Antioxidant enzyme gene expression in response to oxidative stress

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Abstract

Aerobic metabolism produces reactive oxygen species that can damage nucleic acids, proteins, and lipids. These harmful byproducts can be broken down by antioxidant enzymes, the most important of which are catalase, glutathione peroxidase, and superoxide dismutase. Oxidative stress may be induced by environmental or physiological factors, such as the changes that occur in hibernating mammals during periods of arousal, leading to an increase in the levels of reactive oxygen species and potential cell and tissue damage. I have compared the levels of expression for three genes encoding the antioxidant enzymes mentioned previously in BE(2)-C cells that have been subject to oxidative stress (50 μM H_2O_2 for 6 h) and those that have not by using RT-PCR and analyzing the results on a 1% agarose gel. This experiment supported a difference in gene expression between treated and untreated cells for catalase and superoxide dismutase, but not glutathione peroxidase, and sequencing confirmed that the products amplified were those targeted by the primers that were designed. An increase in the expression of genes encoding antioxidant enzymes in response to oxidative stress can protect cells against oxidant damage to cell structures and tissues. The techniques and primers used here to demonstrate such changes in cultured human neuroblastoma cells can be used to examine organisms' responses to oxidative stress, such as by examining tissue samples from active and torpid mammals.

Introduction

Aerobic metabolism generates reactive oxygen species as byproducts due to the incomplete reduction of oxygen; these byproducts can be involved in cell signaling such as the induction of apoptosis and in defense against pathogens, but can also lead to cell damage if present in excessive levels (Morel & Barouki 1999; Apel & Hirt 2004). Antioxidant enzymes protect aerobic prokaryotes, aerotolerant anaerobic prokaryotes, and eukaryotic cells from the detrimental effects of reactive oxygen species such as mutagenesis, DNA strand breakage, damage to membrane lipids, and damage to proteins found in connective tissue (Harris 1992; Scandalios 1993; Toyokuni *et al.* 1995). Three of the most critical antioxidant enzymes are catalase, glutathione peroxidase, and superoxide dismutase, which differ in their cofactors, cellular locations, and mechanisms of action (Harris 1992). Periods of oxidative stress increase cellular levels of reactive oxygen species and may lead to changes in the expression levels of genes encoding antioxidant enzymes; an appropriate balance of such enzymes is necessary to optimize their protective effects (Michiels *et al.* 1994).

Catalase is a heme-containing enzyme found in peroxisomes that catalyzes the dismutation of hydrogen peroxide (H_2O_2) to water and molecular oxygen (Harris 1992). It is a ubiquitous enzyme found in the tissues of most species, and its homotetrameric structure and the active site configurations of each subunit are highly conserved (Zámocký & Koller 1999). Glutathione peroxidase is a selenium-containing enzyme that catalyzes the oxidiation of glutathione to glutathione disulfide, the ratio of which can be used as an indicator of oxidative stress (Carey *et al.* 2003b), and is involved in the metabolism of lipid hydroperoxides and H_2O_2 (Arthur 2000). Superoxide dismutases catalyze the metabolism of superoxide anions to hydrogen peroxide and molecular oxygen using a variety of cofactors such as copper, zinc,

manganese, and iron (Harris 1992). The enzyme of interest for this study, human superoxide dismutase 1, is located in the cytosol and utilizes copper and zinc. The three antioxidant enzymes described are involved in the metabolism of different oxidant substrates and work in concert to protect cells against damage if excessive levels of reactive oxygen species are present, such as when the organisms are subject to oxidative stress.

Certain mammalian species hibernate to conserve energy resources in periods of cold temperatures when food is scarce and thermoregulation is more costly. Hibernation induces oxidative stress in mammals and may damage susceptible tissues due to changes in blood flow caused by necessary periodic arousal and warming (Carey *et al.* 2000). Studies of urate levels in the plasma of hibernators suggest that increases in the production of reactive oxygen species may occur during arousal (Carey *et al.* 2003a). This, in turn, may lead to changes in the expression of antioxidant enzymes in hibernating mammals to prevent cellular damage as body temperature changes. Studies of glutathione peroxidase enzyme activity in 13-lined ground squirrels, however, did not demonstrate a significant difference despite evidence for oxidative stress in hibernators when compared with active individuals (Carey *et al.* 2003b).

