Induction of Photolyase Activity in Wood Frog (Rana sylvatica) Embryos

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Received 1 June 2000; accepted 18 July 2000

ABSTRACT

Rising ultraviolet-B (UVB, 280–320 nm) radiation has been proposed as a factor which may explain nonnormal amphibian population declines. Accordingly research has been directed toward estimating the photolyase activity of several amphibian species in order to predict a species’ resilience to UV damage. Unfortunately, in spite of published research which demonstrated that the activity of one of the principal photorepair enzymes, photolyase, can be induced, these estimates did not address the potential for in vivo induction by environmental factors present in situ. We show here that wood frog (Rana sylvatica) embryos exposed to periods of ambient solar radiation (1) displayed significantly different photolyase activities from embryos exposed to equivalent periods of dark; and (2) were positively correlated with the UVB fluence received in vivo. Such results suggest that previous conclusions regarding the relationship between photorepair and population decline must be reevaluated. Estimating amphibian photorepair is a complicated process, and caution must be exercised when interpreting such data.

INTRODUCTION

Amphibian populations from around the world are experiencing decline and extinction related to anthropogenic pressures such as habitat destruction, degradation and fragmentation. Rising levels of ultraviolet-B (UVB) radiation have been suggested as a causal environmental factor when a decline occurs in the absence of visible habitat destruction. Consequently, research (1–4) has been completed to estimate the photoenzymatic repair ability of several amphibians in order to predict that species’ UVB damage resilience. The principal enzyme involved in photoenzymatic repair is cyclobutane pyrimidine dimer (CBPD) photolyase and its activity can be strongly regulated by environmental factors (5–10). For instance, UV radiation and temperature can induce alterations in photolyase activity (9,11,12). Indeed, published reports dating back to 1987 have documented induced changes in photolyase concentration of amphibian cells (13). Yet despite this and more recent reports (6), studies on the photoreactivating activity of other organisms from natural populations (1) have neither correlated, nor reported, the ambient levels of UVB radiation upon the time of collection, or mentioned that their research design assumes photolyase to be a constituent enzyme that is not altered by the conditions experienced prior to collection. Struck by the dichotomy in the literature, we decided to investigate whether ambient in vivo embryonic conditions could alter our in vitro estimation of photolyase activity. Here we report results which demonstrate that our in vitro measurement of wood frog (Rana sylvatica) embryo photolyase activity was enhanced by in vivo exposure to ambient UV radiation. Embryos exposed to 2, 4 and 10 h of ambient solar radiation displayed photolyase activities that were significantly different and positively correlated with the UVB fluence each embryo mass had received. Embryos exposed to equivalent dark periods were not significantly different from each other.

Since most other studies utilizing animal tissues from naturally occurring organisms have neither considered nor measured environmental variables that can induce photolyase activity (UV radiation, temperature and hydrogen peroxide [6,14]), our results have important ramifications for other in vitro estimates of amphibian photorepair.

In light of these findings of induction we investigated whether between-species differences could be detected. Using additional photolyase activity data for Ambystoma laterale (blue spotted salamander) Ambystoma maculatum (yellow spotted salamander) and Rana clamitans (green frog), embryos where the UVB fluence for each egg mass was known, we compared these photolyase activities to the hypothesized rate of photolyase activity induction for the wood frog. The results of this test indicate that there likely are intrinsic species-specific differences in photolyase activity. We expect (but have not tested here) that the rates of induction will differ between species and therefore information regarding factors that affect this rate is needed. Between-species comparisons for photolyase activity from the literature which could not account for the potential of ambient induction of photolyase remain a fundamental procedure in the estimation of the photoenzymatic repair abilities of any particular species, but future comparisons should now account for factors that can affect unknown rates of induction (15).
MATERIALS AND METHODS

Eggs from wood frogs were collected in April 1997, and were transported to Trent University where their Gosner stage was recorded (16). UVB readings for each collection day (from 8:00 to 10:00) were obtained from a BE-100 CID UV Measuring Instrument (Vital Technologies Corp., Bolton, Ontario, Canada) located at Trent University (44°21′N, 78°17′W, 100 m elevation). These values were erythemally weighted. Eggs were maintained at 4°C in the dark until experimental exposures occurred. Wood frog embryos were kept for 6 days at 4°C in the dark until the next "sunny" day. At the time of exposure wood frog embryos were approximately Gosner stage 11.

