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Differential gene expression in *Giardia lamblia* under oxidative stress: Significance in eukaryotic evolution

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ABSTRACT

Giardia lamblia is a unicellular, early branching eukaryote causing giardiasis, one of the most common human enteric diseases. *Giardia*, a microaerophilic protozoan parasite has to build up mechanisms to protect themselves against oxidative stress within the human gut (oxygen concentration 60 μM) to establish its pathogenesis. *G. lamblia* is devoid of the conventional mechanisms of the oxidative stress management system, including superoxide dismutase, catalase, peroxidase, and glutathione cycling, which are present in most eukaryotes. NADH oxidase is a major component of the electron transport chain of *G. lamblia*, which in concurrence with disulfide reductase, protects oxygen-labile proteins such as pyruvate: ferredoxin oxidoreductase against oxidative stress by sustaining a reduced intracellular environment. It also contains the arginine dihydrolase pathway, which occurs in a number of anaerobic prokaryotes, includes substrate level phosphorylation and adequately active to make a major contribution to ATP production.

To study differential gene expression under three types of oxidative stress, a *Giardia* genomic DNA array was constructed and hybridized with labeled cDNA of cells with or without stress. The transcriptomic data has been analyzed and further validated using real time PCR. We identified that out of 9216 genes represented on the array, more than 200 genes encoded proteins with functions in metabolism, oxidative stress management, signaling, reproduction and cell division, programmed cell death and cytoskeleton. We recognized genes modulated by at least ≥2 fold at a significant time point in response to oxidative stress.

The study has highlighted the genes that are differentially expressed during the three experimental conditions which regulate the stress management pathway differently to achieve redox homeostasis. Identification of some unique genes in oxidative stress regulation may help in new drug designing for this common enteric parasite prone to drug resistance. Additionally, these data suggest the major role of this early divergent ancient eukaryote in anaerobic to aerobic organism evolution.

1. Introduction

Giardia lamblia, a biflagellate gastro-intestinal parasite in Diplomonadida causes giardiasis that affects 300 million people worldwide (Ortega and Adam, 1997). *Giardia* is a microaerophillic organism and does not usually tolerate elevated oxygen level. In the upper intestinal lining, where this organism generally resides, the O_2 concentration has been measured at 60 μM (Atkinson, 1980). On the other hand *Giardia* is capable of tolerating 0–50 μM dissolved O_2 (Lloyd et al., 2000). The detailed mechanism, by which the parasite could aid in the detoxification of reactive oxygen species (ROS) produced during an oxidative stress, is still not clear. In addition to this, some of the conventional enzymes of detoxifying ROS, such as superoxide dismutase (SOD), catalase, peroxidase, glutathione, glutathione reductase are absent in *G. lamblia* (Brown et al., 1995). However, it possesses a prokaryotic H_2O-producing NADH oxidase, a membrane associated NADH peroxidase, a broad-range prokaryotic thioredoxin reductase-like disulfide reductase and the low molecular weight thiols, L-cysteine, thioglycolate, sulfite and coenzyme-A (Brown et al., 1998). Cysteine is the major low molecular weight thiol in *G. lamblia* and reduced thiols serve as a defense against oxidative stress and as a mechanism to maintain a reduced intracellular environment. L-Cysteine has antioxidant properties, and is used for biosynthesis of glutathione, which is found in most eukaryotes including humans.

Oxidative stress triggers a range of physiological, pathophysiological, and adaptive responses in cells either as a result of cellular damage or

Abbreviations: ROS, reactive oxygen species; NADH, (reduced) nicotinamide adenine dinucleotide; PCD, programmed cell death protein like protein; TYIS-33, tryptone–yeast extract–iron–serum-33; PBS, phosphate buffered saline; PCR, polymerase chain reaction; HEPES, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid; NADPH, (reduced) nicotinamide adenine dinucleotide phosphate; ATP, adenosine tri-phosphate; VSP, variant specific surface protein; SOD, superoxide dismutase; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; DCF, 2′,7′-dichlorofluorescein; SSC, standard saline citrate; dUTP, deoxyuridine triphosphate; dTTP, deoxythymidine triphosphate; RT-PCR, real time PCR; GSH, glutathione; SRP-64, signal recognition particle-64; PCV, protein for cell viability.

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through a specific signaling molecule. These responses ultimately modulate transcriptional outputs to influence cell fate and disease processes. In the past two decades, a number of transcription factors and signaling pathway have been identified and delineated to mediate critical transcriptional responses to oxidative stress. These examples demonstrate the importance as well as the complexity of how alterations in intracellular ROS are converted into discrete and reproducible alterations in gene expression.

Recently, it has become possible to decipher transcriptional programs of an organism by studying gene expression en masse (Brown and Botstein, 1999). Differences in cell types or states are correlated with changes in the mRNA levels of many genes (DeRisi et al., 1997). DNA-microarray technology provides an opportunity to look simultaneously at changes in gene expression in thousands of genes under different physiological conditions (DeRisi and Iyer, 1999). In the present study, we performed DNA microarray analysis of gene expression in stressed *G. lamblia* trophozoites by H_2O_2, metronidazole and also given stress with a cysteine–ascorbate deprived medium. The differentially regulated genes fall into five groups of functionally related proteins. These functional categories are: (i) metabolic enzymes; (ii) structural proteins; (iii) kinases and phosphatases; (iv) cell cycle and proliferation controller; and (v) cell death regulators.

2. Materials and methods

2.1. Maintenance of cultures

G. lamblia trophozoites were maintained in TYIS-33 medium that was supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), and 10% adult bovine serum, according to the methods of Diamond et al. (1978). All of the experiments were conducted with trophozoites that had been harvested during the logarithmic phase of growth.

