[Frontiers in Bioscience S4, 1556-1567, June 1, 2012]

Transcriptomic analysis reveals pH-responsive antioxidant gene networks

Rodrigo Juliani Siqueira Dalmolin[1], Daniel Pens Gelain[1], Fabio Klamt[1], Mauro Antonio Alves Castro[1], Jose Claudio Fonseca Moreira[1]

[1]Unidade de Bioinformatica, Centro de Estudos em Estresse Oxidativo, Departamento de Bioquimica, Universidade Federal do Rio Grande do Sul (UFRGS), Rua Ramiro Barcelos 2600-anexo, Porto Alegre 90035-003, Brazil

TABLE OF CONTENTS

1. ABSTRACT

Reactive oxygen species (ROS) are produced in different physiological conditions. In response to ROS imbalance cells activate oxidative stress defenses, which include more than 60 antioxidant genes. It has been suggested that gene products associated with ROS detoxification can work coordinately, acting as an antioxidant-defense network. However, the functional overlap among oxidative stress defenses and other related cell functions makes difficult the characterization of this network. We previously described a network-based model to characterize the interactions existing among different antioxidant gene products and their substrates. Here, we test whether this network-based model of human antioxidant genes can respond to different physiological conditions. We used a systems biology approach applied to the analysis of two independent gene expression datasets: transcriptomes from HeLa cells and primary astrocytes maintained under hypoxic conditions and transcriptomes from SKGT4 cells exposed to low pH environment. We found that the proposed gene network model responds selectively to both hypoxia and acidosis. We anticipate that this antioxidant gene network model can be helpful to describe stress-responsive expression profiles in different cell types.

2. INTRODUCTION

Reactive oxygen species (ROS) are produced during normal cellular metabolism. ROS mediate diverse physiological responses by acting in several signaling pathways (1-3). In addition, ROS overproduction may lead to cell oxidative damage, playing a causative role in numerous diseases (4). To maintain the homeostasis between ROS production and detoxification, cells developed a complex antioxidant apparatus including endogenous and exogenous components.

The most classical enzymatic antioxidant defense includes catalase, superoxide dismutase and glutathione peroxidase (5-6). They are representative members of enzyme families that have many other constituents (*e.g.* GPX family members). In addition, non-enzymatic components (such as thiol-containing proteins) play essential role in endogenous antioxidant defenses (7-8).

Although gene products associated with antioxidant defenses are in general investigated individually, increasing evidences indicate that they work coordinately as a network (9-12). However, the functional overlap among antioxidant defenses and a number of related cell functions – together with cell

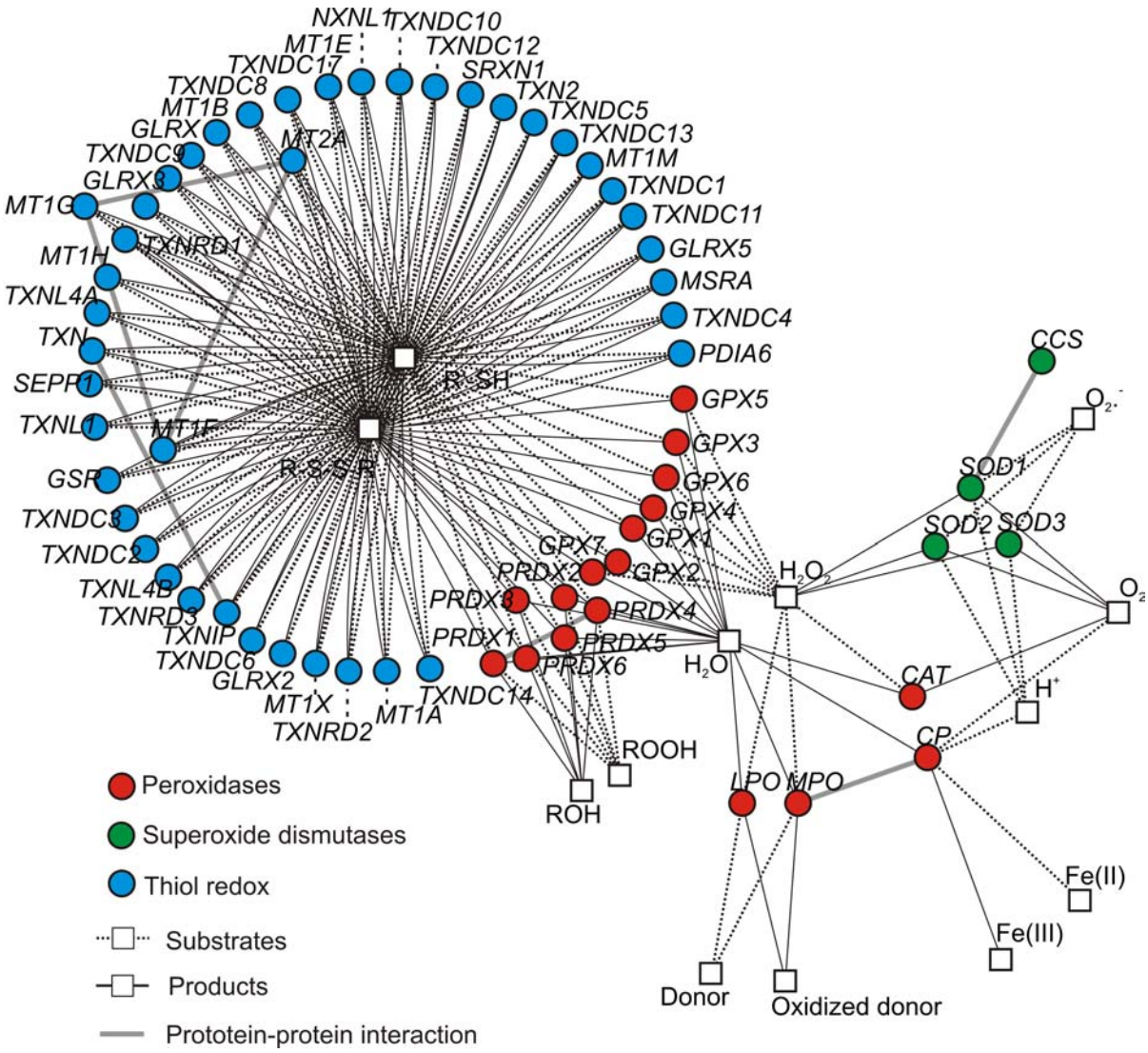

Figure 1. Network-based model of human antioxidant genes. Edges correspond to interactions existing among different antioxidant genes and their substrates as defined previously (8). All genes with prime antioxidant function are considered together with their substrate/product reactions to construct the network interactions (adapted from Gelain et al. (8) and available in www.ufrgs.br/icbs/hag).

compartmentalization of antioxidant key elements – makes difficult the precise characterization of such network. One possible approach to study complex and entangled networks is the use of gene expression data from high-throughput experiments (13).

Gene expression networks are frequently linked with the traditional concept of regulatory pathways that describe cascade of regulatory events and provide the basis for genetic network reconstruction (14). However, such traditional concept has its limitations, since it aims to study properties of individual network components rather than consider all components and their interactions simultaneously (15-16). In order to evaluate the systemic properties of antioxidant gene networks we previously described a network-based model of human antioxidant genes characterizing the interactions existing among different antioxidant key components and its substrates (Figure 1) (7-8). In this model, all genes with prime antioxidant function are considered together with their substrate/product reactions to construct the network interactions.

