μl aliquots of varying radioactivity in duplicate were added to the wells of the spot plate, and then 50 μl of 2 N H_2SO_4 was added. A glass fiber disk initially presoaked with 175 μl of a 2% solution of barium hydroxide was placed over each well. After 30 min, the disks were removed from the wells, and the amount of absorbed radioactivity was determined. During this procedure, the spot plates were maintained at 37° in a water bath and were covered with a glass plate. The total amount of [^{14}C]$NaHCO_3$ added to the wells of the spot plate was ascertained by applying aliquots of different dilutions directly to presoaked glass fiber disks and then counting the radioactivity.

For evaluating the effectiveness of alpha-DFMO in inactivating retinal ODC activity following intravitreal administration, four rabbits were utilized for each timepoint and for each concentration of alpha-DFMO. After anesthetizing the rabbit with intramuscular xylazine and ketamine hydrochloride, 100 μl of alpha-DFMO dissolved in sterile balanced salt solution (BSS) was injected slowly into the rabbit vitreous with a 25-gauge needle utilizing a Zeiss operating microscope, while the fellow eye served as control. Isolated retinas at each timepoint were homogenized and centrifuged, and the resulting crude supernatant fraction was analyzed as previously described for ODC activity in duplicate.

Evaluation of toxicity

Twelve animals were used to obtain evidence of alpha-DFMO toxicity on ocular tissues. The left eye of each rabbit was injected with 100 μl of one of the following: sterile balanced salt solution (BSS), 8 mM alpha-DFMO, or 80 mM alpha-DFMO. Four animals were utilized in each group. Toxicity was evaluated by indirect examination of the retina, by measurement of intraocular pressure, and by electroretinography.

Ocular tissue examination. Gross evidence of toxicity affecting the anterior segment was assessed by using the slitlamp and monitoring intraocular pressure with an applanation tonometer. A series of five intraocular pressure measurements of each eye was made at the same time of day, and the results were averaged. The fundus of the retina was examined by using the indirect ophthalmoscope on days 1, 3, 7 and 14 after intraocular injection.

ERG measurements. As a functional test to ensure that a significant visual loss had not occurred after drug treatment, bilateral simultaneous electroretinogram (ERG) measurements were performed on all twelve animals at the beginning of the study prior to intravitreal administration of either BSS or alpha-DFMO. The rabbits were anesthetized with intramuscular ketamine and xylazine, and the pupils were dilated with a single drop of phenylephrine (10%). This anesthetic mixture has no measurable effect on the ERG. The topical anesthetic proparacaine hydrochloride (0.5%) was administered, and a bipolar Burian–Allen contact lens was placed in each eye. Each rabbit was pre-adapted for 3 min and then dark-adapted for 15 min. A series of ten Gansfeld Xenon flashes at scotopic intensity spaced at 5-sec intervals were utilized in order to produce tracings on the oscilloscope screen that could be photographed and then measured. Three tracings were superimposed at each intensity. The ERG was obtained at both 24 hr and 14 days after injection. For the evaluation of the ERG, the peak-to-peak amplitude of the b-wave was measured, and the preinjection ERG was compared to eyes treated with either alpha-DFMO or BSS.

RESULTS

Under the conditions of the described assay, the quantity of $^{14}CO_2$ produced per mg protein by crude rabbit retinal supernatant was proportional to the time of incubation ranging from 30 to 90 min (Fig. 1). In our hands, the efficiency of the $^{14}CO_2$ collection by the barium hydroxide-soaked disks was essentially quantitative ranging from 90 to 95% as determined by the amount of $^{14}CO_2$ trapped by the disks in comparison to what was added initially into the well of the spot plate. These results correspond to those reported earlier in the literature [16].

Retinal ODC activity following a 30-min preincubation with various concentrations of alpha-DFMO was also determined (Fig. 2). ODC activity in the presence of 10^{-5} M alpha-DFMO was 34 ± 6.9% (SD) that of control, whereas preincubation with 10^{-6} M alpha-DFMO resulted in an ODC activity level that was 81 ± 3% of normal (N = 3). However, exposure to 10^{-8} M alpha-DFMO resulted in 94 ± 2% ODC activity of control values.

