J Endocrinol Reprod 9 (1)(2) : 11-18 (2005)
JER 54

THE BIOLUMINESCENT DINOFLAGELLATE *LINGULODINIUM POLYEDRUM* AS A TEST ORGANISM FOR PRO-ANDANTIOXIDANT EFFECTS

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SUMMARY

In the bioluminescent, marine dinoflagellate *Lingulodinium polyedrum* [syn. *Gonyaulax polyedra*], light emission is sensitive to oxidative stress in a dual way: (a) The nocturnal glow maximum, which depends on the melatonin metabolite 5-methoxytryptamine (5-MT), is decreased by 5-MT depletion following oxidation of its precursor melatonin, which easily undergoes radical reactions; attenuations of the glow maximum are typical for sublethal oxidative stress. (b) Since luciferin availability is triggered by proton translocation to the scintillons (bioluminescent microsources which form cytoplasmic intrusions into acidic vacuoles), lethal oxidative stress causes strong rises in bioluminescence ("dying peaks") as a consequence of cytoplasmic acidification, which results from impaired proton pumping into vacuoles under ATP deficiency and/or proton leakage through damaged membranes. Sublethal concentrations of paraquat or buthionine sulfoximine decrease the circadian glow peak, in a dose-dependent fashion. These effects can be reverted by exogenous melatonin, although this indoleamine itself does not stimulate but rather slightly diminishes light emission. After a lethal dose of hydrogen peroxide, a dying peak appears, which can be rapidly terminated by adding catalase to the medium. Sublethal and lethal effects of the endogenous oxidotoxin 3-hydroxykynurenine can be distinguished on the basis of the differently timed glow and dying peaks.

Key words: Bioluminescence; Buthionine sulfoximine; 3-Hydroxykynurenine; *Lingulodinium*; Melatonin; Oxidative stress; Paraquat.

INTRODUCTION

Among eukaryotic unicells, a particularly large body of data exists on oxidative stress and antioxidative protection in the marine bioluminescent dinoflagellate *Lingulodinium polyedrum* [syn. *Gonyaulax polyedra*]: All relevant antioxidant enzymes, oxidative catabolism, many radical scavengers – including melatonin which is present in high concentrations – and oxidative damage have been studied in much detail, usually on a chronobiological basis (1, 2). *L. polyedrum*, representing a chronobiological model organism, is also known for its circadian rhythmicity of bioluminescence, and the mechanisms triggering light emission have been identified. In brief, the luminescent system is composed of a host of luminescent microsources ("scintillons"), which form cytoplasmic intrusions into a system of acidic vacuoles. It is a remarkable feature of this system that conducted proton action potentials are propagating at the intracellular vacuolar membrane (1); as soon as the action

potential is arriving at a scintillon, protons entering its interior associate with a luciferin-binding protein, which undergoes a conformational change, thereby releasing luciferin; this mechanism makes the substrate available to the luciferase and light is emitted (3). This explains why luminescence can be experimentally stimulated by protonophores (4, 5). Two other features of the system are relevant for understanding changes in light emission. ATP is not required for the luciferase reaction in this organism, but rather for a V-type proton ATPase pumping protons into the interior of the vacuole system (6). Moreover, bioluminescence is strongly stimulated by a melatonin metabolite, 5-methoxytryptamine (5, 7, 8). The circadian glow maximum at the end of subjective night depends on the presence of 5-MT, which, in this circadian phase, attains its maximum due to high rates of melatonin deacetylation (6, 9, 10).

As a consequence of these properties of the luminescent system, damage to cells or interference with 5-MT levels leads to the following changes in light emission. Any treatment causing cytoplasmic acidification, either due to proton leakage through a damaged vacuolar membrane or ATP deficiency impairing the action of the V-type H^+-ATPase, will strongly stimulate bioluminescence. This is usually seen after any kind of lethal stress and the pattern of light emission has been called a dying peak, which, in a well designed experiment, can be easily distinguished from physiological maxima of light emission (11). On the other hand, a decrease in 5-MT concentration attenuates the glow peak, an effect which has been demonstrated by blocking the biosynthetic pathway by various tryptophan hydroxylase inhibitors and carefully investigating restoration of this peak by 5-MT precursors including melatonin (9, 10). Decrease in light emission can also be expected under conditions of moderate oxidative stress not yet leading to a dying peak, since various sublethal treatments with oxidants and oxidotoxins cause strong declines in 5-MT concentration, in correspondence to the disappearance of its precursor melatonin (12, 13). This effect is well explicable on the basis of melatonin's properties as a free-radical scavenger (5, 14-18). Although the structural analog 5-MT can also be destroyed by oxidants, its radical-scavenging capacity is – surprisingly – considerably smaller than that of melatonin (15, 19). In the present investigation, we shall demonstrate the suitability of *L. polyedrum* for distinguishing sublethal and lethal forms of oxidative stress by measuring bioluminescence, which may be regarded as a convenient screening procedure for substances with unknown pro- or antioxidant properties.