Here, we accessed the gene expression activity of the antioxidant gene network components in HeLa cells and primary astrocytes submitted to hypoxic condition to test our network-based model. We also analyzed the gene expression activity of the antioxidant gene network in esophageal adenocarcinoma SKGT4 cells exposed to low pH environment. We have found that the proposed antioxidant gene network responds selectively to hypoxia in HeLa cells and low pH treatment in SKGT4 cells.

3. METHODS

3.1. Network-based model of human antioxidant genes

The network-based model of human antioxidant genes has been previously described (7-8) and is provided at the Human Antioxidant Genes (HAG) database (www.ufrgs.br/icbs/hag). The HAG website shows a manually curated set of genes involved in antioxidant defenses. The gene list is classified as following: (1) genes whose products are widely accepted as being primarily antioxidant enzymes; (2) genes whose products may not be antioxidant enzymes, but are also recognized as having a primary antioxidant function; (3) genes whose products are recognized as having a primary role in other physiologic processes, but are known to physically interact with reactive species, also converting them into less or non-toxic products, through redox reactions carried by functional groups; and (4) genes whose products were described to maintain exclusive, specific interactions with the gene products described above, being essential for the antioxidant function of such proteins. In total, 63 antioxidant genes are listed in the gene network, which is subdivided according to three categories: *i*) peroxidases (17 genes), *ii*) superoxide dismutases (4 genes), and *iii*) thiol redox (42 genes). These gene sets hereby are referred to as Groups of Functionally Associated Genes (GFAG). Each gene in the network is identified according to the approved HGNC gene symbol (Figure 1) (17). The network showing edge relationships was handled in Medusa software (18). Functional categories are discriminated by different colors and protein data are crossed with substrates in order to integrate the network. For this purpose we considered the substrates annotated for each gene product in KEGG database (19).

3.2. Data selection

Microarray data of HeLa cells were obtained from GEO database, series GSE3051 (http://www.ncbi.nlm.nih.gov/projects/geo/). This dataset comprises 6 arrays (Affymetrix GeneChip Human Genome U133 Plus 2.0 Array), as previously described (20). The experimental design provides three independent sample-sets grown under normoxic (95% air-5% CO2) and three independent sample-sets grown under hypoxic conditions (1% O2-5% CO2-94% N2) for 24 hours. Microarray data of human primary astrocytes follow the same experimental design (GEO database, series GSE3045). Microarray data of esophageal adenocarcinoma SKGT4 cell line were also obtained from GEO database, series GSE2144 (http://www.ncbi.nlm.nih.gov/projects/geo/). This dataset comprises 10 arrays (Affymetrix GeneChip Human Genome U133 Array Set HG-U133A), as previously described (21). This study was originally designed to identify pathways involved in the progression of gastro-esophageal reflux disease through Barrett's esophagus to adenocarcinoma. The SKGT4 esophageal cells were stimulated with Low pH environment (pH=6.5) for 30, 120, 180 and 240 minutes (n=2 for each treatment time; further details please see (21) and GEO database). Each sample contemplates 50 antioxidant genes listed in HAG website. Microarray data are provided as normalized raw signal (MAS5.0) (20-21).

3.3. Statistical analysis

The statistics used to describe the activity and diversity of a given gene set has been previously described (13). Briefly, to obtain a quantitative measure to characterize the regulation of each GFAG in the samples we accessed the information content using Shannon's entropy (22-23) defined as follows. Consider M as the number of genes in the GFAG α (α = 1,…, n). For a given GFAG, we can define $s(i,\alpha)$ as being the signal of a given gene i, (i = 1,…, $M\alpha$), whose sum for a given α adds up to $N\alpha$. The contribution $p(i,\alpha)$ of the signal of gene i to the total signal of the α-GFAG is

$$p(i,\alpha) = \frac{s(i,\alpha)}{N_\alpha} \quad , \quad (1)$$

such that $\sum_i p(i,\alpha) = 1$ ..... $H\alpha$, hereby called diversity, is defined as

$$H_\alpha = -\frac{1}{\ln(M_\alpha)} \sum_i^{N_\alpha} p(i,\alpha) \ln p(i,\alpha) \quad , \quad (2)$$

where we have divided all terms by the normalization factor $\ln(M\alpha)$ to guarantee that $0 \le H\alpha \le 1$. In this way we can compare different GFAGs that may present different numbers of genes. Finally, in order to normalize the quantities by groups of genes, taking as reference the diversity of the signal of the control, we define the relative diversity $h\alpha$ for any given GFAG as

$$h_\alpha = \frac{H_\alpha^e}{H_\alpha^e + H_\alpha^\gamma} \quad , \quad (3)$$

where H_α^e - H_α^γ ..... experiment and control (*i.e.* GFAGs from treatment and control microarrays). In analogy, the relative gene expression activity $n\alpha$ of the α-GFAG is defined as

$$n_\alpha = \frac{N_\alpha^e}{N_\alpha^e + N_\alpha^\gamma} \quad , \quad (4)$$

where N_α^e and N_α^γ are, respectively, the gene expression activity of experiment and control. To determine the significantly altered GFAGs we used resampling analysis with replacement (bootstrapping) with 50.000 replications in order to estimate the sampling distribution of both $h\alpha$ and $n\alpha$ in the microarray dataset (this bootstrap number guarantee convergence without fluctuations). Given that this analysis considers genes in the context of functional groups, the statistical design is set to compare groups of genes (*i.e.* GFAGs). Therefore, to understand the significance boundaries, it is essential to observe that the bootstrap distribution produces random groups with the same size of the GFAGs (*i.e.* the number of genes seeded in the bootstrap is the same of the GFAG tested). This procedure can detect other aspects of the transcriptome

