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Use of Fluorogenic Probes To Differentiate between Hydrophilic and Lipophilic Antioxidant Activity in a Fish Cell Line

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ABSTRACT: In finfish aquaculture, dietary antioxidants have been shown to improve indicators of general fish health and to inhibit the oxidative deterioration of polyunsaturated fatty acids. To facilitate the characterization of novel antioxidants or antioxidant mixtures, we developed assays for antioxidant activity in a fish cell line. We used 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) to determine the protective effects of a panel of representative antioxidant compounds against the formation of reactive oxygen species (ROS) under conditions that promote oxidative stress, whereas protective effects against lipid peroxidation were measured using the thiobarbituric acid reactive substances (TBARS) assay and a novel implementation of 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid (C_{11}-BODIPY$^{581/591}$). We found that the highly hydrophilic antioxidant, sodium ascorbate, inhibited H_2DCFDA oxidation but had no effect on lipid peroxidation, whereas the highly hydrophobic antioxidant, α-tocopherol, potently inhibited lipid peroxidation but did not prevent H_2DCFDA oxidation. The data suggest that a single assay is not sufficient for estimating antioxidant activity in cultured fish cells.

KEYWORDS: *finfish, antioxidant activity, hydrophilic, lipophilic*

INTRODUCTION

High levels of free radicals and reactive oxygen species (ROS) in cells and tissues can result in oxidative damage to proteins, nucleic acids, membrane phospholipids, and other cellular components. Antioxidants obtained from the diet play an important role in protecting against such damage by scavenging free radicals or neutralizing ROS, thereby complementing endogenous antioxidant systems.

The inclusion of antioxidants in animal feeds is commonplace and is normally intended to serve one of two purposes. First, antioxidants prevent the oxidative deterioration of lipids (often referred to as rancidity) in manufactured feeds, particularly the polyunsaturated fatty acids (PUFA). Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (*t*-BHQ) are commonly used for this purpose.[1] Second, supplementation of antioxidants with known biological activity prevents deficiency syndromes and promotes the general health of animals. In the case of finfish aquaculture, dietary antioxidants have been shown to improve indicators of general health[2,3] and inhibit lipid peroxidation in vivo.[4−7] Furthermore, carry-over of lipid-soluble antioxidants to harvested fish fillets helps to prevent lipid peroxidation during storage.[8,9]

The steady increase in demand for fish oil for use in aquaculture feeds[10] has prompted intensive research into alternative lipid sources such as plants, terrestrial animals, or algae. Because of differing PUFA profiles and natural antioxidant content, the requirement for supplementary antioxidants is likely to change according to the source of the lipids in manufactured feeds. Large-scale diet trials involving live fish, although important for investigating dietary requirements for different species, are expensive and time-consuming. We therefore identify a need for a cell-based experimental model suitable for estimating the potential protective effects of antioxidants against cellular oxidative stress and lipid oxidation in fish. Such a model would aid in the preliminary characterization of new antioxidants, combinations of antioxidants, or natural extracts, prior to undertaking feeding trials in live fish.

A range of tests have been developed that rely on the use of redox-sensitive fluorescent dyes to estimate ROS content in living cells (see ref 11 for a comprehensive review). To facilitate the rapid screening and characterization of antioxidants with potential as finfish aquaculture feed additives, we have developed assays that use redox-sensitive fluorescent compounds to measure the ability of antioxidants to prevent ROS formation and lipid peroxidation in a fish cell line, EPC. Decreases in cellular ROS in response to antioxidants were measured using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), which is used widely as a nonspecific indicator of ROS levels in cultured mammalian cells (see, e.g., ref 12). To detect the protective effects of antioxidants against lipid peroxidation in EPC cells, we used the redox-sensitive fluorescent fatty acid 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid (C_{11}-BODIPY$^{581/591}$), which has shown promise as an indicator of lipid peroxidation in cultured mammalian cells.[13−15] We compared the results of the C_{11}-BODIPY$^{581/591}$ assay with the more conventional TBARS assay[16,17] to investigate the suitability of C_{11}-BODIPY$^{581/591}$ as an indicator of lipid peroxidation in cultured fish cells.

Using a panel of representative antioxidant compounds, we show that the accurate determination of the antioxidant activity of a given compound in cultured fish cells depends on the

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aqueous solubility of both the antioxidant and the fluorescent indicator.

