Genetic dissection of systemic autoimmune disease in Nrf2-deficient mice

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Li, Jiang, Thor D. Stein, and Jeffrey A. Johnson. Genetic dissection of systemic autoimmune disease in Nrf2-deficient mice. Physiol Genomics 18: 261–272, 2004. First published June 1, 2004; 10.1152/physiolgenomics.00209.2003.—Systemic lupus erythematosus (SLE) is an autoimmune disease with immune-complex deposition that affects multiple organs. Previous studies have suggested the involvement of oxidative stress and apoptosis in SLE, but no clear link to etiology has been established. Here we show that mice deficient in a transcription factor responsible for controlling the expression of numerous detoxification and antioxidant genes develop an autoimmune disease with multiple organ pathologies that closely resembles human SLE. Aged female mice with a knockout of nuclear factor, erythroid-derived 2, like 2 (nrf2) are prone to develop antibodies against double-stranded DNA and the Smith antigen as well as IgG, IgM, and C3 deposition in kidney, liver, heart, and brain. Prior to the development of autoimmune antibodies and organ pathology, oxidative damage occurs in the liver and kidney as indicated by the increased levels of the DNA oxidation marker 8-hydroxydeoxyguanosine and the later increase in the lipid peroxidation product malondialdehyde. Gene expression profiles demonstrate an early decrease in numerous antioxidant and detoxification genes in the livers and altered levels of cytokines and T and B cell-specific genes in the spleens of nrf2 knockout mice. These data strongly suggest that a deficiency in detoxification and increased oxidative stress can result in the development of a systemic autoimmune disease.

SYNOPSIS

Systemic lupus erythematosus (SLE) is a recurring and remitting autoimmune disorder that primarily affects women. Uncontrolled production of autoantibodies to DNA, histones, nucleolar antigens, and/or ribonucleotide-binding proteins are a key feature of the disease and are believed to mediate much of the pathology observed in SLE. Gene knockouts in mice suggest the involvement of multiple pathways in the etiology of SLE. For instance, increased oxidative stress (20), increased apoptosis of lymphocytes and neutrophils (13, 16), decreased clearance of apoptotic debris (37, 49), and a dysregulation of T and B lymphocyte functions all may be involved in the development of the autoimmunity associated with SLE. Disruptions in specific genes involved in many of the above processes are sufficient to lead to development of an SLE-like syndrome in transgenic mice. However, it is unclear what the natural progression of SLE may be in humans.

It has been suggested that SLE is caused by an overload of apoptotic cells (1). High levels of extracellular DNA or other nuclear macromolecules not normally exposed to the immune system may lead to autoantigen presentation and autoantibody production. This can occur by a decreased clearance of circulating DNA or other cellular components such as occurs in both mice and humans with a knockout of the DNA-degrading enzyme deoxyribonuclease I (37, 49) or the complement protein C1q (5, 43). Alternatively, high levels of oxidative stress, either by drugs or toxins or by decreased antioxidant enzymes, may increase the number of apoptotic or dying cells, which then overwhelm normal clearance mechanisms. Increased oxidative stress via an interaction of environmental toxins and genetically compromised antioxidant pathways may lead to the development of human SLE.

Several markers for oxidative stress are elevated in patients with SLE, including oxidized glutathione (42), the lipid peroxidation product malondialdehyde (MDA) (2, 42), and the DNA oxidation adduct 8-hydroxydeoxyguanosine (8OHdG) (3). Dietary intake of antioxidants was decreased in a population of SLE patients (2), and vitamin E as well as a mixture of vitamin E, vitamin C, and β-carotene improved kidney function and reduced the levels of autoantibodies in MRL/lpr SLE mice (45, 46). These data implicate the involvement of oxidative stress in SLE pathogenesis and suggest that antioxidant therapies may help ameliorate the disease.

Nuclear factor, erythroid-derived 2, like 2 (NRF2) is a basic leucine zipper transcription factor. As a heterodimer, NRF2 can bind to a NF-E2/AP-1 repeat sequence in the promoter of the β-globin gene (36) as well as the similar antioxidant response element core sequence present in the promoters of many antioxidant and detoxification genes (23, 38, 44). Thus NRF2 is a key transcription factor important for cellular protection against oxidative stress and chemical-induced damage in liver (7, 17, 29), lung (8), and brain (31, 34). Nrf2 knockout mice would be expected to possess increased oxidative damage in these organs and therefore may be susceptible to the development of SLE. In fact, it has previously been shown that aged nrf2 knockout mice develop autoantibodies and a severe glomerulonephritis (50). Here we examine multiple organs that can be affected in SLE and establish a time course of oxidative stress and autoimmune development. We show autoantibody production and multi-organ involvement consistent with SLE in aged female nrf2 knockout mice. Gene expression profiles of the liver and spleen demonstrate an early deficiency in antioxidant genes and an immune dysregulation specific for female nrf2 knockout mice.