MATERIALS AND METHODS

L. polyedrum was grown as previously described in a variant f/2 seawater medium (20), under a light/dark cycle of 12:12 h, at 20°C. Chemicals were obtained from Sigma, Deisenhofen/Taufkirchen, Germany. Melatonin was predissolved in a very small volume of DMSO, as previously described (7), and further diluted with medium; the other compounds were directly dissolved in the medium. Bioluminescence was measured in a modified, temperature-controlled scintillation spectrometer (Packard model 3330), at 20°C, operated at settings optimized for detecting light emitted from dinoflagellates (21). In long-term experiments (cf. Figs. 1 and 3), medians of 10-min intervals were calculated. Cells were transferred to darkness and measurements started at ZT 6 (Zeitgeber time 6 hours), in the case of the 3-hydroxykynurenine experiments at ZT 12. This different timing was chosen for optimally distinguishing between the different forms of bioluminescence peaks. Melatonin was given at ZT 6, H_2O_2, paraquat and buthionine sulfoximine at ZT 7, 3-hydroxykynurenine at ZT 12 (for dosage see figures). Catalase (8,000 U/10 ml sample) was added at times indicated in Fig. 1.

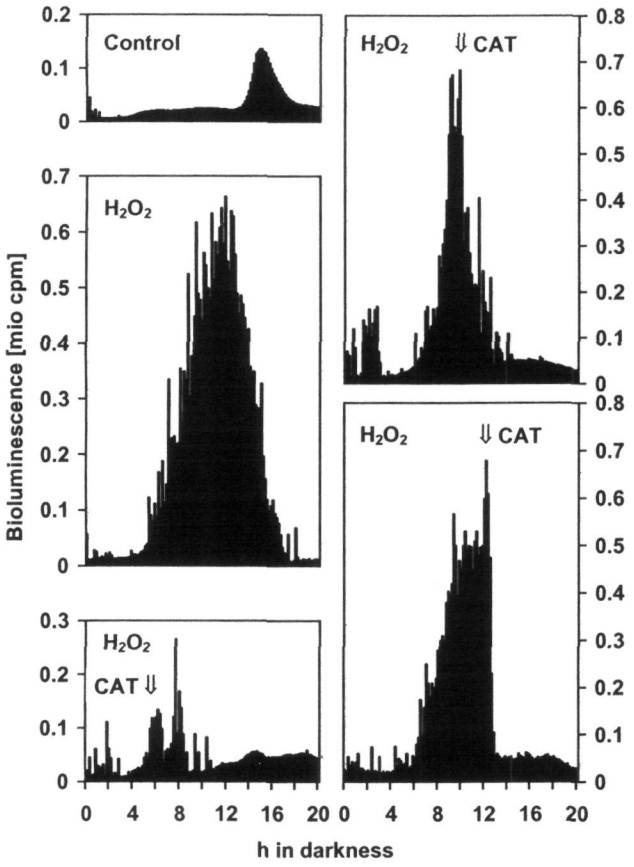

Fig. 1. Effects of lethal oxidative stress by H_2O_2 on bioluminescence of *L.polyedrum* and termination of the dying peak by catalase. 80 µm H_2O_2 was added at 2 – 7 (1h after after start of experiment), catalase (800 U/ml) at times indicated by arrows. The control exhibits a circadian maximum of spontaneous glow activity after 14.5 h of the experiment.

RESULTS

Exposure of cells to 80 µM H_2O_2 caused severe damage to the cells detectable by a dying peak, which was clearly distinguished from the circadian glow maximum, with regard to both timing and height (Fig. 1). Such a type of experiment was only successful at low cell density, since a higher number of dinoflagellates is capable of efficiently destroying H_2O_2 up to 300 µM (22). For this reason, absolute luminescence values remained relatively low in the runs shown in Fig. 1. Addition of catalase to the medium strongly counteracted the dying peak. When the enzyme given at the beginning of this peak (after 5 h of exposure), the rise in luminescence was largely prevented, although the somewhat noisy pattern observed during the first hours after addition of catalase indicated that cells were still slightly irritated by after-effects of the oxidant (Fig. 1). In this experiment, most of the cells were rescued from lethal oxidative damage (about 94% moving cells of controls). When catalase was given later, after 8 or 11 h of exposure to H_2O_2, the dying peak of luminescence was rapidly terminated (Fig.1). Again, the percentage of surviving cells approximately corresponded to the section of the dying peak which was suppressed by catalase (about 72 or 20% of moving cells compared to controls).

