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MODIFICATION OF OXIDATIVE STRESS ON GENE EXPRESSION PROFILING IN THE RAT INFARCTED HEART

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Abstract

Cardiac oxidative stress is developed following myocardial infarction (MI) particularly in the first week of MI. The influence of reactive oxygen species (ROS) on gene expression profiling and molecular pathways in the infarcted myocardium remains uncertain and is explored in the present study. Rats with MI were treated with or without antioxidants for one week. Normal rats served as controls. Cardiac oxidative stress and gene profiling were investigated. Compared to normal hearts, malondialdehyde (MDA), a marker of oxidative stress, was significantly increased in the infarcted myocardium, which was significantly suppressed by antioxidants. Microarray assay showed that over a thousand genes were differentially expressed in the infarcted myocardium. Antioxidants significantly altered the expression of 159 genes compared to untreated MI rats. Ingenuity pathway analysis (IPA) indicated that multiple pathway networks were affected by antioxidants, including those related to cell movement, growth/development, death, and inflammatory/fibrotic responses. IPA further identified that these changes were primarily related to NFκB, p38 MAPK, and ERκ1/2 pathways. Hub genes were identified in the associated gene networks. This study reveals the gene networks associated with cardiac oxidative stress postMI. These observations indicate that ROS regulate various molecular and cellular actions related to cardiac repair/remodeling through multiple gene networks.

Keywords

Myocardial infarction; cardiac repair/remodeling; oxidative stress; gene expression

INTRODUCTION

ROS, such as superoxide (O_2^-), hydroxyl (OH^-), nitric oxide (NO) and peroxynitrite ($ONOO^-$) are simple molecules characterized by the presence of unpaired electrons. ROS

can be produced intracellularly by electron leakage from the mitochondria during oxidative phosphorylation and by activation of several cellular enzymes, including NADPH oxidase, xanthine oxidase, and nitric oxide synthase (1–4). Living cells have both enzymatic and non-enzymatic defense mechanisms to balance the multitude of oxidative challenges presented to them. The enzymatic subgroup includes superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSHPx) (5–6). The non-enzymatic group includes a variety of biological molecules, such as vitamins E and C (7).

ROS have emerged as important in many pathophysiological processes. Their effects can be beneficial or deleterious at the site of production. In addition to their well-known damaging effects, ROS also have signaling roles, acting as second messengers that modulate the activity of diverse intracellular signaling pathways and transcription factors (8–9). ROS may influence signal transduction pathways through changes in the activity of redox-sensitive protein kinases; through altering the activity of redox-sensitive transcription factors; and/or through direct effects on enzymes or receptors (8–9).

Oxidative stress occurs when ROS production is enhanced and/or antioxidant reserve is depleted. Following acute MI, cardiac oxidative stress is developed, particularly in the infarcted myocardium. NADPH oxidase is a major source of O_2^- in the infarcted myocardium (10–11). Furthermore, inducible nitric oxide synthase, a major source of NO in the repairing tissue, is significantly increased in the infarcted myocardium (12), leading to enhanced NO production. As a result, ROS production is elevated in the infarcted myocardium and contributes to the development of oxidative stress in the infarcted heart. Impaired antioxidant capacity also plays a role in oxidative stress in the infarcted heart. Singal and colleague have shown evidence of the progressive decrease of SOD, catalase, and GSHPx activity as well as vitamin E levels in the infarcted rat heart (13). Cardiac glutathione levels are also decreased in patients with acute MI (14).

Oxidative stress is most evident in the infarcted myocardium during the first week postMI (10). ROS have been proven to be one of the stimulators of cardiac repair/remodeling. Antioxidant treatment reduced vascular density, apoptosis, inflammation and fibrosis in the infarcted myocardium (15–19). However, the underlying molecular basis is not fully explored. In the current study, we sought to determine the effects of ROS on gene expression profiles and signaling pathways at the infarcted myocardium in the early stage of MI. Our hypothesis is that ROS regulate various molecular and cellular actions of cardiac repair/remodeling via multiple gene networks.

MATERIAL AND METHODS

Animal Model

Left ventricular anterior transmural MI was created in 8-week-old male Sprague-Dawley rats (Harlan, Indianapolis, IN) by permanent ligation of the left coronary artery with silk ligature. Rats were anesthetized, intubated and ventilated with a rodent respirator. After left thoracotomy, the heart was exposed and 7-0 silk suture placed around the left coronary artery. The vessel was ligated, which results in 40–45% left ventricular infarction, the chest closed and lungs reinflated using positive-end expiratory pressure (20). Pharmacological

intervention with antioxidants was used to suppress O^- in the infarcted heart. The following animal groups were included: 1) normal rats; 2) rats with MI; and 3) rats with MI treated with combination of antioxidants, tempol (a free radical scavenger) (Sigma, St. Louis, MO) and apocynin (a NADPH oxidase inhibitor) (Sigma, St. Louis, MO), each 120mg/kg/day given by gavage twice a day for 7 days (n=6/group). The combined treatment with two antioxidants can suppress ROS production as well as enhance ROS clearance, thus maximizing the antioxidative effect. Cardiac function was monitored, hearts were then removed, rinsed in cold normal saline, frozen in isopentane with dry ice, and kept at -80°C . This study was approved by the University of Tennessee Health Science Center Animal Care and Use Committee.

