Disruption of Dihydronicotinamide Riboside:Quinone Oxidoreductase 2 (NQO2) Leads to Myeloid Hyperplasia of Bone Marrow and Decreased Sensitivity to Menadione Toxicity*

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Dihydronicotinamide riboside (NRH):quinone oxidoreductase 2 (NQO2) is a flavoenzyme that catalyzes the reductive metabolism of quinones. To examine the in vivo role of NQO2, NQO2-null (NQO2−/−) mice were generated using targeted gene disruption. Mice lacking NQO2 gene expression showed no detectable developmental abnormalities and were indistinguishable from wild-type (NQO2+/+) mice. However, NQO2-null mice exhibited myeloid hyperplasia of the bone marrow and increased neutrophils, basophils, eosinophils, and platelets in the peripheral blood. Decreased apoptosis of bone marrow cells and circulating granulocytes contributed to myeloid hyperplasia and hyperactivity of bone marrow in NQO2-null mice. The hematological changes in NQO2−/− mice were specifically associated with loss of the NQO2 gene because histological analysis of various tissues including spleen, thymus, blood cultures, and urine analysis demonstrated no sign of infection. NQO2-null mice also demonstrated decreased toxicity when exposed to menadione or menadione with NRH. These results establish a role for NQO2 in protection against myelogenous hyperplasia and in metabolic activation of menadione, leading to hepatic toxicity. The NQO2-null mice are a model for NQO2 deficiency in humans and can be used to determine the role of this enzyme in sensitivities to toxicity and carcinogenesis.
significant differences exist in relative affinities (substrate specificities have been observed for NQO1 and NQO2, acetin, are inhibitors of NQO2 (25). Benzo[c]ibacron blue, and phenindone (25). Flavones, including quer-
resistant to typical inhibitors of NQO1, such as dicoumarol, protein are 54 and 49% similar to the human liver cytosolic human, rat, or mouse NQO1. The human NQO2 cDNA and
constructed using the pPNT vector having the positive selection neomycin I; X
I fragment containing a deletion of exon 3 of the NQO2 gene was constructed. The
constructed targeting construct pPNT-mouse NQO2 gene. This targeting construct upon
transfection in ES cells replaced exon 3 of the endogenous gene with a neocassette by homologous recombination. The 1.0-kb PCR probe to screen the ES cells for homologous recombination is shown.

human, rat, or mouse NQO1. The human NQO2 cDNA and protein are 54 and 49% similar to the human liver cytosolic NQO1 cDNA and protein, respectively (24). Recent studies have revealed that NQO2 is different from NQO1 in its cofactor requirements (25, 26). NQO2 uses dihydricotiamidine ribo-
side (NRH) rather than NADPH as an electron donor. Another
difference between NQO1 and NQO2 is that NQO2 is resistant to typical inhibitors of NQO1, such as dicoumarol, cibacron blue, and phenindone (25). Flavones, including quer-
cetin, are inhibitors of NQO2 (25). Benzo[a]pyrene is another
known inhibitor of NQO2 (26). Even though overlapping sub-
strate specificities have been observed for NQO1 and NQO2, significant differences exist in relative affinities (Km) for the various substrates (25). Analysis of the crystal structure of NQO2 revealed that NQO2 contains a specific metal binding site that is not present in NQO1 (27). The human NQO2 gene has been localized precisely to chromosome 6p25 (6). The hu-
man NQO2 gene locus is highly polymorphic (6, 24). The in vivo role of NQO2 in the detoxication of quinones remains unknown.

In the present report, we used homologous recombination in embryonic stem cells to disrupt the NQO2 gene and generate knockout (NQO2—/–) mice. NQO2—/– mice showed no detectable developmental abnormalities and were indistinguishable from wild-type (NQO2+/+) or heterozygous (NQO2+/–) mice. However, NQO2—/– mice exhibited myeloid hyperplasia of the bone marrow. The myeloid hyperplasia caused a significant increase in granulocytes in the peripheral blood of NQO2—/– mice. Further analysis showed that decreased apoptosis of myeloid cells in bone marrow and granulocytes in the peripher-

al blood contributed to increased bone marrow myeloid cell hyperplasia and hyperactivity in NQO2—/– mice. Interest-
ingly, these studies also revealed that unlike NQO1, the loss of NQO2 leads to decreased sensitivity to menadione toxicity. This indicates that NQO2 plays a role in the metabolic activa-
tion of menadione.