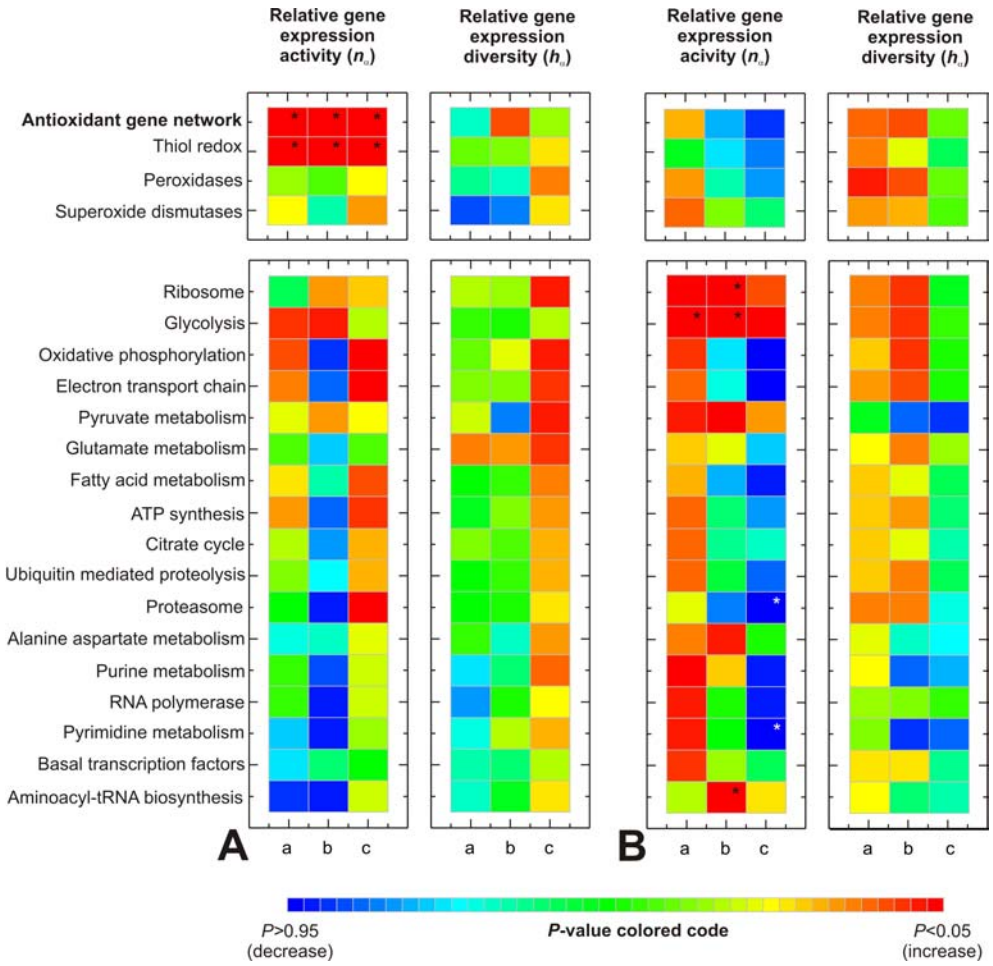

Figure 2. Antioxidant gene network function in HeLa cells and primary astrocytes maintained under hypoxic conditions. Relative gene expression activity ($n\alpha$) and diversity ($h\alpha$) of antioxidant genes (upper panels) and essential cell functions (lower panels) in HeLa cells (a) and human primary astrocytes (b) maintained under hypoxic conditions (1% O2-5% CO2-94% N2) for 24 h in three independent experiments (a, b, and c: n=3). The panels are color-coded according to the P-value obtained in bootstrap analysis for each Group of Functional Associated Genes (GFAG) compared with normoxic maintained groups. The raw P-values from the bootstrap analysis were controlled for multiple comparisons by False Discovery Rate (FDR) analysis. * Significant differential expression with FDR<0.05.

functioning, possible not detect in the typical single-gene analysis. The raw P-values from the bootstrap analysis were controlled for multiple comparisons by False Discovery Rate (FDR) analysis (24). This procedure was used to identify GFAGs exhibiting significant differential gene expression profiles with a FDR no greater than 5% (*i.e.* a 5% FDR indicates that among all GFAGs identified as being differentially expressed, 5% of them are truly not significant). All data analysis was carried out in the ViaComplex software (25).

4. RESULTS

4.1. Antioxidant gene network activity in hypoxic conditions

The relative gene expression activity ($n\alpha$) and diversity ($h\alpha$) of the antioxidant genes in HeLa cells and human primary astrocytes are presented in Figure 2. In HeLa cells (Figure 2a, upper panels), the antioxidant gene activity is significantly altered in all three independent experiments (a, b and c). It shows that HeLa cells submitted to hypoxia have increased the gene expression activity when compared to normoxic controls (P_a=0.01504, FDR_a=0.025; P_b=0.02216, FDR_b=0.025; P_c=0.0062, FDR_c=0.025), and this increase is concentrated in thiol redox functions (P_a=0.0056; FDR_a=0.0125; P_b=0.005, FDR_b=0.0125; P_c=0.00392, FDR_c=0.0125). In order to test whether these results are associated with large-scale and non-specific gene expression changes we also quantified the gene expression activity of several essential pathways (Figure 2a, lower panels). According to KEGG database (19), this panel provides at least one pathway that may characterize the gene expression status of the following cell metabolic functions: *i*) carbohydrate metabolism, *ii*) energy metabolism, *iii*) lipid metabolism, *iv*) nucleotide metabolism, *v*) amino acid metabolism, vi) transcription, vii) translation, and *viii*) folding, sorting and degradation.