MATERIALS AND METHODS

Cell Culture. The EPC cells used in this study were obtained from the Australian Animal Health Laboratory, CSIRO Livestock Industries, Geelong, Australia. The cells were maintained in Liebovitz' L-15 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), and 20 mM HEPES buffer (pH 7.4) (Sigma), in filter-capped 75 cm^2 flasks (Nunc) at 25 °C under a standard atmosphere. The cells were passaged every 3−7 days.

The EPC cell line was first reported as an isolate from a skin lesion on carp (*Cyprinus carpio*),[18] but the stocks held by the American Type Culture Collection (ATCC accession CRL-2872) and many laboratories around the world are now known to have originated from fathead minnow (*Pimephales promelas*).[19] To confirm the species identity of the lineage used in the current study, a fragment of the gene for mitochondrial cytochrome *c* oxidase subunit I (coxI) was amplified by PCR as previously described,[20] sequenced and checked against public databases using BLAST.[21]

Determination of Cellular ROS Using the DCFH Assay. EPC cells were seeded into 96-well black opaque plates with optically clear well bases (Corning Life Sciences) at a density of 3×10^4 cells per well and incubated for 24 h at 25 °C. A dilution series of each antioxidant was prepared in sterile tubes using a suitable solvent. Complete growth medium containing 10% (v/v) FBS was added and the solution mixed thoroughly before an appropriate volume was transferred to the wells of cell culture plates. The final concentration of solvent (usually ethanol) in the growth medium did not exceed 0.1% (v/v). Solvent controls and cell-free controls were included in each experiment. Cells were incubated in the presence or absence of antioxidants for 24 h at 25 °C, washed twice with phosphate-buffered saline, pH 7.4 (Oxoid), containing 1 mM $CaCl_2$ (Ajax Finechem) and 1 mM $MgCl_2$ (Ajax Finechem) (hereafter designated PBS^+), and loaded with 10 μM H_2DCFDA (Invitrogen Molecular Probes) in PBS^+ at 25 °C for 30 min in darkness. The dye solution was removed, and the wells were washed twice with PBS^+. Cells were incubated in serum-free L-15 medium (supplemented as described above) or in L-15 containing 10% (v/v) FBS (supplemented as above). Additional controls consisted of cells treated with solvent only (with and without H_2DCFDA), cells treated with the highest concentration of antioxidant without H_2DCFDA, and cell-free wells (with and without H_2DCFDA). After 4 h at 25 °C, fluorescence was measured from the underside of the wells using a plate spectrofluorometer (BMG Fluostar) fitted with 485 nm excitation and 520 nm emission filters. Data were analyzed using Prism 5 (GraphPad). Significant differences compared to solvent controls (set at $p < 0.05$) were determined using one-way ANOVA followed by Dunnet's post hoc test.

Estimation of Lipid Peroxidation Using the TBARS Assay. EPC cells were seeded into 6-well cell culture plates (Corning Life Sciences) at a density of 5×10^5 cells per well and grown for 24 h at 25 °C. A dilution series of each antioxidant was prepared in sterile tubes using a suitable solvent. Complete growth medium containing 10% (v/v) FBS was added and the solution mixed thoroughly before transferring an appropriate volume to the wells of cell culture plates. The final concentration of solvent (usually ethanol) in the growth medium did not exceed 0.1% (v/v). Solvent controls were included in each experiment. Quantification of thiobarbituric acid-reactive substances (TBARS) in cultured cells was carried out using a procedure based on previously described methods.[20,21] Cells were incubated in the presence of antioxidants for 24 h at 25 °C and washed twice briefly with PBS^+ before 1 h of incubation at 25 °C in PBS^+ containing 0.5 mM cumene hydroperoxide (Sigma) plus 1 μM hemin (Sigma). After two washings with PBS^+, the cells were lysed by incubation for 5 min in 1% (w/v) SDS, and the resulting lysates were transferred to 1.5 mL centrifuge tubes. To determine interassay reproducibility, a standard curve was prepared by serially diluting malonaldeyde bis(dimethyl acetal) (Sigma) in an aqueous solution of 1% (w/v) SDS. An equal volume of 1 M HCl containing 0.375% (w/v) thiobarbituric acid (TBA) (Sigma), 0.005% (w/v) butylated hydroxytoluene (Sigma), and 15% (w/v) trichloroacetic acid (Sigma) was added to each tube, mixed by vortexing, and incubated for 2 h at 75 °C. Insoluble material was removed by centrifugation at 16000*g* for 10 min, and the supernatants were transferred to black opaque 96-well plates in triplicate for fluorescence determination. TBARS were detected by determining fluorescence using 540 nm excitation and 590 nm emission filters in a plate spectrofluorometer (BMG Fluostar). Data were analyzed using Prism 5 (GraphPad). Significant differences compared to solvent controls (set at $p < 0.05$) were determined using one-way ANOVA followed by Dunnet's post hoc test.