An increase in the exposure to reactive oxygen species and subsequent cellular damage has also been demonstrated in studies of cancer (Toyokuni *et al.* 2005). Considering the occurrence of oxidative stress in both cancer cells and mammalian hibernators, techniques used to assay gene expression in response to oxidative stress could be applied to a variety of sample types. The objective of this study, therefore, was to examine the levels of gene expression for the three most critical human antioxidant enzymes – catalase, glutathione peroxidase 1, and superoxide dismutase 1 – in BE(2)-C cells, a neuroblastoma cell line. Neuroblastoma is the most common solid tumor found in children and tumors are often classified into subtypes based on

pathogenicity and prognosis for the afflicted child (Brodeur 2003). The BE(2)-C cells used here are classified as I-type cells, which are phenotypically intermediate between neuroblastic N-type cells and substrate-adherent S-type cells; I-type cells can be induced to differentiate to either cell type and are considered highly malignant (Ross *et al.* 1995). The methods and primers used in this study to examine the levels of antioxidant enzyme gene expression in cancer cells could potentially be applied to tissues from mammalian hibernators.

Materials and Methods

Cell Lines and Treatment

BE(2)-C cells were kindly provided by the Laboratory of Neurobiology at Fordham University. The cells were cultured in MEM with 10% fetal calf serum, 10 μg/mL each of streptomycin and penicillin and incubated at 37°C with 5% CO_2 and humidity. After culturing, the cells were treated by Leleesha Samaraweera with 50 μM H_2O_2 for 6 hours to induce oxidative stress. Treatment with a higher concentration of H_2O_2 or for a longer period of time would have likely induced apoptosis in the BE(2)-C cells.

RNA Extraction

RNA extraction was performed using the RNeasy Plus Mini Kit (QIAGEN) following the manufacturer's protocol and eluting the RNA with 30 μL RNase-free dH_2O. The RNA concentration of each eluate was measured using a spectrophotometer (λ = 260 nm). The eluate of RNA extracted from treated BE(2)-C cells was determined to have an RNA concentration of

135.96 ng RNA/μL and an OD 260/280 reading of 1.723, while the eluate of RNA extracted from untreated cells was determined to have an RNA concentration of 168.68 ng RNA/μL and an OD 260/280 reading of 1.768. These measurements were subsequently used to prepare dilutions of the extracted RNA to final concentrations of approximately 20 ng RNA/μL.

Primers

Three pairs of primers were designed to amplify regions of the genes for three antioxidant enzymes found in human cells – catalase, glutathione peroxidase 1, and superoxide dismutase 1. Sequence information and expected product sizes of both cDNA and genomic DNA are provided (Table 1). Expected sizes of genomic DNA products are provided because the primer pairs designed were capable of amplifying both cDNA and genomic DNA. If a large band was observed on a 1% agarose gel following the RT-PCR reaction, its size would have permitted its identification as genomic DNA.

The forward primer for catalase (GenBank Accession Number NM_001752) was located in exon 11 and the reverse primer was located in exon 13. The forward primer for glutathione peroxidase 1 (GenBank Accession Number NM_000581) was located in exon 1 and the reverse primer was located in exon 2. The forward primer for superoxide dismutase 1 (GenBank Accession Number NM_000454) was located in exon 4 and the reverse primer was located in exon 5. All designed primers were ordered from Invitrogen and diluted upon arrival to a concentration of 10 pmol/μL. Additionally, primers previously designed to amplify a 226 bp region of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) spanning exons 2 to 4 were kindly provided by Bo Liu. The expression of this gene is not expected to change in response to

oxidative stress, allowing it to act as a loading control. A loading control is used to demonstrate that differences observed following agarose gel electrophoresis are due to differences in the expression of the gene of interest, and not to differences in the amount of RNA loaded into each PCR tube.