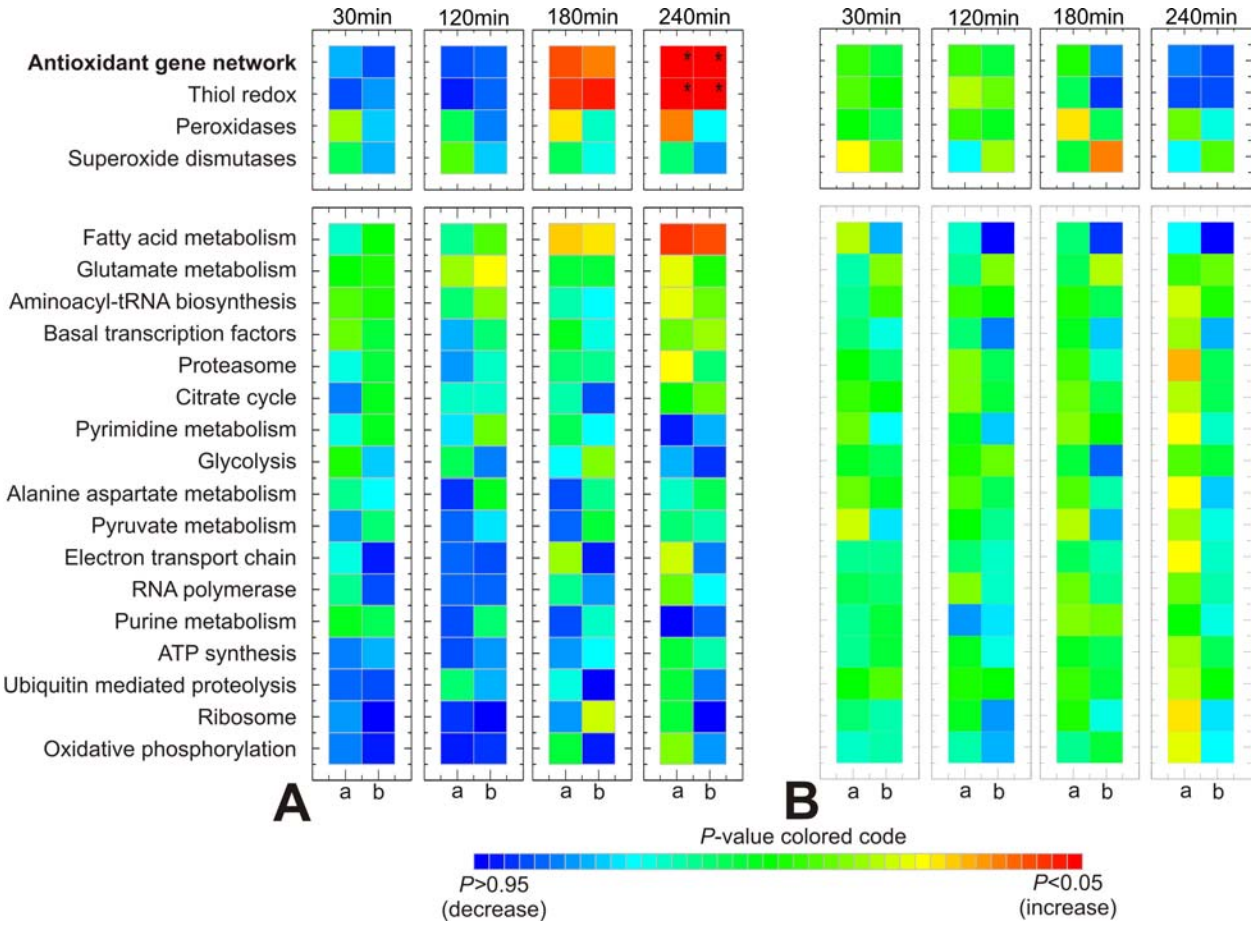

Figure 3. Antioxidant gene network function in SKGT4 cells maintained in low pH environment (pH=6.5). Relative gene expression activity ($n\alpha$) (a) and diversity ($n\alpha$) (b) of antioxidant genes (upper panels) and essential cell functions (lower panels) in 30, 120, 180, and 240 min, in low pH environment, in two independent experiments (a and b: n=2 for each treatment time). The panels are color-coded according to the P-value obtained in bootstrap analysis for each Group of Functional Associated Genes (GFAG) compared with physiologic pH-maintained groups. The raw P-values from the bootstrap analysis were controlled for multiple comparisons by False Discovery Rate (FDR) analysis. *Significant differential expression with FDR<0.05.

Accordingly, neither activity nor gene expression diversity of these essential pathways are significantly altered in HeLa cells submitted to hypoxia when compared to normoxic condition (at an FDR < 5%), and in primary astrocytes only one pathway presents gene expression activity significantly increased at an FDR < 5% (n=2) when compared to the control (Figure 2b, lower panels).

4.2. Antioxidant gene network activity in low pH environment

In Figure 3 (upper panels) we present the relative gene expression activity ($n\alpha$) and diversity ($h\alpha$) of the antioxidant GFAGs in SKGT4 cells maintained under low pH condition (pH=6.5). The antioxidant gene sets seem to respond to the low pH environment in a time-dependent manner, showing a progressive increase that is significantly different from the control at 240 min (FDR < 5%) (Figure 3a, upper panels). This result does not seem to be related to global gene expression changes, as indicated by the same analysis on essential pathways (Figure 3a, lower panels). In addition, the gene expression diversity in SKGT4 cells is not significantly altered (at an FDR < 5%) in any evaluated pathway (Figure 3b).

4.3. Gene expression levels of antioxidant genes

In order to compare the gene expression levels among all experiments we normalized the expression values from all microarrays and ordered all genes according to the normalized expression levels in each cell type (HeLa cells, primary astrocytes, and SKGT4 cells). The result of this ranking is depicted in Figure 4 and suggests that the gene expression pattern is similar among the three cell types, that is, genes with high expression levels in HeLa cells also present high expression values in the other two cell types, and vice versa. However, this observation might be biased due to a possible unbalanced distribution of the GFAGs' members (*i.e.* peroxidases, *s*uperoxide dismutases, or thiol redox). Therefore, next we grouped the antioxidant genes by both GFAG membership and gene rank. Such hierarchical subdivision is listed in Table 1 and the relative gene expression activity is depicted in Figure 5. It shows that the response pattern of antioxidant genes is in fact

Table 1. Antioxidant gene groups

	Gene Symbol	Gene Name	Gene Group
ASTROCYTES			
Group A	TXNL4B	thioredoxin-like 4B	Thiol-redox
	MT1M	metallothionein 1M	Thiol-redox
	TXNRD2	thioredoxin reductase 2	Thiol-redox
	TXNRD3	thioredoxin reductase 3	Thiol-redox
	TXNDC3	thioredoxin domain containing 3 (spermatozoa)	Thiol-redox
Group B	TXN2	thioredoxin 2	Thiol-redox
	TXNDC13	thioredoxin domain containing 13	Thiol-redox
	GLRX	glutaredoxin (thioltransferase)	Thiol-redox
	MT1E	metallothionein 1E	Thiol-redox
	GLRX3	glutaredoxin 3	Thiol-redox
	GSR	glutathione reductase	Thiol-redox
	MT1H	metallothionein 1H	Thiol-redox
	MT1G	metallothionein 1G	Thiol-redox
Group C	MT1X	metallothionein 1X	Thiol-redox
	SEPP1	selenoprotein P, plasma, 1	Thiol-redox
	GLRX5	glutaredoxin 5	Thiol-redox
	TXNL4A	thioredoxin-like 4A	Thiol-redox
	GLRX2	glutaredoxin 2	Thiol-redox
	MSRA	methionine sulfoxide reductase A	Thiol-redox
	TXNDC4	thioredoxin domain containing 4 (endoplasmic reticulum)	Thiol-redox
	MT1F	metallothionein 1F	Thiol-redox
Group D	TXNDC5	thioredoxin domain containing 5	Thiol-redox
	TXNL1	thioredoxin-like 1	Thiol-redox
	MT2A	metallothionein 2A	Thiol-redox
	TXN	thioredoxin	Thiol-redox
	TXNDC9	thioredoxin domain containing 9	Thiol-redox
Group E	PDIA6	protein disulfide isomerase family A, member 6	Thiol-redox
	TXNDC1	thioredoxin domain containing 1	Thiol-redox
	TXNDC14	thioredoxin domain containing 14	Thiol-redox
	TXNIP	thioredoxin interacting protein	Thiol-redox
	TXNRD1	thioredoxin reductase 1	Thiol-redox
Group F	GPX2	glutathione peroxidase 2 (gastrointestinal)	Peroxidases
	MPO	myeloperoxidase	Peroxidases
	LPO	lactoperoxidase	Peroxidases
	GPX5	glutathione peroxidase 5 (epididymal androgen-related protein)	Peroxidases
Group G	GPX7	glutathione peroxidase 7	Peroxidases
Group H	PRDX2	peroxiredoxin 2	Peroxidases
Group I	GPX3	glutathione peroxidase 3 (plasma)	Peroxidases
	GPX1	glutathione peroxidase 1	Peroxidases
	PRDX3	peroxiredoxin 3	Peroxidases
	CP	ceruloplasmin (ferroxidase)	Peroxidases
	CAT	catalase	c
ASTROCYTES			
Group J	PRDX6	peroxiredoxin 6	Peroxidases
	PRDX1	peroxiredoxin 1	Peroxidases
	PRDX4	peroxiredoxin 4	Peroxidases
	GPX4	glutathione peroxidase 4 (phospholipid hydroperoxidase)	Peroxidases
Group K	SOD3	superoxide dismutase 3, extracellular	SOD
Group L	CCS	copper chaperone for superoxide dismutase	SOD
Group M	SOD2	superoxide dismutase 2, mitochondrial	SOD
Group N	SOD1	superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult))	SOD
HELA			
Group A	TXNL4B	thioredoxin-like 4B	Thiol-redox
	TXNDC13	thioredoxin domain containing 13	Thiol-redox
	TXNDC3	thioredoxin domain containing 3 (spermatozoa)	Thiol-redox
Group B	TXN2	thioredoxin 2	Thiol-redox
	GLRX2	glutaredoxin 2	Thiol-redox
	TXNDC4	thioredoxin domain containing 4 (endoplasmic reticulum)	Thiol-redox
	GSR	glutathione reductase	Thiol-redox
	TXNRD2	thioredoxin reductase 2	Thiol-redox
	TXNRD3	thioredoxin reductase 3	Thiol-redox
	MSRA	methionine sulfoxide reductase A	Thiol-redox
Group C	TXNDC1	thioredoxin domain containing 1	Thiol-redox
	MT1M	metallothionein 1M	Thiol-redox
	TXNIP	thioredoxin interacting protein	Thiol-redox
	GLRX5	glutaredoxin 5	Thiol-redox
	TXNL1	thioredoxin-like 1	Thiol-redox
	TXNL4A	thioredoxin-like 4A	Thiol-redox
	TXNDC9	thioredoxin domain containing 9	Thiol-redox
	GLRX3	glutaredoxin 3	Thiol-redox
	SEPP1	selenoprotein P, plasma, 1	Thiol-redox
Group D	TXNRD1	thioredoxin reductase 1	Thiol-redox

