Research article

Development of an *in vitro* model of excess intracellular reactive oxygen species

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Abstract

These investigations characterize an *in vitro* model for generating excess intracellular reactive oxygen species (ROS). This novel model may be useful in the identification and delineation of cellular mechanisms associated with aging due to the link between age and excess oxidative events. The human cell line, MCF7, was stably transfected using the pSV3.neo plasmid housing a gene encoding the *Aequorea victoria* green fluorescent protein (GFP). Transfected cells were analyzed for maintenance of GFP over time, showing stability of the GFP gene. These studies demonstrate that the presence of fluorescing GFP significantly increases intracellular ROS, creating oxidative stress in these cells. Antioxidant supplementation was evaluated to determine the effectiveness of intracellular H₂O₂ reduction. The results demonstrate that supplementation with a potent antioxidant, such as reduced glutathione, protects cells from oxidative damage by decreasing intracellular concentrations of H₂O₂. This model for intracellular generation of excess ROS establishes a clear method by which the utility of antioxidant supplementation to protect against intracellularly generated reactive oxygen species may be evaluated.

Introduction

Oxidative stress has been defined as a disturbance in the cellular pro-oxidant/antioxidant systems in favor of the former (Sies 1985), and involves responses to oxidants or agents that cause oxidation via intracellular reduction and oxidation reactions (Wiese et al. 1995). The final result of pro-oxidant reactions is reactive oxygen intermediates, or reactive oxygen species (ROS), including superoxide and hydroxyl radicals. In eukaryotic systems, the hydroxyl radical is easily produced from H₂O₂ either by exposure to ultraviolet light (Inouye and Tsuji 1994) or by interaction with transition metal ions. Of these, the most important *in vivo* transition metal is iron. The hydroxyl radical can initiate macromolecule oxidation,

potentially causing DNA strand breaks or indiscriminately oxidizing virtually any organic molecule (Halliwell and Gutteridge 1999). Perhaps not coincidentally, these types of cellular damage increase dramatically in aged cells, and oxidative stress has been shown to be significantly increased in age-related diseases such as Alzheimer's and Progeria (Butterfield et al. 2001).

Due to the increased intracellular ROS resulting from utilization of oxygen as an electron acceptor during oxidative phosphorylation, eukaryotic cells have developed effective mechanisms of defense against potentially destructive ROS. One such mechanism is simply to release ROS into the extracellular environment (Halliwell and Gutteridge 1999). Cultured human fibroblasts, stimulated endothelial cells,

and phagocytic cells have all been reported to release low levels of these superoxide radicals (Murrell et al. 1990). A second method utilized by mammalian systems to deal with excess intracellular ROS is to eliminate them by interaction with specific antioxidants (Halliwell and Gutteridge 1999; Ozben 1998). These intracellular antioxidants are either synthesized or transported into the cell, and include molecules that can react with and eliminate ROS before they are able to oxidatively damage vital cellular components (Ozben 1998). The most efficient method for eliminating excess ROS is by catalysis, and mammalian cells have evolved families of antioxidant enzymes for this purpose. Certain levels of ROS inevitably escape catalytic conversion, however, and remain in the cell. To this end, antioxidant compounds including vitamin C, vitamin E, ubiquinone, and uric acid may be sacrificed to protect cellular components from oxidative damage (Davies 1995; Tsien 1998; Halliwell and Gutteridge 1999). The delicate balance between enzymatic mechanisms for reducing ROS and intracellular levels of ROS is normally strictly controlled by supplying cells with essential nutrients and by minimizing the effects of substances that stimulate ROS (Murrell et al. 1990).

The consequences of pro-oxidant/antioxidant imbalance favoring oxidative stress include tissue, DNA, and membrane damage and have been implicated in numerous disease states. Currently, investigations of oxidative stress primarily focus on initiating cellular events by adding ROS to culture media, rather than on the effects of endogenously generated intracellular ROS levels which are believed to be a primary cause of cellular changes in many disease states (Butterfield et al. 2001). To this end, these investigations were initiated with the goal of establishing a model for generating excess intracellular ROS. This model facilitates investigation of a proper intracellular pro-oxidant/antioxidant balance and the effects of imbalance on cellular proliferation, stabilization, and maintenance while demonstrating a potential to test substances' antioxidant activity.