Malondialdehyde Assay

The left ventricle from the normal heart and infarcted myocardium from the infarcted heart were dissected and homogenized. After centrifugation, supernatant MDA concentration was determined colorimetrically using a commercially available kit (Oxis International Inc, Foster City, CA, USA). Duplicate aliquots of supernatant were incubated at 45°C with 7.7 mM *N*-methyl-2-phenylindole in acetonitrile/methanol and 15.4 mM methanesulfonic acid. After clarification by centrifugation, absorbance was measured at 586 nm using 1,1,3,3-tetramethoxypropane as a standard.

RNA Isolation and Microarray Analysis

Total RNA from the left ventricle of the normal heart and infarcted myocardium from the infarcted heart was isolated using TRIzol reagent (Invitrogen, Grand Island, NY) and purified by the RNeasy MiniElute Cleanup kit (Qiagen, Valencia, CA). The concentration and quality of the extracted RNA were determined by ND-1000 Spectrometer (NanoDrop) and Agilent Bioanalyzer (Agilent, Santa Clara, CA), respectively. High quality total RNA with a RIN (RNA integrity score) number of more than seven was used to generate cDNA and cRNA with the Illumina® TotalPrep™ RNA amplification kit (Ambion, Grand Island, NY). The procedure consisted of reverse transcription with an oligo(dT) primer bearing a T7 promoter using Array-Script™, a reverse transcriptase (RT) engineered to produce higher yields of first-strand cDNA than wild type enzymes. ArrayScript catalyzed the synthesis of full-length cDNA and ensured production of reproducible microarray samples. The cDNA then underwent second-strand synthesis and clean-up to become a template for *in vitro* transcription with T7 RNA polymerase and biotin UTP, which generates multiple copies of biotinylated cRNA. After purification, the purity and concentration of cRNA was ascertained using ND-1000 Spectrometer (NanoDrop). High quality cRNA was then used with the Illumina direct hybridization array kits. cRNA sample (1.5μg) was hybridized on RatRef-12 expression beadchip for 16 hours in a multiple step procedure according to the manufacturer's instructions. The chips were then washed, dried, and scanned on the Bead Array Reader (Illumina, San Diego, CA), and raw data were generated using GenomeStudio 3.4.0 (Illumina, San Diego, CA). Normalization for the raw data was performed using Illumina Genome Viewer 3.2.9. Six rats per group were used for the RNA isolation and profiling. The statistical difference of the genes between the normal and MI or MI +AT groups were analyzed by paired t-test, with a significant level of $p<0.05$.

Analysis of Molecular Networks

IPA was used to identify biologically relevant networks affected by antioxidants. Selected genes with unique gene identifiers (Agilent probe set ID) and their corresponding fold-change values were uploaded as a tab-delimited text file where the gene identifiers were mapped to its corresponding gene object in the Ingenuity database. These were then called “focus genes” and used as starting points to query the database to generate biological networks with a statistical score provided for each network, indicating the probability of the focus genes within a network being due to random chance (a score 2 signified less than 1% chance that the focus genes were randomly linked) (21).

RT-PCR

Total RNA was extracted from cardiac tissue using Trizol Reagent (Invitrogen, Carlsbad, CA). The RNA was treated with DNase by using TURBO DNA-free kit (Ambion, Austin, TX), and purified with RNeasy Mini Kit (Qiagen Inc, Valencia, CA). The purification, concentration and integrity of the RNA were examined with NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE), and Agilent Bioanalyzer (Agilent Technologies, Foster City, CA), respectively. cDNA was prepared from total RNA using a High Capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, CA). The gene-specific probes and primer sets for VCAM, MT3, MMP12, and CD8A were deduced using Universal ProbeLibrary Assay Design software (<https://www.roche-applied-science.com>). VCAM, MT3, MMP12, and CD8A mRNA levels were detected and analyzed on an LightCycler 480 System (Roche, Indianapolis, IN) under the following cycling conditions: 1 cycle at 95 °C for 5 min and then 45 cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 10 s. The PCR mix contained 0.2 µl of 10 µM primers, 0.1 µl of 10 µM Universal library probe, 5 µl of LC 480 master mix (2X), 2 µl of template cDNA and RNase-free water to 10 µl. TATA box-binding protein has been selected as endogenous quantity control. Fold change were used to compare the difference between two groups.

Echocardiographic Assessment of Left Ventricular Function

Rats were lightly anesthetized with isoflurane at a constant volume and anesthetic time. Transthoracic echocardiography was performed in the left lateral decubitus position using an echocardiographic system (Sonos 4500 with a 7- to 11-MHz transducer). The parasternal short-axis view under two-dimensionally (2D) M-mode at the level of the papillary muscle was recorded, and the left ventricle diastolic and systolic diameters were measured to calculate fraction shortening in accordance with the guidelines of American Society of Echocardiography.

Statistical analysis

Statistical analyses of MDA, RT-PCR and echocardiography data were performed using analysis of variance. Values are expressed as mean $\pm$ SEM, with $P < 0.05$ considered significant. Multiple group comparisons among controls and each group were made by Scheffe's F -test.