Induction experiments with wood frog embryos were conducted on 29 April 1997 from 10:00 A.M. until 8:00 P.M. Three groups of 50.0 mg of eggs, with jelly intact, were isolated in 500 mL plastic containers containing purified river water. These egg masses were then placed either in the dark at 5°C, or exposed to natural sunlight while maintained at 5°C. Maintaining eggs in both dark and light allowed us to test whether the photolyase activity was intrinsic to the embryo, or whether exposure to ambient light induced photolyase activity. By maintaining each treatment at a constant temperature we intentionally prevented the comparison of any temperature-driven changes in photolyase activity, and ensured that eggs did not age during treatments. Wood frog egg egg masses were removed from dark or ambient light conditions after 2 h (12:20 P.M.), 4 h (2:20 P.M.) and 10 h (8:20 P.M.) of exposure and were immediately frozen at −80°C until protein purification took place.

In an attempt to resolve the difference between the UV-induced photolyase activity levels from photolyase activity levels that are endogenous to the tissue in question, blue spotted salamander, yellow spotted salamander and green frog eggs were exposed to ambient UV light on 26 May 1997 and immediately frozen at −80°C in 50 mL containers. These values were compared to the wood frog induction data.

We created cell-free extracts from amphibian embryos following previously described methods (4,17,18) which allow for optimum isolation of proteins in the photolyase size range. Transformation efficiencies were calculated as has been previously described (17). Variations from these reports are described elsewhere (M. Alex Smith et al., personal communication) and the methods are only outlined here.

Briefly, 50 mg of egg replicate were frozen at −80°C. About 10 mL of 2% t-cysteine was then added to each 50 mL container and embryos were shaken at room temperature for between 1 and 3 h. Embryos were washed two times in cold phosphate buffered saline (8 g NaCl, 0.2 g Na2HPO4, 0.24 g KH2PO4 in 1 L of distilled H2O, pH 7.4) and were then centrifuged at 3000 rpm for 15 min in a KompSpin 21.50 rotor in a Beckman J2MC high speed centrifuge. The supernatant was removed and the packed cell volume (PCV) was estimated. Four times PCV of buffer I (10 mM tris pH 7.4, 1 mM MgCl2, 0.5 mM EDTA, 2 mM DTT and 50% glycerol) was added and the mixture was incubated on ice for 20 min. Embryos were lysed and the resulting solution was then placed in a flask, where four times PCV of buffer II (50 mM tris, 10 mM MgCl2, 2 mM DTT, 25% sucrose wt/vol and 50% glycerol vol/vol) was added. One times the PCV of saturated deoxycyromonucleic acid (DNase)-free ammonium sulfate was added. The solution was centrifuged at 20000 rpm for 5 h at 4°C. The supernatant was decanted and 0.33 g of Sigma-Aldrich Canada (Oakville, Ontario, Canada) Dnase-free ammonium sulfate per 1.0 mL of supernatant was added. While stirring, 100 mL 1 N NaOH per 10 g of ammonium sulfate was added. This solution was then centrifuged at 10000 rpm for 30 min at 4°C. The resulting pellet was resuspended in 1/40th the measured supernatant volume of storage/dialysis buffer (25 mM N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid, 100 mM KCl, 12 mM MgCl2, 0.5 mM EDTA, 2 mM DTT and 16% glycerol vol/vol) and dialyzed overnight in tubing of molecular weight 14000.

The dialysate was centrifuged for 10 min at 10000 rpm at 4°C. The supernatant was loaded onto a Sigma chromatographic column with Blue Sepharose CL-6B, equilibrated with 0.1 M KCl and buffer B (50 mM tris-HCl pH 7.4, 1 mM EDTA, 10 mM 2-mercaptoethanol and 20% vol/vol glycerol), and washed with 0.1 M KCl and buffer B with 0.6 g/L adenosine triphosphate. The protein rich fractions were collected, pooled and dialyzed for 4–6 h against photolyase storage buffer (50 mM tris pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM DTT and 50% glycerol). The resulting dialysate was collected, and frozen at −20°C until used in the bacterial transformation assay.