Materials and methods

Cellular transfection

The expression vector, pPGKGFP, previously described by Miller et al. (2000), with slight modification, was

utilized for cellular transfection. Specifically, the original expression vector was modified by excision of the two-estradiol response elements. Utilizing the PGK promoter, the vector contains the enhanced green fluorescent protein, followed by a bovine poly-A, on a pSV3 background. The modified expression vector was prepared in large quantities according to the manufacturer's protocol (NucleoBond Plasmid Maxi Kit, Clontech, Palo Alto, CA) and checked for integrity by sequencing; pPGKGFP was linearized and transfected into MCF-7 cells (American Type Culture Collection, Rockville, MD). Transfected cells were maintained at 37 °C under 5% CO₂ in standard minimal essential medium (MEM; Gibco BRL, Grand Island, NY) supplemented with 1 mM sodium pyruvate (Sigma, St. Louis, MO), 1 µg/ml insulin (Sigma), 1.25 µg/ml Fungizone® (Gibco BRL), and 0.1 mg/ml gentamycin sulfate (Sigma). Electroporation of linearized pPGKGFP into MCF-7 cells was carried out as previously described (Miller et al. 2000) utilizing a BTX Transfector 300 (Biotechnologies and Experimental Research, Inc., San Diego, CA), according to methods established by Ausubel et al. (1994). Transfected cells were allowed to recover for a total of 40 min before being added to MEM (Gibco BRL, Grand Island, NY).

These cells were allowed to grow to confluency before selection by fluorescence activated cell sorting (FACS). Immediately prior to sorting, the cells were trypsinized, pelleted, and resuspended in phosphate buffered saline (PBS). Cells were analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer, equipped with the sorting option, using CellQuest (Becton Dickinson) acquisition software. Green fluorescence from the GFP was collected through a 530/30-nm bandpass filter with log amplification. For sorting, a region was set to include only GFP-positive cells, and sorting was performed using the 'single cell' setting which aborts if additional cells are present in the sort envelope. The GFP-positive cells were designated as MCF-ARE cells, and used to examine the effect of GFP on cellular proliferation.

Growth curves

The growth rates of MCF-ARE and MCF-7 cells were determined in MEM (Gibco BRL, Grand Island, NY), without phenol red and supplemented with 10% heat

inactivated, charcoal dextran treated, fetal bovine serum (HICDFBS), non-essential and essential amino acids (Gibco BRL), 1 mM sodium pyruvate (Sigma, St. Louis, MO), 1 μ g/ml insulin (Sigma), 1.25 μ g/ml Fungizone[®] (Gibco BRL), and 0.1 mg/ml gentamycin sulfate (Sigma). All cells were seeded into 60-mm culture dishes at 10 ml of 10,000 cells/ml. After 4 h, the medium was removed, and replaced by stripped, supplemented MEM. Cells attached to the dish at this stage of growth were harvested with trypsin, counted using a Nuebauer hemacytometer, and were reported as day 0. Every two days during the experiment the cells were counted in triplicate and supplied fresh media. For the growth curves designed to determine the benefit of supplemental antioxidants, cells were seeded, counted, and supplemented with either 2 mM reduced glutathione (Boehringer Mannheim, Germany), or the glyconutrient, Ambrotose[®], at 0.05 mg/ml (Mannatech Inc.[™], Coppell, TX).

The mean and SEM were calculated for experimental groups, consisting of a minimum of three independent experiments each. Statistical analysis for each experimental group used Fisher's protected least significant difference (LSD), or Hsu's test in those sample sets determined to be independent. Followed by multiple comparisons with the best (MCB), the population means among treatment groups were compared, yielding the mean significantly larger than the others.

Measurement of reactive oxygen species

Hydrogen peroxide measurements were taken in Costar 96 well tissue culture plates (Corning, Inc., Corning, NY), using a Bio-Tek FL600 fluorescent plate reader (Bio-Tek Instruments, Winooski, VT) with excitation/emission wavelengths at 530/25 and 590/35, respectively. Assays were performed with Molecular Probes' Amplex[™] Red Hydrogen Peroxide Assay kit (Eugene, OR) according to the manufacturer's instructions. MCF-7 cells were seeded and grown as described above and on growth day three were trypsinized and collected. After counting, they were centrifuged to remove excess trypsin, and resuspended in lysis buffer (10 mM Tris·Cl, 0.1 M EDTA, 0.5% SDS) at a concentration of 185 cells/ μ l lysis buffer. Cells were incubated at room temperature for 1 h and 100 μ l total cellular lysate was utilized for an Amplex[™] Red reaction. Each data point was tested

in a minimum of six separate reactions together with positive and negative controls.

