Selective Rod Degeneration and Partial Cone Inactivation Characterize an Iodoacetic Acid Model of Swine Retinal Degeneration

Wei Wang,1,2 Juan Fernandez de Castro,1,2 Eric Vukmanic,1 Liang Zhou,1 Douglas Emery,1 Paul J. DeMarco,1,3 Henry J. Kaplan,1 and Douglas C. Dean1,4,5,6

PURPOSE. Transgenic pigs carrying a mutant human rhodopsin transgene have been developed as a large animal model of retinitis pigmentosa (RP). This model displays some key features of human RP, but the time course of disease progression makes this model costly, time consuming, and difficult to study because of the size of the animals at end-stage disease. Here, the authors evaluate an iodoacetic acid (IAA) model of photoreceptor degeneration in the pig as an alternative model that shares features of the transgenic pig and human RP.

METHODS. IAA blocks glycolysis, thereby inhibiting photoreceptor function. The effect of the intravenous injection of IAA on swine rod and cone photoreceptor viability and morphology was followed by histologic evaluation of different regions of the retina using hematoxylin and eosin and immunostaining. Rod and cone function was analyzed by full-field electroretinography and multifocal electroretinography.

RESULTS. IAA led to specific loss of rods in a central-to-peripheral retinal gradient. Although cones were resistant, they showed shortened outer segments, loss of bipolar cell synaptic connections, and a diminished flicker ERG, hallmarks of transition to cone dysfunction in RP patients.

CONCLUSIONS. IAA provides an alternative rod-dominant model of retinal damage that shares a surprising number of features with the pig transgenic model of RP and with human RP. This IAA model is cost-effective and rapid, ensuring that the size of the animals does not become prohibitive for end-stage disease. Here, the authors evaluate an alternative rod-dominant model that shares features of the transgenic pig and human RP.

Transgenic pigs carrying a mutant human rhodopsin transgene represent a large animal model of retinitis pigmentosa (RP). This model displays some key features of human RP, but the time course of disease progression makes this model costly, time consuming, and difficult to study because of the size of the animals at end-stage disease. Here, the authors evaluate an alternative rod-dominant model that shares features of the transgenic pig and human RP.

From the Departments of 1Ophthalmology and Visual Sciences, 2Psychological and Brain Sciences, and 3Biochemistry and Molecular Biology, the 4Molecular Targets Program, James Graham Brown Cancer Center, and the 5Birth Defects Center, University of Louisville Health Sciences Center, Louisville, Kentucky.

2These authors contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Supported in part by American Health Assistance Foundation, National Institutes of Health Grants P20 RR018753 and EY015636, Research to Prevent Blindness, and The Commonwealth of Kentucky Research Challenge.

Submitted for publication May 9, 2011; revised July 22, 2011; accepted August 10, 2011.

Disclosure: W. Wang, None; J. Fernandez-de Castro, None; E. Vukmanic, None; L. Zhou, None; D. Emery, None; P.J. DeMarco, None; H.J. Kaplan, None; D.C. Dean, None.

Corresponding author: Douglas C. Dean, University of Louisville Health Sciences Center, 301 E. Muhammad Ali Boulevard, Louisville, KY 40202; dcdean01@louisville.edu.

Copyright 2011 The Association for Research in Vision and Ophthalmology, Inc.
neurons are unaffected by IAA treatment in rabbits and monkeys. Such results suggest that IAA can specifically eliminate photoreceptors in the retina, thereby providing a model in which transplanted photoreceptors may be sufficient to restore a visual transduction pathway in an otherwise undamaged retina.

Here, we examined the effects of IAA on photoreceptor viability and function in the pig to determine whether it might recapitulate some of the features of RP. We found that rods were specifically targeted by IAA in a central-to-peripheral fashion, as in the RHO transgenic pig model. Further, cones were resistant to IAA, leading to a monolayer of cones retaining shortened outer segments in the outer nuclear layer (ONL). These cones were still functional, but they showed a diminished response in the flicker ERG. These results suggest that IAA in the pig can serve as a model for specific loss of rods and that under these conditions cones survive but display a diminished electrophysiological response characteristic of the cells in RP patients.