The transformation assay and dimer repair were based upon previously derived calculations (17). Briefly, dimer repair was calculated as the difference between the relative transformation efficiency for the irradiated plasmid without photorepair and the irradiated plasmid with photorepair.

\[
\text{mean dimer number per plasmid molecule} = \left( -\ln\left( -\text{PRL}/z \right) \right) - \left( -\ln\left( +\text{PRL}/z \right) \right).
\]

These numbers were then standardized for time of exposure to photoreactivating light (PRL), and the ratio of protein to DNA.

RESULTS

The dimer removal characteristics of the wood frog embryos maintained at 4°C in the dark for 2, 4 or 10 h did not significantly differ from each other \((P = 0.483, \text{df} = 2, F = 0.7662)\). In contrast, the wood frog embryos exposed to 2, 4 or 10 h of ambient sunlight differed from each other \((P = 0.001, \text{df} = 1, F = 9.603)\) (Table 1). Photolyase activities were also compared to the total UVB fluence that each treatment had received (Table 1). A correlation analysis of wood frog photolyase activity and total UVB fluence produced a significant \(R\) value (0.99862, \(P < 0.05\)) (Table 1).

The photolyase activity of the salamander and green frog embryos was compared with the wood frog embryos exposed to 2, 4 and 10 h of ambient light (Table 2). A linear fit of wood frog photolyase activity data (where the photolyase activity has been transformed according to \(\log(pa + 1)\) where \(pa = \text{photolyase activity}\), and the 95% confidence intervals of that linear fit suggested that the salamanders were not significantly different from the wood frog pattern. However, the green frog photolyase activity likely was different from the wood frog pattern.

DISCUSSION

We have shown here significant differences between photolyase activity from ambient UV treatments of different du-
We thank Dr. Wayne Evans and Dr. Michael the wood frog rate activity induction and are therefore not significantly different from the confidence interval of the projected rate of wood frog photolyase activities fall within the 95% confidence interval of the projected rate of wood frog photolyase activity induction. The yellow spotted salamander and the blue spotted salamander photolyase activities fall within the 95% confidence interval of the projected rate of wood frog photolyase activity induction and are therefore not significantly different from the wood frog rate of oxidative reactions may have occurred in the plastic containers holding the wood frog embryos while they were exposed to ambient UV radiation. This possibility is tempered by the fact that the water surrounding the embryos was purified river water (Otonabee River) low in DOC and therefore likely not to have had high oxidative production characteristics (20). However, these types of confounding environmental factor represent real world indirect UV stress to the amphibian embryo. In the natural environment, many of the intracellular effects of UV on the developing embryo are likely oxidative and are not simply due to the accumulation of CBPD in DNA.

Our experimental exposure period was for roughly one day-length and therefore this data does not address what happens to photolyase activity levels during the night. However, the embryos maintained in the dark had photolyase activities that were not significantly different from zero (data not shown) and we speculate that levels would decline overnight.

It is unknown whether rates of induction differ between species and therefore between-species comparisons of photolyase activities are important. Such comparisons must be expressed in relation to the environmental factors that are known to regulate photolyase activity and variables that could alter the amount of UV radiation received by an embryo. Amongst others already mentioned, these variables include the UVB absorbance of the embryo jelly and the UVB fluence on a particular day. This fluence is in turn determined by a myriad of factors including latitude, elevation, cloud cover, depth of oviposition and DOC concentration of the water. We suggest that future between-species analyses of photolyase activity property should accommodate changes in both the level of activity and the potential induced rate of change.

The photolyase activity of the wood frog was altered, either directly or indirectly, by exposure to ambient levels of late spring sunlight in south-central Ontario, thus providing evidence of a photoenzymatic reaction that was induced in response to the existing environmental conditions in tissue from the wild. These results should help foster acceptance that when the defining protocol quantifies photolyase activity via an in vitro assay it is crucial to remember that the in vivo conditions may significantly affect that estimation.

Acknowledgements.—We thank Dr. Wayne Evans and Dr. Michael Bidocha at Trent University for providing UVB data and lab space with discussions, respectively. Dr. Erica Nol provided an excellent sounding board for comparison ideas. Dr. Aziz Sancar provided greatly appreciated materials and advice. This research was supported by grants from the Canadian Wildlife Service to M.B., and the Natural Science and Engineering Research Council of Canada to C.K.B.

REFERENCES


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Photochemistry and Photobiology, 2000, 71(4) 577


