Video Article

Characterization of Molecular Mechanisms of *In vivo* UVR Induced Cataract

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URL: https://www.jove.com/video/4016
DOI: doi:10.3791/4016

Keywords: Medicine, Issue 69, Neuroscience, Molecular Biology, Ophthalmology, Immunology, UVR-B, lens, cataract, qRT-PCR, PCR, immunohistochemistry, rat restrainer, animal model

Date Published: 11/28/2012

Citation: Galichanin, K., Talebizadeh, N., Söderberg, P. Characterization of Molecular Mechanisms of *In vivo* UVR Induced Cataract. *J. Vis. Exp.* (69), e4016, doi:10.3791/4016 (2012).

Abstract

Cataract is the leading cause of blindness in the world [1]. The World Health Organization defines cataract as a clouding of the lens of the eye which impedes the transfer of light. Cataract is a multi-factorial disease associated with diabetes, smoking, ultraviolet radiation (UVR), alcohol, ionizing radiation, steroids and hypertension. There is strong experimental [2-4] and epidemiological evidence [5,6] that UVR causes cataract. We developed an animal model for UVR B induced cataract in both anesthetized [7] and non-anesthetized animals [8].

The only cure for cataract is surgery but this treatment is not accessible to all. It has been estimated that a delay of onset of cataract for 10 years could reduce the need for cataract surgery by 50% [9]. To delay the incidence of cataract, it is needed to understand the mechanisms of cataract formation and find effective prevention strategies. Among the mechanisms for cataract development, apoptosis plays a crucial role in initiation of cataract in humans and animals [10]. Our focus has recently been apoptosis in the lens as the mechanism for cataract development [8,11,12]. It is anticipated that a better understanding of the effect of UVR on the apoptosis pathway will provide possibilities for discovery of new pharmaceuticals to prevent cataract.

In this article, we describe how cataract can be experimentally induced by *in vivo* exposure to UVR-B. Further RT-PCR and immunohistochemistry are presented as tools to study molecular mechanisms of UVR-B induced cataract.

Video Link

The video component of this article can be found at https://www.jove.com/video/4016/

Protocol

1. Exposure to Ultraviolet Radiation

1. 15 min before the exposure, anesthetize a female Sprague-Dawley rat with a mixture of 90 mg/kg Ketalar (ketamine) and 10 mg/kg Rompun (xylazine) by intraperitoneal injection.
2. Place the animal in a rat restrainer and tighten the belts until immobilization of the rat without causing a trunk squeeze [13].
3. Instill Mydriacyl (tropicamide), 10 mg/ml, in both eyes of the rat to induce mydriasis.
4. Place the animal so that one eye is positioned against a narrow beam of UVR at 300 nm [14] with 10 nm full width at half maximum and shield the contralateral eye with black tape.
5. Expose the rat unilaterally to sub-threshold dose 1 kJ/m^2 UVR-B at 300 nm for 15 min [15].

2. Dissection

1. After pre-determined post exposure interval (1, 5, 24 and 120 hr), sacrifice the six week old rat (weight < 200 g) by carbon dioxide asphyxiation and dislocation of the cervical vertebrae.
2. Enucleate eyes. Thereafter, put the eye cornea down, posterior side up. Then, punch a minimal hole by applying a 27 gauge cannula and push tangentially to the sclera to avoid damaging the lens that is very close to the sclera. Then use a pair of ophthalmic surgery scissors to cut circumferentially just behind the limbus until the posterior portion of the sclera can be lifted off. Then, lift the lens with a blunt curved forceps.
3. Remove remnants of the ciliary body from the lens equator under a microscope, keeping the lens in balanced salt solution no longer than 5-10 min.