	MT1F	metallothionein 1F	Thiol-redox
	GLRX	glutaredoxin (thioltransferase)	Thiol-redox
	TXN	thioredoxin	Thiol-redox
	TXNDC14	thioredoxin domain containing 14	Thiol-redox
	MT1G	metallothionein 1G	Thiol-redox
	TXNDC5	thioredoxin domain containing 5	Thiol-redox
Group E	MT2A	metallothionein 2A	Thiol-redox
	MT1X	metallothionein 1X	Thiol-redox
	PDIA6	protein disulfide isomerase family A, member 6	Thiol-redox
	MT1E	metallothionein 1E	Thiol-redox
	MT1H	metallothionein 1H	Thiol-redox
HELA			
Group F	GPX3	glutathione peroxidase 3 (plasma)	Peroxidases
	GPX2	glutathione peroxidase 2 (gastrointestinal)	Peroxidases
	GPX7	glutathione peroxidase 7	Peroxidases
	CP	ceruloplasmin (ferroxidase)	Peroxidases
	MPO	myeloperoxidase	Peroxidases
	GPX5	glutathione peroxidase 5 (epididymal androgen-related protein)	Peroxidases
	LPO	lactoperoxidase	Peroxidases
Group G	CAT	catalase	Peroxidases
Group H	GPX1	glutathione peroxidase 1	Peroxidases
	PRDX3	peroxiredoxin 3	Peroxidases
	PRDX2	peroxiredoxin 2	Peroxidases
Group I	PRDX1	peroxiredoxin 1	Peroxidases
	PRDX4	peroxiredoxin 4	Peroxidases
	GPX4	glutathione peroxidase 4 (phospholipid hydroperoxidase)	Peroxidases
	PRDX6	peroxiredoxin 6	Peroxidases
Group J	SOD2	superoxide dismutase 2, mitochondrial	SOD
	CCS	copper chaperone for superoxide dismutase	SOD
	SOD3	superoxide dismutase 3, extracellular	SOD
Group K	SOD1	superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult))	SOD
SKGT4			
Group A	MSRA	methionine sulfoxide reductase A	Thiol-redox
	TXNRD2	thioredoxin reductase 2	Thiol-redox
	TXNRD3	thioredoxin reductase 3	Thiol-redox
	SEPP1	selenoprotein P, plasma, 1	Thiol-redox
	TXNDC3	thioredoxin domain containing 3 (spermatozoa)	Thiol-redox
Group B	TXNDC4	thioredoxin domain containing 4 (endoplasmic reticulum)	Thiol-redox
	TXNL4A	thioredoxin-like 4A	Thiol-redox
	MT1M	metallothionein 1M	Thiol-redox
	GLRX	glutaredoxin (thioltransferase)	Thiol-redox
	TXNL4B	thioredoxin-like 4B	Thiol-redox
	GSR	glutathione reductase	Thiol-redox
	TXNDC13	thioredoxin domain containing 13	Thiol-redox
Group C	MT1F	metallothionein 1F	Thiol-redox
	MT1G	metallothionein 1G	Thiol-redox
	GLRX2	glutaredoxin 2	Thiol-redox
	GLRX3	glutaredoxin 3	Thiol-redox
	GLRX5	glutaredoxin 5	Thiol-redox
	TXN2	thioredoxin 2	Thiol-redox
SKGT4			
Group D	TXNDC5	thioredoxin domain containing 5	Thiol-redox
	TXNDC14	thioredoxin domain containing 14	Thiol-redox
	TXNRD1	thioredoxin reductase 1	Thiol-redox
	MT1E	metallothionein 1E	Thiol-redox
	TXNL1	thioredoxin-like 1	Thiol-redox
	TXNDC9	thioredoxin domain containing 9	Thiol-redox
Group E	MT2A	metallothionein 2A	Thiol-redox
	MT1X	metallothionein 1X	Thiol-redox
	TXNIP	thioredoxin interacting protein	Thiol-redox
	PDIA6	protein disulfide isomerase family A, member 6	Thiol-redox
	TXNDC1	thioredoxin domain containing 1	Thiol-redox
	MT1H	metallothionein 1H	Thiol-redox
	TXN	thioredoxin	Thiol-redox
Group F	GPX7	glutathione peroxidase 7	Peroxidases
	MPO	myeloperoxidase	Peroxidases
	GPX5	glutathione peroxidase 5 (epididymal androgen-related protein)	Peroxidases
	LPO	lactoperoxidase	Peroxidases
Group G	GPX3	glutathione peroxidase 3 (plasma)	Peroxidases
Group H	PRDX2	peroxiredoxin 2	Peroxidases
	CAT	catalase	Peroxidases
	GPX2	glutathione peroxidase 2 (gastrointestinal)	Peroxidases
	PRDX3	peroxiredoxin 3	Peroxidases
Group I	PRDX6	peroxiredoxin 6	Peroxidases

	PRDX4	peroxiredoxin 4	Peroxidases
	CP	ceruloplasmin (ferroxidase)	Peroxidases
	GPX4	glutathione peroxidase 4 (phospholipid hydroperoxidase)	Peroxidases
Group J	PRDX1	peroxiredoxin 1	Peroxidases
	GPX1	glutathione peroxidase 1	Peroxidases
Group K	SOD2	superoxide dismutase 2, mitochondrial	SOD
Group L	SOD3	superoxide dismutase 3, extracellular	SOD
	CCS	copper chaperone for superoxide dismutase	SOD
Group M	SOD1	superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult))	SOD