RESULTS

Oxidative Stress in the Infarcted Myocardium

ROS degrade polyunsaturated lipids, forming stable MDA. The latter is used as a biomarker to measure the level of oxidative stress. Compared to the normal heart, MDA level was significantly increased in the infarcted myocardium, which was significantly suppressed by antioxidant treatment (Figure 1).

Changes of Gene Expressions in the Infarcted Myocardium

Compared to the normal heart, over a thousand genes were differently expressed in the infarcted myocardium in the early stage of MI, including those significantly decreased and increased (data not shown).

Effect of Antioxidants on Cardiac Gene Expression Profiling

Compared with untreated rats with MI, antioxidant treatment significantly suppressed the expression of 159 genes in the infarcted myocardium. However, antioxidants barely increase expressions of genes in the infarcted myocardium. The ingenuity analysis revealed that antioxidants statistically changed 12 pathway networks in the infarcted myocardium by suppressing expressions of various genes in these networks. Each network, summarized in Table 1, includes all genes in that network, genes altered by antioxidants, statistical score and top functions of the network.

Network 1 functions in inflammatory response and cell-to-cell signaling. NF- κ B plays a key role in network 1 (Figure 2). Network 2 controls cellular assembly and organization, cellular function and maintenance and inflammatory response. Actin and CD44 play key roles in network 2 (Figure 2). Network 3 functions in cell death, lipid metabolism and cellular transport. ERK, RAF1 and Ap1 play key roles in network 3 (Figure 2). Network 4 controls cellular movement, cell-to-cell signaling and interaction and tissue development. Integrin and VCAM play key roles in this network (Figure 2).

Network 5 functions in gene and cell cycle. Cyclin A plays a key role in network 5 (Figure 3). Network 6 acts in cell signaling, post-translational modification and cellular compromise. ERK1/2, LAT and SYK play key roles in network 6 (Figure 3). Network 7 regulates cell death, cellular movement, connective tissue development and function. TNF α and MAPK1 play key roles in this network (Figure 3). Network 8 controls drug metabolism, endocrine system development/function and lipid metabolism. Myc plays key role in network 8 (Figure 3).

Network 9 functions in cell-to-cell signaling and interaction and inflammatory response. ICAM1 and TGF β 1 play major roles in network 9 (Figure 4). Network 10 regulates cellular function and maintenance, cellular assembly and organization and cellular movement. IFN- γ plays a key role in this network (Figure 4). Network 11 controls cell signaling, small molecule biochemistry and free radical scavenging. P38 MAPK, VEGF and NADPH oxidase play key roles in this network (Figure 4). Network 12 functions in cell-to-cell

signaling and interaction and immune cell trafficking. IFN- β , Interferon- α and IL12 play key roles in this network (Figure 4).

Gene symbols and names altered by antioxidants are listed on table 2.

Validation of Microarray Results by RT-PCR

The expression of four selected genes (VCAM, MT3, MMP12, and CD8A) was verified between MI and MI-AT group by quantitative RT-PCR. The fold changes in the expression levels of target genes revealed by RT-PCR were similar to those determined by microarray (Figure 5).

Ventricular Function

Compared with the normal heart, the fraction shortening was significantly reduced in the infarcted heart. Antioxidant treatment, however, did not affect the fraction shortening in the infarcted heart (Figure 6).

DISCUSSION

Oxidative stress is developed in the infarcted myocardium, which is most evident in the first week postMI (10). The current study has indicated that multiple signaling pathways and networks are activated in the infarcted myocardium by ROS in the early stage of MI. The top functions of these pathways and networks are as follows.

The Role of ROS on Cardiac Cell Growth, Assembly, Function, and Maintenance

Our data have shown that antioxidant treatment significantly reduces MDA levels in the infarcted myocardium, indicating that cardiac oxidative stress is successfully suppressed in rats receiving antioxidants.

Compared to MI rats, the expression of genes related to cell growth, assembly, function and maintenance are significantly reduced in the infarcted myocardium in rats treated with antioxidants. Our data also reveal that multiple pathway networks are involved in the regulation of ROS on cell growth and function. Antioxidants significantly suppress the majority of gene expressions in network 2. The top functions of the network are related to cellular assembly and organization, cellular function and maintenance. The key molecules in the network include actin and CD44. Actin participates in cell mobility, division and movement; and CD44 is involved in cell–cell interactions, cell adhesion and migration. These data indicate that ROS activate the signaling network in the infarcted myocardium, contributing to cell growth and maintenance during cardiac repair.

In addition, our study also showed that antioxidants significantly altered multiple genes in several other networks, of which the top functions are also related to cell movement, tissue development, cell cycle, and cellular assembly. The key pathways and molecules of these networks include integrin and ERK1/2. Integrin plays a role in cell signaling and regulate cellular shape, motility, and cell cycle. ERKs are involved in functions including the regulation of meiosis, mitosis and post-mitotic functions. These observations indicate that

ROS stimulate cell growth through multiple pathway networks, which have overlapped cellular functions.