METHODS

Animals and Treatment

All methods were approved by the University of Louisville Institutional Animal Care and Use Committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Domestic swine (males; weight range, 12–16 kg; age range, 6–8 weeks) were obtained from Oak Hill Genetics (Ewing, IL). Swine received a single dose of 7.5 (11 pigs) or 12.0 mg/kg (8 pigs) pH balanced IAA immediately after a baseline electrophysiological assessment. Four control animals were injected with normal saline.

Assessments of Retinal Morphology and Function in IAA-Treated Swine

Electrophysiology. Baseline functional assessments consisted of both multifocal ERG (mf-ERG) and full-field ERG (ff-ERG). Before electrophysiological analyses, animals were anesthetized using the protocol previously described by Lalonde et al. Pupils were dilated with topical phenylephrine hydrochloride 2.5% and tropicamide 1%.

The mf-ERG was recorded first at light-adapted levels (VERIS System; Electro-Diagnostic, Inc., Redwood City, CA) and a DTL electrode on the cornea. Reference and ground needle electrodes were placed in the skin above the eye and behind the ear, respectively. A topographic map of the focal retinal response was rendered, including the visual streak, which corresponds to a location 0.5 disc diameters above the optic disc, 1 disc diameter in height, and the full width of the stimulus pattern. An infrared photograph of the fundus was taken before the baseline assessment and kept as a reference to align subsequent evaluations of the fundus.

**FIGURE 1.** IAA causes a central-to-peripheral gradient of photoreceptor loss without affecting the number of inner retinal neurons. (A) Low-power view of a hematoxylin and eosin section extending from the optic disc to the periphery of the retina 12 weeks after 7.5 mg/kg IAA. (B) Higher power views of hematoxylin and eosin sections of different retinal regions. (C) The numbers of ONL and INL nuclei were counted along a 100-μm length of retina in the different regions (*P < 0.05). Scale bar, 20 μm.
The mf-ERG response amplitude was calculated by averaging the N1-P1 amplitude of each mf-ERG trace over the visual streak.

The ff-ERG was then recorded (UTAS ERG System with a BigShot Ganzfeld Stimulator, LKC Technologies, Gaithersburg, MD) and an ERG-jet electrode (Fabrinal SA, La Chaux de Fonds, Switzerland). Ground and reference electrodes were placed behind the ear and on the midline of the forehead, respectively. Recordings were performed following the ISCEV standard for full-field clinical electroretinography.27,28 Ten eyes from five pigs were assessed for each ERG time point.

**Histologic Evaluation.** Pigs were euthanized at the indicated times after IAA injection. Eyes were enucleated, and retinas were isolated and fixed by immersion in 4% paraformaldehyde for 30 minutes. The retina that extended from the dorsal to the ventral margin of the eyecup was bisected at the optic disc along the sagittal plane. Half the retina was dehydrated and embedded in OCT for frozen sections. The other half was processed for paraffin sections. Frozen sections measuring 12 to 14 μm were cut for immunostaining, and 4-μm sections were cut for paraffin sections and hematoxylin and eosin staining. Ten eyes from 10 pigs were used (see Fig. 3), and six eyes from six pigs were used (see Fig. 5).

**Immunohistochemistry Staining.** Slides were dried at room temperature for 30 minutes, washed with phosphate-buffered saline (PBS), and blocked with 0.2% BSA, 4% goat serum, and 0.05% Tween-20 in PBS for 1 hour at room temperature. The samples were then incubated overnight at 4°C or for 1 hour at room temperature with primary antibodies in blocking solution. After primary antibodies were removed and the samples were washed, secondary antibodies were applied for 1 hour at room temperature. The primary antibodies used were mouse anti-rhodopsin (1:300; Millipore, Billerica, MA), rabbit anti-L/M opsin (1:500; Millipore), and anti-neural retina leucine zipper protein (NRL),29 a kind gift from Anand Swaroop (National Eye Institute), diluted 1:1000. Labeled cells were visualized with either Alexa Fluor 488 (1:500; Invitrogen, Carlsbad, CA) or Alexa Fluor 568 (1:500; Invitrogen)-conjugated secondary antibodies. The nuclei were counterstained with DAPI. The images were captured with an inverted fluorescence microscope (Axiovert 200; Zeiss, Thornwood, NY). The immunoreactivity of each antibody was confirmed by immunostaining swine retinal tissue as a positive control. As a negative control, no immunostaining was evident in the absence of the primary antibodies.