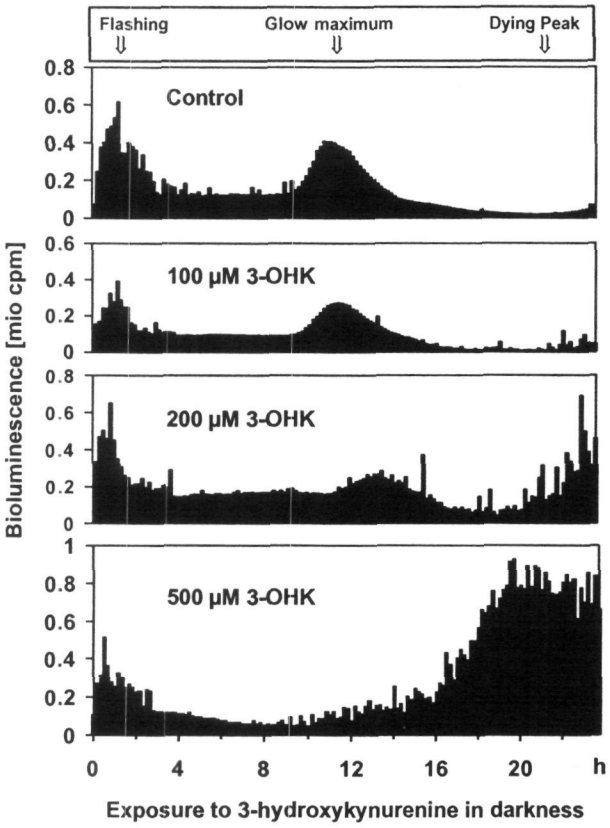

Fig. 2. Effects of various oxidotoxins and melatonin on the size of the circadian glow maximum. PQ = Paraquat ; BSO = buthionine sulfoximine; MEL = melatonin; 3 - OHK = 3 - hydroxykynurenine

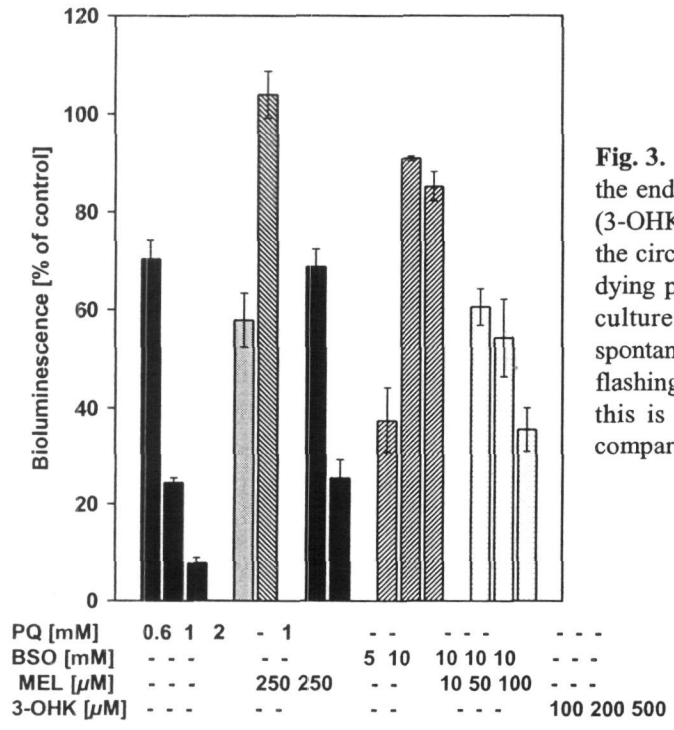

Fig. 3. Sublethal and lethal oxidative stress by the endogenous oxidotoxin 3-hydroxykynurenine (3-OHK) : Concentration–dependent attentation of the circadian glow maximum and appearance of a dying peak at higher doses. In this experiment, a culture was selected showing two modes of spontaneous luminescence, flashing and glow; since flashing is particularly exhibited by colliding cells, this is especially seen at higher cell densities, compared to runs of Fig. 1.