The raw fluorescence data, generated by Kineticalc for Windows, Kc4, (Bio-Tek Instruments, Inc., Winooski, VT), was transferred to a Microsoft Excel worksheet. Average background fluorescence was subtracted from the data values to obtain the change in fluorescence. The mean and SEM were calculated for experimental groups which consisted of a minimum of six independent reactions each. A standard curve of H₂O₂ was evaluated in parallel with the studies. Therefore, final concentrations of reactive oxygen species in the cells were determined by comparison of fluorescence values in the test samples to the standard curve for each independent experiment. Statistical analysis for each experimental group used Fisher's protected least significant difference (LSD), or Hsu's test in those sample sets determined to be independent.

For validation of the H₂O₂ measurements, a cell-free system was also tested for antioxidant and phytochemical (Ambrotose[®]) quenching. Reduced glutathione (Boehringer Mannheim, Germany) was added in concentrations ranging from 0.01 to 10 μ M to Amplex[™] Red solutions containing 4 μ M H₂O₂. Measurements of reduction in total H₂O₂ concentrations were replicated up to 17 times for each test concentration. Similarly, Ambrotose[®] (Mannatech[™], Coppell, TX) was added, with concentrations varying between 0.01 and 0.9 mg/ml, to Amplex[™] Red

Figure 1. MCF-ARE cells. A phase contrast micrograph of MCF-ARE cells superimposed with a fluorescent micrograph of MCF-ARE cells. MCF-7 cells were transfected with the GFP cDNA and passaged 10 $\times$ prior to fluorescent photography.

Figure 2. MCF-7 and MCF-ARE growth curves. Cells were grown in 60 mm culture dishes, in standard MEM with phenol red (Gibco BRL). They were harvested and counted in triplicate every two days. Graph represents the mean and standard error of multiple independent experiments.

solutions containing 4 μM H_2O_2 and measured in replicates.

Results

Cellular transfection

Cells were maintained at 37 °C under 5% CO_2 in standard minimal essential medium as described above. Transfected cells were allowed to grow to

confluency before selection by FACS (data not shown) and were periodically monitored by observation with a phase contrast fluorescence microscope and photographed (Figure 1).

Growth curves

Cells were evaluated over 14 days to detect potential growth differences between the parent cell line, MCF-7, and the stably transfected cell line, MCF-ARE (Figure 2). The MCF-7 growth curve revealed three

Figure 3. Growth of the GFP-expressing MCF-ARE cells in unsupplemented medium, medium supplemented with reduced glutathione, and medium supplemented with Ambrotose®. The parent cell line, MCF-7, is included for comparison and reference to Figure 2. Statistical comparison was carried out initially with Hsu's test, followed by MCB.

Figure 4. Intracellular hydrogen peroxide measurements in untransfected (MCF-7) and transfected cells (MCF-ARE) under various medium supplementations. Bars indicate SEM.

typical phases: (1) lag phase, (2) exponential growth phase, and (3) stationary phase. MCF-ARE cells appeared to grow past confluency without inhibition and had a significant growth advantage ($P = 0.0025$) over untransfected MCF-7 cells (Figure 2). MCF-ARE cells also grew at dramatically different rates depending on medium supplementation (Figure 3). In the first few days after plating it appeared that cell groups supplemented with GSH and the proposed glyconutrient antioxidant, Ambrotose®, exhibited a definitive growth advantage. However, the most remarkable difference in growth rate began at day 11 when the experimental groups diverged, with MCF-ARE cells growing at a markedly increased rate over MCF-7 cells, and cells supplemented with

GSH growing most rapidly. These differences were replicated in each of at least three independent experiments, represented as mean $\pm$ standard error in figures.

Measurement of reactive oxygen species

Reactive oxygen species in MCF-ARE cells were compared to those in the parent cell line, MCF-7, using the Amplex™ Red assay. Additionally, MCF-ARE cells grown in medium supplemented with reduced glutathione or Ambrotose® were compared to the other cell groups. The parent cell line, MCF-7, exhibited a low level of H_2O_2 equivalent to approximately 0.5 μM (Figure 4), which is significantly

Figure 5. A hydrogen peroxide standard curve, as established with the Amplex™ Red Hydrogen Peroxide Assay Kit, shows the range of detection and the assay sensitivity level.