EXPERIMENTAL PROCEDURES

Animals. Nrf2−/− mice were generated by replacing the basic leucine zipper domain with the lacZ reporter construct as described previously (9). Nrf2−/− mice (C57B6/J) were crossed with nrf2−/− mice (C57B6/129SVJ) to generate mice heterozygous for nrf2. Interbreeding these heterozygotes generated the double-knockout mice (nrf2−/−) as well as the corresponding littermate controls (nrf2+/−). Mice were housed in plastic cages on a bedding of wood chips and maintained at ~22°C, ~60% relative humidity, and a 12:12-h light/
dark cycle. The animals were fed with mouse diet 5015 (LabDiet, Richmond, IN), which includes 30 IU/g of vitamin A and 35 IU/kg of vitamin E, and drinking water ad libitum. All animal protocols were approved by the Institutional Animal Use and Care Committee at the University of Wisconsin-Madison.

**Histopathology.** Mice were killed with CO2 and immediately perfused through the heart with phosphate-buffered saline (PBS). All major organs, including spleen, heart, liver, kidney, and brain, were collected and fixed in 4% paraformaldehyde overnight. Spleens were weighed and expressed as a percentage of total body weight. Organs were then sunk in 30% sucrose and frozen in OCT embedding medium (Sakura Finetec, Torrance, CA) at −70°C. Six-micrometer cryosections were stained with hematoxylin and eosin (H&E) and evaluated by light microscopy. All samples were examined for pathology at ×20 and ×40 magnifications by an experimenter blinded to the sample’s genotype. Pathology was determined using several criteria described previously (12), including mononuclear cell infiltration.

**Immunofluorescent evaluation of IgM, IgG, and C3 deposits.** Cryosections from kidney, liver, and brain were pretreated with goat serum and stained with fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG (Vector Laboratories, Burlingame, CA) and C3 (ICN Biomedicals, Aurora, OH) antibodies and Texas Red-labeled anti-human IgG (Vector Laboratories) diluted 1:100. Hoechst 33258 (Sigma, St. Louis, MO) counterstaining was performed before examination by UV fluorescence microscopy.

**Determination of autoantibodies in the serum.** Levels of anti-double-stranded DNA (anti-dsDNA) and anti-Smith antigen antibodies were determined in the sera using specific mouse ELISA kits supplied by Alpha Diagnostic International (San Antonio, TX). Serum was diluted 1:100, and anti-mouse IgG (H/L) conjugated with horseradish peroxidase was used as the secondary antibody. Values were normalized to a normal mouse serum negative control that did not contain antibodies against dsDNA or the Smith antigen. Each sample was run in duplicate, and each data point represents the mean of the two values.

**Lipid peroxidation assay.** MDA was measured with a colorimetric assay (Calbiochem, San Diego, CA) as described previously (19). Absorbance was measured at 586 nm using a microplate reader. A standard curve was used to calculate the concentration (μmol/g) of MDA for each sample. The final MDA level is presented as the average of 3–5 age-matched animals.

**Detection of 8-hydroxydeoxyguanosine.** 8OHdG was detected using standard immunohistochemistry techniques. Frozen sections with a width of 10 μm were taken, and 8OHdG was detected with a 1:200 dilution of the polyclonal antibody against 8OHdG (Chemicon, Temecula, CA). As a control preimmune goat IgG (Vector) was used in place of the primary antibody. The Vectastain Elite ABC kit and avidin–biotin complex (Vector) were used for the entire experimental series. Sections were counterstained with 70% of ethanol. The pellets were resuspended in PBS containing propidium iodide (0.01 mg/ml), Triton X-100, propidium iodide, and RNase (10 μg/ml) and analyzed by a modular flow cytometry system (FACS-calibur; Becton-Dickinson, San Jose, CA). The percentage of apoptotic cells was calculated as follows: % apoptotic cells = (% of cells in subdiploid peak)/(% of cells in subdiploid, diploid, and hyperdiploid peaks) × 100. In tissues, cells with DNA fragmentation were determined by the terminal deoxynucleotidyltransferase incorporation of FITC-12-dUTP into DNA (in situ cell death detection kit; Roche Biochem, Indianapolis, IN).

**Statistical analysis.** All the experimental data shown were repeated at least three times unless otherwise indicated. The autoantibody histology, DNA oxidation, and TUNEL results are representative of the data obtained from at least 4 mice per group. Results are expressed as means ± SE. Unless otherwise stated, statistical significance was determined using an unpaired, two-tailed Student’s t-test, and a P value <0.05 was considered significant.

**RESULTS**

**Phenotype of nrfl2 knockout female mice.** Acute weight loss is a common clinical manifestation of SLE. At 12 mo of age several nrfl2 knockout female mice were substantially smaller in size than their nrfl2 wild-type female littermates. Taken together, the weight of the nrfl2 knockout mice was 27.3 ± 3.6 g (n = 7), whereas the wild-type or heterozygous female mice weigh 44.8 ± 4.9 g (n = 8, P < 0.05). No wild-type or heterozygous mice weighed less than 30 g, whereas 45.5% of the nrfl2 knockout mice were less than 30 g (Table 1). Figure 1A shows a severe case of chronic weight loss in a female nrfl2
knockout mouse compared with a wild-type female littermate. There were no differences in size between knockout and wild-type male mice.