**Experimental Procedures**

Cloning and Sequencing of cDNA and Gene Encoding Mouse NQO2—
The cloning and nucleotide sequence of mouse cDNA and gene encoding NQO2 have been reported previously (25). The structure of the mouse NQO2 gene was found to be similar to that reported for the human
NQO1 gene. Like the human NQO2 gene, the mouse NQO2 gene con-
tains seven exons interrupted by six introns (28; Fig. 1). The splice
junctions and nucleotide sequences in the various exons are conserved
between human and mouse NQO2 genes (28). Exon 1 in both human and mouse is noncoding (28).

Construction of the Targeting Vector—The targeting vector was con-
structed using the pPNT vector having the positive selection neomycin (G418) resistance (29). To disrupt the NQO2 gene, a targeting plasmid containing a deletion of exon 3 of the NQO2 gene was constructed. The
2.1-kb BamHI fragment containing a portion of intron 2 and exon 3 and
a small portion of intron 3 of the NQO2 gene was replaced by the 2.0-kb BamHI fragment from the pPNT vector containing the bacterial phospho-
biosyltransferase II gene conferring G418 resistance (Fig. 1). The targeting vector contained 1.8 kb of homologous 3′-sequence and 4.0 kb of homologous 5′-sequence flanking the neocassette. To construct the
targeting vector, a 1.8-kb BamHI-EcoRI fragment of the mouse NQO2 gene containing a portion of the third intron was first subcloned at the
BamHI-EcoRI sites of the pPNT vector. The recombinant plasmid, designated as pPNT-3′-NQO2, was digested and diphosphorylated. A 4.0-kb EcoRI-EcoRI fragment of mouse NQO2 gene containing the 5′-flanking region, exon 1, intron 1, exon 2, and a portion of intron 2 was isolated, EcoRI-XhoI adapters added, digested with XhoI, and subcloned at the XhoI site of pPNT-3′-NQO2 plasmid to generate targeting construct pPNT-mouse NQO2 gene.

Electroporation, Selection of Embryonic Stem (ES) Cells, and Generation of Chimeric Mice—Genome Systems (St. Louis, MO) mouse ES cells were used for homologous recombination and deletion of exon 3 from the NQO2 gene. The ES cells were thawed and diluted with ES cell medium (HEPES-buffered Dulbecco’s modified Eagle medium, 15% fe-
tal bovine serum, 0.1 mM nonessential amino acids, penicillin-streptomycin, 1,000 units of leukemia inhibitor factor/ ml), pelleted by centrifugation, and resuspended in ES cell medium. The ES cells were plated onto a 60-mm Petri dish previously seeded with mitotically inactive, γ-irradiated mouse embryonic fibroblasts. After 2 days, fresh medium was added to the ES cells, incubated for 4 h, and trypsinized with 0.25% trypsin-EDTA buffered with HEPES, resus-
pended at 2.5 × 10^6/60-mm diameter plates with electroporation buffer (Hanks’ balanced salt solution, 20 mM HEPES buffer, 0.1 mM β-mer-
captoethanol, pH 7.2), and used for electroporation.

Supercoiled targeting pPNT-mouse NQO2 gene plasmid DNA was prepared and linearized with NotI. The DNA was cleaved, precipitated, and resuspended at a concentration of 2 μg/μl sterile distilled water. 50
μg of linearized plasmid was ethanol precipitated and resuspended in 50 μl of electroporation buffer and electroporated in ES cells with a Bio-Rad gene pulser. The electroporation was carried out at 250 V, 250 microfarad capacitance. The electroporated ES cells were plated onto 60-mm Petri dishes containing mitotically inactive mouse embryonic fibroblasts. One day after electroporation, cells were selected with the drug G418 (300 μg/ml). After a 1-week selection, the ES cell clones were picked up under a dissecting microscope, transferred to individual wells of a 96-well plate containing 25 μl of 0.025%
diluted HEPES-buffered trypsin-EDTA, and dissociated by pipetting up and down six times. Dissociated clones were transferred to wells of a 24-well plate containing mitotically inactive mouse embryonic fibro-
blasts. The clones were expanded and trypsinized. One plate was used to make DNA for analysis, and one plate was frozen in 10% dimethyl sulfoxide (MeSO) and 20% fetal bovine serum in ES medium at
−80 °C.

