Age-related impairment of the transcriptional responses to oxidative stress in the mouse heart

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1Department of Genetics and Medical Genetics, 2Department of Statistics, 3Veterans Administration Hospital, 4Department of Medicine and Wisconsin Primate Research Center, University of Wisconsin, Madison, Wisconsin 53706; and 5Department of Pharmacology and Medicine, Vanderbilt University, Nashville, Tennessee 37232-6602

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Edwards, Michael G., Deepayan Sarkar, Roger Klopp, Jason D. Morrow, Richard Weindruch, and Tomas A. Prolla. Age-related impairment of the transcriptional responses to oxidative stress in the mouse heart. Physiol Genomics 13: 119–127, 2003. First published February 25, 2003; 10.1152/physiolgenomics.00172.2002.—To investigate the transcriptional response to oxidative stress in the heart and how it changes with age, we examined the cardiac gene expression profiles of young (5-mo-old), middle-aged (15-mo-old), and old (25-mo-old) C57BL/6 mice treated with a single intraperitoneal injection of paraquat (50 mg/kg). Mice were killed at 0, 1, 3, 5, and 7 h after paraquat treatment, and the gene expression profile was obtained with high-density oligonucleotide microarrays. Of 9,977 genes represented on the microarray, 249 transcripts in the young animal (23, 29, 30). However, this technology has not been used to characterize the transcriptional response to oxidative stress in rodents (2, 10, 50), suggesting that stress responses may also play a role in aging of longer lived species. In mammals, the only intervention that is proven to extend lifespan is caloric restriction (CR) (53), and CR rodents display increased resistance to heat shock (18, 21) and oxidative damage (45).

The endogenous production of reactive oxygen species (ROS), a by-product of cellular respiration, may contribute to the aging phenotype (19). The heart is an organ that is likely to be particularly vulnerable to increases in oxidative stress, since cardiomyocytes depend heavily on mitochondrial function. The ability to cope with cardiovascular injury has been shown to decline with age (23, 29, 30), and many heart-related stresses, such as myocardial ischemia and reperfusion, generate ROS that may contribute to pathology (11). Possibly, the age-associated increase in the production of ROS contributes to the observed decline in the ability to recover from cardiac-related trauma in the aged animal (23, 29, 30).

Previous studies using high-density oligonucleotide arrays have characterized the basal transcriptional response to the aging process in skeletal muscle (25), brain (26), and heart (24) of mice. However, this technology has not been used to characterize the transcriptional response to acute oxidative stress as a function of age. Accordingly, we investigated the transcriptional response to oxidative damage in the heart by challenging 5-mo-old (young), 15-mo-old (middle-aged), and 25-mo-old mice with paraquat. Paraquat is a toxin that reacts with molecular oxygen in vivo to generate ROS in several tissues and has been used previously to elicit oxidative stress in rodents (2, 10, 50). Paraquat ingestion in rats and humans leads to severe heart damage (36, 39) and an increase in the levels of 8-hydroxydeoxyguanosine in the heart, a marker of oxidative DNA damage (48). To identify genes that have differential expression levels between young and old animals after paraquat treatment, we used oligonucleotide arrays that can simultaneously measure mRNA levels of thousands of genes. The gene expression data were then compared between age groups, and statistical methods...
were used to identify age-specific patterns of expression.

MATERIALS AND METHODS

Animal treatments. Details on the methods used to house and feed male inbred C57BL/6N Hsd mice, which have an average lifespan of ~30 mo in our colony, have been described (41). Mice were individually housed and, from 2 mo on, fed 84 kcal/kg which is ~5–10% less than the range of individual ad libitum intakes. We routinely employ this regimen to avoid age-associated obesity in aged animals. Animals were given a single intraperitoneal injection of paraquat at a dose of 50 mg/kg body wt dissolved in PBS 24 h after their last feeding and killed by cervical dislocation 1, 3, 5 and 7 h after injection. Three different animals were used for each time point and age group. All collected hearts were then dissected, placed in a microcentrifuge tube, flash-frozen in liquid nitrogen, and stored at −80°C.

Isoprostane assay. Hearts were dissected, flash-frozen with liquid nitrogen, and kept frozen at −80°C until processed. Lipids from whole heart homogenates were extracted by the method of Polch (see Ref. 34), and isoprostanes were hydrolyzed by chemical saponification, extracted using C-18 and silica Sep-Pak cartridges, purified by thin-layer chromatography and converted to pentafluorobenzyl ester trimethylsilyl ether derivatives. They were quantified by stable isotope dilution techniques using gas chromatography/negative ion chemical ionization/mass spectrometry using [2H4]-8-isopGF2-α as an internal standard as previously described (34).