The spleen may be enlarged in patients with SLE. Similarly, upon postmortem analysis, most 12-mo-old female nrf2 knockout mice possessed a grossly enlarged spleen (Fig. 1C). In fact, the spleen-to-body weight ratio was 27.3 ± 11.8 mg/g (n = 7) in nrf2 knockout mice and 4.5 ± 1.2 mg/g (n = 8, P < 0.05) in wild-type or heterozygous mice (Table 1).

**Autoantibody production and immune complex deposition in multiple organ systems.** Despite the variable clinical presentation of SLE, autoantibody production and deposition, especially with the glomeruli of the kidney, is a defining feature of the disease. We found elevated levels of autoantibodies that recognize dsDNA in the serum of some 12-mo-old female nrf2 knockout mice (1.08 ± 0.21) compared with female wild-type (0.45 ± 0.05, n = 8, P < 0.05) or male wild-type or knockout mice (Fig. 2). In addition, autoantibodies against the Sm small nuclear ribonucleoproteins (Smith antigen) are specific to SLE and are significantly elevated in 12-mo-old female nrf2 knockout mice (0.90 ± 0.14) compared with female wild-type mice (0.49 ± 0.05, n = 8, P < 0.05). There were no significant changes in autoantibody levels at 6 mo of age (Fig. 2).

The complement protein C3 can bind to IgG and IgM complexes, and all three molecules deposit in target organs in SLE. Consistent with a previous report (50), we find IgG, IgM, and C3 deposition within the kidney of aged female nrf2 knockout mice (Fig. 3B). However, we also observed a less substantial, but demonstrable, deposition of IgG, IgM, and C3 in the kidney of 5-mo-old female nrf2 knockout mice (Fig. 3A). A small amount of IgG and IgM also deposited in the glomeruli of male nrf2 knockout mice. No antibody deposition was observed in male or female wild-type littermate controls at either 5 or 12 mo of age.

In the liver, IgG and IgM accumulate around the sinusoids and portal triads of 12-mo-old female nrf2 knockout mice but not female wild-type or male wild-type or knockout mice (Fig. 4A). No IgG or IgM could be detected in the livers of wild-type or knockout mice at 5 mo of age (data not shown). At 12 mo of age, but not 5 mo, substantial mononuclear cell infiltration occurred in the region around the central veins and portal triads only in female nrf2 knockout mice. Some of these cells stained intensely for IgG or IgM, suggesting that they are IgG- or IgM-secreting plasma cells (Fig. 4A).

In the heart, IgG and IgM deposition occurred along the cardiac muscle fibers of 12-mo-old female nrf2 knockout mice (Fig. 4B). Staining was especially intense around the blood vessels of the heart. No staining was observed in wild-type female or wild-type or knockout male mice. Deposition of IgG, IgM, and C3 also occurred within the choroid plexus of the brain of female nrf2 knockout but not wild-type mice (Fig. 4C). Female nrf2 knockout mice with the highest levels of anti-Sm and anti-dsDNA demonstrated the most dramatic autoantibody deposition in the kidney, liver, and heart.

**Organ pathology.** Consistent with antibody complex deposition in the kidney, liver, and heart, SLE-associated pathology also occurs in these organs (Table 1). Within the kidney of many 12-mo-old female nrf2 knockout mice, the glomeruli varied in size. Several glomeruli were atrophic, while others were enlarged. The glomerulonephritis involved segmental hyalinization and sclerosis (Fig. 5D). As described previously (50), there was moderate proliferation of mesangial cells and

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**Table 1. Decreased weight, splenomegaly, and multiple organ pathology in 12-mo-old female nrf2 knockout mice**

<table>
<thead>
<tr>
<th>Nrf2 Genotype</th>
<th>Weight, g</th>
<th>Percent of Animals &lt;30 g</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+ or +/-</td>
<td>44.8±4.9 (n=8)*</td>
<td>0</td>
<td>Kidney: 2/11, Liver: 3/11, Heart: 0/11, Spleen: 1/11, Spleen/Body, mg/g: 4.5±1.2 (n=8)*</td>
</tr>
<tr>
<td>−/−</td>
<td>27.3±3.6 (n=7)*</td>
<td>45.5</td>
<td>8/10, 7/10, 7/10, 6/10, 27.3±11.8 (n=7)*</td>
</tr>
<tr>
<td>P value</td>
<td>0.019</td>
<td></td>
<td>0.030</td>
</tr>
</tbody>
</table>

Values are means ± SE as shown. Values for “Pathology” are the numbers of mice demonstrating pathology out of the total number of mice examined. *The last three nrf2 +/+ and nrf2 −/− mice analyzed for organ pathology were not weighed. P values were calculated by a two-tailed $t$-test.