Also, retinal ODC activity was measured after varying the preincubation time with various concentrations of alpha-DFMO (Fig. 3). Each point is the average of three different retinas assayed in duplicate. Crude retinal supernatant preincubated at 37° with 10^{-8} M alpha-DFMO at various times displayed 91 ± 3% (SD) of ODC activity in comparison with control values. However, after 40 min of preincubation with 10^{-5} M alpha-DFMO, ODC activity was 26 ± 5% of control level.

Intraocular administration of various concentrations of alpha-DFMO resulted in inhibition of ODC activity to varying degrees (Fig. 4). For each timepoint and each concentration, four rabbits were utilized. The non-treated fellow eye served as control. Actual control values of the fellow eye ranged from 126.77 ± 3.5 to 180.52 ± 10.55 pmol CO_2/mg/hr at different timepoints, so that experimental values were expressed as percent of control for that particular time, thus allowing for comparison between experiments. Both 24 and 48 hr after injection of 100 μl of 80 mM alpha-DFMO, retinal ODC activity was less than 5% of control levels; 100 μl of 8.0 mM alpha-DFMO depressed the activity to 66.80 ± 7.8% and 41.50 ± 2.1% after 24 and 48 hr respectively. With administration of 100 μl of 0.8 mM alpha-DFMO, retinal ODC activity was depressed to 82.6 ± 15.5% and 56.5 ± 12.02% of control values after 24 and 48 hr respectively.

To determine whether retinal ODC activity would recover to control levels after administration of 100 μl of 80 mM alpha-DFMO, ODC activity was determined at 1, 2, 4, 7 and 16 days later (Fig. 5). By 4 days, the activity had risen to 40.0 ± 5.1% of normal values, which returned to normal level by day 7 ($t = 1.95$; $P < 0.05$; $df = 6$) and remained at that level 16 days later ($t = 1.75$; $P < 0.05$; $df = 6$).

Examination of rabbits treated with alpha-DFMO intravitreally revealed no gross or severe toxicity following administration of alpha-DFMO. Intraocular pressure remained normal, and no fundoscopic changes in the retina were observed at the 14-day follow-up period after injection of either 8 or 80 mM alpha-DFMO. ERG recordings following

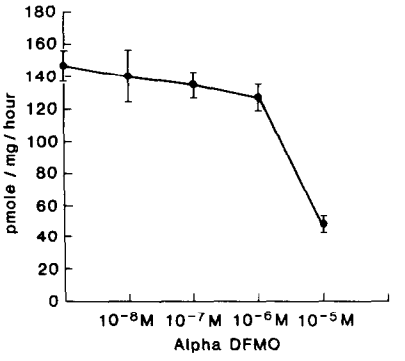

Fig. 2. Retinal ODC activity after preincubation with various concentrations of alpha-DFMO. Activity was measured after a 30-min preincubation at 37° with four different concentrations of alpha-DFMO ranging from 10^{-8} M to 10^{-5} M. The $^{14}CO_2$ was collected and determined. Formation of $^{14}CO_2$ was linear with protein concentration within the range used in the assay. Each point is the average of three retinal samples assayed in duplicate. Values are given in pmol $^{14}CO_2$ formed/mg protein/hr ± SD).

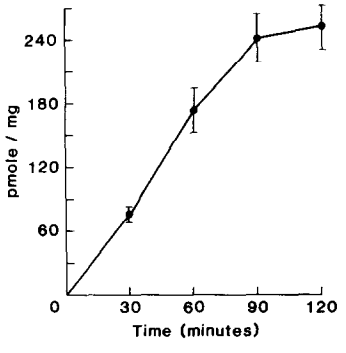

Fig. 1. Determination of linear range for assay of ODC activity. The amount of $^{14}CO_2$ released per mg of protein after varying the time of incubation between 30 and 120 min is demonstrated. At 30-min intervals, the disks were removed, and the $^{14}CO_2$ absorbed onto the disks was determined. Each point is the average of three different samples assayed in duplicate. This experiment was performed twice, and the results were averaged (±SD).