Estimation of Lipid Peroxidation Using C_{11}-BODIPY$^{581/591}$. Cells were seeded into wells of black opaque 96-well plates with optically clear bases (Corning) at a density of 1×10^4 cells per well and grown for 24 h. After incubation for a further 24 h in the presence or absence of various antioxidant compounds as described above (in the paragraph on H_2DCFDA assay), cells were washed twice with PBS^+ and incubated with 2 μM C_{11}-BODIPY$^{581/591}$ (Invitrogen) in PBS^+. After 30 min of loading, unincorporated dye was removed by three washings with PBS^+. After initiation of oxidation of the probe by incubating cells in PBS^+ containing 100 nM hemin and 0.5 mM cumene hydroperoxide (or an equal concentration of other peroxides as shown), oxidation of cell-associated C_{11}-BODIPY$^{581/591}$ was measured by high-throughput microscopy using the InCell Analyzer 1000 (GE Life Sciences). Images were captured prior to treatment (cells were incubated in PBS^+ during imaging) and after 30 min in the presence of cumene hydroperoxide plus hemin. Four fields per well were captured using two filter sets: 480 nm excitation/535 nm emission for the oxidized form of C_{11}-BODIPY$^{581/591}$ and 565 nm excitation/620 nm emission for the reduced form. Exposure times for each channel were kept constant across all wells. Pixel intensities were measured using ImageJ,[22] omitting background fluorescence and saturated pixels by setting thresholds for the analysis (which were applied identically across all images from a given experiment). Pixel intensities for each image were expressed as integrated density (the product of area and mean pixel intensity). The degree of inhibition of C_{11}-BODIPY$^{581/591}$ oxidation by antioxidant pretreatment was expressed as a percentage of the signal in the green channel in solvent controls. A minimum of three independent experiments were performed, each with wells in triplicate. Data were analyzed using Excel (Microsoft) and Prism 5 (GraphPad). Significant differences compared to solvent controls (set at $p < 0.05$) were determined using one-way ANOVA followed by Dunnet's post hoc test.

Cell Viability Determination. Cell viability was determined using a Neutral Red uptake assay, essentially as previously described[23] with slight modifications. Briefly, a stock solution of 1 mg/mL Neutral Red (Sigma) was prepared in ethanol and diluted in standard growth medium (Liebovitz' L-15 supplemented as above) to a concentration of 10 μg/mL. Cells were incubated in this medium for 2 h at 25 °C and then washed for 1 min with 10% (w/v) $CaCl_2$ and 4% (v/v) formaldehyde. Cell-associated Neutral Red was solubilized in an aqueous solution of 50% (v/v) ethanol and 10% (v/v) acetic acid for 5 min on an orbital shaker. Absorbance at 550 nm was read using a plate spectrofluorometer (BMG Fluostar).

RESULTS

Detection of Antioxidant Activity of Hydrophilic Compounds in EPC Cells using the DCFH Assay. We investigated the capacity of various antioxidants to inhibit DCFH oxidation in EPC cells subjected to conditions that promote oxidative stress. We saw a time-dependent increase in fluorescence in H_2DCFDA-loaded EPC cells incubated in reduced-serum media, which was inversely correlated with FBS concentration (Figure 1). This indicated that under serum deprivation conditions, reactive species capable of oxidizing DCFH were generated in EPC cells. We therefore investigated

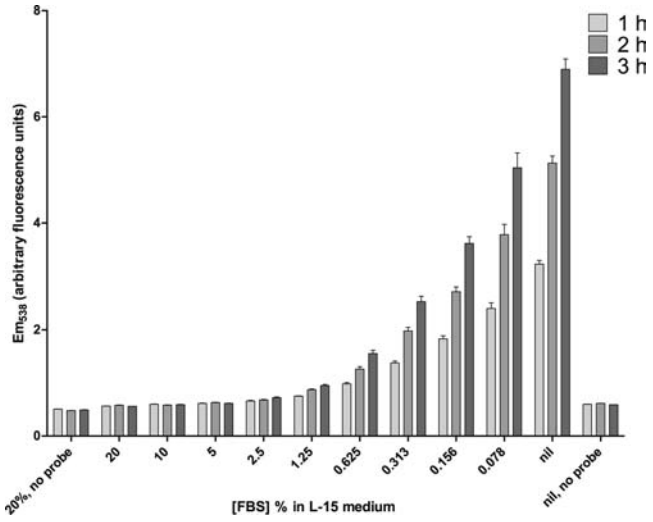

Figure 1. ROS production in serum-deprived EPC cells as measured using the DCFH assay. Cells were loaded with 10 μM H_2DCFDA for 30 min and washed thoroughly before incubation in L-15 medium containing various concentrations of FBS. Fluorescence attributable to oxidized DCFH was measured from the underside of the plate after the times shown. Data are the means ± SE from three replicate wells.