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Fig. 1. Phenotypic changes that occur in some female nrf2 knockout mice. A: at 12 mo of age some female nrf2 knockout mice are considerably smaller than their female wild-type littermates. B: genotyping was done to determine the presence of nrf2 wild-type (+/+ ) or knockout (−/− ) constructs. C: the spleens of nrf2 wild-type and heterozygous mice are normal sized. Female nrf2 knockout mice have grossly enlarged spleens. Half of the spleen is shown.
proliferation of the capillary tufts in the larger glomeruli. Some blood vessels were surrounded by thick accumulations of primary lymphocytes. Interstitial mononuclear infiltration also was evident (Fig. 5B), and tube-shaped proteinaceous material occasionally was observed in tubule segments in nrf2 knockout mice. Furthermore, the presence of kidney pathology (Table 1) has a significant positive correlation with the ELISA levels of either anti-Sm or anti-double-stranded (anti-dsDNA) antibody levels between female nrf2 knockout (KO) and wild-type (WT) was statistically significant (*P < 0.05).

Fig. 2. Female nrf2 knockout mice possess elevated levels of autoantibodies at 12 (n = 8) to 18 mo (n = 3) (c) but not 6 mo (n = 6; c) of age. ELISA was performed for anti-dsDNA and anti-Smith antigen antibodies, and the absorbance (A_{450}) was measured and normalized to a normal mouse serum negative control. Bars indicate the mean values for 12-mo-old mice. The difference of the mean anti-Smith antigen and anti-double-stranded (anti-dsDNA) antibody levels between female nrf2 knockout (KO) and wild-type (WT) was statistically significant (*P < 0.05).

Fig. 3. IgG, IgM, and C3 deposit in the glomeruli of the kidney in female nrf2 knockout mice. Sections from 5- and 12-mo-old mice were stained with an anti-mouse IgG antibody (green) and counterstained with the DNA-binding dye Hoechst 33258 (blue). Other sections were stained with an anti-mouse IgM (red) antibody, an anti-mouse C3 antibody (green), and Hoechst (blue). Female and male wild-type mice demonstrated no antibody staining. A: 5-mo-old female nrf2 knockout mice stained intensely for IgG and less intensely for IgM and C3, whereas male nrf2 knockout mice had much lower levels of IgG and IgM deposited in their glomeruli. B: at 12 mo of age female nrf2 knockout mice stain intensely for IgG, IgM, and C3 within their glomeruli. Bar (in A and B, bottom right) = 50 μm.
frequently surrounded the central veins, portal veins, and bile ducts. Some hepatocytes demonstrated cytoplasmic vacuolation and cell swelling consistent with a progression toward cell death. These morphological changes were seldom observed in female wild-type or male wild-type or knockout mice and became more obvious in mice older than 12 mo. In the heart of 12-mo-old female knockout mice, mononuclear infiltration occurred in the mesothelial surfaces and myointerstitium area, resulting in myocarditis. Myocardial fibrillar loss and vacuolation also was observed, indicating myocardial degeneration (Fig. 5, J and L). In the brain, no observable morphological changes, including mononuclear cell infiltration or neuronal loss, were found in the hippocampus or frontal cortex of female nrf2 knockout mice (data not shown).

Oxidative stress. Lipid peroxidation is one measure of the extent of damage induced by reactive oxygen species (ROS). The formation of MDA is a direct measure of lipid peroxidation. The levels of MDA in the liver and kidney of wild-type and nrf2 knockout male and female mice were measured at 6 and 12 mo of age. In the female mice at 12 mo of age the nrf2 knockout mice had significantly elevated MDA levels compared with the female wild-type mice in both the liver and kidney (Table 2). The male nrf2 knockout mice had significantly elevated MDA levels only in the liver at 12 mo of age (Table 2). Levels of MDA were increased in female nrf2 knockout mice at 6 mo of age in both the liver and kidney, but these differences were not statistically significant. There were no significant changes in the MDA levels of 6-mo-old male mice (Table 2).

Oxidative damage by hydroxyl radicals can modify DNA bases. 8OHdG is one such base formed from oxidative damage to DNA, and it can be detected using an antibody raised against

<table>
<thead>
<tr>
<th>A Liver</th>
<th>B Heart</th>
<th>C Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female W.T.</td>
<td>Female W.T.</td>
<td>Female W.T.</td>
</tr>
<tr>
<td>Female nrf2 K.O.</td>
<td>Female nrf2 K.O.</td>
<td>Female nrf2 K.O.</td>
</tr>
</tbody>
</table>

Fig. 4. IgG, IgM, and C3 deposit in the liver, heart, and brain of 12-mo-old female nrf2 knockout mice. Sections from 12-mo-old mice were stained with an anti-mouse IgG antibody (green) and counterstained with the DNA-binding dye Hoechst 33258 (blue). Other sections were stained with an anti-mouse IgM (red) antibody, an anti-mouse C3 antibody (green), and Hoechst (blue). Female (shown) and male (not shown) wild-type mice demonstrated no antibody staining. A: dramatic staining for IgG and IgM occurs around the central and portal veins of the liver of female, but not male, nrf2 knockout mice. Here several IgM-positive cells can be seen surrounding the central vein of a female nrf2 knockout mouse. A small amount of staining for IgG and IgM, but not C3, occurs in male nrf2 knockout mice. B: staining for IgG occurs along the myocardial fibers of the heart in female, but not male, nrf2 knockout mouse. A small amount of staining for IgM and C3 also occurs around the blood vessels of the myocardium in female nrf2 knockout mouse. C: the choroid plexus of female nrf2 knockout mouse stains intensely for IgG and less intensely for IgM and C3. Bar (in A–C, bottom right) = 50 µm.
Table 2. Malondialdehyde levels in kidney and liver of male and female nrf2 wild-type (+/+) and knockout (−/−) mice