3. Quantitative RT-PCR

1. Place a lens into a mix of 350 µl RA1 lysis buffer (NucleoSpin RNA II total RNA isolation kit) and 3.5 µl β-mercaptoethanol in a 2 ml Eppendorf tube.
2. Keep the lens in this mix during 30 min at room temperature.
3. Homogenize the lens with a needle until only hard nucleus of the lens is left from the lens (cortex and capsule of the lens are lysed in the lysis buffer). Remove the nucleus from the mix.
4. Store samples immediately at -70 °C.
5. Thaw the samples and follow the protocol "Total RNA purification from cultured cells and tissue" (NucleoSpin RNA II total RNA isolation kit).
6. Store RNA samples at -70 °C.
7. To verify sufficient removal of DNA from each sample, run a PCR under the following conditions (step 1: 95 °C for 2 min; step 2 for 40 cycles: 95 °C for 20 sec, 55 °C for 20 sec, 72 °C for 20 sec; step 3: 72 °C for 7 min) with p53 DNA specific primers, forward 5'-ACCCTCTGACCTTTTTCCCA-3' and reverse 5'-TGCTGGGATCTTAGGCACTC-3' and Taq DNA polymerase (dNTPack), according to the manufacture protocol. The expected PCR product is 243 base pairs.
8. Run a 1.5 % agarose gel electrophoresis to detect DNA specific PCR product of 243 base pairs. To prepare 1.5 % agarose gel in TBE buffer take 3.75 g of agarose and solve it in 250 ml of TBE buffer. Add ethidium bromide (0.5 mg/ml) 500 µl in 500 ml of 1.5 % agarose gel solution. Heat the mixture in a microwave owen and stir to solve the agarose.

None of the samples has to reveal any DNA specific PCR products on 1.5 % agarose gel electrophoresis.

9. Measure the concentration of RNA samples (1 µl) and the ratio of sample absorbance at 260 nm (RNA) and 280 nm (protein) in a NanoDrop ND-1000 spectrophotometer. If the ratio RNA to protein of RNA sample absorbance is 2.0 or more then the RNA sample is pure. If the ratio of RNA sample absorbance is lower than 2.0, redo RNA purification step 3.5.
10. Take a volume of RNA sample corresponding to 1 µg and synthesize cDNA following the protocol 1st Strand cDNA synthesis Kit (1st Strand cDNA synthesis Kit for RT-PCR).
11. Store cDNA samples at -20 °C (for a period of 1 and more years, store at -70 °C).
12. Run quantitative real-time PCR on iCycler MyiQ Single Color Real Time PCR detection system. Load triplicates on 96-well plate with 1 µl cDNA sample, TaqMan Gene Expression Master Mix, TaqMan Gene Expression Assay for caspase 3, according to manufacture instructions. Load triplicates on another 96-well plate with 1 µl cDNA sample, TaqMan Gene Expression Master Mix, TaqMan Gene Expression Assay for 18s, according to manufacture instructions (TaqMan Gene Expression Assay Protocol). Dilute in series a fraction of cDNA from three randomly chosen non-exposed lenses. Run serial dilutions together with the cDNA from samples in a 96-well plate.
13. Use the standard curve method for quantification of the results. The number of cycles at threshold fluorescence is used as the measurement in MyiQ software. A standard curve expressing number of cycles at threshold as function of relative concentration of calibrator is established for the serial dilutions in each plate. cDNA from three non treated lens samples was used for calibration. Relate the threshold number of cycles for each sample to the standard curve to obtain the relative concentration of the sample cDNA measured. Finally, get expression level of the target genes by relating the relative concentration of the target cDNA to the internal control 18s cDNA.

4. Immunohistochemical Staining

1. Fixation
    1. Dissect the eye in PBS (Phosphate Buffered Saline; pH 7.4).
    2. Put the eye in a tube filled with 4 % paraformaldehyde (PFA) in ice cold for 20 min. Prepare PFA 4 % the day before. Store at +4 °C until use. PFA is fresh for approximately one week.
    3. Remove PFA with a micropipette. Then, wash with ice cold PBS for 20 min.
    4. Put the eye in sucrose 30 % at +4 °C over night.
    5. Put the eye in a cup filled with Optimal Cutting Temperature (OCT)-medium (Tissue-Tek). Put the eye in a suitable position for sectioning.
    6. Freeze in OCT-medium over dry ice.
    7. Store at -70 °C until use.