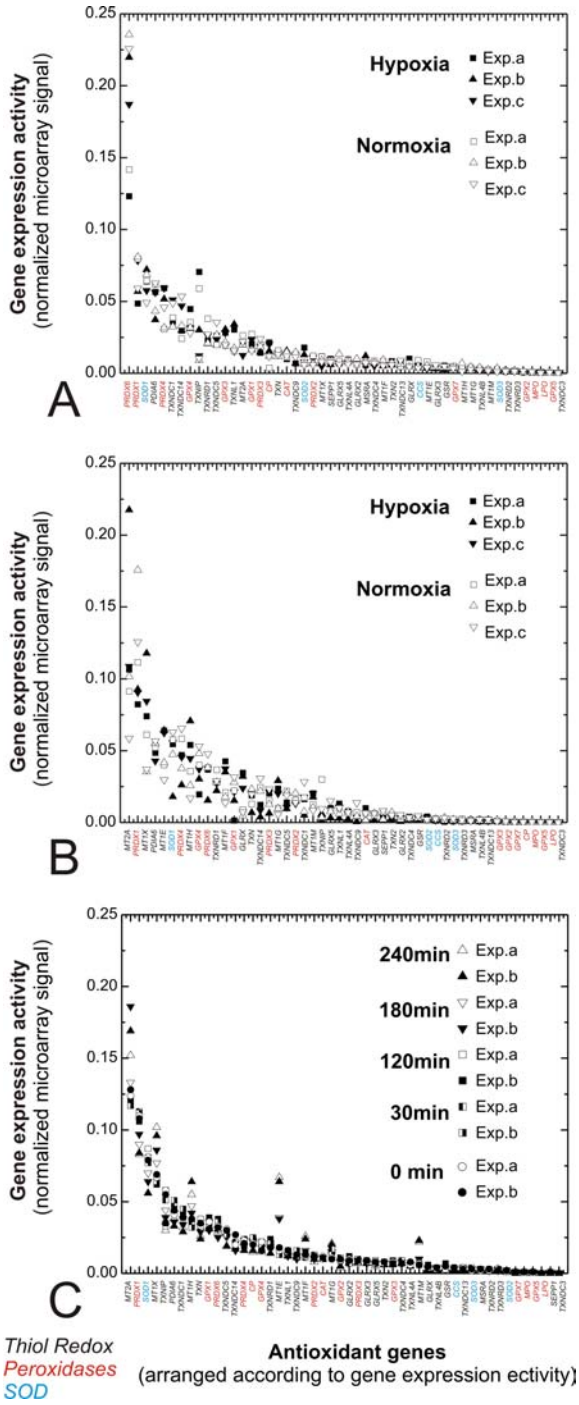

Figure 4. Gene expression activity level of antioxidant genes. The microarray signal of the antioxidant genes have been normalized for each sample and then the antioxidant genes of primary astrocytes (a), HeLa cells (b), and SKGT4 cells (c) have been ordered according to the expression level.

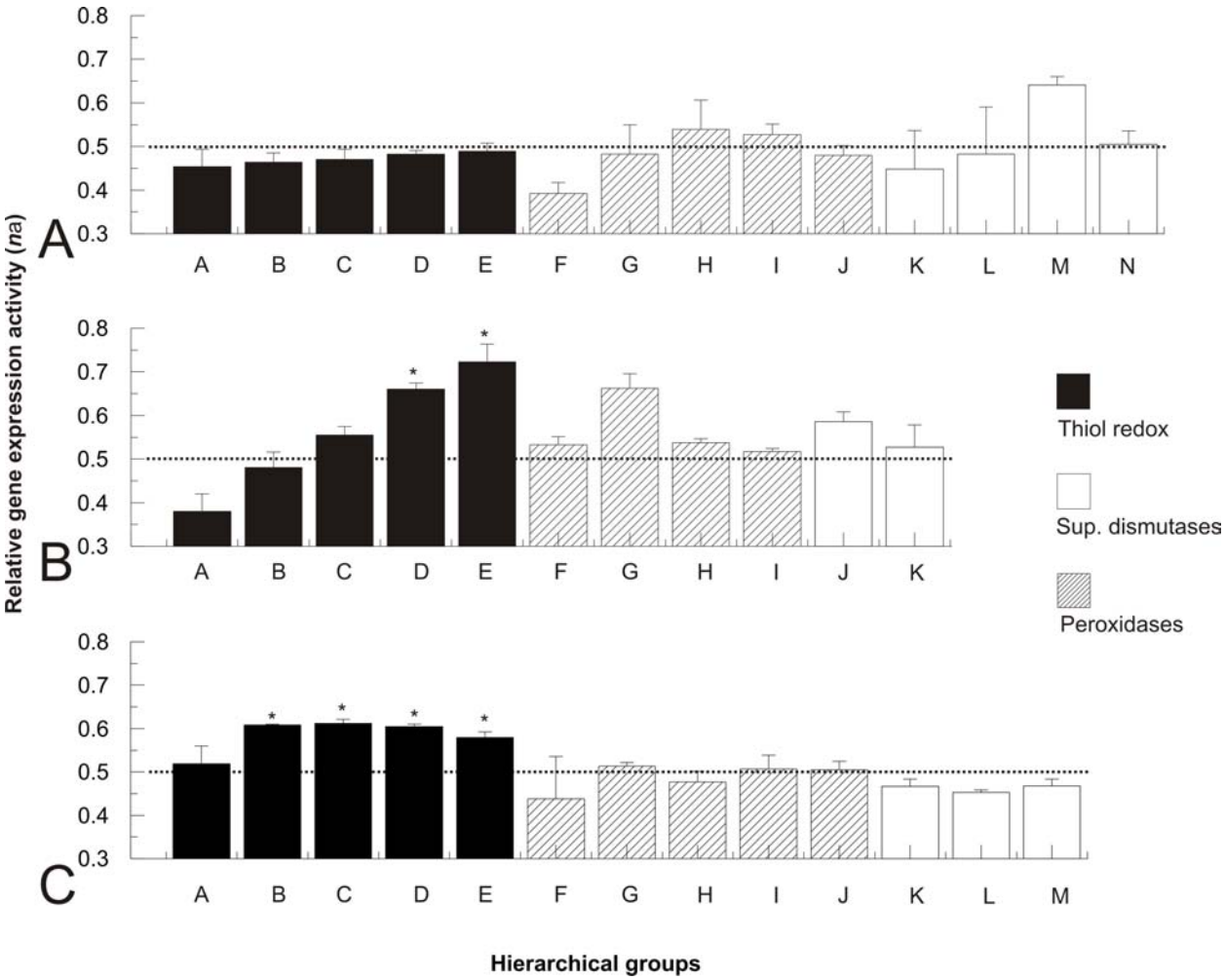

Figure 5. Gene expression activity of hierarchical groups. Relative gene expression activity of hierarchical groups in primary astrocytes maintained under hypoxic conditions for 24h (a), in HeLa cells maintained under hypoxic conditions for 24h (b), and in SKGT4 cells maintained in low pH environment for 240min (c). Stippled line indicates the equality point between control and experiment. Data expressed by average (±SEM).*P <0.05; significance after hierarchical groups analysis (*i.e. post-hoc* test for thiol-redox with 50,000 bootstrapping).

significant different among the three cell types: (i) primary astrocytes do not respond to hypoxic treatment (Figure 5a), (ii) HeLa cells show thiol-redox activation predominantly in two groups (Figure 5b), and (iii) SKGT4 cells present a homogeneous thiol-redox up-regulation (Figure 5c). Taken together, these results indicate that the thiol-redox gene group shows the most responsive antioxidant function when considering hypoxic condition (HeLa cells) and low pH environment (SKGT4 cells).