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age, mo</th>
<th>Organ</th>
<th>Nrf2 Genotype</th>
<th>n</th>
<th>MDA, μmol/g</th>
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<tbody>
<tr>
<td>Female</td>
<td>6</td>
<td>kidney</td>
<td>+/+</td>
<td>4</td>
<td>1.37±0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>liver</td>
<td>−/−</td>
<td>4</td>
<td>1.65±0.18</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>kidney</td>
<td>+/+</td>
<td>4</td>
<td>1.33±0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>liver</td>
<td>+/+</td>
<td>4</td>
<td>2.19±0.14*</td>
</tr>
<tr>
<td>Male</td>
<td>6</td>
<td>kidney</td>
<td>+/+</td>
<td>5</td>
<td>1.88±0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>liver</td>
<td>−/−</td>
<td>3</td>
<td>1.80±0.19</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>kidney</td>
<td>+/+</td>
<td>4</td>
<td>1.69±0.16</td>
</tr>
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</table>

Values are means ± SE; n = number of mice assayed. *Malondialdehyde (MDA) levels that are significantly increased in nrf2−/− mice compared with the corresponding value for nrf2+/+ mice (P < 0.05).

Female nrf2 knockout mice demonstrate pathology in multiple organs at 12 mo of age. Sections from the kidney, liver, and heart from aged nrf2−/− (left) and nrf2−/− (right) female mice were stained with hematoxylin and eosin. A–D: interstitial mononuclear infiltration (B, arrows) was evident in nrf2 knockout mice. Segmental hyalinization occurs within a glomerulus (D, arrow). E–H: aged nrf2 wild-type female mice showed a mild to moderate fatty liver (E and G). The nrf2 knockout female mice demonstrated severe lymphocytic infiltration and vasculitis surrounding the central vein (CV) (F, arrow) and portal tracts (H, arrow). I–L: compared with age-matched nrf2 wild-type mice (I and K), aged nrf2 knockout mice show wavy fibers with elongation and narrowing in a longitudinal section of the left ventricular wall (J). In a cross-sectional view, the myocardial fibrillar loss and vacuolation indicating myocardial degeneration also was evident (L). The wide spaces between the cardiac fibers contained scattered mononucleocyte infiltration (J and L, arrows) with a severe edema. Bar in B = 200 μm, in D = 50 μm, and in H and L = 100 μm.
wild-type mice (Fig. 7, E and F). Female nrf2 knockout mice also possessed numerous TUNEL-positive cells within the mononuclear cell infiltrates of the kidney at 12 mo of age (Fig. 7, A and B).

Cell culture introduces a number of stresses on cells, including oxidative stress. Concordantly, splenocytes from female nrf2 knockout mice are more susceptible than wild-type splenocytes to the cell death induced by culturing. Cultured splenocytes demonstrated increased spontaneous apoptosis at 6 and 48 h in vitro in female nrf2 knockout mice as determined by propidium iodide staining and then cell-cycle analysis through flow cytometry (Fig. 8). However, we observed no difference in spontaneous apoptosis at 24 h. This time-dependent toxicity may be due to the different sensitivities of various cell populations within these cultures. After subtraction of spontaneous apoptosis, 1 μM camptothecin, an inhibitor of topoisomerase, induced apoptosis in an additional 2.45 ± 0.77% in splenocytes of female nrf2 wild-type mice and 15.19 ± 3.50% in female nrf2 knockout mice after 24 h (P < 0.05, n = 3).

Gene expression changes. By 12 mo of age the organ pathology in female nrf2 knockout mice can be severe, including hepatitis and cirrhosis in the liver and splenomegaly in the spleen. Therefore, to determine the gene expression changes that precede histopathology, we used livers and spleens from 5-mo-old mice, which are histologically normal. The expression levels of numerous genes involved in a myriad of pathways are altered by a knockout of nrf2 in the liver. Consistent with liver’s function as a detoxification organ and the role of nrf2 as a transcription factor, many detoxification genes are downregulated in both male and female nrf2 knockout mice (Table 3). These include many classes of glutathione S-transferases, aldehyde dehydrogenases, and flavin-containing monoxygenases.