The ES cells with homologous recombination were detected by genomic Southern analysis using a 1.0-kb PCR product from 5′-flanking region as probe (the position of the probe is shown in Fig. 1). Positive ES cell clones with homologous recombination and deletion of exon 3 were detected in a ratio of 9:170 ES cell clones. The ES cells from the positive clones were injected into blastocysts, and chimeric animals were then bred to produce F1 mice. Thawing and preparation of the ES cells for microinjection were done according to the Genome Systems protocol.
Briefly, a vial of mouse embryonic fibroblasts was thawed, expanded, and plated on six-well plates in preparation for the thawing of the ES cell clones. The cells in the 24-well plates were thawed in a water bath and moved to the six-well plates. Two days later, the cells were trypsinized and replated. The clones were either expanded to 25-cm² flasks or used directly from the six-well plates for microinjection (depending on the growth rate). The morning of the microinjection, the medium was changed 4 h prior to delivery to the Baylor College of Medicine Microinjection Core Facility. 2.5–5 h after media replacement, the cells were washed with PBS, trypsinized, and centrifuged at 1,500 rpm. The cells were resuspended in 500 μl of STO medium and placed on ice. The core facility injected at least 50 embryos with each of the nine ES cell clones. Southern blots were used to identify germ line transmission of the homozygous and heterozygous animals and to produce homozygous knockout mice that do not express the NQO2 gene.

NQO2+/−, NQO2−/−, and wild-type mice were housed in the Baylor College of Medicine Center for Comparative Medicine. The animals were kept in polycarbonate cages, maintained with a 12-h light/dark cycle, a temperature of 24 ± 2 °C, a relative humidity of 55 ± 10%, and a negative atmospheric pressure. The mice were fed standard rodent chow and acidified tap water ad libitum. Animals received humane care throughout the experiment according to the American Association of Laboratory Animal Care guidelines for animal welfare.

Generation of Probe to Detect Homologous Recombination in ES Cells and Germ Line Transmission in Mice—The probe to test the ES cells for homologous recombination with the targeting construct is shown in Fig. 1. The PCR kit was purchased from Invitrogen, and forward/reverse primers were used for PCR analysis of the 1.0-kb probe of the 4.3-kb EcoRI fragment in the promoter region of the mouse NQO2 gene. The nucleotide sequence of the forward primer was 5′-GGAGAACACA-GAACCGGCG-3′; that of the reverse primer was 5′-CAATCCAATT-AGACTTTT-3′. The denaturation step was done at 94 °C, reannealing at 40 °C, and extension at 72 °C. 30 cycles were done, followed by a 7-min extension.

Southern and Northern Blot Analysis—DNA was isolated from ES cells and mouse tails by the procedure described by Laird et al. (30). DNAs were digested overnight with NotI, eletrophoresed on 1.0% agarose gels, blotted, and hybridized with 2.0-kb neocassette probes. The blots were washed and autoradiographed. In a related experiment, the DNAs from NQO2+/+, NQO2+/−, and NQO2−/− mice were digested with BamHI, run on agarose gel, blotted, and hybridized with the 2.0-kb neocassette probes. The blots were washed and autoradiographed.

Total RNA was isolated from various tissues including liver, kidney, testis, and lung by the procedures described (31, 32). 80 μg of RNA was subjected to electrophoresis on a 1.0% agarose gel containing formaldehyde, blotted, and hybridized with 165 bp of exon 3 probe from the mouse NQO2 gene (31, 32).