whether prior incubation of the cells with antioxidants could inhibit DCFH oxidation under such conditions. The antioxidants used in this study (sodium ascorbate, α-tocopherol, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and *tert*-butyl hydroquinone (*t*-BHQ)) were chosen as representatives of different classes of antioxidant compounds. According to the Neutral Red uptake assay, performed on the same cells following the DCFH assay, cell viability was not significantly affected by preincubation with any of the antioxidants tested (Figure 2, white bars), showing that any observed reduction in fluorescence signal in response to increasing antioxidant concentration was not due to reduced cell numbers occurring as a result of cytotoxicity. We found that sodium ascorbate, Trolox, and, to a lesser extent, *t*-BHQ significantly inhibited DCFH oxidation in serum-deprived EPC cells in a concentration-dependent manner (Figure 2, black bars). Conversely, α-tocopherol did not inhibit DCFH oxidation, even at extremely high concentrations, prompting us to investigate other assays for detecting the activity of this and other nonpolar antioxidants in EPC cells.

α-Tocopherol Prevents TBARS Formation in Peroxide-Challenged EPC Cells. To assess the effects of antioxidants on lipid peroxidation in EPC cells, we incubated cells for 24 h in the presence of various antioxidant concentrations, subjected the cells to conditions that promote lipid peroxidation, and used the TBARS assay to detect secondary lipid peroxidation products. We utilized the cumene hydroperoxide/hemin system to initiate lipid peroxidation to replicate the conditions others have utilized to promote the oxidation of C_{11}-BODIPY$^{581/591}$ dye in cultured cells.[13−15] We found that TBARS formation in EPC cells treated with cumene hydroperoxide and hemin was potently inhibited by pretreatment with α-tocopherol, whereas Trolox and *t*-BHQ did not significantly inhibit TBARS formation under the conditions tested (Figure 3).