Because only female nrf2 knockout mice develop an autoimmune disorder, we focused on those genes that passed our criteria for significant changes in female mice. Two female nrf2 knockout mice were compared with two female wild-type littermates for a total of four comparisons. We assigned a rank based on a Wilcoxon signed rank test for each comparison and summed the ranks as outlined in the methods. To identify sex-specific patterns, the same procedure was performed with male mice. SOM clustering using those genes that passed our criteria for significant changes in the female nrf2 knockout mice generates sex-specific and nrf2-specific gene clusters in the liver (Fig. 9). SOM clustering of genes increased in female nrf2 knockout mice generated a cluster that includes those genes that are specifically upregulated in female, but not male, nrf2 knockout mice (cluster 4, Fig. 9A). These genes include the genes for cathepsin E and histocompatibility 2, T region locus 23—two genes involved in antigen presentation. In addition, the gene for immunoglobulin κ-chain variable 8 is involved in the generation of anti-DNA autoantibodies and is upregulated solely in the female nrf2 knockout mice. S100 calcium binding protein A8 is a potent chemotaxic agent for neutrophils and monocytes, and this gene is upregulated in...
female nrf2 knockout mice before significant mononuclear cell infiltration occurs. Other genes in this cluster include cytochrome P-450s, genes involved in fatty acid metabolism, a gene for a proteasome subunit, and DNA binding genes (Fig. 9A).

SOM clustering of genes decreased in female nrf2 knockout mice generated additional clusters (Fig. 9B). Cluster 1 includes those genes decreased in both female and male nrf2 knockout mice. These include nrf2 itself as well as numerous detoxification genes also listed in Table 2. In addition, the expression levels of some immune-related genes are decreased (Fig. 9B).

A severe immune dysregulation within the spleens of female nrf2 knockout mice is suggested by the changes in expression of numerous immune-related genes (Supplemental Table S1, available at the Physiological Genomics web site). Consistent with the later development of hyperplasia in the spleen, the genes for two proteins that drive the proliferation and differentiation of B cells, B-cell differentiation antigen Lyb-2 and B lymphocyte-induced maturation protein, are upregulated in the spleens of nrf2 knockout mice. In SLE, CD4 and CD8 T cells overexpress Fc receptor, IgE, high-affinity I, γ-polypeptide (FceRIγ) (18). Similarly, FceRIγ is upregulated in female nrf2 knockout mice. In contrast, genes for three proteins that play a role in CD8 T cell function, the CD8 antigen α- and β-chains and cathepsin C, are downregulated in nrf2 knockout mice. A few detoxification genes are downregulated in the spleens of nrf2 knockout mice, including the genes for 1-Cys peroxiredoxin, thioredoxinase 2, and glutathione peroxidase 4. Finally, consistent with the increased susceptibility of splenocytes from female nrf2 knockout mice to spontaneous and camptothecin-induced apoptosis, the pro-apoptotic gene T-cell death associated gene is upregulated and the anti-apoptotic gene apoptosis inhibitor 6 is downregulated (Supplemental Table S1). The altered expression levels of several genes in the liver and spleen were confirmed by RT-PCR and support the microarray data (Supplemental Fig. S1). However, the differential expression of many genes has yet to be confirmed.

For the complete microarray data see the GEO database (http://www.ncbi.nlm.nih.gov/geo; accession number GSE867).

**DISCUSSION**

Here we show that female mice deficient in an antioxidant and detoxification enzymes develop a multi-organ autoimmune disorder. Some of the events that can occur in SLE are manifested in the nrf2 knockout mice, such as splenomegaly, vasculitis, glomerulonephritis, hepatitis, and myocarditis. Antibodies against dsDNA and the Smith antigen are specific for SLE and are elevated in female nrf2 knockout mice. Prior to the development of these pathologies and autoantibodies, markers of DNA oxidation and lipid peroxidation are dramatically increased. Furthermore, splenocytes isolated from female nrf2 knockout mice demonstrate increased spontaneous and camptothecin-induced apoptosis. Finally, gene

Fig. 7. The number of cells undergoing spontaneous apoptosis is increased in the kidney, liver, and spleen of 12-mo-old female nrf2 knockout mice. A and B: some cells within the mononuclear cell infiltrate of the kidney stained positively for TUNEL. Inset: a magnification of the cells in the small square of B. No mononuclear cell infiltrate occurred in the female wild-type mice, and no TUNEL-positive cells were observed (A). C and D: an occasional TUNEL-positive cell could be seen within the liver of wild-type mice (C). However, many more TUNEL-positive cells were observed in the livers of female nrf2 knockout mice and were frequently found within the mononuclear cell infiltrate (D, arrow). E and F: in 12-mo-old nrf2 knockout mice (F) the number of TUNEL-positive splenocytes also was increased compared with wild-type mice (E). Bar in F = 200 μm; bar in inset of B, = 50 μm.

![Image](http://physiolgenomics.physiology.org/)

Fig. 8. Splenocytes from female nrf2 knockout mice show accelerated spontaneous apoptosis in a time-dependent manner. Top: splenocytes harvested from 9- to 12-mo-old nrf2 knockout and wild-type mice were cultured in vitro for the amount of time indicated. Spontaneous apoptosis was calculated based on the sub-G1 peak, also called the apoptotic peak, generated by cell cycle analysis through propidium iodide staining. The final apoptotic rate was determined after subtraction of the value at the zero time point. The bars are means ± SE of four mice. *Statistically significant differences (P < 0.05). Bottom: the cell cycle analysis of one pair of female wild-type (left) and knockout (right) mice, demonstrating increased spontaneous apoptosis in the nrf2 knockout mouse 48 h after culturing.