Genomic PCR—NQO2 gene-PCR primers (forward, 5′-GTAAGAAAG-GTGTCCTATCGTGC-3′; reverse, 5′-ATCACATTGTGCGCCCTTG-3′) were used to amplify 155 bp of exon 3 to genotype the wild-type, NQO2−/−, and NQO2−/− mice. Similarly, neo-PCR primers (forward, 5′-GTATCCGATGGAACGGCCGT-3′; reverse, 5′-AATACGGC-GAGCAACCCT-3′) were used to amplify 250 bp of neocassette. 0.5 μg of genomic DNA from the wild-type, NQO2+/−, and NQO2−/− mice and the above sets of primers was used in a PCR (1× PCR buffer, 2.5 mM MgCl₂, 200 μM dNTPs, and 100 pmol of primers) under the following conditions: 94 °C for 10 min, 25 cycles of 94 °C for 1 min followed by 62 °C for 1 min, and 62 °C for 3 min using an Invitrogen PCR kit. PCR-amplified products were separated on 2% agarose-ethidium bromide gels and photographed. The PCR-amplified products were run on agarose gels, blotted, and hybridized to 32P-labeled NQO2 exon 3 and neocassette probes by procedures described previously (24). The membranes were washed and autoradiographed.

Western Blot Analysis and NQO2 Activity—The various tissues (liver, kidney, testis, and lung) from wild-type, NQO2+/+, NQO2−/−, and NQO2−/− mice were homogenized in 50 mM HEPES containing 0.25 M sucrose and centrifuged at 105,000 × g for 1 h to obtain cytosolic fractions. The various cytosolic fractions were analyzed for the presence or the absence of NQO2 protein by Western blot analysis as described previously using antibodies against purified rat liver NQO1 protein (33). The rat NQO1 antibody is known to cross-react with mouse and human NQO1 proteins. Cytosolic proteins were isolated from the ECL (Amersham Biosciences) reagents by the procedure suggested by the manufacturer. Benzothiazopyrene-sensitive NQO2 activity was measured in all cytosolic fractions by a method reported earlier (25, 28). Briefly described, the wild-type and NQO2−/− mice were sacrificed by cervical dislocation. The liver, kidney, testis, and lung were surgically removed and washed briefly in cold PBS. The organs were homogenized in 250 mM sucrose and 50 mM Tris, pH 7.4, and centrifuged at 100,000 × g for 1 h. The Bradford protein assay (Bio-Rad) was used to determine the protein concentration of the supernatant. The supernatant was used for NQO2 enzyme analysis. NRH was synthesized by adding 1,000 units of calf intestinal alkaline phosphatase (Sigma) to 500 μl of 10 mM nicotinamide mononucleotide (Sigma) in PBS. The reaction was allowed to proceed for 15 min at room temperature. 10 μl of the NRH was added to 50 mM Tris, pH 7.4, 100 μM dichloroindophenol and, and cytosolic extract in a 1-ml standard cuvette. The decrease in absorbance was followed at 600 nm for 1 min with a Beckman DU640 spectrophotometer. Cytosol extract concentration was calculated from a curve produced with 0.08−0.15 A change in absorbance. 1. The specific activity of NQO2 was calculated from the change in A/μg of protein.

Histology and Pathology Analysis—NQO2−/− and wild-type mice were euthanized, and the various tissues were surgically removed. The tissues were placed in 10% neutral buffered formalin (Fischer Scientific) for 1 week. After this period, the tissues were then embedded in paraffin and cut into 4-μm sections. The sections were placed onto slides and stained with hematoxylin and eosin (Richard-Allan Scientific, Kalamazoo, MI). Standard blood, urine, and serology analyses were also performed on wild-type and NQO2−/− mice to demonstrate that mice were free of infection.

Bone Marrow Blot Analysis—NQO2−/− and wild-type mice were anesthetized with isoflurane (Vedco, St. Joseph, MO) followed by intraperitoneal injections of a combination anesthetic (2.14 mg of ketamine, 0.43 mg of xylazine, and 70 μg of acepromazine). The animals were opened, and 0.5 ml of blood was withdrawn from the heart with a 22-gauge needle. The syringes were coated with 0.5 ml EDTA to prevent coagulation. The blood was immediately placed into EDTA-coated tubes (Microtainer, Fisher Scientific) and mixed thoroughly. The blood samples were analyzed by a Technicon H1 analyzer.

Bone Marrow NQO2 Protein and Activity—The adult wild-type and NQO2−/− mice were sacrificed and their femurs removed. The bones were cut and marrow flushed out and homogenized in a buffer containing 1 M sucrose, 1 mM EDTA, and 100 μM N-acetyl-L-cysteine. The cells were washed with PBS. The reaction was allowed to proceed for 15 min at room temperature. The blood was immediately placed into EDTA-coated tubes (Microtainer, Fisher Scientific) and mixed thoroughly. The blood samples were analyzed by a Technicon H1 analyzer.