2. Sectioning
    1. Position the eye for cryo-sectioning in an appropriate plane.
    2. Cut three 5 µm thick mid-sagittal sections from a central portion of each lens. Discard at least 6 sections between sequential sections to avoid staining the same nuclei in different sections.
    3. Once a section has been cut, gently place a slide on top of it. The section should then stick to the slide.
    4. Leave the slides to air-dry before use. Slides can be stored at -20 °C until required.

3. Fluorescence immunohistochemistry
    1. Let samples attain room temperature (5-10 min).
    2. Draw edges around the specimens with a PAP-pen (Invitrogen).
    3. Let the border dry for a while (10 min).
    4. Label the microscope slides.
    5. Rehydrate in 1 × PBS for 15 min.
    6. Permeabilize in standard block solution for 30 min.
    7. Add primary antibody (rabbit polyclonal, cleaved caspase-3 antibody Asp175 9661; Cell Signalling Technology, Inc) diluted 1:3,000 in standard block solution.
    8. Store in a humidified chamber at +4 °C over night.
    9. Wash in PBS by pipetting (3 × 5 min) or by immersing in a large bath (>15 min, 1-2 changes).

10. Add secondary antibody (anti rabbit secondary antibody with a specific absorption/emission spectrum) diluted 1:300 in the standard block solution.
11. Wash in PBS by pipetting (3 × 5 min) or by immersing in a large bath (>15 min, 1-2 changes).
12. Wipe off PAP-pen smears.
13. Store in a humidified chamber at room temperature for 3 hr. Avoid exposure to light.
14. Add a drop of Vectashield and place a cover slip on the slide. Avoid making bubbles.
15. Let the Vectashield harden (~20 min).
16. Keep sections in the dark until analysis.
17. All control sections are processed in the absence of primary antibody.
18. Look for the results within a few hours under a fluorescence microscope.
19. Count epithelial cell' nuclei from one side nuclear bow to other side nuclear bow of each lens. Apply the standard blue filter to see all lens epithelial nuclei in blue and a standard filter that fits the emission of the secondary antibody to see the caspase-3 positive nuclei in green.
20. Record the number of all lens epithelial nuclei and the number of positive nuclei. Count the cells three times for each section.

Representative Results

The various sources of variation in the measurements were estimated with an analysis of variance and it was found that considering three measurements per animal the variance for measurements was on the order of 15 % of that for animals. Thus, considering whole lens analysis, it is not possible to increase the precision. Orthogonal testing elucidated a statistically significant contrast for caspase-3 message between 120 hr latency interval versus shorter latency intervals.

In vivo UVR exposure induces caspase-3 expression.

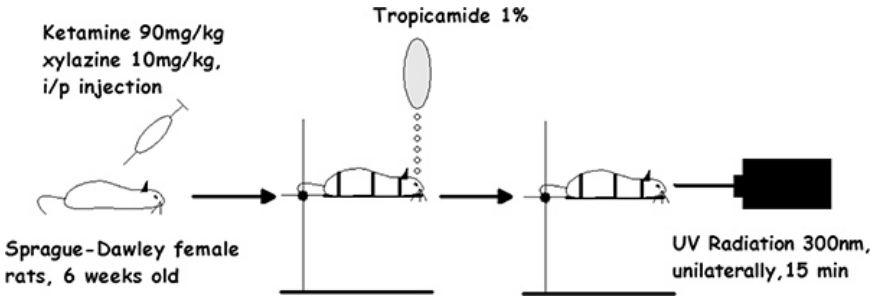

Figure 1. Schematic of the flow of exposure to ultraviolet radiation.