5. DISCUSSION

Free radicals and other reactive oxygen species are generated in several cellular sites during normal metabolism. One of the major physiological sources of ROS is the mitochondrial electron transport chain (26). The oxidative phosphorylation represents the main pathway in ATP generation in most eukaryotic cells. However, a wide number of cells can survive at low oxygen tension by using the glycolysis pathway to generate ATP. Gene expression is adjusted to oxygen availability by several mechanisms, including regulation of transcription activity by hypoxia-inducible factor α1 (HIF-α1). HIF-α1 regulates the expression of a wide number of genes, including many involved in ROS control (27). In the present work, we evaluated the relative gene expression activity of antioxidant genes in HeLa cells and primary human astrocytes maintained under hypoxic conditions. A network describing the associations among those genes has been proposed by Gelain *et al.* (7-8), and includes 63 components involved in codifying antioxidants products, grouped into functional categories.

According to Figure 2a, hypoxic treatment significantly increases the relative expression of the antioxidant genes in HeLa cells and this up-regulation is

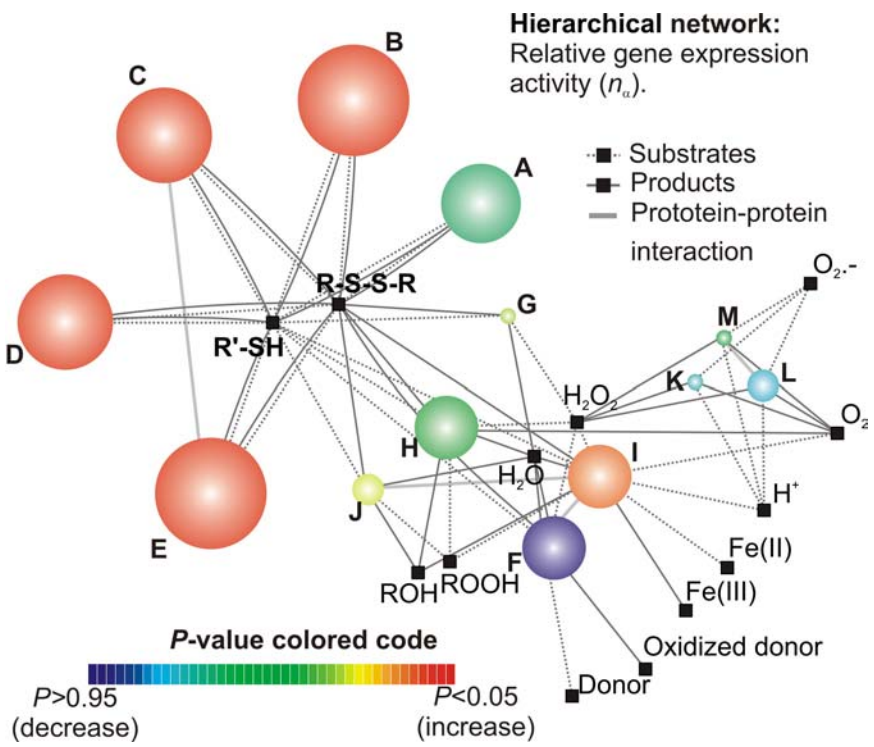

Figure 6. Network of SKGT4 cells response to acidosis. The hierarchical groups are color-coded according acidosis treatment response. The size of the spheres is proportional to number of genes in each hierarchical group.

not followed by a general increase in transcriptional activity. Surprisingly, even glycolysis genes (which could be expected to be upregulated under hypoxic conditions) are not altered in HeLa cells. This is in contrast with primary astrocytes, which shows significant changes in glycolysis genes, but no significant alterations in the antioxidant GFAGs (Figure 2b). This result is consistent with the original paper that produced the transcriptomes from HeLa cells and astrocytes (20). According to Mense & cols (20), primary astrocytes present significant increase in the induction of genes involved in glycolysis, glucose transport, and lactate transport when compared to HeLa cells. The authors suggest that this gene expression pattern might contribute to the higher resistance of astrocytes to hypoxia, indicating that the oxygen-dependent gene expression regulation is cell type specific. It is possible that the unchanged status of the antioxidant gene groups in astrocytes is related to its ability to deal with anaerobic metabolism. In contrast, HeLa cells represent a highly proliferative tumor cell type that requires additional energy supply, obtained mainly through oxidative phosphorylation. Therefore, conditions of low O_2 tension might cause severe cellular stress in this cell type when compared to human astrocytes, which might be better adapted to hypoxic conditions. In some extent, it is possible that such cellular stress may modulate the thiol-redox apparatus expression, as showed in Figure 2a.

Cells have mechanisms that regulate the intracellular pH (pH_i), such as the Na^+/H^+ antiport. However, an intense glycolytic activity may lead to acidosis if an excessive H^+ production overcomes the cellular pH_i regulating systems. Thus, a common hypoxic consequence is cellular acidosis, which may trigger a selective gene expression response (21). To determine the relative expression of the antioxidant genes submitted to acidosis, we accessed microarray data from esophageal adenocarcinoma SKGT4 cells maintained under low pH conditions. As shown in Figure 3a, gene expression levels of thiol-redox members are increased in these cells. It shows that the antioxidant gene network (mainly thiol-redox genes) in SKGT4 cells is responsive to low pH environment, suggesting that cellular acidosis can be one of the conditions able to up regulate the expression of thiol-redox genes. Additionally, the gene expression diversity in SKGT4 cells is not altered in any experimental condition, indicating that the observed gene expression changes are due to a coordinated response (not caused by isolated gene expression peaks), which reinforces the idea that antioxidant genes work as a system.

The contrast among the relative gene expression patterns from HeLa, astrocytes and SKGT4 cells rises two interesting observations: (i) the most expressed genes are consistently overlapped among the three cell types and (ii) the functional subdivision of the gene rank shows that the thiol-redox response to hypoxia and acidosis does not seems to be merely related to the gene expression levels. As a summary of this last observation, in Figure 6 we provide a network representation of the antioxidant response to low pH treatment in the SKTG4 cell line, which showed the most consistent thiol-redox response to acidosis. In this sense, some key points might be useful to

contemplate these results. Firstly, there are a number of thiol-redox genes codifying peptidic but non-enzymatic antioxidant products, such as metallothioneins. Contrasting to SODs and peroxidases, thiol-redox components are not controlled by an equivalent post-translational regulatory process (such as enzymatic activity). In this condition transcriptional regulation may acquire an essential role for thiol-redox functioning. Secondly, the interconnectivity between multiple thiol/disulfide couples on redox environments are maintained at stable but non-equilibrium steady states in which the abundance of antioxidant-active components (*i.e.* thioredoxins and glutaredoxins) can modulate the redox network function (28). Removing a thiol-redox component will necessarily perturb the overall thiol-redox homeostasis of the cell, which is a typical trait of biological networks (29). As the thiol-redox network presents more components and is more interconnected than the other antioxidant functions (7-8), it is likely that such genes are collectively more responsive to redox environments. In addition, thiol-containing molecules represent the most important redox buffers within cells. Taking into account that alterations in pH status affect the cellular redox state (30), the increased expression of thiol-redox genes described here is consistent with a physiological response to maintain the redox state homeostasis in cells submitted to acidosis.

6. CONCLUSIONS

In this work we provide evidences that the human antioxidant gene network described by Gelain *et al* (8) can respond selectively to oxidative stress stimulus. We anticipate that this network-based model will be particularly useful not only to describe stress-responsive expression profiles but also to detect other aspects of transcriptome functioning possibly not detected in the typical single-gene analysis.

7. ACKNOWLEDGMENTS

We acknowledge the Brazilians funds CNPq, CAPES, and PROPESQ/UFRGS.