Flow Cytometric Analysis of Bone Marrow Cytosplasts from Wild-type and Apoptosis—NQO2−/− and wild-type mice were euthanized. The femurs were surgically removed from the animals. The heads of each femur were removed, and the bone was then flushed with ~1.0 ml of cold PBS containing 1.0 mM EDTA. After two PBS washes, the bone marrow cells were suspended in annexin V binding buffer to a concentration of 1 × 10⁶ cells/ml. The bone marrow cells were mixed with differentiation marker antibody PE anti-mouse Ly-6G (Gr-1), leukocyte common antigen antibody Cy-Chrome anti-mouse CD45, and annexin V-FITC and incubated in ice for 30 min. All of the antibodies were from Pharmingen. Assays for the determination of myeloid/leukocyte counts and apoptotic cells were essentially performed as described by the manufacturer and measured using Coulter® Epics XL-MCL flow cytometer (Beckman-Coulter Co., Miami, FL).

Flow Cytometric Analysis of Blood Granulocyte Counts and Apoptosis—0.5 ml of blood from wild-type mice and NQO2−/− mice was collected by cardiac stick. Blood was collected in EDTA-coated tubes to avoid clotting. 100 μl of the blood was added to a 75-μl glass tube containing 2.5 mM NADH and 0.5 μl of annexin V-PE (Roche Diagnostics). The annexin V-PE was added to the blood in a 1:1 ratio. The samples were incubated in the dark for 20 min. The blood was then pelleted by centrifugation at 1,500 g for 10 min. Following centrifugation, 50 μl of the supernatant was removed from each embryo. The pellets were resuspended in 500 μl of cold PBS. 0.43 mg of xylazine, and 70 μl of acepromazine. The animals were opened, and 0.5 ml of blood was withdrawn from the heart with a 22-gauge needle. The syringes were coated with 0.5 ml EDTA to prevent coagulation. The blood was immediately placed into EDTA-coated tubes (Microtainer, Fisher Scientific) and mixed thoroughly. The blood samples were analyzed by a Technicon H1 analyzer.
**RESULTS**

**Production of NQO2+/− Mice**

The structure of the targeting vector pPNT-mouse NQO2 gene is shown in Fig. 1. This construct was used successfully to generate NQO2+/− mice. The 5′- and 3′-homologous genomic sequences were 4.0 and 1.8 kb long, respectively. In the targeting vector, a 2.1-kb BamHI fragment containing exon 3 of the NQO2 gene was used to replace the 2.0 kb of neocassette. This replacement was engineered to delete 55 amino acids from the NQO2 protein. This design would effectively disrupt NQO2 gene function. The selection to delete exon 3 was based on a previous observation that deletion of exon 3 from the NQO2 cDNA resulted in the loss of NQO2 protein in transfected COS1 cells (28).

DNA from selected ES cells was analyzed by Southern blotting to screen for homologous recombinants (Fig. 2A). The digestion of DNA from ES cells (NQO2+/+) and wild-type mice with NcoI and hybridization with a 1.0-kb PCR fragment from the mouse NQO2 gene revealed the presence of a 23-kb band (Fig. 2A and B). Of the 170 ES clones analyzed, nine NQO1+/− heterologous ES cell clones were identified. The presence of an 11-kb NcoI fragment in a genomic Southern blot (Fig. 2A) indicated that exon 3 is replaced by the neocassette, as depicted in Fig. 1. The homologous recombination-positive ES cells were used to generate chimeric mice, and germ line transmission was detected. 15 chimeric pups were obtained from the microinjection of nine different ES-positive clones. 14 of the 15 mice were male. Large portions of these mice were white or brown. Interestingly, only one of these 15 mice led to germ line transmission after breeding. The observation that 14 of 15 chimeric mice were male is highly intriguing. However, it remains unknown at present whether the loss of NQO2 has any role in gender linkage.