RT-PCR and Product Analysis

Using materials from the OneStep RT-PCR Kit (QIAGEN), a master mix was prepared for each pair of primers containing 5 μL 5X buffer, 1 μL dNTP mix, 1 μL enzyme mix, 10 pmol forward primer, 10 pmol reverse primer, and 15 μL RNase-free dH_2O per reaction. After preparation, 24 μL of the master mix was added to PCR tubes, and 1 μL (20 ng) of RNA extract was added to each PCR tube for a total reaction volume of 25 μL. The PCR tubes were then placed in a thermal cycler set as follows: 50°C for 30 minutes and 95°C for 15 minutes to carry out reverse transcription; cycles of 94°C, 55°C, and 72°C for 30 seconds each to denature the cDNA, anneal primers to the denatured cDNA strands, and elongate the fragments of cDNA, respectively; 72°C for 10 minutes to complete the elongation of cDNA products, and a holding temperature of 4°C until RT-PCR products could be analyzed using agarose gel electrophoresis. Reaction mixtures containing primers to amplify antioxidant enzymes were allowed to run for 45 cycles, while reaction mixtures containing primers to amplify GAPDH were allowed to run for 30 cycles.

After the addition of 10 μL loading dye to each PCR tube, RT-PCR products were analyzed by loading 8 μL of each reaction mixture on a 1% agarose gel prepared with ethidium

bromide and allowing the gel to run for 45 minutes at 130 Volts. Bands were visualized using ultraviolet light.

RT-PCR Product Purification and Sequencing

After bands of the expected sizes for each antioxidant enzyme were viewed on a 1% agarose gel, RT-PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN) following the manufacturer's protocols. The double stranded DNA concentration of each purified product was determined using a spectrophotometer (λ = 260 nm) and mixtures to be sent for sequencing were prepared by combining 20 ng cDNA, 10 pmol primer, and dH_2O to a total volume of 12 μL. Samples of untreated BE(2)-C cells were sent to Genewiz, Inc. for sequencing and the alignment of sequenced DNA was verified using the NCBI Nucleotide BLAST search tool.

Results

Complementary DNA was generated and amplified using RNA purified from BE(2)-C cells that were either treated with H_2O_2 to induce oxidative stress or were not treated. Primers designed to amplify portions of three antioxidant enzymes – catalase, glutathione peroxidase 1, and superoxide dismutase 1 – did amplify the expected fragments as bands produced were of the sizes predicted when using the NCBI PrimerBLAST search tool (Figure 1). Products generated were not from genomic DNA, which the primers used also would have been able to amplify, as the predicted band sizes for such products were much larger than those obtained (Table 1). RT-

PCR products from untreated cells were then purified and sent for sequencing, and resulting sequences were assessed using the NCBI Nucleotide BLAST database (Figure 2). The sequenced RT-PCR products for catalase, glutathione peroxidase 1, and superoxide dismutase 1 were 98%, 100%, and 99% homologous with the targeted human genes, respectively. The high sequence homology confirmed that the primers used had amplified the regions desired.

Differences in band density between treated and untreated BE(2)-C cells were observed after two sets of RT-PCR reactions for both catalase (Figure 1A) and superoxide dismutase 1 (Figure 1C), but were not observed for glutathione peroxidase 1 (Figure 1B). Band density was consistent for GAPDH across all reactions (Figure 1). GAPDH was used as a loading control to show whether differences observed were due to gene expression or rather in the amount of RNA added to each RT-PCR mixture. If the appearance of GAPDH bands had differed between treated and untreated cells, observed differences in antioxidant enzyme bands would not be compelling as they could be due to error in the preparation of RT-PCR mixtures rather than differences in expression in response to oxidative stress.

Discussion

Oxidative stress can lead to an increase in the presence of reactive oxygen species in cells, which may subsequently lead to an increase in the expression of genes encoding antioxidant enzymes (Michiels *et al.* 1994). Differences in band density between treated and untreated BE(2)-C cells when amplifying portions of both catalase and superoxide dismutase suggest that these enzymes may be differentially expressed in cells subjected to oxidative stress compared with those that are not. No difference was observed in the expression of glutathione

peroxidase, which is consistent with Carey and colleagues' results obtained when examining the activity of this enzyme in torpid and active ground squirrels (2003b). The differences in the enzymes' responses to oxidative stress may be due to the balance that must be achieved among the critical antioxidant enzymes to enhance protection of the cell under stressful conditions (Michiels *et al.* 1994).

Future studies could include the use of real-time RT-PCR to more accurately quantitate changes in gene expression, and could include different levels of oxidative stress by adjusting the treatment protocol used. Adjusting the treatment protocol so that cells are exposed to different degrees of oxidative stress may further elucidate when antioxidant enzymes respond to stress by increasing gene expression and what level of stress would be cytotoxic. Additionally, whole organism studies using model species such as *Caenorhabditis elegans* and the use of tissues from hibernating and active mammals would further elucidate cellular fluctuations in response to physiological stressors.