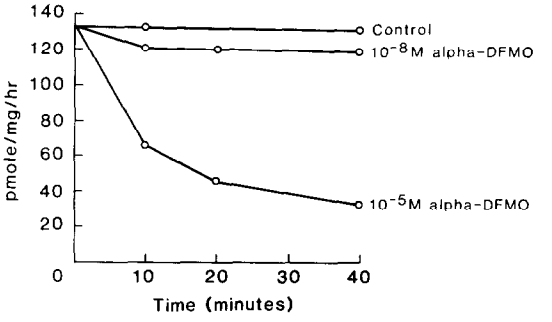

Fig. 3. Retinal ODC activity after various preincubation times with alpha-DFMO. Activity was defined as pmol $^{14}CO_2$ formed/mg protein/hr. The final concentration of alpha-DFMO was either 10^{-8} M or 10^{-5} M. Each point is the average of three retinas assayed in duplicate.

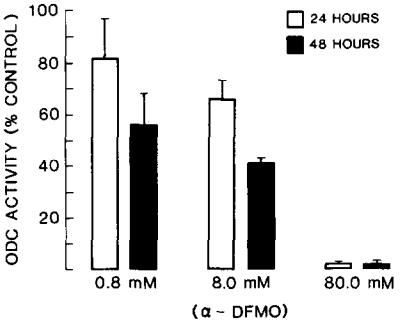

Fig. 4. Retinal ODC activity after intraocular administration of alpha-DFMO. Following a single intravitreal injection of 100 μl alpha-DFMO at various concentrations, retinal ODC activity was measured at both 24 and 48 hr later. Four animals per timepoint were utilized at each concentration of alpha-DFMO. The resulting supernatant fraction was assayed immediately in duplicate. In each case, the fellow non-treated eye served as control.

administration of 80 mM alpha-DFMO were within control levels at 14 days, but at 24 hr displayed no discernible b-wave. BSS-treated retinas were within normal ranges at both 24 hr and 14 days after intravitreal administration.

DISCUSSION

This study demonstrates that adult rabbit retinal ODC activity can be inactivated by alpha-DFMO under both *in vitro* and *in vivo* conditions. The present data constitute the first report of alpha-DFMO administration directly in the vitreous and the resulting determination of retinal-associated ODC activity. Indirect ophthalmoscopic examination of the retina, along with electroretinographic measurements after intraocular administration of 80 mM alpha-DFMO, suggests that no gross toxicity occurred in the retina over the observation period of 2 weeks. However, the short-term effect of alpha-DFMO in deleting the b-wave, which did not occur in the sham injection with BSS, demonstrates that a reversible functional impairment had occurred after drug treatment. Regardless, the significance of these observations relates to the possible utility of alpha-DFMO (or a similar analogue) for determining the possible role of ODC and polyamines in intraocular proliferative diseases. Recent work [12] demonstrates that retinal-associated ODC activity was elevated 2-fold over normal levels during epiretinal membrane formation in a rabbit model.

Additional studies indicate that non-neuronal cells enter the vitreous at a retinal hole or detachment and are thought to follow a gradient of chemoattractants [18, 19]. Whether such chemotactic agents are derived from serum, injured retinal tissue, or the vitreous itself currently is unclear. In addition, existence of growth factors may encourage the hypertrophy and proliferation of these cells [20]. Extensive work has been done with corticosteroids [21] and anti-neoplastic agents such as 5-fluorouracil [22] and daunomycin [23] in attempts to inhibit cell proliferation without retinal toxicity [24]. Studies utilizing liposomes as a drug delivery system, to either prolong exposure of epiretinal cells to the drug, or to specifically deliver their contents to these cells, thus potentially decreasing retinal toxicity, appear promising [25].

Further research utilizing alpha-DFMO will delineate more clearly what physiological role both polyamines and ODC may play in such intraocular proliferative diseases. The ability of alpha-DFMO to inactivate ODC activity *in vivo* may provide therapeutic intervention at the time of surgery or trauma to prevent the onset of proliferative vitreoretinopathy (PVR). The fact that recovery of ODC activity requires several days could be fortuitous in allowing time for wound healing to occur without concurrent proliferative processes. Experiments currently are underway to determine whether the progression of PVR in a rabbit model [11] is either prevented or delayed following intraocular administration of alpha-DFMO.