**Statistical Analysis**

Results are reported as mean ± SE. ANOVA and Student’s t-test were used for the determination of statistical significance among treatment groups.

**RESULTS**

**IAA Causes a Central-to-Peripheral Gradient of Photoreceptor Loss without Affecting the Number of Inner Retinal Neurons**

IAA was injected into the ear veins of pigs at 6 to 8 weeks of age (weight range, 12–16 kg) at concentrations of 7.5 mg/kg or 12 mg/kg. After 12 weeks, the eyes were removed, and sagitally cut sections were stained with hematoxylin and eosin (Figs. 1A, 1B). We studied three areas of the retina: the central...
retina (1 mm dorsal to the optic disc), the visual streak (2 mm superior to the optic disc), and the peripheral retina (1 mm posterior to the ora serrata). Nuclei located in the ONL were counted along a 100-μm linear distance in each region (Fig. 1C). Similar numbers of nuclei were evident in the ONL in each retinal area in the untreated animals. The ONL in the central retina and the streak was greatly diminished after injection of 7.5 mg/kg IAA. Surprisingly, the ONL in the peripheral retina was only modestly diminished. Injection of 12 mg/kg IAA also led to a similar loss of the ONL in the central retina and visual streak with only a modest loss in the peripheral retina. As a control, IAA had little effect on the number of inner nuclei located in the inner nuclear layer (INL) in any of the retinal regions (Figs. 1A–C).

Identification and Organization of Rods and Cones in the Swine Outer Nuclear Layer

Next, we asked whether IAA might have a differential effect on rod and cone photoreceptors. First, it was necessary to identify rod and cone cell nuclei within the ONL. Frozen sections of central and peripheral retina were immunostained for the rod specification transcription factor, neural retina leucine zipper protein (NRL), which is present in rod nuclei. Sections were double immunostained for the cone-specific, long-wavelength opsin 1 (OPN1LW) and medium wavelength opsin 1 (OPN1MW) or for the short-wavelength S opsin (OPN1SW); the latter makes up approximately 16% of the cones in the pig (Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7849/-/DCSupplemental). NRL was uniformly expressed in the nuclei of cells in the inner rows of the ONL, but the outermost rows of the ONL were devoid of NRL in both the central and the peripheral retina (Figs. 2A–D; Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7849/-/DCSupplemental). Accordingly, cell bodies in these outer rows of the ONL immunostained for cone opsins (Figs. 2A–D; Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7849/-/DCSupplemental). Cone outer segments (OS) also immunostained brightly for cone opsins, as did cone pedicles, the synaptic terminals between cones and cone bipolar cells (Figs. 2A–D). In most species cones are concentrated in the outer half of the ONL, but these results demonstrate that in the pig cones are completely segregated from rods and that they constitute the outermost rows of the ONL; this organization is evident in both the central and the peripheral retina.

The numbers of cone and rod nuclei were then counted in the central and peripheral retina. The number of cones was similar in these regions, but there were approximately 25% fewer rods in the peripheral than in the central retina (Figs. 3A, 3B).

IAA Causes a Selective Loss of Rods in the Central Retina

After IAA treatment, central and peripheral retina sections were immunostained for NRL and cone opsins to determine whether rods or cones were selectively affected by IAA (Figs. 3A–C, 4). The number of cones was not affected by either 7.5 mg/kg or 12 mg/kg IAA in either the central or the peripheral retina. By contrast, rods were dramatically diminished in the central retina with 7.5 mg/kg IAA, and they were even further diminished in this region with the 12-mg/kg dose (Figs. 3A–C, 4). IAA had less effect on rod number in the peripheral retina. The 7.5-mg/kg dose decreased the number of rods by approximately 35% in the peripheral retina, whereas the higher dose of 12 mg/kg led to an approximately 70% decrease in rod number. Next, we analyzed the time course of effect of IAA on rod number (Fig. 3C). Rod number was diminished by 7.5 mg/kg and 12 mg/kg IAA 3 weeks after treatment. The number of rods decreased further by 5 weeks. Thus, the initial treatment with IAA and the resultant block in glycolysis led to a progressive loss of rods with time.