Tissue preparation and oligonucleotide array hybridization. Total RNA was extracted from frozen tissue using the TRIzol reagent (Life Technologies, Grand Island, NY). Polyadenylate [poly(A)+] RNA was purified from the total RNA with oligo-dT-linked Oligotex resin (Qiagen, Valencia, CA). One microgram of poly(A)⁺ RNA was converted into double-stranded cDNA (ds-cDNA) by using the SuperScript Choice System (Life Technologies) with an oligo-dT primer containing a T7 RNA polymerase promoter (Genset, La Jolla, CA). The ds-cDNA was extracted with phenol-chloroform-isoamyl alcohol and precipitated with pellet paint coprecipitant (Novagen, Madison, WI). Biotin-labeled RNA was synthesized in vitro using a high-yield RNA transcription labeling kit (BioArray; Enzo, Farmingdale, NY). The biotin-labeled antisense cRNA was purified using the RNEasy affinity column (Qiagen) and fragmented randomly. The hybridization cocktail (200 µl) containing 10 µg of fragmented cRNA was injected into the MG-U74A array (Affymetrix, Santa Clara, CA). The GeneChip was placed in a 45°C oven at 60 rpm for 16 h. After hybridization, the GeneChips were washed and stained in a fluidic station (model 800101, Affymetrix) with signal amplification protocol using antibody. DNA GeneChips were scanned at a resolution of 3 µm twice using a Hewlett-Packard GeneArray Scanner (model 900154, Affymetrix), and the averaged images were used for further analysis.

Data analysis. The Affymetrix MG-U74A array was built on the base of the cDNA sequences from the UniGene (8/96 and build 4.0) and TIGR (build 1.0 beta) databases. This array allows measurement of mRNA levels for 12,588 genes. Approximately 16 probe pairs of oligonucleotide probes in a probe set (total of 32 probes; 16 for perfect match and 16 for mismatch control probes) are used to measure the transcript level of a gene. Each probe pair consists of a perfect match (PM) probe and a mismatch probe (MM), which allows direct subtraction of cross-hybridization signals after background subtraction. In February 2001, Affymetrix disclosed that ~21% of probe sets in MG-U74A array were defective. As a result of this finding, 2,611 probe sets were masked and removed from the analysis. GeneChip Analysis Suite 5.0 was used to analyze the image data. Affymetrix software determines the presence of mRNA in samples and computes the signals of probe sets. The software calculates differences and ratios between perfect match and mismatch signals, which are representative of the hybridization levels of their targets in each probe set. These values are integrated into a decision matrix to determine whether the transcript is detected in the sample. The average of the differences between perfect match and mismatch signals (after removing the outliers beyond 3 standard deviations) is used to estimate relative mRNA levels of the transcripts. Signals in each image are normalized to minimize an overall variability in hybridization intensities by a global scaling method. Global scaling is the computational technique in which the average signal of all probe sets in an image is scaled to a target average intensity by multiplying a scaling factor. This scaling factor is multiplied to each probe set signal to give the raw signal intensity. Since for each gene we are more interested in the fold changes than in the absolute intensities, the scaled data were further normalized by dividing all raw measurements for each gene by the mean raw signal intensity for the same gene as measured in the three control animals (nontreated) for that particular age group to give the normalized expression.

Statistical analysis. Each gene was considered separately when attempting to classify it as paraquat responsive. All genes considered absent as determined by Affymetrix software for all measured time points in all age groups were eliminated from our analysis (4,463 probe sets from a total of 9,977 were eliminated based on this criteria). A gene was classified as paraquat responsive in young mice as follows: a one-way analysis of variance (ANOVA) model was fit to the intensities obtained for that gene from the young mice, with 5 treatment conditions, namely, 0, 1, 3, 5, and 7 h post-paraquat, and 3 replicates for each condition. Genes that had a small P value (less than 0.01) and had at least one present call for one of the time points were categorized as paraquat responsive. This procedure was also used to classify a gene as paraquat responsive in middle-aged and old mice. For a gene to be considered paraquat responsive in all age groups, its P value, as determined by ANOVA for each specific age group, had to be less than 0.01 in two of the three ages and less than 0.05 in the remaining age group.