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Tables and Figures

Table 1

Primer Pair	mRNA Position	Sequence (5' → 3')	Expected Size (cDNA)	Genomic DNA
CAT	1446-1468	F - CAGAGGAAACGTCTGTGTGAGAC	195 bp	3107 bp
	1640-1620	R - CAAGTGAGATCCGGACTGCAC		
GPX	338-357	F - CGCCAAGAACGAAGAGATTC	224 bp	836 bp
	561-543	R - CAACATCGTTGCGACACAC		
SOD	139-156	F - CTAGCGAGTTATGGCGAC	272 bp	7527 bp
	410-392	R - CATTGCCCAAGTCTCCAAC		
GAPDH	108-125	F - GAAGGTGAAGGTCGGAGT	226 bp	433 bp
	333-314	R - GAAGATGGTGATGGGATTTC		

Table 1: Primers used in this study, including mRNA position, sequence information, and predicted band size for both mRNA products and genomic DNA products.

Figure 1

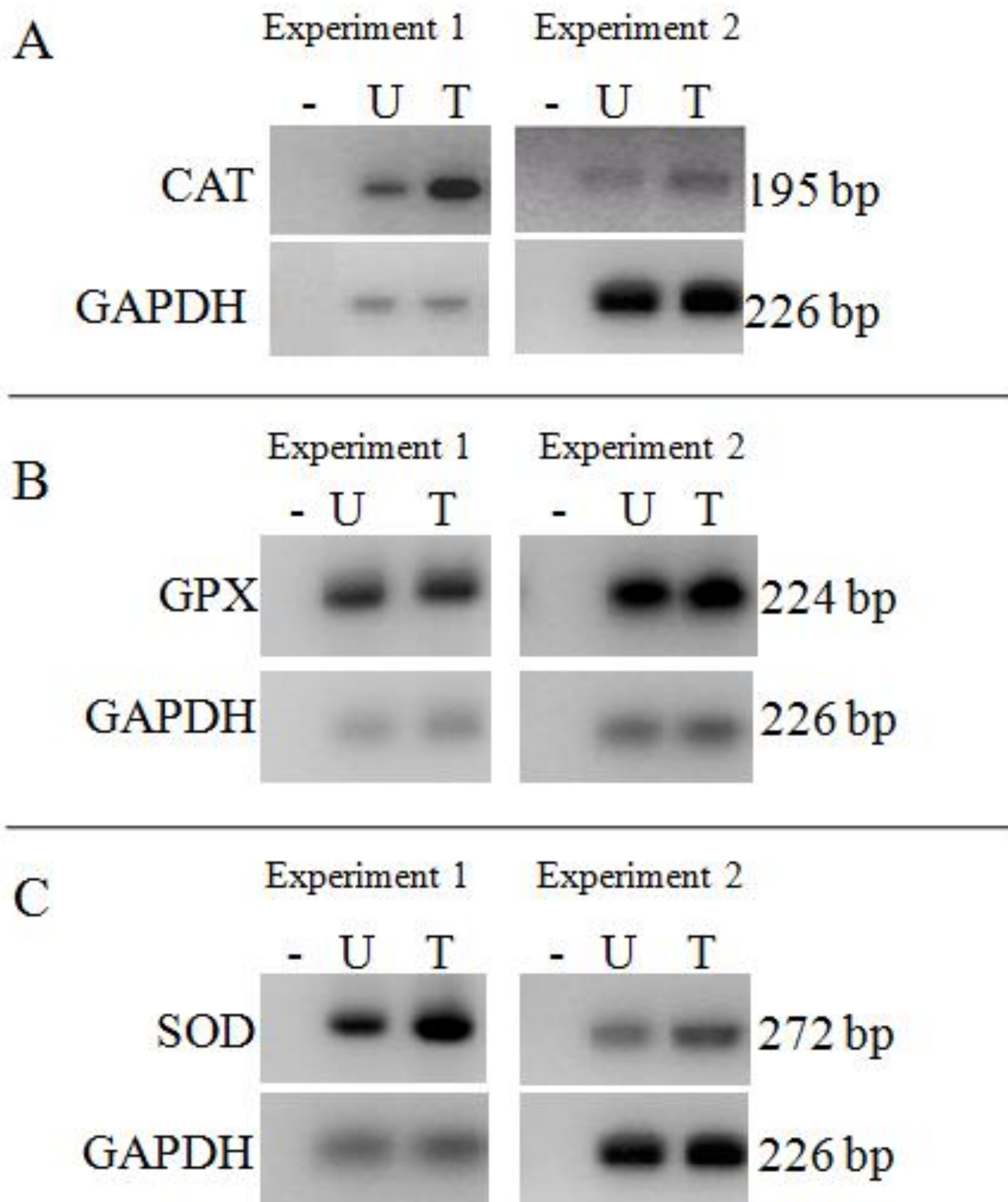

Figure 1: RT-PCR amplification of the genes encoding human enzymes (A) catalase, (B) glutathione peroxidase 1, and (C) superoxide dismutase 1, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control in all instances. Treated (T) cells were exposed to 50 μM H_2O_2 for 6 hours, while untreated (U) cells were not exposed to H_2O_2.

Figure 2

A

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Query  8     ATCGCATTCTTAGGCGTCTCAGCATTGTACTTGTCCAGAAGAGCCTGGATGTGGCTCCCG  67
             ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1609  ATCGCATTCTTAGGCTTCTCAGCATTGTACTTGTCCAGAAGAGCCTGGATGTGGCTCCCG  1550

Query  68    TAGTCAGGGTGGACCTCAGTGAAGTTCTTGACCGCTTTCTTCTGGATGAAAATTTGTGCA  127
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1549  TAGTCAGGGTGGACCTCAGTGAAGTTCTTGACCGCTTTCTTCTGGATGAAAATTTGTGCA  1490

Query  128   TCCTTCAGGTGGCCGGCAATGGTCTCACACAGACGTTTCCTCTG  171
             ||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  1489  TCCTTCAGGTGGCCGGCAATGTTCTCACACAGACGTTTCCTCTG  1446
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B