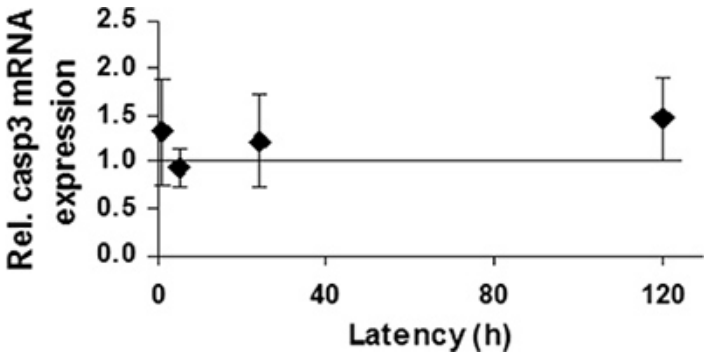

Figure 2. Evolution of caspase-3 (casp3) mRNA expression in the crystalline lens after *in vivo* exposure to 1 kJ/m^2 UVR at 300 nm. Error bars are 95 % confidence intervals for mean ratio of casp3 mRNA/18s rRNA between exposed lens and contralateral not exposed lens. The means, above and below the black line at 1 rel. unit, respectively, represent up- and down-regulation of the casp3 gene.

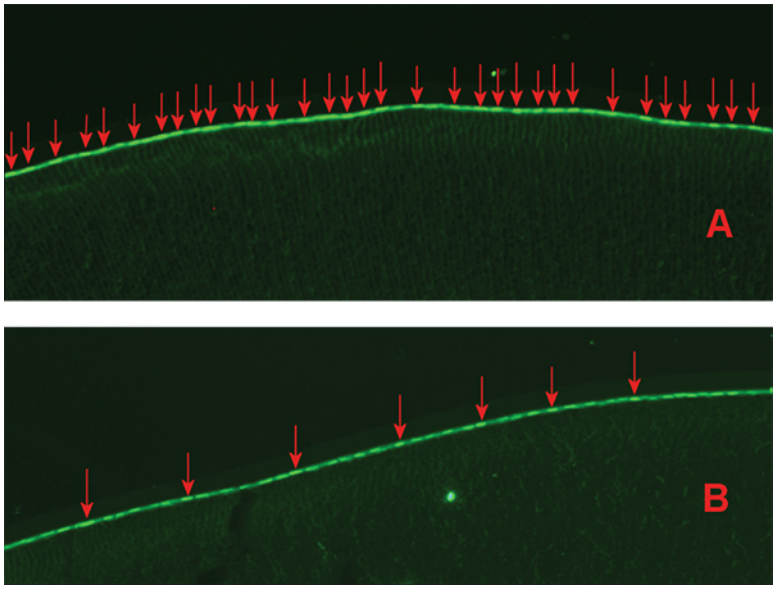

Figure 3. Caspase-3 expression (A) in an exposed lens, 24 hr after exposure and (B) in a non-exposed lens. Arrows show labeled cells.

Discussion

This paper describes methods that enable studying molecular events occurring during UVR-B induced cataract.

Considering, that most information available for *in vivo* UVR induced cataract was derived from experiments on albino Sprague-Dawley rats [7, 16, 17, 18, 19], we decided to use the albino Sprague-Dawley rat in the current study. Age of the rats was six week old. The gender was chosen to be female because, in contrast to males, females have less allergenic urine. Moreover, there is no difference in gender in relation to severity of UVR induced cataract [20].

All animals were kept and treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Ethical permission was obtained from the Uppsala Animal Experiments Ethics Committee, protocol number C 29/10. Rats are obtained from a commercial breeder (Taconic, Denmark).

A narrowband UVR source was chosen in order for the UVR to be spectrally well defined. Maximum sensitivity of the rat lens to UVR-B is around 300 nm. The dose 1 kJ/m^2 was chosen to be below the threshold dose [15]. The exposure time of 15 min was selected to induce maximum damage to the lens [21]. The dose of exposure to UVR-B and post-exposure time can vary depending on the experimental design.

Paired organ eye has its advantage in terms of statistics and ethics in use of animals in research. Unilateral exposure of the eye enables to use one side as exposed and another side as control in one animal thus it reduces number of animals by half compared to unpaired organ.