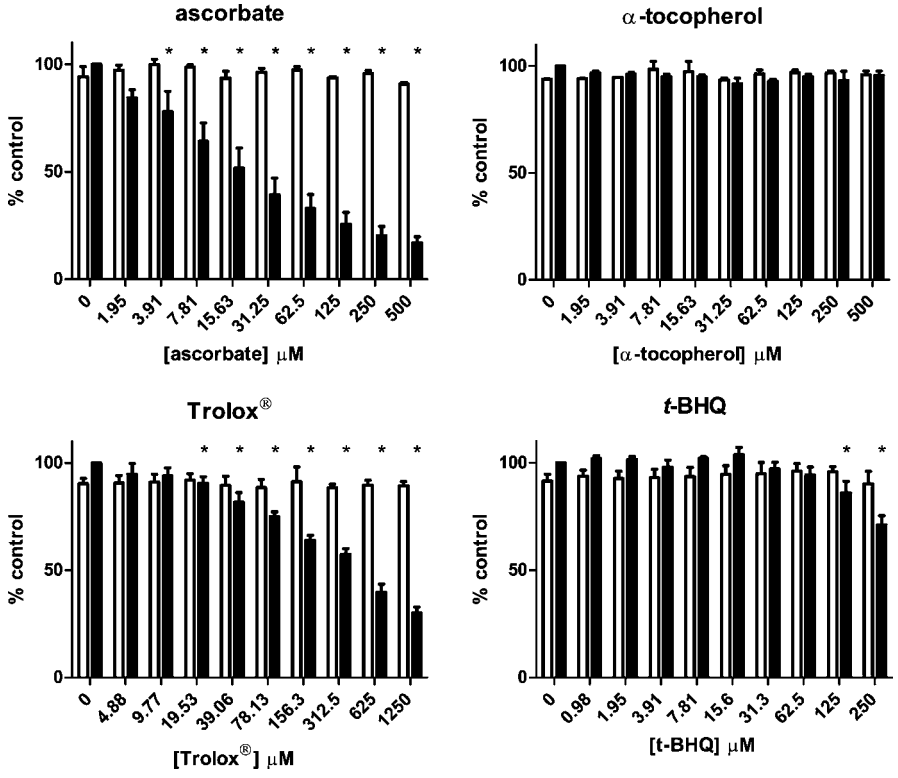

Figure 2. ROS production in antioxidant-pretreated EPC cells as measured using the DCFH assay. After 24 h in the presence of various antioxidants, cells were washed, loaded with H_2DCFDA for 30 min, and then washed again before incubation for 4 h in serum-free L-15 medium. ROS production (black bars) was determined using the H_2DCFDA assay. Cell viability (white bars) was determined using the Neutral Red uptake assay. Data are the normalized means ± SE from three independent experiments each performed in triplicate. ∗ indicates a significant difference ($p < 0.05$) from the solvent control according to a one-way ANOVA followed by Dunnet's post hoc test.

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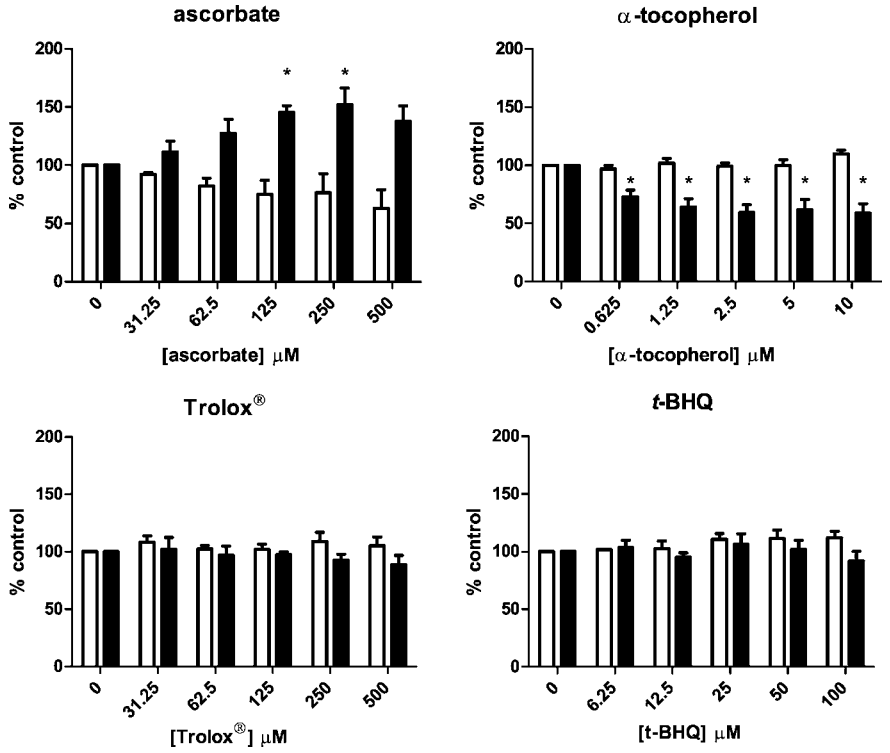

Figure 3. Lipid peroxidation as determined using the TBARS assay. EPC cells were pretreated for 24 h with various antioxidants before being exposed to 0.5 mM cumene hydroperoxide plus 1 μM hemin for 1 h. The cells were then washed and TBARS (black bars) determined in cell lysates. Cell viability (white bars) was determined in duplicated plates using the Neutral Red uptake assay. Data shown are the normalized means ± SE from three independent experiments. ∗ indicates a significant difference ($p < 0.05$) from the solvent controls according to a one-way ANOVA followed by Dunnet's post hoc test.

Interestingly, ascorbate treatment caused a significant increase in apparent TBARS content, with cell viability also diminishing as ascorbate concentration increased. Ascorbate is known to undergo redox cycling to form a number of radical species in cell culture media, with the concomitant production of hydrogen peroxide,[24,25] and has also been observed to contribute to lipid peroxidation in a liposome-based model system utilizing iron in the Fe^{3+} form.[26] However, it cannot be ruled out that the increase in signal may result from a possible interaction between TBA and one or more products of ascorbate oxidation. It should also be noted that the ascorbate concentrations tested were extremely high (maximum of 0.5 mM), so are not likely to be physiologically relevant.