2.2. Activation of trophozoites with oxidative stress

In the present study, three conditions have been chosen to generate oxidative stress in the trophozoites *in vitro*. First, hydrogen peroxide (H_2O_2) is a very well known chemical reagent that can generate free oxygen radicals very easily (Lindley et al., 1988). Secondly, metronidazole is a commonly used drug against giardiasis in most developing countries. Its mode of action involves free nitro radical generation within the organism (Brown et al., 1998). The third, is the modified medium that is devoid of cysteine and ascorbate. Cysteine has been found to protect *G. lamblia* trophozoites from thiol-blocking reactants, indicating a role as a reducing agent for the protection of crucial thiol groups. Ascorbic acid also protects the trophozoites under high PO_2 (Tekwani and Mehlotra, 1999). Dose and time kinetics of the three different oxidative stress generating conditions has been standardized following the IC_{50} values according to Lindley et al. (1988) and Sadhu et al. (2004). Finally, from standardized data, 0.1 μM H_2O_2 and 1 μg/ml metronidazole have been administered to all the experiments mentioned below.

2′,7′-Dichlorodihydrofluorescein diacetate (DCFDA) assay was performed to observe the ROS generation in each of the above cases. Briefly, 5×10^6 trophozoites of *Giardia* for each set (control and three oxidative stressed sets) were collected and dissolved in PBS. 2 μl of DCFDA (stock 100 μM) was added in each case. Then all the sets were incubated for 15 min at 37 °C in the dark. The samples were washed thrice and finally slides were prepared for confocal study.

2.3. Genomic shotgun library

Genomic DNA was isolated from 10^8 *G. lamblia* trophozoites using the method of Huber et al. (2001). RNA contamination was removed by RNase digestion for 30 min at 37 °C. Genomic DNA was sheared for 30, 60, 80, and 120 s by use of a nebulizer (Invitrogen) which was connected to the laboratory's compressed-air line. The sheared DNA was blunt-end repaired by use of T4 DNA and Klenow polymerases, and the ends were dephosphorylated by using calf intestinal phosphatase, to create a suitable substrate for TOPO cloning (Invitrogen). DNA fragments were ligated into PCR4 Blunt-TOPO vector (Invitrogen). The cloning reaction was performed for 5 min at room temperature, with undiluted and with 3- and 9-fold serially diluted blunt-end DNA; 3.3 ml of precipitated TOPO cloning reaction was electroporated into 50 ml of One Shot TOP10 Eletrocomp *Escherichia coli* (Invitrogen), and the transformants were incubated overnight on agar plates in the presence of X-gal and 100 mg/ml ampicillin. To check the quality, specificity and integrity of the genomic DNA library, ~250 clones were randomly chosen and sequenced (data not shown). Gene repetition and fragmentation were at minimum level.

2.4. G. lamblia microarray

Individual white colonies from the fresh random-fragment genomic DNA library were transferred to a 600 μl solution of Luria–Bertani medium (with 100 μg/ml ampicillin) in individual wells of a 96-well plate and were incubated for 18 h at 37 °C; 1 μl from each well was used for amplification of the insert by PCR, using the M13 forward and reverse primers. After analysis of the PCR products on 1% agarose gels, the products were consolidated onto 96-well plates. The PCR products from the 96-well plates were precipitated, washed, and transferred to 384-well plates and were printed on the aminisilane-coated glass slides, by use of the GeneMachine®. The concentration of each DNA product in 3× standard saline citrate (SSC: 1× SSC is 0.15 mol/l NaCl, 0.015 mol/l sodium citrate) printing solution was 200 ng/μl, which produced acceptable spot quality. After the DNA solution was deposited on every slide, the tips were washed with 0.5× SSC and dried, and the process was repeated for the next set of DNA samples, with the new spots offset a small distance (200 mm) relative to previous spots, to produce a high-density grid. Microarrays were printed under controlled environmental conditions (*i.e.*, a temperature of 19 °C and a relative humidity of 50%). At the end of the print run, the slides were allowed to dry for ~12 h.

2.5. RNA isolation, labeled cDNA preparation, and microarray hybridization

Total RNA was isolated from ~5×10^7 trophozoites by use of a TRIZOL kit (Invitrogen) and as directed by the manufacturer. Optical density readings were taken at 260 nm and 280 nm. The integrity of total RNA was checked in denatured formaldehyde gel, according to standard protocol (Sambrook et al., 1989). Fluorescently labeled cDNA copies of the total RNA pool were prepared from both oxidative stress-induced and non-induced *G. lamblia*. In brief, 30 μg of total RNA was reverse transcribed by using Stratascript reverse transcriptase (Stratagene), 10 μg/μl of oligo-dT 0.1 M dithiothreitol, and a 3:2 ratio of amino-allyl dUTP to dTTP, for 2 h at 42 °C. After hydrolysis of the RNA, the samples were purified by the use of a Qiagen® column. The monofunctional NHS-easte Cy-3 and Cy-5 dyes were coupled with the cDNA, and the unincorporated/quenched Cy-dyes were removed by the use of a column; the Cy-5 labeled sample was eluted into the tube containing the corresponding Cy-3 labeled sample. The two cDNA pools to be compared were mixed and were applied to the array in a hybridization mixture containing 3× SSC, 25% SDS, and 25 mmol/l HEPES (Ph 7.0). Hybridization took place in the Hybstation from Genomic Solutions® by setting a standardized protocol.

2.6. Data acquisition, analysis and sequencing

Arrays were scanned by use of ScanExpressHT (Perkin-Elmer); images were acquired by scanning with the Cy3 and Cy5 channels at a resolution of 5 mm. The Cy5/Cy3 fluorescence ratios and log10-transformed ratios