RESULTS

Detection of lipid peroxidation in mouse hearts. To determine whether paraquat treatment induced oxidative stress in mouse hearts, we assayed cardiac tissue for the presence of P(2)-isoprostanes. Isoprostanes are generated from the free radical catalyzed peroxidation of arachidonic acid and have previously been used to accurately assess the oxidative status in vivo (34). Basal levels of isoprostanes were 1.5 ± 0.1 ng/g in the control old mouse hearts and 1.1 ± 0.2 ng/g in the control young mouse hearts, and levels increased significantly (Student’s t-test, P < 0.05) in both young and old age groups following 7 h of paraquat treatment (2.3 ± 0.4 and 2.2 ± 0.1 ng/g, respectively) (Fig. 1). Although basal levels of isoprostanes were greater in the old vs. the young hearts, this difference was not significant. Interestingly, we observed no increases in tissue carbonyl groups, a marker of oxidative protein damage.
damage, as measured by 2,4-dinitriphenylhydrazine staining (data not shown).

Paraquat-responsive genes. Of 9,977 genes probed on the oligonucleotide microarray, we identified 249 genes in the young mice, 298 genes in the middle-aged mice, and 256 genes in the old mice with differential expression levels in response to paraquat over the 7-h time course (ANOVA, \( P < 0.01 \)). Tables containing all genes induced at each age group are available as a data supplement, published online at the Physiological Genomics web site. Among these induced genes, a total of 55 transcripts were determined to be paraquat responsive for all age groups (Table 1). We consider these transcripts to be the most statistically robust set of genes involved in the cardiac paraquat response, since differential expression in response to paraquat is observed in all study groups.

Among these 55 common genes, the FK506-binding protein 5 (Fkbp5) encoding gene had the largest fold change in both the young and old groups (greatest fold change post-paraquat was 12.2-fold and 24.5-fold in the young and old, respectively). The FK506-binding protein is tightly associated with the cardiac sarcoplasmic reticulum Ca\(^{2+}\)-release channel [ryanodine receptor type 2 (RyR2)], and it is likely that Fkbp5 inhibits the signaling of calcineurin through its association with this receptor (4). Possibly, Fkbp5 activation in response to paraquat may be related to the involvement of calcineurin in activating the immune response during cardiac stress (33). Paraquat induced metallothionein 1 and 2, two low-molecular-mass proteins that bind to free metal ions and regulate cellular copper and zinc metabolism. Metallothionein, which protects cells against oxidative insults, can be induced by a variety of stressors, including glucocorticoids (17), heavy metals (1), ischemia-reperfusion (8), and ionizing and UV irradiation (6).

The observed induction of several genes appears to be associated with a protective metabolic stress response in the heart. Bel-XL is an anti-apoptotic protein that allows cells to maintain oxidative metabolism during cellular stress by allowing continued transport of metabolites across the outer mitochondrial membrane (49). The 5′-nucleotidase activity controls the production of adenosine in the heart through dephosphorylation of AMP (28). Adenosine receptors may play a key role in the adaptive response to altered O\(_2\) delivery or increased metabolic activity (20). Pyruvate dehydrogenase kinase 4 (PDK4) is a key element involved in fuel selection. PDK4 inhibits pyruvate dehydrogenase and thus minimizes carbohydrate oxidation by preventing the flow of glycolytic products into the tricarboxylic acid cycle (5).

Aging is associated with altered transcription of immediate early genes following oxidative stress. A major class of genes displaying increased expression after paraquat injection was the immediate early response genes (IEGs). As the first response to damage, IEG protein products initiate a coordinated cascade of adaptive gene expression in response to a given stimulus (9). We found that young mice displayed a greater number of IEGs induced compared with middle-aged and old mice (12 genes in the young, 7 genes in the middle-aged, and 5 genes in the old), suggesting age-related impairment in this response (Table 2). Interestingly, many of the IEGs that showed age-related impairment in the transcriptional response have been shown to be dependent on mitogen-activated protein kinase kinase (MAPKK) signaling for expression including zfp36, btg2, cyr61, nr4a1, ptpn16 (22), and atf3 (7). The average normalized expression of the MAPKK-dependent IEGs increased significantly in the young and middle-aged hearts 1 h after paraquat injection and then returned to similar levels of normalized expression as found in the old animals 5 h after the initial oxidative insult (Fig. 2). The lower induction of IEG gene expression in older mice was apparently not due to constitutively higher levels of expression in old mouse hearts, since the average raw expression for these genes was nearly identical in the young and old groups (average raw expression of 4,198 and 4,340 fluorescent units, respectively).