Cell growth, movement and assembly are active in the infarcted myocardium, particularly in the first week postMI. MI induces loss of myocardium and blood vessels. The growth of new blood vessels (angiogenesis) and scar tissue at the site of myocyte loss, preserve structural integrity and is central to the heart's recovery. Angiogenesis requires endothelial cell proliferation, migration, adhesion and lumen formation. Myofibroblasts play a major role in scar formation in the infarcted myocardium (22–23). These cells are not residential cells in the heart, appear in the infarcted myocardium soon after MI, rapidly proliferate and become a major cell type in the infarcted myocardium. Our previous study has demonstrated that angiogenic and fibrogenic responses are most evident in the infarcted myocardium within the first week postMI, which is spatially and temporally coincident with the development of oxidative stress (24–25). ROS have been shown to increase vascular density and fibrosis (24, 26). Thus, enhanced ROS in the infarcted myocardium may stimulate endothelial cell and myofibroblast proliferation and movement through multiple pathway networks and facilitate angiogenesis and scar formation in the early stage of MI.

The Role of Cardiac ROS on Inflammatory and Fibrotic Responses

The current study has further revealed that antioxidants significantly alter the expression of multiple genes related to inflammatory response. Several pathways and networks are found to be involved in the regulation of ROS on inflammation. Inflammatory response initiates the process of cardiac repair following MI. Our data have shown that ROS play a role in immune cell trafficking in the infarcted myocardium, which facilitates inflammatory cell infiltration. Our study further reveals that antioxidants significantly suppress the majority of genes in network 1 with top functions of inflammatory response. NF- κ B is a key molecule in this network. NF- κ B is a transcription factor, which maintains an inactive form bound to its inhibitory subunit Ikappa B under normal conditions. It is activated by various local substances, including ROS (27). Upon activation, NF- κ B stimulates inflammatory and immune responses and cellular growth by increasing the expression of specific cellular genes. Its activation has been demonstrated in various models of myocardial ischemia and reperfusion (28–29). Activated NF- κ B triggers gene expression of interstitial and vascular adhesion molecules, leading to leukocyte infiltration into the infarcted myocardium, as well as monocyte chemoattractant protein-1, and inducing recruitment of mononuclear cells. It also triggers gene expression of proinflammatory cytokines, such as TNF- α and interleukins and initiates inflammatory response (30). Our findings have confirmed that ROS promote inflammatory response through the NF- κ B pathway. The current study has further shown that antioxidants also suppress multiple genes in other networks with top function of inflammatory response. This observation indicates that ROS regulate inflammation via numerous pathway networks.

The current study has also revealed that antioxidants suppress the expression of various genes in the network with the top function of connective tissue development, demonstrating the role of ROS on fibrous tissue formation in the infarcted myocardium in the early stage of MI. The previous studies have indicated the up-regulation of ROS on collagen synthesis in

the pathological conditions (31–32). *In vitro* studies have further stated that ROS promotes fibroblast proliferation and type I collagen gene expression in cardiac fibroblasts (33). Scar formation is a major feature of cardiac repair, which is required to maintain heart integrity following MI. However, the effect of ROS on fibrous tissue formation may be harmful to the heart. ROS have been reported to promote interstitial fibrosis in the noninfarcted myocardium, contributing to ventricular dysfunction (4). Thus, ROS play both beneficial and deleterious effects on fibrous tissue formation in the infarcted heart.

The Role of ROS on Cardiac Gene Expression, Cell Signaling and Cell-to-cell Signaling

Another important effect of ROS we observed in the study is its regulation on gene expression, cell signaling and cell-to-cell signaling in the infarcted myocardium. Antioxidants significantly reduced the expression of a number of genes in several pathway networks, which have overlapping functions in gene expression, cell signaling and cell-to-cell signaling. The key molecules of these networks include NF- κ B, integrin, EKR1/2, TGF- β 1, p38MARK and interferon. The data indicate that ROS stimulate gene expression, cell signaling and cell-to-cell signaling through multiple pathway networks. These molecular and cellular functions are involved in various reactions related to cardiac repair/remodeling postMI. The alteration of ROS on gene expression and cell signaling has been reported in various cell types. ROS increase the expression of genes related to atherosclerosis and vascular remodeling in endothelial cells (34). Hydrogen peroxide is found to increase extracellular matrix gene expression via TGF- β 1 signaling pathway in human mesangial cells (35). NADPH oxidase-derived ROS have been reported to stimulate VEGF and PDGF signaling pathways in smooth muscle cells (36). Therefore, ROS stimulate gene expression and cell signaling in various cell types and pathological conditions.

The Role of Antioxidants on Ventricular Function

Our study has shown that ventricular dysfunction is developed in rats with MI at one week postMI. Antioxidant treatment, however, did not impact ventricular function in the infarcted heart at the early stage of MI. ROS have both beneficial and detrimental effect on the infarcted heart. It promotes cardiac repair, which is constructive to cardiac recovery. On the other hand, oxidative stress also induces myocardial remodeling, including myocyte apoptosis, hypertrophy and interstitial fibrosis in the noninfarcted myocardium, which may contribute to the development of ventricular dysfunction. Antioxidant supplement have been effective in the treatment of animal paradigms; however, the data for the possible benefits of treatment for patients with heart failure have failed to demonstrate convincing benefits (37).