Figure 6. Hydrogen peroxide quenching by reduced glutathione in a cell-free analysis with Amplex™ Red.

different ($P = 0.0025$) from the 2- μM level of H_2O_2 in transfected MCF-ARE cells. Supplementation of transfected cells with 2 mM GSH reduced the H_2O_2 concentration to about 0.75 μM . Although reduced glutathione, GSH, lowered the level of H_2O_2 in transfected cells to levels closer to those of the parent cell level, Ambrotose® slightly increased the H_2O_2 level to 3.25 μM .

Validation of ROS evaluation

To verify the effectiveness of medium supplementation on intracellular H_2O_2 reduction, we analyzed H_2O_2 quenching in a cell-free system, utilizing the Amplex™ Red Hydrogen Peroxide Assay (Molecular Probes). The standard curve (Figure 5) indicated saturation of the assay at approximately 8–10 μM H_2O_2 with sensitivity as low as 0.5 μM H_2O_2 . Both reduced glutathione and Ambrotose® were tested for H_2O_2 quenching. Medium supplemented with GSH (2 mM) showed a statistically significant reduction ($P < 0.05$) of total H_2O_2 , from 4 μM to less than 0.5 μM (Figure 6). Increasing the concentration of GSH above 2 mM continued to decrease the overall concentration of H_2O_2 , with the total reduction closely approaching elimination of H_2O_2 at a GSH concentration of 10 mM. Hydrogen peroxide quenching with Ambrotose® was more variable than GSH but reduced the H_2O_2 concentration from 4 to about 2.5 μM with significant differences between both 0 and

0.01 mg/ml Ambrotose®, and 0 and 0.9 mg/ml ($P < 0.05$) Ambrotose®.

Discussion

Data presented here indicate that a primary difference between the transfected GFP-expressing MCF-ARE, and untransfected, MCF-7, cell lines is in the level of intracellular ROS produced (Figure 4). MCF-ARE cells have a significantly higher level ($P = 0.0032$) of H_2O_2 than do MCF-7 cells, presumably as a result of the continuous production and folding of green fluorescent protein in the transfected, GFP-expressing cells. Due to the fact that the cells were cleansed of their extracellular medium before cell lysis and H_2O_2 measurement, the resulting levels should accurately reflect intracellular levels of H_2O_2 , as has been demonstrated in previous investigations by the manufacturer (Molecular Probes). Considering the nature of GFP folding, this result would be expected. GFP chromophore formation is thought to be dependent on the oxidation of Tyr66 (Heim et al. 1994), and requires an increased oxidative state to fluoresce. Reducing agents, such as iron sulfide (FeSO_4) and sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$), convert GFP to a nonfluorescent form, while weaker reducing agents such as β -mercaptoethanol and reduced glutathione, GSH, do not affect its fluorescence (Inouye and Tsuji 1994). After treatment with the

stronger reducing agents and loss of fluorescence, GFP reverts to its fluorescent state upon exposure to molecular oxygen. These data support the proposal that GFP undergoes a reversible oxidation–reduction reaction in the presence of molecular oxygen, and, assuming the proposal is correct, suggests that strong reducing agents would be expected to de-colorize GFP by reducing oxidized Tyr66 which would reduce the chromophore (Hoffman et al. 1984; Inouye and Tsuji 1994). One expected consequence of GFP oxidation is the release of H_2O_2 in 1 : 1 stoichiometry with mature GFP (Davies 1995). The H_2O_2 by-product created during fluorophore formation might also explain occasions when high-level expression of GFP can be damaging to the cell (Liu et al. 1999). Further, some GFPs seem to express most readily when targeted to peroxisomes and mitochondria (Rizzuto et al. 1996), the organelles most adept at coping with reactive oxygen species such as H_2O_2 .

Gradients of H_2O_2 across membranes are formed when the membranes separate the production and consumption sites of H_2O_2 (Boveris and Cadenas 2000) due to the high permeability of H_2O_2 across cellular membranes (Hoffman et al. 1984; Liu et al. 1999). Therefore, cells expressing GFP may attempt equilibrium across the cellular membrane by depositing H_2O_2 into the culture medium. This deposition, in turn, is demonstrated by the significant growth difference between transfected and untransfected cell lines (Figure 3) and has been noted by other investigators as well. Burdon et al. (1989) indicated that low concentrations (10 nM–1 μM) of H_2O_2