Heterozygous mice from the F1 generation were normal and were interbred to generate homozygous NQO2−/− mice. The DNA from heterozygous and homozygous mice was analyzed for the presence of mutant allele(s) of the NQO2 gene carrying the deletion of exon 3 (Fig. 2B). The absence of the 23-kb NcoI band and the presence of the 11-kb NcoI band clearly indicate that homozygous NQO2−/− mice were born (Fig. 2B). The absence of exon 3 was also confirmed by genomic PCR of exon 3 from wild-type, NQO2+/−, and NQO2−/− mice (Fig. 2B, Genomic PCR, left panel) and hybridization with an exon 3 probe (Fig. 2B, Genomic PCR, right panel). The NQO2 gene exon 3 was amplified from the wild-type and NQO2+/− mice. However, this amplification was absent in NQO2−/− mice. The presence of a neocassette was confirmed by digestion of genomic DNA with BamHI followed by Southern hybridization with NQO1 cDNA and neocassette probes (Fig. 2C). The presence of neocassettes in NQO2+/− and NQO2−/− mice was confirmed further by PCR amplification and hybridization with neocassette probes (Fig. 2C, Genomic PCR). The wild-type DNA did not hybridize to the neocassette probes (Fig. 2C). These results clearly indicate that a 2.0-kb neocassette has replaced...
the 2.1-kb BamHI fragment containing exon 3 from the mouse NQO2 gene.

Analysis of NQO2−/− Mice

Viability and Fertility—The NQO2−/− mice were found to be normal in appearance and showed no discernible differences in their weight, development, or behavior compared with their wild-type littermates. This was true for both male and female mice. At 6 weeks of age, NQO2+/+, NQO2+/−, and NQO2−/− mice were killed for gross and histological examination. The organs and tissues examined histologically included liver, kidney, testis, ovary, lung, colon, stomach, duodenum, spleen, thymus, lymph nodes, heart, brain, and skeletal muscle. No obvious anatomical differences in these organs were seen. In addition, the knockout mice appeared to have normal reproductive capacity.

Northern Analysis—Analysis of RNA from four different tissues (liver, kidney, testis, and lung) by hybridization with the exon 3 probe indicates that NQO2 mRNA is present in wild-type and absent in NQO2−/− mice (Fig. 3A). The NQO2 mRNA levels were lower in NQO2+/− mice tissues than those observed in the wild-type mice (data not shown).

Analysis of NQO2 Protein—Because NQO2 is highly homologous to NQO1 (28), antibodies against NQO1 were used to detect NQO2 expression levels (24). Analysis of cytosolic proteins from the various tissues (liver, kidney, lung, and testis) by SDS-PAGE and Western blotting showed absence of 26-kDa NQO2 protein in NQO2−/− mice (Fig. 3B). In similar experiments, NQO2 protein was detected in all of the tissues of wild-type mice and NQO+/− mice. However, the NQO+/− mice had intermediate amounts of NQO2 protein, between that of the NQO2−/− and wild-type mice. Western analysis of the various tissues with NQO1 antibody also revealed that levels of the 32-kDa NQO1 band were not altered significantly among wild-type, NQO2+/+, and NQO2−/− mice. Western analysis also indicated the presence of a 30-kDa cross-reacting band in all tissues of wild-type, NQO1+/+, and NQO1−/− mice (Fig. 3B). This is a nonspecific band and has been detected previously (19).

NQO2 Activity—The levels of benzo(a)pyrene-sensitive cytosolic NQO2 activity in the various tissues of wild-type, NQO2+/+, and NQO2−/− mice are shown in Fig. 4. Among the four tissues tested, the highest levels of cytosolic NQO2 activity were observed in liver, followed by kidney, testis, and lung of wild-type mice. Lung from wild-type mice showed only one-third the NQO2 activity observed in liver. The kidney, testis, and lung showed almost a complete absence of NQO2 activity in NQO2−/− mice; however, NQO2−/− liver showed some NQO2 activity, ~20% of that observed in the wild-type mice. Heterozygous NQO2+/− mice tissues showed intermediate levels of NQO2, between that of NQO2+/+ and wild-type animals.