Aging is associated with an impairment in the induction of stress-response genes: GADD45, MAP3K6, and JunB. Another class of genes preferentially induced in the young was the GADD45 gene family. GADD45 was initially identified as a gene whose transcription is rapidly induced in cells treated with DNA-damaging agents (15, 38). Using a yeast two-hybrid system to screen a placenta library, Takekawa and Saito (47) previously isolated three GADD45-like cDNAs (GADD45α, GADD45β, and GADD45y) that encode for three similar GADD45 proteins that bind to MAP3K4, a protein which mediates activation of both p38 and JNK pathways in response to external stimuli (16). The levels of

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1. The Data Supplement for this article is available online at [http://physiolgenomics.physiology.org/cgi/content/full/13/2/119](http://physiolgenomics.physiology.org/cgi/content/full/13/2/119/DC1).
Table 1. Common paraquat-responsive genes in young (5 mo old), middle-aged (15 mo old), and old (25 mo old) C57BL/6 mouse hearts

<table>
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<tr>
<th>Identifier</th>
<th>Gene Name</th>
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<th>Middle-aged</th>
<th>Old</th>
<th>Function</th>
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<tbody>
<tr>
<td>U16959</td>
<td>FK506 binding protein 5 (51 kDa)</td>
<td>12.2(7)</td>
<td>2.3(5)</td>
<td>24.5(7)</td>
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<td>L00039</td>
<td>Myelocytomatosis oncogene</td>
<td>11.4(3)</td>
<td>5.4(3)</td>
<td>17.2(5)</td>
<td>immediate early response</td>
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<tr>
<td>K02292</td>
<td>Metallothionein 2</td>
<td>10.0(7)</td>
<td>11.5(7)</td>
<td>13.0(7)</td>
<td>antioxidant response</td>
</tr>
<tr>
<td>AW048937</td>
<td>Cyclin-dependent kinase inhibitor 1A(P21)</td>
<td>5.6(7)</td>
<td>2.3(7)</td>
<td>6.5(7)</td>
<td>cell cycle</td>
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<tr>
<td>L35049</td>
<td>Bcl2-like</td>
<td>3.9(7)</td>
<td>1.9(7)</td>
<td>3.0(7)</td>
<td>apoptosis</td>
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<tr>
<td>V00835</td>
<td>Metallothionein 1</td>
<td>3.5(7)</td>
<td>5.3(7)</td>
<td>6.6(7)</td>
<td>antioxidant response</td>
</tr>
<tr>
<td>L44678</td>
<td>Zinc finger protein 36</td>
<td>2.9(5)</td>
<td>3.2(5)</td>
<td>3.1(3)</td>
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<td>M61007</td>
<td>CCAAT/enhancer binding protein (C/EBP), beta</td>
<td>2.8(3)</td>
<td>1.7(7)</td>
<td>4.3(7)</td>
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<tr>
<td>AW046181</td>
<td>Serum/glucocorticoid regulated kinase</td>
<td>2.7(5)</td>
<td>2.2(7)</td>
<td>2.7(5)</td>
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<td>AI846938</td>
<td>Herpud1</td>
<td>2.4(7)</td>
<td>1.7(7)</td>
<td>2.7(7)</td>
<td>unfolded protein response</td>
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<tr>
<td>M74570</td>
<td>Alcohol dehydrogenase family 1, subfamily A2</td>
<td>2.2(7)</td>
<td>1.4(7)</td>
<td>3.2(7)</td>
<td>antioxidant response</td>
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<tr>
<td>AI843106</td>
<td>Ser/2A regulated PA26 nuclear protein</td>
<td>2.0(7)</td>
<td>2.2(7)</td>
<td>2.4(7)</td>
<td>genotoxic response</td>
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<tr>
<td>AI85318</td>
<td>Cardiac morphogenesis</td>
<td>1.8(7)</td>
<td>1.7(5)</td>
<td>1.5(7)</td>
<td>stress response</td>
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<tr>
<td>U00830</td>
<td>Oxidative stress induced</td>
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<td>1.9(7)</td>
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<td>AF103875</td>
<td>ATP-binding cassette, sub-family G (WHITE), member 2</td>
<td>1.6(7)</td>
<td>1.7(7)</td>
<td>2.0(7)</td>
<td>drug resistance</td>
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<tr>
<td>M63801</td>
<td>Gap junction membrane channel protein alpha 1</td>
<td>0.4(7)</td>
<td>0.4(7)</td>
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**Growth Factor Response/Signaling**