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1The Supplementary Material for this article (Supplemental Table S1 and Supplemental Fig. S1) is available online at http://physiolgenomics.physiology.org/cgi/content/full/00209.2003/DC1.
Multiple mechanisms may account for the breakdown of tol-

To the thymus and anergy and suppression in the periphery.

dead cells such as keratinocytes and senescent or damaged

tibodies bind injured cells (41). In fact, autoantibodies have

line (50).

A low level of autoimmunity exists in normal individuals

expression profiles provided disease-specific genetic changes

that may underlie the etiology of the disease. These results are

supported by the demonstration of glomerulonephritis and

autoantibody production in a distinct nrf2 knockout mouse

line (50).

A low level of autoimmunity exists in normal individuals

(14). For instance, in normal rats with a brain injury, autoan-
tibodies bind injured cells (41). In fact, autoantibodies have

been demonstrated to promote the phagocytosis and removal of
dead cells such as keratinocytes and senescent or damaged
erythrocytes (24, 26). However, in the normal state, some level
of tolerance is maintained through processes such as deletion
in the thymus and anergy and suppression in the periphery.

Multiple mechanisms may account for the breakdown of tol-
erance that occurs in autoimmune diseases. For instance, ge-
netic mutations that interfere with the above tolerance controls
can lead to autoimmunity (e.g., Fas/Fas ligand). In addition, an
immune response can be mounted against foreign antigens that
mimic self antigens. Antibody diversification can then lead to
a full-blown autoimmune attack (32). Alternatively, exposure
and processing of proteins normally unexposed to the immune
system (cryptic epitopes) by antigen-presenting cells can break
tolerance and lead to the development of autoimmune antibod-
ies. For instance, immunization of normal mice with cryptic
peptides from a small nuclear ribonucleoprotein, but not the
native protein, led to autoreactive T cell proliferation and
autoantibody production (4). An increase in oxidative stress
and cell death may increase the levels of normally cryptic

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proteins such that they are exposed to the immune system in amounts sufficient to elicit an autoimmune response. In fact, several SLE autoantigens, including nucleosomal DNA and small nuclear ribonucleoproteins, have been demonstrated clustered on the surface of apoptotic cells (6). In addition, the generation of ROS and local inflammation can lead to the production of costimulatory cytokines that break anergy in autoreactive T cells. Finally, the ROS generated by cell death increase their immunogenicity. In fact, autoantibodies that specifically recognized MDA have been found in the lupus-prone MRL/lpr mice, suggesting that lipid peroxidation can alter the immunogenicity of self peptides (25, 48).

Oxidative stress and the development of SLE. Several markers of oxidative damage were increased in the female nrf2 knockout mice at both 5 and 12 mo of age. The generation of ROS may come from a variety of sources. For instance, normal cellular metabolism and/or exogenous toxins ingested normally in the diet will generate ROS. Multiple antioxidant and detoxification genes and proteins have been developed by the cell to deal with the potentially pathologic production of ROS. A knockout of nrf2 compromises a substantial portion of these detoxification mechanisms and thus leads to increased cellular oxidative damage. In addition, the dysregulation of the immune system and the deposition of antinuclear antibodies within multiple organs, including the liver and kidney, may activate neutrophils and monocytes and lead to increased ROS generation. Male nrf2 knockout mice have a dramatic decrease in the levels of a number of antioxidant and detoxification genes, and they do not develop SLE nor produce or deposit substantial levels of autoantibodies in the liver. However, at 12 mo of age MDA levels are substantially and significantly upregulated within the livers of male nrf2 knockout mice. Therefore, in the male liver, oxidative stress is likely a result of the nrf2 deficiency and not autoantibody generation and immune cell activation. Furthermore, though MDA levels are not significantly altered in the livers of 5-mo-old mice, levels of 8OHdG are dramatically increased within the livers of 5-mo-old female nrf2 knockout mice. IgG and IgM deposition within the liver was detected in 12-mo-old but not 5-mo-old female nrf2 knockout mice. Altogether, this demonstrates that the oxidative damage that occurs within the livers of nrf2 knockout mice preceeds autoantibody deposition, mononuclear cell infiltration, and autoimmune cell activation. This sequence of events together with the well-established role of NRF2 in protection against oxidative injury (7, 8, 31, 34) strongly suggests that increased oxidative stress can result in the development of SLE.