Limitations of the Study

Our previous findings have shown that cardiac oxidative stress is most evident in the first week postMI. Accordingly, the current study was focused on the regulation of ROS on cardiac gene expression profiling and cardiac function at day 7 postMI. However, the effect of ROS on cardiac gene networks and cardiac function may vary at different stages postMI.

In summary, antioxidant treatment significantly suppresses expression of numerous genes related to cell growth, inflammatory/fibrogenic responses, gene expression and cell

signaling in the infarcted myocardium in the early stage of MI. These findings suggest ROS play a role in various molecular and cellular functions involved in cardiac repair/remodeling through multiple pathways networks.

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Figure 1.
Cardiac MDA content: Compared with that of normal hearts (CTL), MDA level was significantly increased at the site of MI postMI, which was significantly suppressed by antioxidant treatment (AT). * $p < 0.05$ vs CTL; † $p < 0.05$ vs MI

Figure 2.

Genes in the infarcted myocardium suppressed by antioxidants in network 1–4. Network 1 acts in inflammatory response and cell-to-cell signaling. Network 2 functions in cellular assembly and organization, cellular function and maintenance and inflammatory response. Network 3 works in cell death, lipid metabolism and cellular transport. Network 4 controls in cellular movement, cell-to-cell signaling and interaction and tissue development.

Network 5

Network 6

Network 7

Network 8

Figure 3.

Genes in the infarcted myocardium suppressed by antioxidants in network 5–8: Network 5 controls in gene and cell cycle. Network 6 functions in cell signaling, post-translational modification and cellular compromise. Network 7 controls in cell death, cellular movement, connective tissue development and function. Network 8 acts in drug metabolism, endocrine system development/function and lipid metabolism.

Network 9

Network 10

Network 11

Network 12

Figure 4.

Genes in the infarcted myocardium suppressed by antioxidants in network 9–12. Network 9 controls in cell-to-cell signaling and interaction and inflammatory response. Network 10 operates in cellular function and maintenance, cellular assembly and organization and cellular movement. Network 11 works in cell signaling, small molecule biochemistry and free radical scavenging. Network 12 functions in cell-to-cell signaling and interaction and immune cell trafficking.

Figure 5.
Validation of microarray results by RT-PCR: RT-PCR confirmed the relative differences between MI and MI+AT observed in microarray.

Figure 6. Ventricular function following MI in response to antioxidants. Cardiac function was impaired postMI. Antioxidants did not alter ventricular function in the infarcted heart in the early stage of MI.

Table 1

Antioxidants Suppressed Gene Expressions of Pathway Networks in the Infarcted Myocardium PostMI (Bold: altered genes; ↓: suppressed genes)