Evaluation of C_{11}-BODIPY$^{581/591}$ as a Probe for the Estimation of Lipid Peroxidation in EPC Cells. The fluorescent dye-conjugated fatty acid C_{11}-BODIPY$^{581/591}$ changes fluorescence spectral properties upon oxidation, shifting from red to green fluorescence.[27] This compound has shown promise as an indicator of lipid peroxidation in living cells due to its accumulation in membranes, where it can be oxidized by incubation with organic peroxides in the presence of transition metal ions.[13−15] Because others have reported the use of this probe to estimate lipid peroxidation in rat fibroblasts in a 96-well plate format,[14] we sought to develop a similar assay using the EPC fish cell line. Using a fluorescence-capable plate reader, we found that signal intensities corresponding to the reduced form of C_{11}-BODIPY$^{581/591}$ (540 nm excitation/590 nm emission; the "red channel") were high, but despite extensive optimization of conditions, the proportion of oxidized C_{11}-BODIPY$^{581/591}$ (485 nm excitation/520 nm emission; the "green channel") remained low and was effectively below the detection threshold of the instrument (data not shown), resulting in relatively high background readings in the green channel. To visually confirm uptake of the probe into cellular membranes and to develop an alternative method of accurately detecting the oxidized form of C_{11}-BODIPY$^{581/591}$, we decided to pursue an approach based on automated fluorescence microscopy using the InCell Analyzer 1000 (GE Life Sciences).

Automated fluorescence microscopy using the InCell Analyzer 1000 revealed that the hemin-catalyzed oxidation of cell-associated C_{11}-BODIPY$^{581/591}$ occurred readily in response to cumene hydroperoxide or *tert*-butylhydroperoxide, but poorly in response to hydrogen peroxide (Figure 4A). This is consistent with the work of Drummen et al.,[13] who observed a relatively low rate of C_{11}-BODIPY$^{581/591}$ oxidation in small unilamellar phosphatidylcholine vesicles in the presence of 0.5 mM copper sulfate in response to 2 mM hydrogen peroxide compared to that observed after treatment with 2 mM cumene hydroperoxide. The oxidation of C_{11}-BODIPY$^{581/591}$ in the EPC cell line required a relatively high concentration of cumene hydroperoxide compared to the levels reported by other groups using a rat fibroblast cell line (500 μM in our study as opposed to 50 μM in ref 14), suggesting that the oxidative stability of the probe may differ between cell types.

Incubating cells with α-tocopherol potently inhibited the subsequent oxidation of C_{11}-BODIPY$^{581/591}$ in the presence of cumene hydroperoxide and hemin, whereas Trolox and ascorbate did not prevent oxidation of the probe at the concentrations tested (Figure 4B). The observed lack of antioxidant activity of Trolox in this assay is intriguing, because this compound is reported to partition between the lipid and aqueous phases in liposomes.[28] Despite the apparent decrease