In this protocol we used anesthesia as a method for animal immobilization. However, other alternative, rat restrainer [13], which we designed in our laboratory, can be used for immobilization of unanaesthetized animals. Rats have to be conditioned to the rat restrainer prior the UV exposure. This device allows well controlled repeated exposures when anesthesia is not recommended due to its side effects. Here, we used the rat restrainer as a positioning and holding device for anesthetized animals.

The rat restrainer is made of wood and is available in several items in our laboratory. We clean our restraining device before each animal is positioned on it. Wood blocks, guttering and wood shelters are used as enrichment in the rat cages. Such enrichment is approved by Uppsala Animal Experiments Ethics Committee and Federation of European Laboratory Animal Science Associations (FELASA).

The lens has to be dissected in BSS in short period of time (5-10 min). This restriction allows the lens to stay clear and transparent.

qRT-PCR

The time of 30 min for keeping the lens in the RA1 lysis buffer is enough to disrupt the lens capsule and cortex. The lens nucleus remains hard within 30 min. The lens nucleus, or organelle-free zone is considered to be a transcription non-active part of the lens. Therefore, the nucleus is removed from the sample in order to increase the signal/noise mRNA expression ratio.

The DNA specific primer used for RNA purity (absence of DNA) control was selected arbitrarily to be p53-primer. Any generally occurring coded sequences of DNA of particular animal genome could be chosen. Both lenses were analyzed with PCR for 10 animals per post-exposure interval and for each lens, caspase-3 RNA content was determined in three independent measurements.

RT-PCR enables to measure the mRNA of interest. The reverse transcriptase converts mRNA into complementary DNA which is then amplified by PCR. Measurements were quantified using the standard curve obtained by amplifying serially diluted samples of the cDNA of interest. The advantages of applying a standard curve are that the standard curve provides a reliable way to calculate the uncertainty of the concentration, and that the standard curve provides quantitative measurements of the mRNA of interest. Alternatively, the relative content of the mRNA of

interest could be estimated by directly comparing the number of cycles required to obtain a standardized fluorescence signal, the Ct-method. The main drawback of the calibration curve is that it requires usage of more genomic products than the Ct method.

Immunohistochemical Staining

Immunohistochemistry was used to study spatial distribution of active caspase-3 in lens epithelial cells.

The eyes were frozen immediately to -70 °C to stop all ongoing biochemistry. The eyes were then stored at the same temperature for preservation.

Immunohistochemistry is associated with two general problems; the specificity of the primary antibody and non specific binding of the antibody. The antibody should be acquired from a well controlled source, thus guaranteeing the specificity. If possible, tissue containing the epitope should be stained as a positive control. In order to outrule non-specific staining, if possible, the epitope should be blocked before staining with the specific antibody, negative control. Further, non specific staining can be minimized by establishing the optimal antibody concentration and reaction time. By staining both tissue exposed to UVR and tissue not exposed to UVR, it is possible to establish the UVR induced epitope, here caspase-3. To facilitate the counting of cells expressing caspapse-3 signal the fluorescence microscope image was digitally recorded. In order to minimize variation of background noise, we used fixed settings for all microscope photographs.

The intensity of signal to background fluorescence varies on a continuous scale. Therefore, a threshold for significant staining has to be set. However, the average fluorescence may vary spatially within the section observed making it difficult to use an absolute threshold for significant staining. Therefore, usually the judgment of specific staining relies on an experienced observer and the absolute outcome of specific staining depends on the opinion of the experienced observer but will be consistent for that observer. For this reason, only one experienced observer was used. To improve signal caused by the experimental variable, exposure to UVR, as compared to noise, the photograph of each section was counted three times.

If possible, immunohistochemistry should be verified by western blot thus confirming the existence of epitopes with the expected molecular size.

Disclosures

No conflicts of interest declared.

Acknowledgements

This work was supported by the Karolinska Institutet KID-funding, Swedish Radiation Protection Authority, Karolinska Institutet Eye Research Foundation, Gun och Bertil Stohnes Stiftelse, St. Erik's Eye Hospital Research Foundation, Ögonfonden, Konung Gustav V:s och Drottning Viktorias Frimurarstiftelse.

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