ID	Molecules in Network	Score	Focus molecules	Top functions
1	↓ ADRM1 , ↓ ALCAM , Angiotensin II receptor type 1, B-cell receptor, ↓ B4GALT1 , ↓ BCL10 , C8, ↓ CARD9 , ↓ CCL3L1/CCL3L3 , ↓ CCNB2 , ↓ CORO1A , Elastase, ETS, Fcgr2, ↓ GDF15 , ↓ GLRX , ↓ GPX1 (includes EG:14775), ↓ GSR , Igg3, IgG2a, IgG2b, ↓ IL18BP , Inflammasome (Nalp1, Asc, Casp1, Casp4), ↓ LITAF , ↓ MT3 , NFkB (complex), NfκB-RelA, ↓ PRDX5 , ↓ PYCARD , ↓ SCLY , Sod, ↓ SPN , TEC/BTK/ITK/TKX/BMX, ↓ TXN (includes EG:116484), Vla-4	37	20	Inflammatory Response, Cell-To-Cell Signaling and Interaction, Hematological System Development and Function
2	↑ ACTA1 , ↓ ACTB , Actin, ↓ AIF1 (includes EG:11629), ↓ ARHGDI1B , ↓ ATP6V1B2 , ↓ BID , ↓ CAPZA2 , ↓ CASP1 , ↓ CASP6 , Caspase, Caspase 3/7, CD3, ↓ CD44 (includes EG:100330801), Cofilin, ↓ CTSK , Cytochrome c, Erm, F Actin, Hsp90, ↓ LIPA , Mapk, ↓ MSN , ↓ NPC1 , ↓ PFN1 , Pkc(s), Plc beta, ↓ RAC1 , Ras homolog, Rho gdi, ↓ RPS7 , ↓ S100A8 , ↓ SNCA , ↓ SNX1 , ↓ TTEC	33	21	Cellular Assembly and Organization, Cellular Function and Maintenance, Inflammatory Response
3	14-3-3, ↓ ACSL5 , Ap1, ↓ AP2A2 , ↓ BLVRA , Calcineurin protein(s), CaMKII, collagen, Creb, ↓ EMB , ERK, ↓ FABP4 , ↓ GCLC , ↓ GSTP1 , Hsp27, ↓ KCNN4 , LDL, ↓ M6PR , MAP2K1/2, Mek, ↓ MGST1 , NFAT (complex), ↓ OSTF1 , PDGF BB, ↓ PICALM , PP2A, Ppp2c, ↓ PRKCD , Proinsulin, ↓ RAF1 , ↓ RETSAT , ↓ RPS6KA1 (includes EG:20111), SAA, Sos, ↓ WNT4	30	17	Cell Death, Lipid Metabolism, Molecular Transport
4	Akt, ↓ ALOX15 , Alpha Actinin, Alpha catenin, Calpain, Collagen type I, Collagen type III, Collagen type IV, Collagen(s), ↓ CYBB , ↓ ENPP2 , Fibrinogen, Focal adhesion kinase, Integrin, Integrin alpha 4 beta 1, Integrin alpha V beta 3, Integrin α 5, ↓ ITGA4 , ↓ ITGAD , ↓ ITGB7 , JINK1/2, Kallikrein, Laminin, Lfa-1, ↓ MMP12 , Pdgf (complex), Pld, Rap1, ↓ RASSF5 , ↓ SDCBP , ↓ SLC3A2 , ↓ SLC7A7 , ↑ THBS4 , ↓ VAPA , ↓ VCAM1	23	14	Cellular Movement, Cell-To-Cell Signaling and Interaction, Tissue Development
5	Adducin, ↓ ALAS2 , Alpha tubulin, ↓ ATF2 , ↓ ATF3 , ↓ BTK , ↓ CCNA2 , Cdc2, ↓ CEBPA , Ck2, Cyclin A, Cyclin B, E2f, Estrogen Receptor, Growth hormone, ↓ GTF2B , ↓ HBB , Hdac, HISTONE, Histone h3, Histone h4, Jnk, Lamin b, ↓ LYL1 , ↓ MAX , N-cor, ↓ NCOA3 , PEPCK, RNA polymerase II, Rock, ↓ SLC31A1 , ↓ SP1 , ↓ SPP1 (includes EG:20750), Thymidine Kinase, VitaminD3-VDR-RXR	22	14	Cancer, Gene Expression, Cell Cycle
6	Adaptor protein 1, BCR, CD8, ↓ CD37 , ↓ CD8A , ↓ CTLA4 , ↓ ELF1 , ERK1/2, Fc gamma receptor, Fcgr1, Fcgr3, FCGR1A/2A/3A, ↓ FCGR3A , ↓ HCST , Ige, IgG1, IL-2R, ↓ LAT , ↓ LCP2 , MHC Class I (complex), Nfat (family), PI3K p85, PLC gamma, Profilin, Ptk, PTPase, ↓ PTPN6 , ↓ PTPN7 , ↓ PTPRC , SHP, ↓ SLA , ↓ SYK , SYK/ZAP, ↓ TYROBP , VAV	21	14	Cell Signaling, Post-Translational Modification, Cellular Compromise
7	↓ 1600029D21Rik , ↓ AKR1B15 , ALOX15B, ↓ C3AR1 , CD300C, CD300LD, ↓ CIAO1 , DLEU1, E2F4, ERBB2, FGF2, ↓ FPGT , ↓ FXDY2 , ↓ GOLPH3 , GPR77, IGF1R, ↓ KIAA0101 , KRT34, LAMP3, LSR, MAPK1, Na+, K+ - ATPase, NDST1, ↓ NINJ1 , NMBR, ↓ NME6 , PEBP4, PRKCDBP, RAB18, ↓ SGPL1 , ↓ SMU1 , SPOCK1, TNF, TNFSF11, ZFP36L2	19	12	Cell Death, Cellular Movement, Connective Tissue Development and Function
8	↓ 4732456N10Rik , ASAH2, ATAD2, BAI1, beta-estradiol, ↓ C6orf108 , ↓ CDCA3 , CDKN1A, ceramide, CLYBL, COX6A1, COX7A2L, ↓ CTBS , ERF, Gm4617/Ptma, GNPAT, GNS, ↓ GPR88 , GPRC5A, GRB2, HNF4A, LEP, ↓ LEPROT , LPHN1, MSMB, MYC, ↓ NAAA , ↓ OPLAH , RAB40B, ↓ SHANK2 , SIGLEC12, SOCS3, STAT, ↓ Tceb2 , ↓ ZNRD1	17	11	Drug Metabolism, Endocrine System Development and Function, Lipid Metabolism
9	20s proteasome, 26s Proteasome, Alp, ATPase, Calmodulin, ↓ CCR1 , CHEMOKINE, FSH, GPR21, GPR34, GPRC5A, Gsk3, ↓ ICAM1 , ↓ Ifi47 , Ikb, IKK (complex), IRG, ↓ ITGAD , ↓ ITGAM (includes EG:16409), Mmp, NIT1 (includes EG:27045), ornithine decarboxylase, Pka, ↓ PSME2 , PSME4, ↓ PTAFR , ↓ SQSTM1 , SULF4A1, TCR, Tgf beta, ↓ TGFB1 (includes EG:21803), ↓ TPST2 , Tubulin, Ubiquitin, ↓ USP15	17	11	Cell-To-Cell Signaling and Interaction, Inflammatory Response, Hematological System Development and Function
10	AAK1, Adaptor protein 2, ↓ AIF1 (includes EG:11629), ↓ AP2A2 , APP, ↓ ARSB , Clathrin, ↓ CLIC1 , CTSH, CXCR6, EPN1, EPN2, EPN3, GGA3, IFI27, IFI30, IFNG (includes EG:15978), ISG20, ↓ LAPTM5 , LDLRAP1, ↓ M6PR , ↓ MBNL1 , MFGE8, MT-ATP6, ↓ NADSYN1 , ↓ PICALM , RAMP1, SLC11A1, SLC40A1, SMARCA4, SNAP91, TNNT2, TPP1 (includes EG:1200), TREM2, ↓ VKORC1L1	14	10	Cellular Function and Maintenance, Cellular Assembly and Organization, Cellular Movement