8. REFERENCES

1. H. Liu, R. Colavitti, I. I. Rovira, T. Finkel: Redox-Dependent Transcriptional Regulation. *Circ Res*, 97(10):967-974 (2005)

2. S.G. Rhee: Cell Signaling: H_2O_2, a necessary evil for cell signaling. *Science*, 312(5782):1882-1883 (2006)

3. E. A. Veal, A. M. Day, B. A. Morgan: Hydrogen peroxide sensing and signaling. *Mol Cell*, 26(1):1-14 (2007)

A. L. Jackson, L. A. Loeb: The contribution of endogenous sources of DNA damage to the multiple mutations in cancer. *Mutat Res-Fund Mol M*, 477(1-2):7-21 (2001)

5. F. Dal Pizzol, F. Klamt, M. S. Benfato, E. A. Bernard, J. C. F. Moreira: Retinol supplementation induces oxidative stress and modulates antioxidant enzyme activities in rat Sertoli cells. *Free Radical Res*, 34(4):395-404 (2001)

6. M. R. de Oliveira, R. B. Silvestrin, T. M. Souza, J. C. M. Moreira: Therapeutic vitamin A doses increase the levels of markers of oxidative insult in Substantia nigra and decrease locomotory and exploratory activity in rats after acute and chronic supplementation. *Neurochem Res*, 33(3):378-383 (2008)

7. R. J. S. Dalmolin, D. P. Gelain, M. A. A. Castro: Human Antioxidant Genes. *http://www.ufrgs.br/icbs/hag/index.htm 2008*.

8. D. P. Gelain, R. J. S. Dalmolin, V. L. Belau, J. C. F. Moreira, F. Klamt, M. A. A. Castro: A systematic review of human antioxidant genes. *Front Biosci*, 144:457-463 (2009)

9. D. Bonatto: A systems biology analysis of protein-protein interactions between yeast superoxide dismutases and DNA repair pathways. *Free Radical Bio Med*, 43(4):557-567 (2007)

10. S. Casagrande, V. Bonetto, M. Fratelli, E. Gianazza, I. Eberini, T. Massignan, M. Salmona, G. Chang, A. Holmgren, P. Ghezzi: Glutathionylation of human thioredoxin: A possible crosstalk between the glutathione and thioredoxin systems. *P Natl Acad Sci Usa*, 99(15):9745-9749 (2002)

11. Finkel T. Oxidant signals and oxidative stress. *Curr Opin Cell Biol*, 15(2):247-254 (2003)

12. J. Haendeler, J. Hoffmann, V. Tischler, B. C. Berk, A. M. Zeiher, S. Dimmeler: Redox regulatory and anti-apoptotic functions of thioredoxin depend on S-nitrosylation at cysteine 69. *Nat Cell Biol*, 4(10):743-749 (2002)

13. M. A. A. Castro, J. C. M. Mombach, R. M. C. de Almeida, J. C. F. Moreira: Impaired expression of NER gene network in sporadic solid tumors. *Nucleic Acids Res*, 35(6):1859-1867 (2007)

14. M. M. Xiong, J. Y. Zhao, H. Xiong: Network-based regulatory pathways analysis. *Bioinformatics*, 20(13):2056-2066 (2004)

15. H. V. Westerhoff, B. O. Palsson: The evolution of molecular biology into systems biology. *Nat Biotech*, 22(10):1249-1252 2004

16. I. Borodina, J. Nielsen: From genomes to *in silico* cells via metabolic networks. *Curr Opin Biotech*, 16(3):350-355 (2005)

17.H. M. Wain, M. J. Lush, F. Ducluzeau, V. K. Khodiyar, S. Povey: Genew: the Human Gene Nomenclature Database, 2004 updates. *Nucleic Acids Res*, 32:D255-D257 (2004)

18.S. D, Hooper, P. Bork: Medusa: a simple tool for interaction graph analysis. *Bioinformatics*, 21(24):4432-4433 (2005)

19.M. Kanehisa, S. Goto, M. Hattori, K. F. Aoki-Kinoshita, M. Itoh, S. Kawashima, T. Katayama, M. Araki, M.

Hirakawa: From genomics to chemical genomics: new developments in KEGG. *Nucleic Acids Res*, 34:D354-D357 (2006)

20. S. M. Mense, A. Sengupta, M. Zhou, C. Lan, G. Bentsman, D. J. Volsky, L. Zhang: Gene expression profiling reveals the profound upregulation of hypoxia-responsive genes in primary human astrocytes. *Physiol Genomics,* 25(3):435-449 (2006)

21. S. P. Duggan, W. M. Gallagher, E. J. P. Fox, M. M. Abdel-Latif, J. V. Reynolds, D. Kelleher: Low pH induces co-ordinate regulation of gene expression in oesophageal cells. *Carcinogenesis,* 27(2):319-327 (2006)

22. M. A. A. Castro, T. T. G. Onsten, R. M. C. de Almeida, J. C. F. Moreira: Profiling cytogenetic diversity with entropy-based karyotypic analysis. *J Theor Biol*, 234(4):487-495 (2005)

23. M. A. A. Castro, T. G. H. Onsten, J. C. F. Moreira, R. M. C. de Almeida: Chromosome aberrations in solid tumors have a stochastic nature. *Mutat Res-Fund Mol M*, 600(1-2):150-164 (2006)

24. Y. Benjamini, Y. Hochberg: Controlling the false discovery rate - A practical and powerful approach to multiple testing. *J Roy Stat Soc B Met,* 57(1):289-300 (1995)

25. M. A. A. Castro, J. L. Rybarczyk-Filho, R. J. S. Dalmolin, M. Sinigaglia, J. C. F. Moreira, J. C. M. Mombach, R. M. C. de Almeida: ViaComplex: software for landscape analysis of gene expression networks in genomic context. *Bioinformatics*, 25(11):1468-1469 (2009)

26. I. Ohsawa, M. Ishikawa , K. Takahashi, M. Watanabe, K. Nishimaki, K.Yamagata, K. Katsura, Y. Katayama, S. Asoh, S. Ohta: Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nat Med*; 13(6):688-694 (2007)

27. F. J. Giordano: Oxygen, oxidative stress, hypoxia, and heart failure. *J Clin Invest*, 115(3):500-508 (2005)

28. M. Kemp, Y. M. Go, D. P. Jones: Nonequilibrium thermodynamics of thiol/disulfide redox systems: A perspective on redox systems biology. *Free Radical Bio Med*, 44(6):921-937 (2008)

29. M.B. Toledano, C. Kumar, N. Le Moan, D. Spector, F. Tacnet: The system biology of thiol redox system in Escherichia coli and yeast: Differential functions in oxidative stress, iron metabolism and DNA synthesis. *FEBS Lett,* 581(19):3598-607 (2007)

30. F. Q. Schafer, G. R. Buettner: Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radical Bio Med*, 30(11):1191-1212 (2001)

Key Words: Human Antioxidant Genes, HAG Database, Gene Network, System Biology, Reactive Oxygen Species, Free radicals.

Send correspondence to: Rodrigo Juliani Siqueira Dalmolin, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos 2600-anexo, Porto Alegre, RS, Brazil, Tel: 55-51-33085578, Fax:55-51-33085540, E-mail:rodrigo.dalmolin@ufrgs.br