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<td>AF064748</td>
<td>S3-12 protein</td>
<td>5.7(7)</td>
<td>3.0(7)</td>
<td>3.2(7)</td>
<td>growth factor response</td>
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<tr>
<td>M70642</td>
<td>Connective tissue growth factor</td>
<td>4.6(3)</td>
<td>5.2(5)</td>
<td>7.2(5)</td>
<td>immediate early gene</td>
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<tr>
<td>U50413</td>
<td>Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1</td>
<td>3.5(7)</td>
<td>2.0(7)</td>
<td>2.3(7)</td>
<td>adipocyte differentiation</td>
</tr>
<tr>
<td>AF009246</td>
<td>RAS, dexamethasone-induced 1</td>
<td>3.2(7)</td>
<td>3.1(7)</td>
<td>9.5(7)</td>
<td>GTP binding</td>
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<tr>
<td>AF051945</td>
<td>Cardiac morphogenesis</td>
<td>2.5(7)</td>
<td>2.1(7)</td>
<td>2.1(5)</td>
<td>growth factor response</td>
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<tr>
<td>U28656</td>
<td>Eukaryotic translation initiation factor 4E binding protein 1</td>
<td>2.0(7)</td>
<td>1.9(7)</td>
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**Metabolic/Catabolic**

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<td>AJ001418</td>
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<td>L12059</td>
<td>5’ nucleotidase</td>
<td>2.8(5)</td>
<td>1.9(7)</td>
<td>1.9(3)</td>
<td>AMP catabolism</td>
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<tr>
<td>Y08027</td>
<td>ART3 gene</td>
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<td>2.2(7)</td>
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<td>AF084466</td>
<td>Ras-like GTP-binding protein rad</td>
<td>0.2(7)</td>
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**Transcription Regulation**

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<td>D49473</td>
<td>SRY-box containing gene 17</td>
<td>3.7(7)</td>
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<td>embryogenesis</td>
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<td>AF072240</td>
<td>Methyl-CpG binding domain protein 1</td>
<td>3.4(7)</td>
<td>2.4(7)</td>
<td>4.1(7)</td>
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<td>D42124</td>
<td>V-maf, cytoplasmic, light chain I</td>
<td>1.6(7)</td>
<td>1.7(7)</td>
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<td>induction of iron-regulating genes</td>
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**Miscellaneous**

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<tr>
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<td>L02914</td>
<td>Aquaporin 1</td>
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<td>AW123904</td>
<td>GABA(A) receptor-associated protein like 1</td>
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<td>1.9(7)</td>
<td>1.8(7)</td>
<td>unknown</td>
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<td>AF029992</td>
<td>Period homolog (Drosophila)</td>
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<td>AI844532</td>
<td>splicing factor 3b, subunit 1, 155 kDa</td>
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<td>AW184677</td>
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<td>1.8(3)</td>
<td>1.8(7)</td>
<td>1.6(5)</td>
<td>vesiculur transport</td>
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<td>U71205</td>
<td>RAS-like protein expressed in many tissues</td>
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<td>1.8(7)</td>
<td>1.6(7)</td>
<td>calmodulin binding</td>
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**Unknown**

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<td>4.7(7)</td>
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<td>2.9(5)</td>
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<td>AI849135</td>
<td></td>
<td>1.6(5)</td>
<td>1.5(3)</td>
<td>1.9(7)</td>
<td>unknown</td>
</tr>
<tr>
<td>AW124678</td>
<td></td>
<td>1.4(7)</td>
<td>1.7(7)</td>
<td>1.7(5)</td>
<td>unknown</td>
</tr>
<tr>
<td>AW125272</td>
<td></td>
<td>0.6(5)</td>
<td>0.7(5)</td>
<td>0.6(7)</td>
<td>unknown</td>
</tr>
<tr>
<td>AA958903</td>
<td></td>
<td>0.4(7)</td>
<td>0.5(7)</td>
<td>0.4(5)</td>
<td>unknown</td>
</tr>
</tbody>
</table>

Presented in this table are the 55 common genes that changed significantly in response to paraquat at all ages. The largest or smallest normalized measurement is shown in the table along with the corresponding time point in which it occurs for that particular age group. There were 48 common genes that increased and 7 common genes that decreased in mRNA levels for the majority of the paraquat time course.
all the GADD45 transcripts increased significantly in the young hearts following paraquat treatment, two of which were also significantly induced in the middle-aged hearts (GADD45α and GADD45γ), but no induction was found in the old hearts (Fig. 3).