ID	Molecules in Network	Score	Focus molecules	Top functions
11	AMPK, ↓ APOBEC1 , ↓ CCL13 , ↓ CSTB , ↓ CTSC , G protein alpha, G protein beta gamma, ↓ GMFG , GOT, Gpcr, GST, hCG, HDL, Hsp70, IgG, IL1, ↓ IL1RN , Insulin, Lh, NADPH oxidase, Nos, P38 MAPK, p85 (pik3r), Pdgfr, PLC, Pro-inflammatory Cytokine, ↓ RAC2 , Rac, Ras, Sapk, Sfk, Shc, SRC, ↓ TRPV2 , Vegf	11	8	Cell Signaling, Small Molecule Biochemistry, Free Radical Scavenging
12	↓ ARG1 , Arginase, atypical protein kinase C, ↓ CCL7 , ↓ CXCL9 , Gm-csf, HLA-DR, ↓ HMGB2 , Ifn, IFN alpha/beta, IFN Beta, Ifn gamma, IFN TYPE 1, Ifnar, Iga, Igm, IL23, IL12 (complex), IL12 (family), Immunoglobulin, Immunoproteasome Pa28/20s, Interferon alpha, JAK, MHC CLASS I (family), MHC Class II (complex), NFkB (family), Nfkb1-RelA, PI3K (complex), ↓ PSMA5 , ↓ PSMB8 , STAT5a/b, TH2 Cytokine, ↓ TLR2 , Tlr, Tnf	9	7	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking

Table 2**Cardiac Genes Altered by Antioxidant Treatment**

Symbol	Gene Name	Symbol	Gene Name
1600029D21Rik	RIKEN cDNA 1600029D21 gene	ITGAD	integrin, alpha D
4732456N10Rik	RIKEN cDNA 4732456N10 gene	ITGAM	integrin, alpha M
ACSL5	acyl-CoA synthetase long-chain family member 5	ITGB7	integrin, beta 7
ACTA1	actin, alpha 1, skeletal muscle	KCNN4	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4
ACTB	actin, beta	KIAA0101	KIAA0101
ADRM1	adhesion regulating molecule 1	LAPTM5	lysosomal protein transmembrane 5
AIF1	allograft inflammatory factor 1	LAT	linker for activation of T cells
AKR1B15	aldo-keto reductase family 1, member B15	LCP2	lymphocyte cytosolic protein 2
ALAS2	aminolevulinate, delta-, synthase 2	LEPROT	leptin receptor overlapping transcript
ALCAM	activated leukocyte cell adhesion molecule	LIPA	lipase A, lysosomal acid, cholesterol esterase
ALOX15	arachidonate 15-lipoxygenase	LITAF	lipopolysaccharide-induced TNF factor
AP2A2	adaptor-related protein complex 2, alpha 2 subunit	LYL1	lymphoblastic leukemia derived sequence 1
APOBEC1	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1	M6PR	mannose-6-phosphate receptor
ARG1	arginase, liver	MAX	MYC associated factor X
ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	MBNL1	muscleblind-like splicing regulator 1
ARSB	arylsulfatase B	MFGE8	milk fat globule-EGF factor 8 protein
ATF2	activating transcription factor 2	MGST1	microsomal glutathione S-transferase 1
ATF3	activating transcription factor 3	MMP12	matrix metalloproteinase 12
ATP6V1B2	ATPase, H ⁺ transporting, lysosomal 56/58kDa, V1 subunit B2	MSN	Moesin
B4GALT1	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1	MT3	metallothionein 3
BCL10	B-cell CLL/lymphoma 10	NAAA	N-acyl ethanolamine acid amidase
BID	BH3 interacting domain death agonist	NADSYN1	NAD synthetase 1
BLVRA	biliverdin reductase A	NCOA3	nuclear receptor coactivator 3
BTK	Bruton agammaglobulinemia tyrosine kinase	NINJ1	ninjurin 1
C3AR1	complement component 3a receptor 1	NME6	non-metastatic cells 6
C6orf108	chromosome 6 open reading frame 108	NPC1	Niemann-Pick disease, type C1
CAPZA2	capping protein, alpha 2	OPLAH	5-oxoprolinase (ATP-hydrolysing)
CARD9	caspase recruitment domain family, member 9	OSTF1	osteoclast stimulating factor 1
CASP1	caspase 1, apoptosis-related cysteine peptidase	PFN1	profilin 1
CASP6	caspase 6, apoptosis-related cysteine peptidase	PICALM	phosphatidylinositol binding clathrin assembly protein
CCL13	chemokine (C-C motif) ligand 13	PRDX5	peroxiredoxin 5
CCL21	chemokine (C-C motif) ligand 21	PRKCD	protein kinase C, delta