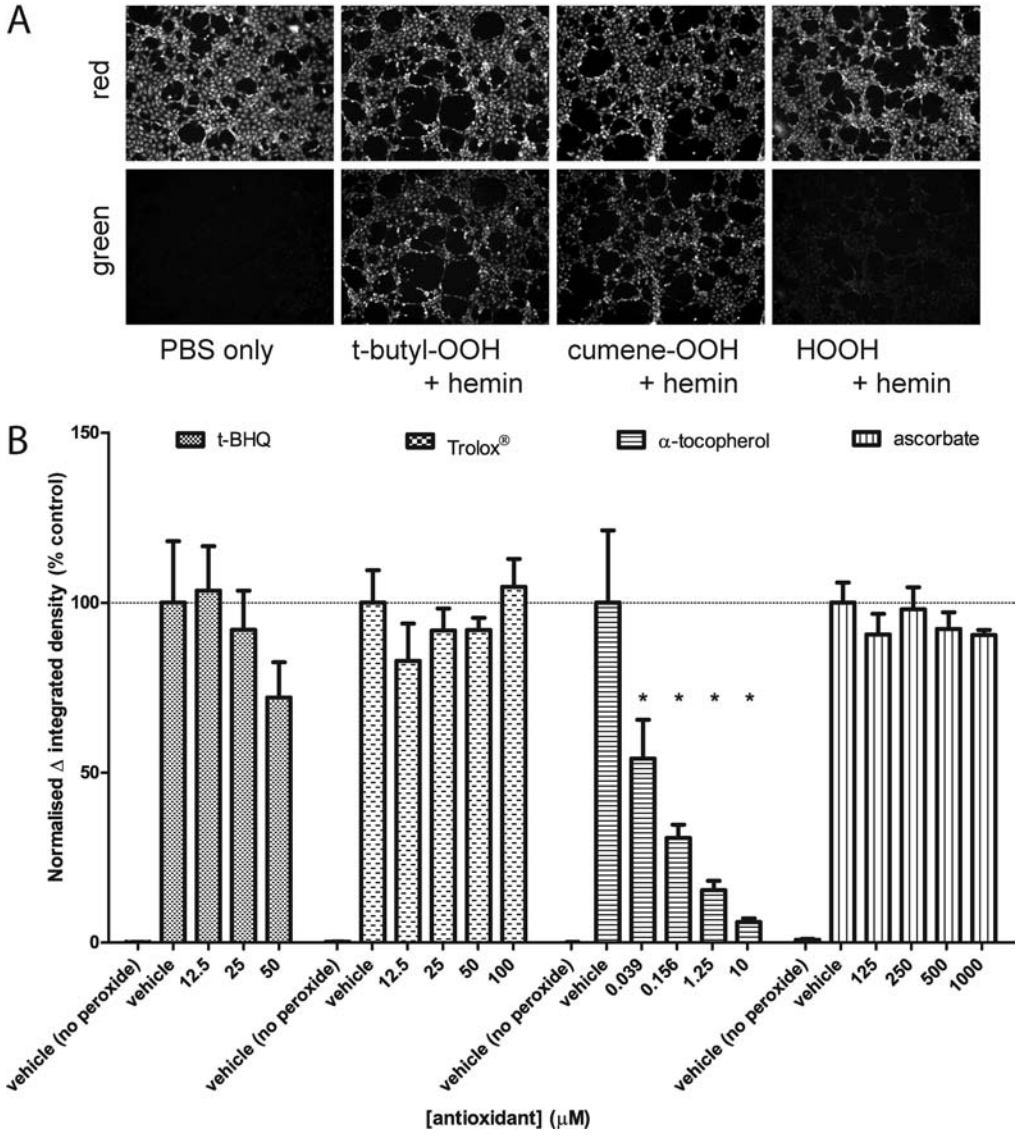

Figure 4. Lipid peroxidation as determined using C_{11}-BODIPY$^{581/591}$ oxidation. (A) EPC cells were loaded with C_{11}-BODIPY$^{581/591}$ and then washed thoroughly before incubation with 0.5 mM cumene hydroperoxide, *tert*-butylhydroperoxide, or hydrogen peroxide in the presence of 100 nM hemin. After 30 min, images were captured using filter sets for red and green fluorescence. (B) EPC cells pretreated with various antioxidants for 24 h, washed, loaded with C_{11}-BODIPY$^{581/591}$ for 30 min, and then washed thoroughly again and imaged in the red and green channels were analyzed by high-throughput microscopic analysis. Cells were then exposed to 0.5 mM cumene hydroperoxide plus 100 nM hemin for 30 min and imaged again. Data shown are normalized mean changes in integrated density (product of area and intensity) ± SE in the green channel (480 nm excitation/535 nm emission) from three replicate wells of a representative experiment. ∗ indicates a significant difference ($p < 0.05$) from the solvent control according to one-way ANOVA followed by Dunnet's post hoc test.

in fluorescence intensity in the green channel response to increasing *t*-BHQ concentrations, a visual inspection of images in both the red and green channels revealed that the decrease in signal was probably due to cell death and subsequent detachment from well surface rather than inhibition of C_{11}-BODIPY$^{581/591}$ oxidation.

In summary, α-tocopherol was the only compound tested here that potently inhibited C_{11}-BODIPY$^{581/591}$ oxidation in EPC cells, an observation that was consistent with the results we obtained using the TBARS assay.

DISCUSSION

In this study, we evaluated methods for detecting and quantifying the antioxidant activity of exogenously applied compounds in cultured fish cells, with the view to developing a rapid means of screening novel compounds or complex mixtures for potential use as antioxidants in finfish aquaculture feeds. Cell-based assays are desirable for this purpose as they can indicate cytotoxicity and provide insight into cellular uptake of antioxidant compounds in addition to determining antioxidant potency.

A number of redox-sensitive fluorescent indicators are commonly employed for ROS detection in cell-based models of oxidative stress (for reviews, see refs 11 and 29). In particular, H_2DCFDA (cleaved by endogenous cellular esterases to H_2DCF, commonly known as DCFH) is widely used for this purpose. DCFH is not oxidized by hydrogen peroxide in the absence of ferrous iron or superoxide,[30] but is particularly sensitive to hydroxyl radicals[30−32] and peroxynitrite.[33] The polar nature of DCFH means that it is not likely to