Rods in the Peripheral Retina Show Diminished Outer Segments with RHO Redirected to Cell Bodies after IAA Treatment

RHO is normally directed to rod outer segments, but it becomes redirected to cell bodies as rods lose OS in models of retinal degeneration. Although rods persisted in the peripheral retina after IAA treatment, they showed an IAA concentration-dependent loss of OS with RHO redirected to cell bodies (Fig. 4).

![Figure 3](http://tvst.arvojournals.org/) IAA causes a dose-dependent loss of rods in a central-to-peripheral retinal gradient without affecting the number of cones. (A) Representative immunostaining of central (C) and peripheral (P) retinal sections for NRL to identify rods 12 weeks after injection of 7.5 mg/kg IAA. Arrows: same position in adjacent sections. (B) Quantification of the effects of IAA on rod and cone nuclei in the central and peripheral retina. NRL immunostaining was used to identify rods and L/M opsin immunostaining was used to identify cones (see Figs. 4, 5). The numbers of rods and cones in 100-μm length sections of retina were counted as in Figure 1. (C) Rod and cone numbers in central retina at 3, 5, 8, and 12 weeks after IAA treatment compared with baseline (*P < 0.05). Scale bar, 20 μm.
IAA Causes a Concentration-Dependent Loss of Cone OS, a Decrease in Cone OS Length, and a Loss of Cone Pedicles

As noted, IAA did not affect cone cell number in either the central or the peripheral retina (Figs. 3A–C, 4). However, because IAA led to loss of OS on rods persisting in the peripheral retina (Fig. 4), we wondered whether IAA might also be affecting cone OS. Coimmunostaining for NRL and cone L/M opsin allowed identification of cone cell bodies, cone inner segments, and cone OS (Fig. 5A). We then used such immunostaining to assess cone OS number after IAA treatment. Although 7.5 mg/kg IAA did not affect cone OS number in either the central or the peripheral retina, the higher dose of 12 mg/kg IAA led to a dramatic loss of cone OS in the central retina, but cone OS was diminished only by approximately 50% in the peripheral retina (Fig. 5B).

Cones are also relatively resistant in the pig transgenic model, expressing a mutant human RHO, but the remaining cones exhibit shortened OS.16 In human RP, shortened cone OS correlates with a diminished flicker ERG.31 Therefore, we used immunostaining, as in Figure 5A, to measure the length of cone OS after IAA treatment. Although the number of cone OS was not affected by 7.5 mg/kg IAA, the length of cone OS was diminished by approximately 60% in the central retina; how-
ever, this concentration of IAA had only a modest effect on cone OS length in the peripheral retina (Fig. 5C). The 12-mg/kg dose of IAA led to a dramatic decrease in cone OS length in both the central and the peripheral retina (Fig. 5C).

Along with the diminished number and length of cone OS after treatment with 12 mg/kg IAA, cone pedicles were diminished in a central-to-peripheral gradient (Figs. 4, 5D). By contrast, cone pedicles were unaffected in either the central or the peripheral retina after the 7.5-mg/kg dose of IAA (Figs. 4, 5D).

IAA Treatment Leads to a Concentration-Dependent Loss of Rod Function

The results demonstrate a dramatic loss of rods in the central retina after IAA injection, and rods that remained in the periphery had diminished OS and redirected RHO to the cell bodies. These morphologic changes suggested that IAA caused a loss of rod function. Dark-adapted scotopic ff-ERG was used to assess rod function after IAA injection. We found that rod ERG signals were \( \frac{1}{2} \) of untreated controls 2 weeks after injection of 7.5 mg/kg IAA, and there was not significant change in the rod ERG out to 12 weeks (Fig. 6). In addition, the injection of 12 mg/kg IAA led to a further diminished rod ERG signal, and this reduced signal remained constant until 12 weeks (Fig. 6). With both doses of IAA, rods were still evident histologically at 3 weeks (Fig. 3C), but their loss of ERG by 2 weeks suggested they lost function before the cell bodies disappeared.