The only MAPK-encoding gene found to be paraquat responsive by our ANOVA criteria was map3k6. MAP3K6 is a member of a MAPK cascade that is known to activate two closely related but distinct stress-responsive parallel pathways: the c-Jun N-terminal kinase (JNK) or stress-activated kinase (SAPK) pathway and the p38 kinase pathway. Overexpression of MAP3K6 has been shown to activate the JNK but not the p38 kinase pathway (51). This gene, found to be significantly induced in young animals only, had the highest normalized expression of all paraquat-responsive genes (20-fold). Levels of c-jun and jund expression remained constant over the experimental time course for both age groups, whereas levels of junb mRNA increased considerably in all age groups following paraquat. Junb expression in the young and middle-aged group had a peak normalized expression of 7.9 and 6.7, respectively, 1 h after paraquat (ANOVA, \( P < 0.05 \)). The increase in junb expression was reduced (3.6-fold) and more variable (ANOVA, \( P < 0.20 \)) in older mice, and its peak normalized expression was delayed (5 h postinjection) compared with the young and middle-aged animals.

**Age-related alterations in the expression of antioxidant genes.** The mechanism of paraquat toxicity involves the generation of oxidative stress, and therefore we decided to investigate the expression levels of antioxidant enzymes in the heart of treated and untreated animals (Table 3). There were 10 genes out of a total of

---

**Table 2. IEGs identified as paraquat responsive (ANOVA, \( P < 0.05 \))**

<table>
<thead>
<tr>
<th>Immediate Early Gene</th>
<th>Young</th>
<th>Middle-Aged</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCAAT/enhancer binding protein (C/EBP), beta</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Myelocytomatosis oncogene</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Connective tissue growth factor</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Serum/glucocorticoid regulated kinase</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Zinc finger protein 36</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>B-cell translocation gene 2, anti-proliferative</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Cysteine-rich protein 61</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Activating transcription factor 3</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Growth arrest and DNA-damage-inducible 45 beta</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>TGFβ inducible early growth response</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>Nuclear receptor subfamily 4, group A, member 1</strong></td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>Protein tyrosine phosphatase, non-receptor type 16</strong></td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

All immediate early genes (IEGs) were paraquat responsive in young mice, and this number declined with age. Genes in bold represent IEGs dependent on MAPKK signaling for expression.

---

**Fig. 2.** Age-associated changes in expression profiles of MAPKK-dependent immediate early response genes (IEGs) in the hearts of young and old mice after induced oxidative stress. A: lines represent the averaged normalized expression ± SE for 6 IEGs paraquat responsive in the young [zfp36, btg2, ptpn16, cyr61, nr4a1, and atf3] and 3 other IEGs measured on this microarray [junb, krox-24, and ptgs2] shown to be dependent on MAPKK signaling for expression (7, 22) in the hearts of young, middle-aged, and old mice. No significant differences were found in the constitutive levels of these genes when comparing the average raw intensity levels for young control vs. old control mice. The averaged normalized expression for these genes is significantly higher (\(^*\) Wilcoxon signed ranks test, \( P < 0.05 \) for young mice vs. old mice) in the heart of young animals 1 and 3 h after paraquat treatment, but similar to old hearts by the 5-h time point. c-fos, another IEG whose transcription is stimulated by MAPKK signaling, is not measured on our array and therefore not included in the graph.

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22 antioxidant genes expressed that were considered to be paraquat responsive (ANOVA, \(P < 0.05\)). Only the expression of metallothionein 1 and 2 were induced significantly across all ages due to the paraquat treatment. Moreover, the induction of glutathione s-transferase, alpha 3 was specific to the young class of mice, the induction of glutathione peroxidase 1 and peroxiredoxin 4 was restricted to the middle-aged group, and superoxide dismutase 1 induction was limited to the oldest group of mice. Because age-related differences in constitutive levels of these genes could affect the animals’ ability to cope with induced oxidative stress, we decided to also investigate the basal levels of expression of these genes in the hearts of young and old mice. Interestingly, of the 10 genes that showed a significant difference (Student’s t-test, \(P < 0.05\)) in basal levels of expression between young and old control mice, 8 of these genes (\(gpx4\), \(gstp2\), \(mt1\), \(prdx1\), \(prdx2\), \(prdx5\), \(sod1\), and \(sod2\)) had decreased mRNA levels in the older animals.