In Figure. 2, several treatments are summarized in which moderate forms of oxidative stress were applied. Accordingly, a dying peak either did not appear at all or later than the circadian glow maximum. Paraquat, an electron interceptor generating both H_2O_2 and $\cdot O_2^-$, was tolerated by the cells up to concentration of 1 mM for several days and 2 mM for more than a day. Correspondingly, a dose-dependent decrease of the circadian glow maximum was observed (Fig. 2). Melatonin, in this case at a dose of 0.25 mM, fully restored the glow peak, although this indoleamine alone did not stimulate bioluminescence *per se* (Fig. 2); rises in bioluminescence upon melatonin administration are only observed under conditions leading to enhanced formation of 5-MT via induction of aryl acylamidase (23). Buthionine sulfoximine, causing oxidative stress by interfering with glutathione metabolism, was tolerated by *L. polyedrum*, at least, up to 20 mM, while 30 mM resulted in long-term toxicity (24). Again, the glow peak was suppressed in a concentration-dependent manner. After exposure to 10 mM buthionine sulfoximine, the circadian maximum of luminescence was restored by much lower concentrations of melatonin (50 or 100 µM; cf. Fig. 2).

We also tested the effects of 3-hydroxykynurenine, an endogenous oxidotoxin (25, 26), which is formed and metabolized by *L. polyedrum* (27). When administered in supraphysiological amounts, the substance caused a concentration-dependent attenuation of the glow peak (Figs. 2 and 3). Higher concentrations turned out to be lethal after prolonged exposure and resulted in an additional dying peak, which appeared later than the glow maximum. Therefore, this metabolite allows to follow sublethal and subsequent lethal effects in a single run. Examples for the time- and dosage-dependent changes in the circadian glow peak and the appearance of the dying peak are shown in Figure. 3.

DISCUSSION

The results presented demonstrate the suitability of *L. polyedrum* for studying oxidative stress on the basis of luminescence measurements. This method offers a convenient tool for automatically recording damage to cells, already in cases of sublethal toxicity when no impairment of vitality is yet observed and oxidative damage would otherwise only be detected by time-consuming measurements of oxidized biomolecules. Moreover, the dinoflagellate allows a clear distinction between lethal and sublethal oxidative stress, as indicated by differently timed changes in light emission.

Moreover, this system can be used for studying the efficacy of antioxidants, as exemplarily shown by the catalase experiments. Similar data were obtained using melatonin as an antioxidant after challenging cells by H_2O_2; at high, but physiologically possible concentrations, melatonin was capable of suppressing the dying peak (11), a result, which may be biologically relevant because *L. polyedrum* can be exposed to oxidative stress in conjunction with decreased temperatures not allowing enzyme inductions, and since this organism, in which tryptophan availability is not a limiting factor, can transiently augment its melatonin content up to millimolar levels under these conditions (28, 29).

Melatonin's capability of antagonizing the effects by paraquat and buthionine sulfoximine (Fig. 2) have, however, to be interpreted under two different aspects. On the one hand, melatonin should decrease the sublethal oxidative stress exerted by these oxidotoxins, but on the other hand, it would replenish the source of 5-MT formation which is depleted by the oxidants (10, 12). Moreover, we demonstrate that melatonin, under conditions applied, does not stimulate bioluminescence (Fig. 2), so that the restoration of a fully expressed glow peak can be explained as the superposition of melatonin's dual roles, as an antioxidant and a precursor of 5-MT, the inducer of light emission required at the end of subjective night. The moderate decrease of the glow peak by melatonin should not be misinterpreted in terms of redox metabolism, but rather reflects competition with its structural analog 5-MT for binding sites regulating proton transfer. However, at the end of subjective night, 5-MT

formation from exogenous melatonin should take place at a sufficiently high rate because aryl acylamidase attains its circadian maximum in this phase (10, 30).

The suitability of bioluminescence measurements in *L. polyedrum* for screening redox-active compounds with regard to prooxidant and other toxicological effects is strongly supported by the effects of 3-hydroxykynurenine as well as by results on the melatonin-derived kynuramines AFMK and AMK and on the melatonin analog indole-3-propionic acid. The absence of prooxidant or other toxic side effects of AFMK and AMK became obvious by unchanged luminescence patterns (31). The redox-dependent formation of an ultratoxin from the radical scavenger indole-3-propionic acid was discovered, after we had been alerted by changes in bioluminescence (32).

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