Symbol	Gene Name	Symbol	Gene Name
CCL3L1/CCL3L3	chemokine (C-C motif) ligand 3-like 1	PSMA5	proteasome (prosome, macropain) subunit, alpha type, 5
CCL7	chemokine (C-C motif) ligand 7	PSMB8	proteasome (prosome, macropain) subunit, beta type, 8
CCNA2	cyclin A2	PSME2	proteasome activator subunit 2
CCNB2	cyclin B2	PTAFR	platelet-activating factor receptor
CCR1	chemokine (C-C motif) receptor 1	PTPN6	protein tyrosine phosphatase, non-receptor type 6
CD37	CD37 molecule	PTPN7	protein tyrosine phosphatase, non-receptor type 7
CD44	CD44 molecule	PTPRC	protein tyrosine phosphatase, receptor type, C
CD8A	CD8a molecule	PYCARD	PYD and CARD domain containing
CDCA3	cell division cycle associated 3	RAC1	ras-related C3 botulinum toxin substrate 1
CEBPA	CCAAT/enhancer binding protein, alpha	RAC2	ras-related C3 botulinum toxin substrate 2
CIAO1	cytosolic iron-sulfur protein assembly 1	RAF1	v-raf-1 murine leukemia viral oncogene homolog 1
CLIC1	chloride intracellular channel 1	RASSF5	Ras association (RalGDS/AF-6) domain family member 5
CORO1A	coronin, actin binding protein, 1A	RETSAT	retinol saturase
CSTB	cystatin B (stefin B)	RPS6KA1	ribosomal protein S6 kinase, 90kDa, polypeptide 1
CTBS	chitinase, di-N-acetyl-	RPS7	ribosomal protein S7
CTLA4	cytotoxic T-lymphocyte-associated protein 4	S100A8	S100 calcium binding protein A8
CTSC	cathepsin C	SCLY	selenocysteine lyase
CTSH	cathepsin H	SDCBP	syndecan binding protein (syntenin)
CTSK	cathepsin K	SGPL1	sphingosine-1-phosphate lyase 1
CXCL9	chemokine (C-X-C motif) ligand 9	SHANK2	SH3 and multiple ankyrin repeat domains 2
CYBB	cytochrome b-245, beta polypeptide	SLA	Src-like-adaptor
ELF1	E74-like factor 1	SLC31A1	solute carrier family 31, member 1
EMB	Emigin	SLC3A2	solute carrier family 3, member 2
ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2	SLC7A7	solute carrier family 7, member 7
EPN2	epsin 2	SMU1	smu-1 suppressor of mec-8 and unc-52 homolog
FABP4	fatty acid binding protein 4, adipocyte	SNCA	synuclein, alpha
FCGR3A	Fc fragment of IgG, low affinity IIIa, receptor	SNX1	sorting nexin 1
FPGT	fucose-1-phosphate guanylyltransferase	SP1	Sp1 transcription factor
FXYD2	FXYD domain containing ion transport regulator 2	SPN	Sialophorin
GCLC	glutamate-cysteine ligase, catalytic subunit	SPP1	secreted phosphoprotein 1
GDF15	growth differentiation factor 15	SQSTM1	sequestosome 1
GLRX	glutaredoxin (thioltransferase)	SYK	spleen tyrosine kinase
GMFG	glia maturation factor, gamma	Tceb2	transcription elongation factor B, polypeptide 2
GOLPH3	golgi phosphoprotein 3 (coat-protein)	TFEC	transcription factor EC

Symbol	Gene Name	Symbol	Gene Name
GPR88	G protein-coupled receptor 88	TGFB1	transforming growth factor, beta 1
GPX1	glutathione peroxidase 1	THBS4	thrombospondin 4
GSR	glutathione reductase	TLR2	toll-like receptor 2
GSTP1	glutathione S-transferase pi 1	TNNT2	troponin T type 2 (cardiac)
GTF2B	general transcription factor IIB	TPST2	tyrosylprotein sulfotransferase 2
HBB	hemoglobin, beta	TRPV2	transient receptor potential cation channel, subfamily V, member 2
HCST	hematopoietic cell signal transducer	TXN	Thioredoxin
HMGB2	high mobility group box 2	TYROBP	TYRO protein tyrosine kinase binding protein
ICAM1	intercellular adhesion molecule 1	USP15	ubiquitin specific peptidase 15
Ifi47	interferon gamma inducible protein 47	VAPA	vesicle-associated membrane protein-associated protein A
IL18BP	interleukin 18 binding protein	VCAM1	vascular cell adhesion molecule 1
IL1RN	interleukin 1 receptor antagonist	VKORC1L1	vitamin K epoxide reductase complex, subunit 1- like 1
ITGA4	integrin, alpha 4	WNT4	wingless-type MMTV integration site family, member 4
		ZNRD1	zinc ribbon domain containing 1