**Table 3. Basal levels of expression of oxidative stress-related genes in young, middle-aged and old hearts**

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Gene</th>
<th>Average Basal Expression (Raw)</th>
<th>Young vs. Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>U49430</td>
<td>Ceruloplasmin</td>
<td>788</td>
<td>1,164</td>
</tr>
<tr>
<td>X03920</td>
<td>Glutathione peroxidase 1 (M)</td>
<td>3,150</td>
<td>3,139</td>
</tr>
<tr>
<td>U13705</td>
<td>Glutathione peroxidase 3</td>
<td>24,388</td>
<td>26,482</td>
</tr>
<tr>
<td>D87896</td>
<td>Glutathione peroxidase 4</td>
<td>8,477</td>
<td>8,544</td>
</tr>
<tr>
<td>X65021</td>
<td>Glutathione S-transferase, alpha 3(Y)</td>
<td>0.06</td>
<td>1.051</td>
</tr>
<tr>
<td>L06047</td>
<td>Glutathione S-transferase, alpha 4 (Y, M)</td>
<td>1.060</td>
<td>1.051</td>
</tr>
<tr>
<td>J03952</td>
<td>Glutathione S-transferase, mu 1</td>
<td>20.648</td>
<td>31.976</td>
</tr>
<tr>
<td>J04696</td>
<td>Glutathione S-transferase, mu 2</td>
<td>8.057</td>
<td>8.229</td>
</tr>
<tr>
<td>J03953</td>
<td>Glutathione S-transferase, mu 3</td>
<td>6.037</td>
<td>7.413</td>
</tr>
<tr>
<td>U24428</td>
<td>Glutathione S-transferase, mu 5</td>
<td>2.456</td>
<td>1.979</td>
</tr>
<tr>
<td>X53451</td>
<td>Glutathione S-transferase, pi 2</td>
<td>11.986</td>
<td>13.071</td>
</tr>
<tr>
<td>X98055</td>
<td>Glutathione S-transferase, theta 1</td>
<td>1.246</td>
<td>1.024</td>
</tr>
<tr>
<td>AF054670</td>
<td>Heme oxygenase 2 (Y, M)</td>
<td>962</td>
<td>847</td>
</tr>
<tr>
<td>AI835051</td>
<td>Metallothionein 1 (Y, M, O)</td>
<td>26,268</td>
<td>22,223</td>
</tr>
<tr>
<td>K02236</td>
<td>Metallothionein 2 (Y, M, O)</td>
<td>7,347</td>
<td>5,495</td>
</tr>
<tr>
<td>AB023564</td>
<td>Peroxiredoxin 1</td>
<td>26,090</td>
<td>26,184</td>
</tr>
<tr>
<td>AP032714</td>
<td>Peroxiredoxin 2</td>
<td>26,718</td>
<td>29,950</td>
</tr>
<tr>
<td>U96746</td>
<td>Peroxiredoxin 4 (M)</td>
<td>1,164</td>
<td>984</td>
</tr>
<tr>
<td>AP093857</td>
<td>Peroxiredoxin 5 (M, O)</td>
<td>5,734</td>
<td>4,486</td>
</tr>
<tr>
<td>M35725</td>
<td>Superoxide dismutase 1, soluble (O)</td>
<td>11,288</td>
<td>14,856</td>
</tr>
<tr>
<td>L35528</td>
<td>Superoxide dismutase 2, mitochondrial</td>
<td>9,335</td>
<td>7,905</td>
</tr>
<tr>
<td>U38281</td>
<td>Superoxide dismutase 3, extracellular</td>
<td>3,201</td>
<td>4,434</td>
</tr>
</tbody>
</table>

Average raw signal intensity (as measured in the three control animals for each age group) for genes typically associated with the antioxidant response probed for on the U74A array. The last column in the table gives the \(P\) values for genes in which the basal level of expression was significantly different (Student’s t-test, \(P < 0.05\)) between the control hearts of young and old mice. Glutathione synthetase, glutathione S-transferase, alpha 2, mu 6, and theta 2 had no measured expression in any of the time points for all ages and are not included here. Catalase and heme oxygenase 1 are not probed for on this microarray. NSC, no significant change; Y, paraquat-induced change in expression levels in young (ANOVA, \(P < 0.05\); M, paraquat-induced change in expression levels in middle-aged (ANOVA, \(P < 0.05\); O, paraquat-induced change in expression levels in old (ANOVA, \(P < 0.05\)).
DISCUSSION