Bone Marrow and Peripheral Blood Analysis—Consistent with the analysis of other tissues, bone marrow showed a complete absence of NQO2 (Fig. 5A). NQO2 levels in NQO2+/− mice were approximately half that detected in the wild-type mice. As with other tissues (Fig. 3B), the NQO1 protein levels were essentially unchanged in bone marrow from wild-type, NQO2+/+, and NQO2−/− mice. NQO2 activity was detected in the bone marrow from wild-type and NQO2+/− mice but absent in NQO2−/− mice (Fig. 5A). Analysis of the peripheral blood from NQO2+/+ and wild-type mice revealed significant increases in the number of neutrophils, eosinophils, basophils, and platelets in NQO2−/− animals (Table I). The number of red blood cells and monocytes showed no significant changes, whereas the numbers of WBCs and lymphocytes were significantly lower in the NQO2−/− mice (WBCs, p < 0.01;
NQO2 Disruption and Myeloid Hyperplasia

A. Bone Marrow

I. Western

NQO1

NQO2

II. NQO2 Activity

III. Myeloid Cells

IV. Apoptosis in Myeloid Cells

B. Blood

I. Granulocytes

II. Apoptosis in Granulocytes

Fig. 5. **Bone marrow and blood analysis.** A, bone marrow analysis. Wild-type (NQO2+/+) and NQO2-null (NQO2−/−) mice were sacrificed and their femurs removed. The bones were cut and marrow flushed out and analyzed. I, Western analysis of bone marrow. The bone marrow was homogenized in an appropriate buffer and cytosol prepared by standard techniques (19). 700 µg of the proteins was run on SDS-PAGE (10% gel), Western blotted on ECL nitrocellulose membranes, and probed with polyclonal antibodies against NQO1, II, benzo(a)pyrene-inhibitable NQO2 activity in bone marrow. The cytosolic fractions were analyzed for NQO2 activity by procedures described previously (25, 28). The NQO2 activity is presented as nmol of 2,6-dichlorophenolindophenol reduced/min/mg of protein. III and IV, percentage of Gr-1-positive (myeloid cells) and annexin V-positive (apoptotic cells) in bone marrow of wild-type and knockout mice. Wild-type and NQO2−/− mice were anesthetized, femurs were surgically removed, and bone marrow was flushed gently with sterile PBS. After PBS washes, the cells were suspended in the annexin V-FITC binding buffer to a concentration of 1 × 10^6 cells/ml. The cells were incubated with annexin V-FITC, myeloid differentiation marker Gr-1 antibody, and leukocyte common antigen CD45 antibody. The assay for the determination of myeloid and leukocyte count and apoptotic cells was essentially performed as described by the manufacturer and measured using Coulter® Epics XL-MCL flow cytometer. B, blood analysis. Blood was withdrawn from the mice by cardiac stick and analyzed for total granulocyte counts and apoptotic granulocytes by procedures as described above for bone marrow.

lymphocytes, p < 0.005). Flow cytometric analysis of the bone marrow from NQO2−/− mice revealed a significant increase in myeloid cells (Fig. 5AIII, p < 0.025). The studies further demonstrated a decrease in apoptosis of myeloid cells in bone marrow (Fig. 5AIV, p < 0.01). Flow analysis also showed an increase in granulocytes and a significant decrease in apoptosis of granulocytes in the peripheral blood (Fig. 5B, I and II).

Menadione Toxicity

**Survival of Animals**—To determine the susceptibility of NQO2−/− mice to menadione toxicity in the absence and presence of NRH, we performed the following experiments. Exposure of wild-type and NQO2−/− mice to different concentrations of menadione revealed a dose-dependent response for survival of animals. All of the NQO2−/− and wild-type mice

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**Table I**

Peripheral blood cell count of wild-type and NQO2−/− mice

| Blood cells     | Wild-type  | NQO2−/−  |
|-----------------|------------|----------|
| WBC^a^          | 1.3 ± 0.4  | 0.6 ± 0.2 (0.01) |
| RBC^b^          | 7.7 ± 0.5  | 7.7 ± 0.6 (NS) |
| Platelets^c^    | 979 ± 201  | 1280 ± 90 (0.025) |
| Neutrophils^c^  | 12.3 ± 2.6 | 19.9 ± 5.2 (0.025) |
| Eosinophils^c^  | 1.8 ± 0.4  | 2.2 ± 0.6 (0.1)  |
| Basophils^c^    | 0.5 ± 0.1  | 2.2 ± 1.6 (0.05) |
| Monocytes^c^    | 1.9 ± 0.4  | 1.9 ± 1.4 (NS)   |
| Lymphocytes^c^  | 82 ± 5.3   | 69.8 ± 4.3 (0.005) |

^a White blood cells, ×10^3/µl.