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Query  2    GTCGCTGGGAGCTGGCAGGGCCTCCCGCAGGAAGGCGAAGAGAGGGTGCGCCCCCGCACC  61
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  494  GTCGCTGGGAGCTGGCAGGGCCTCCCGCAGGAAGGCGAAGAGAGGGTGCGCCCCCGCACC  435

Query  62   GTTCACCTCGCACTTCTCGAAGAGCATGAAGTTGGGCTCGAACCCACCACCAGGCCGGAC  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  434  GTTCACCTCGCACTTCTCGAAGAGCATGAAGTTGGGCTCGAACCCACCACCAGGCCGGAC  375

Query  122  GTACTTGAGGGAATTCAGAATCTCTTCGTTCTTGGCG  158
            |||||||||||||||||||||||||||||||||||||
Sbjct  374  GTACTTGAGGGAATTCAGAATCTCTTCGTTCTTGGCG  338
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C

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Query  14   GCGACGGCCCAGTGCAGGGCATCATCAATTTCGAGCAGAAGGAAAGTAATGGACCAGTGA  73
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  180  GCGACGGCCCAGTGCAGGGCATCATCAATTTCGAGCAGAAGGAAAGTAATGGACCAGTGA  239

Query  74   AGGTGTGGGGAAGCATTAAAGGACTGACTGAAGGCCTGCATGGATTCCATGTTCATGAGT  133
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  240  AGGTGTGGGGAAGCATTAAAGGACTGACTGAAGGCCTGCATGGATTCCATGTTCATGAGT  299

Query  134  TTGGAGATAATACAGCAGGCTGTACCAGTGCAGGTCCTCACTTTAATCCTCTATCCAGAA  193
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  300  TTGGAGATAATACAGCAGGCTGTACCAGTGCAGGTCCTCACTTTAATCCTCTATCCAGAA  359

Query  194  AACACGGTGGGCCAAAGGATGAAGAGAGGCATGTTGGAG-CTTGGGCAATG  243
            ||||||||||||||||||||||||||||||||||||||| |||||||||||
Sbjct  360  AACACGGTGGGCCAAAGGATGAAGAGAGGCATGTTGGAGACTTGGGCAATG  410
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Figure 2: Partial alignment of purified RT-PCR products and previously published sequences for human enzymes (A) catalase, (B) glutathione peroxidase 1, and (C) superoxide dismutase 1. A search using the NCBI Nucleotide BLAST database revealed high sequence homology.