Our study provides the first gene expression profile associated with oxidative stress in the heart and suggests that aging is associated with defects on the transcriptional induction of specific cellular signaling pathways. Specifically, we observed an impaired transcriptional activation of IEG genes dependent on MAPKK signaling which also includes the JNK pathway (Fig. 4). The lack of MAPKK-dependent IEG induction in the old mouse hearts suggests an attenuated response in this pathway, which is likely to result in less induction of downstream targets. Additionally, only young and middle-aged mouse hearts display significant changes in the expression of GADD45-like genes which are mediators of stress-activated protein kinase signaling (JNK). Further evidence for an age-related change in paraquat-induced JNK signaling in the heart comes from examining the expression levels of components and targets of this pathway. Map3k6 (a signal-regulated kinase of the JNK pathway) and junb (a downstream target of stress-activated signaling) both exhibit higher levels of normalized expression following paraquat injection in the young mice compared with the old. Previous studies agree with our findings, having shown reduced levels of expression of the IEGs c-fos, c-myc, and c-jun in aged rat hearts following hemodynamic stress (43, 46). Some investigators have suggested that JNK signaling mediates apoptosis in cultured adult rat cardiac myocytes in response to induced oxidative stress (3), while other groups have reported conflicting results for the role of JNK signaling in cardiac myocyte apoptosis. Dougherty et al. (12) reported transfection of myocytes subjected to hypoxia and reoxygenation with JNK pathway interfering plasmid vectors increased the rates of apoptosis nearly twofold compared with control myocytes, whereas Minamino et al. (32) found that embryonic stem (ES) cell-derived cardiac myocytes defective for JNK signaling were extremely sensitive to hydrogen peroxide-induced apoptosis. It is conceivable that the observed early increase in expression of JNK-related genes in the young animals provides increased protection in the heart against induced oxidative stress compared with that of their old counterparts.

It is possible that the age-related differences in the transcriptional response to paraquat in the mouse heart are due to elevated levels of oxidative damage in the aged myocardium. The concentration of the oxidative adduct of guanosine, 8-hydroxy-2’-deoxyguanosine, has been shown to be increased in the hearts of aged mice (44). One possibility is that the response to oxidative damage is already activated in the older mice, and therefore signaling pathways, such as the MAPK pathways, are chronically activated, displaying less induction following exogenous oxidative stress. We did not find evidence to support this hypothesis at the transcriptional level, since the basal level of IEGs and GADD45 mRNA is similar in young and old animals. We also observed that paraquat treatment does not lead to higher lipid peroxidation by-products in the heart of aged mice, compared with young mice. Possibly, other types of oxidative damage may be higher in the hearts of the aged, paraquat-treated mice. Interestingly, we observed that genes encoding the ribosomal proteins acidic ribosomal phosphoprotein PO, S4, S19, S28, L5, L7, and L21 were uniquely upregulated in the old vs. young hearts following paraquat exposure (see the Data Supplement, at the Physiological Genomics web site). It is unclear why transcription of these ribosomal protein genes would increase following paraquat treatment, but it may signify more ribosomal biogenesis in response to the damaging agent. Possibly, the induction of these and other genes exclusively induced in the old mice represent unique metabolic adaptations to oxidative or metabolic stress in the aged heart.

An important issue that should be considered when examining our data is that experimental conditions in this study are designed to induce an acute oxidative stress response. In contrast, chronic oxidative stress is likely to have long-term effects on gene expression that are distinct from the acute effects described here. We find that some antioxidant genes display lower basal levels of expression in old hearts, including gpx4, gstp2, mtl1, prdx1, prdx2, prdx5, sod1, and sod2, suggesting a lower ability to cope with oxidative stress with age in
the heart. Interestingly, a previous study has shown that aging in the heart is associated with augmentation of cardiac IL-6 induction following endotoxin challenge (42). Possibly, decreased antioxidant gene expression and increased expression of pro-inflammatory factors contribute to oxidative stress-mediated injury in aged hearts and contribute to the inability to rapidly induce specific transcriptional responses.

We also note that our data reveal a number of paraquat-inducible genes that were not previously known to respond to oxidative stress. These include several genes involved in metabolic adaptation, signal transduction, and cell growth. Other genes that respond to paraquat have no known homology or function (Table 2). Eventual elucidation of the roles of these genes as components of the antioxidant response may increase our understanding of cellular oxidative damage defense mechanisms in the heart. Because paraquat depletes NADPH in addition to superoxide generation (14), it is also possible that some genes induced by this toxin represent a metabolic stress response and are therefore unrelated to the oxidative stress response. Indeed, the induction of pyrurate dehydrogenase kinase 4, 5'-nucleotidase, and act 3 genes in all age groups is consistent with this hypothesis. We also note that our study represents a first step in understanding transcriptional mechanisms of age-related inability to cope with oxidative stress in the heart. Future studies will involve characterization of specific pathways at the biochemical and cellular level to identify the cell type specificity and the molecular basis of the age-related transcriptional impairment described here.

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REFERENCES