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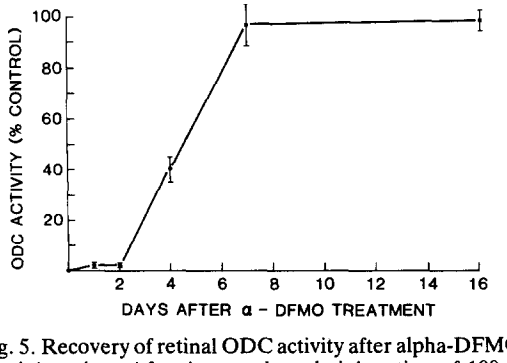

Fig. 5. Recovery of retinal ODC activity after alpha-DFMO administration. After intraocular administration of 100 μl of 80 mM alpha-DFMO dissolved in sterile balanced salt solution, retinal ODC activity was determined over a period of 16 days. Four animals were used for each timepoint, and each retina was analyzed in duplicate.

REFERENCES

1. A. E. Pegg and P. P. McCann, *Am. J. Physiol.* **243**, C212 (1982).
2. J. R. Fozard, M. L. Part, N. J. Prakash, J. Grove, P. J. Schechter, A. Sjoerdsma and J. Koch-Wester, *Science* **208**, 505 (1980).
3. A. Sjoerdsma and P. J. Schechter, *Clin. Pharmac. Ther.* **35**, 287 (1984).
4. T. A. Slotkin, F. J. Seidler, P. A. Trepanier, W. L. Whitmore, L. Lerea, G. A. Garnes, S. J. Weigel and J. Bartolome, *J. Pharmac. exp. Ther.* **222**, 741 (1982).
5. G. D. Luk, L. J. Marton and S. B. Baylin, *Science* **210**, 195 (1980).
6. P. S. Sunkara, N. J. Prakash, G. D. Mayer and A. Sjoerdsma, *Science* **219**, 851 (1983).
7. A. E. Pegg, J. Seely and I. S. Zagon, *Science* **217**, 68 (1982).
8. R. Machemer and H. Laqua, *Am. J. Ophthal.* **80**l, 1 (1975).
9. R. Machemer, D. L. Van Horn and T. M. Aaberg, *Am. J. Ophthal.* **85**, 181 (1978).

10. D. M. Fastenberg, K. R. Diddie, K. Dorey and S. J. Ryan, *Am. J. Ophthal.* **93**, 565 (1982).
11. P. E. Cleary and S. J. Ryan, *Br. J. Ophthal.* **63**, 306 (1979).
12. V. Renardel de Lavalette, B. Miller, C. G. Wong and S. J. Ryan. *Curr. Eye Res.* **5**, 101 (1986).
13. F. G. De Mello, U. Bachrach and M. Nirenberg, *J. Neurochem.* **23**, 847 (1976).
14. M. A. Grillo, T. Fossa and U. Dianzani, *Int. J. Biochem.* **15**, 1081 (1983).
15. S. Macalone and A. Calatroni, *Life Sci.* **23**, 683 (1978).
16. D. S. Lapointe and R. J. Cohen, *Analyt. Biochem.* **109**, 291 (1980).
17. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
18. P. A. Campochiaro, J. A. Jerdan and B. M. Glaser, *Archs Ophthal.* **102**, 1830 (1984).
19. P. A. Campochiaro and B. M. Glaser, *Archs Ophthal.* **103**, 576 (1985).
20. P. A. Campochiaro, J. A. Jerdan, B. M. Glaser, A. Cardin and R. G. Michels, *Archs Ophthal.* **103**, 1403 (1985).
21. Y. Tano, G. Sugita, G. Abrams and R. Machemer, *Am. J. Ophthal.* **89**, 131 (1980).
22. M. S. Blumenkranz, A. Ophir, A. Claflin and A. Hajek, *Am. J. Ophthal.* **94**, 458 (1982).
23. M. Kirmani, M. Santana, N. Sorgente, P. Wiedemann and S. J. Ryan, *Retina* **3**, 269 (1983).
24. R. H. Steinberg, *Expl Eye Res.* **43**, 695 (1986).
25. W. H. Stern, T. D. Heath, G. P. Lewis, C. J. Guerin, P. A. Erickson, N. G. Lopez and K. Hong, *Invest. Ophthal. vis. Sci.* **28**, 907 (1987).