added to culture medium were effective in stimulating *in vitro* growth of rat fibroblasts while Wiese and colleagues (1995) reported that 3 μM and 15 μM H_2O_2 in media stimulated cell growth and division in Chinese hamster ovary cells, HA-1. As may be expected for cellular proliferation to be enhanced, gene expression is also altered under low level H_2O_2 exposure in HL-525 cells, with increased expression of *c-jun*, *c-fos* (Datta et al. 1992; Nose et al. 1991; Shibamura et al. 1988), and *c-myc* (Crawford et al. 1988). One of the earliest events following a proliferative stimulus is the expression of the *c-fos* gene (Lau and Nathans 1987; Verma et al. 1986). Following up-regulation of expression, *c-fos*, *c-myc* and *c-ras* begin exerting their effects on transcriptional regulation within the cell (Chiu et al. 1988; Piechaczyk et al. 1986; Ricketts and Levinson 1988) and *egr-1*, or early growth response-1 (Datta et al. 1992) initiates processes essential for proliferation.

Knowing that the growth patterns and intracellular ROS levels of MCF-ARE suggested increased levels of H_2O_2 , we were interested in determining the effects of known and potential antioxidants on the redox status and growth potential of MCF-ARE cells. Specifically, we selected reduced glutathione (GSH) for medium supplementation because of its well-characterized mechanism of H_2O_2 reduction in mammalian cells (Davies 1995). Having demonstrated a remarkable ability for quenching H_2O_2 in a cell-free system analysis (Figure 6), 2 mM GSH was used to supplement medium for MCF-ARE cells. GSH-supplemented cells significantly decreases intracellu-

Figure 7. Hydrogen peroxide quenching by Ambrotose®, in a cell-free analysis with Amplex™ Red.

lar H_2O_2 levels ($P < 0.05$; Figure 4), indicating that GSH acted as an antioxidant in this system. This was expected, but has not to our knowledge been demonstrated intracellularly. Cellular proliferation data corroborate these results by demonstrating a growth advantage for GSH-supplemented cells over the other experimental groups. While small quantities of H_2O_2 demonstrate growth stimulation in MCF-ARE cells over unsupplemented MCF-ARE cells, excess quantities of H_2O_2 would be expected to be damaging to the cells, as has been reported for other cell types (Wiese et al. 1995). In this study, the GSH appears to maintain levels of H_2O_2 compatible with increased growth; however, the cellular proliferation did not return to a level identical to the parental cell line, MCF7. The proliferation result is also as expected due to the observation that the overall H_2O_2 levels were not completely reverted to the parental levels (Figure 4), but remained slightly higher although not significantly different. It is possible that with increased concentrations of GSH, the intracellular H_2O_2 levels could be further reduced and return both the intracellular levels and proliferation capacity to that of the parental line.

Having verified the capacity of GSH to quench H_2O_2 in media, and demonstrated the ability of GSH to dramatically decrease intracellular H_2O_2 levels in MCF-ARE cells, we examined the potential of Ambrotose[®], a novel mixture of naturally occurring sugars and phytochemicals, for H_2O_2 quenching capacity. In a cell-free system Ambrotose[®] at 0.1 mg/ml significantly reduced the levels of H_2O_2 ($P < 0.05$) (Figure 7). The phytochemical, however, did not demonstrate antioxidant activity, as it failed to decrease intracellular ROS levels and to enhance cellular proliferation over unsupplemented MCF-ARE cells (Figure 4). Since Ambrotose[®] was effective in reducing levels of H_2O_2 in the cell-free system but apparently lacked activity in MCF-7 and MCF-ARE cells, it is proposed that this novel mixture of phytochemicals is not effectively transported *in vitro* into cells of these specific mammalian lines. Clearly, more characterization of the compound is in order if it is to be utilized as an *in vivo* antioxidant in mammalian systems.

In conclusion, these studies demonstrate that cells expressing fluorescing GFP have a significantly higher concentration of intracellular H_2O_2 than cells that do not express GFP. Further, we show that moderately elevated intracellular H_2O_2 levels significantly stim-

ulate proliferation in MCF-ARE cells. In addition, supplementation of the medium with a potent antioxidant, GSH, protected the cells from oxidative damage by decreasing intracellular concentrations of H_2O_2 . Reduced glutathione may aid in the survival and longevity of cells with increased intracellular ROS levels, such as cells expressing GFP. Additional studies are in order, as data presented here suggest that gene expression changes associated with increased intracellular ROS are to be expected in cells expressing GFP.

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