Splenocytes from female nrf2 knockout mice demonstrate an increased sensitivity to cell death induced by the DNA-damaging drug camptothecin. This in vitro susceptibility to DNA damage corresponds to the in vivo accumulation of 8OHdG and increased apoptosis in the liver and spleen of female nrf2 knockout mice. Thus a deficiency in detoxification and antioxidant genes may lead to oxidative stress, oxidative DNA damage, and increased apoptosis in multiple cell types. Although mutations in Fas and Fas ligand lead to a lymphoproliferative disorder that resembles some aspects of SLE in mice, no mutations in Fas have been identified in human SLE patients. In fact, T-cells and neutrophils isolated from SLE patients have elevated levels of Fas and undergo spontaneous apoptosis (13, 16, 21). Recently, it has been shown that knockout or inactivation of NRF2 increases Fas-induced apoptosis, whereas nrf2 overexpression protects HeLa cells from Fas-induced death (28). Consistent with this, in female nrf2 knockout mice, both splenocytes and the mononuclear cells infiltrates in the kidney and liver undergo spontaneous apoptosis in vivo and in vitro. Taken together with the autoantibody production, glomerulonephritis, and the multi-organ IgG, IgM, and C3 deposition and mononuclear cell infiltration, these data demonstrate that the female nrf2 knockout mice are a repre-
sentative model of human SLE. In fact, nrf2 may be involved in human susceptibility to SLE: a genome-wide search of human patients with SLE revealed a linkage with a region in chromosome 2q that includes nrf2 (22). Other known factors related to the nrf2 signaling pathway (nrf1, nrf3, maG, maI, maK, and keap1) are not located within currently identified SLE candidate loci. However, further study on the potential contributions of polymorphisms in nrf2 as well as other genes involved in this detoxification pathway is warranted.

Male and female differences. SLE occurs with a prevalence as high as 1 in 2,500 people but predominantly affects women (35). SLE is especially prevalent in women during their child-bearing years (9:1 female to male ratio) and during pregnancy, suggesting a role of female hormones in development of the disease. Most mouse models of SLE also exhibit an increased susceptibility of females to the disease. The sex-specific decreases in glutathione S-transferase α-1 and -2 and μ-1, -2, and -3 have been shown previously in the livers of nrf2 knockout mice (10) and are in agreement with our study (Table 3). Interestingly, the constitutive activity of glutathione S-transferase was reduced in both wild-type and knockout female mice compared with the corresponding male mice (10). On the other hand, male nrf2 knockout mice possess a severe deficiency of detoxification enzymes in the liver, increased oxidative stress in liver, and display some IgG and IgM deposition in the kidney, but they do not develop the mononuclear cell infiltration in the liver, kidney, or heart nor the glomerulonephritis observed in females. In addition, only one male nrf2 knockout mouse had significant levels of antibodies against dsDNA or Smith antigen (Fig. 2). Therefore, because oxidative damage occurs in both male and female mice, but the progression to SLE only occurs in female mice, estrogens or the expression of female-specific genes may be necessary to break immune tolerance to self antigens. Oxidative stress is likely necessary, but not sufficient, for the development of SLE.

In fact, SLE is likely a multifactorial disease. Previous studies have shown that mice with the 129Sv/J × C57BL/6 background are susceptible to autoimmune disease (5, 47), suggesting that genetic factors on this background may combine with the nrf2 knockout to contribute to autoimmunity. Pollard et al. (40) reported that exposure to mercury accelerated systemic autoimmunity in BXSB mice (autoimmune-prone), whereas the C57BL/6 mice (not autoimmune-prone) were resistant, suggesting that exposure to an environmental toxicant enhances susceptibility to systemic autoimmunity in genetically susceptible individuals (40). It is difficult to know how genetic factors may modify the disease phenotype in female nrf2 deficient mice, but the altered gene expression in the liver and spleen offers some clues.

Genetic alterations that precede full-blown SLE. At 5 mo of age the liver histology of nrf2 knockout mice is normal. However, gene expression analysis reveals numerous genetic alterations. Consistent with the role of nrf2 in the transcription of detoxification genes, both male and female nrf2 knockout mice have numerous antioxidant and detoxification genes downregulated. SOM clustering reveals a subset of genes specifically upregulated in female, but not male, nrf2 knockout mice (Fig. 9A, cluster 4). These include genes that encode for proteins important in antigen presentation and antibody generation. For instance, cathepsin E is an aspartic protease and may be important in antigen presentation mediated by the major histocompatibility complex (MHC) class II in microglia (39). Finally, prior to the mononuclear cell infiltration that occurs in the livers of female nrf2 knockout mice, the gene for S100 calcium binding protein A8 (s100a8) is upregulated. S100A8 is a potent chemotactic agent for neutrophils and monocytes both in vitro and in vivo (15, 30). In addition, S100A8 can alter neutrophil shape and size, suggesting its involvement in leukocyte margination and transmigration into tissues (11). Therefore, the upregulation of s100a8 in the liver of 5-mo-old female nrf2 knockout mice likely mediates some of the subsequent mononuclear cell infiltration and may be involved in the progressive immune dysregulation and autoantibody production that occurs in these mice.

Conclusions. Multiple mechanisms have been implicated in the development of SLE, and many of these are reproduced in female nrf2 knockout mice. The early decrease in detoxification genes, increased oxidative damage, and increased apoptosis in these mice argue strongly that an impairment in detoxification can lead over time to the development of systemic autoimmunity. Targeting the initiating events through activation of NRF2 pathways and other antioxidant therapies may help prevent the development of human SLE.

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272

OXIDATIVE STRESS PRECEDES LUPUS DEVELOPMENT