Cone ERG Signals Show a Biphasic Response to IAA

Photopic ff-ERG was used to assess cone signaling after IAA injection. As with rod signals, the cone response was significantly diminished 2 weeks after IAA injection to approximately 20% of control with the 7.5-mg/kg concentration and to approximately 10% of control with the 12-mg/kg concentration (Fig. 6). Unlike the rod response, which remained suppressed out to 12 weeks, the cone response rebounded to approximately 50% of control with 7.5 mg/kg IAA and to approximately 30% of control with 12 mg/kg IAA (Fig. 6).

The photopic mf-ERG was used to assess the cone response on the visual streak near the central retina. As with the full-field response, the mf-ERG cone response was diminished with both 7.5 and 12 mg/kg IAA 2 weeks after injection, and the response significantly rebounded to approximately 70% of control with 7.5 mg/kg IAA and to approximately 50% of control with 12 mg/kg IAA (Fig. 6). We were unable to assess the peripheral retina using the mf-ERG.

Cones Show a Diminished Response in Flicker ERG after IAA Injection

As noted, shortened OS and a diminished flicker ERG are early markers of loss of cone function in RP. We then examined the temporal response of cones to rapid flashes using flicker ERG. As with the photopic ff-ERG response (Fig. 6), the full-field flicker ERG response was diminished 2 weeks after IAA injection, and it rebounded at 5 weeks and remained similar out to 12 weeks (Fig. 6). Nevertheless, overall flicker ERG was more diminished than the photopic ff-ERG, implying that the cones are less able to respond to the stress of the rapid temporal response imposed by flicker. Representative waveforms for ff-ERG, mf-ERG, and flicker ERG are shown in Supplementary Fig. S2 (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7849/-/DCSupplemental).

DISCUSSION

There is mounting interest in using the pig as a model for retinal disease and stem cell transplantation therapy. The RHO transgenic model recapitulates important features of the disease, including selective loss of rods and the subsequent diminished function of cones highlighted by shortened outer segments and diminished flicker ERG. However, this transgenic model is costly to maintain. By the time of end-stage disease at 9 months of age, the animals are very large (weight 500 pounds), making assessment and stem cell transplantation difficult.

The IAA model we describe here shares a surprising number of features with the transgenic model. Rods are lost after IAA treatment whereas cones are not; this is similar to the rod-specific pattern of loss seen in the transgenic model and in RP. In the transgenic model and RP patients, this rod sensitivity is logical because the RHO mutation is targeted to rods and the subsequent defects in cones are then secondary to rod loss. In the IAA model, there is a similar pattern of rod-specific
loss, but this rod sensitivity is likely caused by differences in metabolic demands between rods and cones, with the cone defects a result of rod loss as in the transgenic model and in RP. Interestingly, there is a gradient of rod loss, with rods being eliminated in the central retina while the most peripheral retina is relatively unaffected after IAA. The reason for the relative resistance of rods in the peripheral retina is unclear, but this same pattern of rod resistance in the periphery is also seen in the RHO transgenic pig model. In addition, as in the transgenic model and in RP, cones that are retained after IAA exhibit shortened OS (and, at the higher IAA concentration, a loss of pedicles), and they display a reduced flicker ERG, implying that the cone signaling defects become more pronounced when the cells are challenged temporally with rapid flashes.

ERG signals from rods were diminished at 2 weeks after IAA injection, and they remained diminished until 12 weeks. These results are consistent with the loss of rods that we observed histologically. By contrast, cone ERG signals dipped at 2 weeks after IAA injection but rebounded significantly by 5 weeks, and this recovery was maintained to 12 weeks. We suggest that the dip in response at 2 weeks reflects the initial covalent modification to GAPDH and the resultant block of glycolysis when IAA is injected. Although rods die from this initial block in glycolysis, cones do not. We suggest that once IAA is cleared, cones can resynthesize GAPDH and restore glycolysis and that the cone defects are likely a secondary result of the surrounding rod loss as discussed.

Importantly, the time course for retinal changes seen with IAA is much more rapid than with the transgenic model, allowing for easy evaluation and surgery. Thus, the IAA model in the pig may provide a relatively rapid and cost-effective alternative swine model of rod degeneration and cone dysfunction that can be easily manipulated and assessed in transplantation experiments. Such a model may then provide a means for collecting data and establishing experimental conditions that can, in turn, be applied to the transgenic model in more long-term experiments.

Acknowledgments

The authors thank Giurong Liu for histologic sections and Anand Swaroop for the NRL antibody.

References