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traverse the membranes of organelles such as lysosomes and peroxisomes and is hence widely considered as an indicator of cytosolic ROS. This is supported by studies showing that DCFH can be readily oxidized in the absence of ROS via mechanisms involving cytochrome *c* liberated from mitochondria or iron released from lysosomes after targeted disruption.[31] In the present study, the uptake of Neutral Red by serum-deprived cells was not markedly different from that observed in controls, suggesting that cell viability and lysosomal integrity were not adversely affected by serum deprivation during the assay period (because Neutral Red accumulates primarily in lysosomes[34]). Whereas the specific species responsible for DCFH oxidation in the present study is unknown, it is clear that incubating EPC cells in the presence of the relatively polar antioxidant compounds ascorbate and Trolox prevents the subsequent oxidation of the probe, suggesting that this assay is well suited to the study of water-soluble antioxidants in cultured fish cells. Conversely, the lack of activity exhibited by α-tocopherol in the DCFH assay suggests that this test, as implemented here, is unsuitable for detecting the activity of nonpolar antioxidant compounds in the EPC cell line.

Although widely criticized for its lack of specificity,[29] the TBARS assay[17,35] is widely used as an indicator of lipid peroxidation in tissues, plasma, and cell-based assays. The potent inhibitory effect of α-tocopherol on TBARS formation observed in EPC cells is consistent with other reports of α-tocopherol activity in cell-based TBARS assays (e.g., TBARS formation was strongly inhibited in a rat astroglioma cell line by 24 h pretreatment with 10 μM α-tocopherol[36]). However, the low sensitivity of the TBARS assay and the requirement for the incubation of samples at relatively high temperatures prevented the practical implementation of this assay in a 96-well plate assay format. We therefore investigated whether C_{11}-BODIPY$^{581/591}$ would be suitable as an indicator of lipid peroxidation in EPC cells in a 96-well plate assay, as has been reported for other cell types.[14] We found that the C_{11}-BODIPY$^{581/591}$ was well suited to this purpose. In our hands, however, the use of automated microscopy to measure the oxidized form of the probe provided vastly more reliable results than those we could obtain with a microplate reader.

In practice, the use of C_{11}-BODIPY$^{581/591}$ as an indicator of lipid peroxidation had a number of advantages over the TBARS assay. First, at least with our methodology, the dynamic range of the C_{11}-BODIPY$^{581/591}$-based assay was significantly greater than that of the TBARS assay. Second, the C_{11}-BODIPY$^{581/591}$-based assay was easily implemented in a 96-well plate format and is therefore well suited to use in high-throughput applications, whereas the low sensitivity of the TBARS assay meant that a large number of cells were required for each experimental condition, preventing the use of the 96-well plate format. Third, using C_{11}-BODIPY$^{581/591}$ combined with automated microscopy, cytotoxicity is clearly apparent during imaging of the plate, or alternatively can be easily determined in the same plate by performing a cell viability assay, whereas duplicate plates are required for the determination of cell viability in the case of the TBARS assay due to the destructive nature of the procedure.

One point of concern was the differing responses to ascorbate in the two lipid peroxidation assays. High ascorbate concentrations resulted in increased apparent cellular TBARS levels in the presence of cumene hydroperoxide and hemin, whereas the oxidation of C_{11}-BODIPY$^{581/591}$ under similar conditions was not affected by ascorbate. Because the rate of oxidation of C_{11}-BODIPY$^{581/591}$ in cultured cells exposed to cumene hydroperoxide has been determined to be similar to that of polyunsaturated fatty acids,[15] it seems likely that the increased signal in the TBARS assay may be due to the formation of a nonspecific TBA reaction product.

The requirement for high-throughput imaging will likely limit the use of the C_{11}-BODIPY$^{581/591}$ assay in the form presented here, because access to high-content screening instruments is not common in the average laboratory. However, the use of a plate reader in a similar assay has been reported by others,[14] suggesting that differences in equipment, and possibly differences in cellular endogenous antioxidant systems in various cell types, may allow the successful implementation of more traditional methods.

We have shown that the apparent potency of antioxidants in cultured fish cells depends on the method used and is related to the aqueous solubility of the antioxidant. In the case of assays based on redox-sensitive fluorescent indicator dyes, the apparent antioxidant activity will be affected by the solubility of all relevant components: the dye, the oxidative species utilized to promote redox changes in the dye, and the antioxidant under investigation. Although this has been extensively discussed in the context of chemical assays for antioxidants that protect against lipid peroxidation,[37] further work is needed in the case of cell-based assays. We conclude that a single cell-based assay for antioxidant activity is not likely to be easily developed, and the best approach for characterizing novel antioxidant compounds or screening complex mixtures for antioxidant activity is to employ a number of assays specific for different classes of antioxidants.

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