^b Red blood cells, ×10^6/µl.

^c Percentage of WBCs.

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**Fig. 6.** Percent survival of mice exposed to menadione without and with NRH. The wild-type and knockout mice were injected intraperitoneally with different doses of menadione dissolved in MeSO. It included 0 (MeSO control), 10, and 20 mg of menadione/kg of body weight. MeSO and menadione were injected without (A) and with (B) 20 mg of NRH/kg of body weight. 15 animals were injected in each group. The various doses were injected once a day for 3 consecutive days. Survival of the animals was followed every day of the experiment. All of the animals in both groups receiving 10-mg doses of menadione/kg of body weight survived. However, significant differences in the survival of NQO2−/− and wild-type mice were observed with 20 mg of menadione/kg of body weight. 75% of mice in both groups receiving 10-mg doses of menadione/kg of body weight and NRH survived. However, significant differences in the survival of animals between NQO2−/− and wild-type were observed with 20 mg of menadione and NRH/kg of body weight. All of the wild-type mice died compared with 50% death of NQO2−/− mice.
survived with 10 mg of menadione/kg of body weight (Fig. 6A). A significant difference in the survival of animals between wild-type and NQO2−/− mice was observed with a 20-mg/kg dose of body weight dose of menadione (Fig. 6A). The survival rate determined to be 100% for NQO2−/− and 20% for wild-type mice with 20 mg of menadione/kg of body weight. The survival rate of NQO2−/− mice was 75 and 50% with 10 and 20 mg of menadione with NRH/kg of body weight, respectively (Fig. 6B).

The wild-type mice showed a survival rate similar to that of NQO2−/− mice with 10 mg of menadione with NRH/kg of body weight. However, all of the wild-type mice died with 20 mg of menadione with NRH/kg of body weight.

Assessment of Hepatic Damage—To assess liver damage after exposure to menadione, we measured the levels of alanine aminotransferase and aspartate aminotransferase in serum of wild-type and NQO2−/− mice (Fig. 7). The levels of alanine aminotransferase and aspartate aminotransferase in serum of wild-type and NQO2−/− mice treated with Me2SO (vehicle control) and menadione in the absence and presence of NRH are shown in Fig. 7A (without NRH) and 7B (with NRH). The levels of these enzymes more or less did not change in wild-type or NQO2−/− mice treated with 10 mg of menadione/kg of body weight (Fig. 7A). However, 20 mg of menadione/kg of body weight produced elevated levels of aspartate aminotransferase and alanine aminotransferase in serum of both wild-type and NQO2−/− mice (Fig. 7A). Interestingly, the magnitude of elevation was less in the NQO2−/− mice compared with wild-type mice. The inclusion of NRH with 10 mg of menadione/kg of body weight dramatically elevated the enzyme levels of wild-type mice (p < 0.001), whereas marginal increases were observed in NQO2−/− mice (Fig. 7B). The magnitudes of the increases were highly significant in wild-type mice compared with marginal increases in NQO2−/− mice. The data at 20 mg of menadione with NRH/kg could not be compared because all of the wild-type mice died with this treatment.

**DISCUSSION**

Several lines of evidence indicate that modification of the NQO2 gene by replacing exon 3 with the neo-cassette resulted in a null mutation. NQO2 mRNA and protein were not detected in NQO2−/− mice. The NQO2 activity dropped from very high levels in kidney, testis, and lung of wild-type mice to almost zero levels in NQO2−/− mice. The NQO2 activity also dropped significantly (>80%) in liver. We believe that residual amounts of NQO activity observed in liver and other tissues are the result of NQO2-related protein(s) rather than NQO2. This is clearly evident from the fact that the NQO2 protein was absent in all tissues of NQO2-null mice as determined by Western analysis. Interestingly, the levels of NQO1 protein remained unaltered in all the tissues tested in NQO2−/− mice compared with wild-type mice. This indicates that the amount of NQO1 did not increase to compensate for the loss of NQO2 protein in NQO2−/− mice. The <30-kDa band detected between the NQO1 and NQO2 bands is nonspecific and has been reported previously (19).

The loss of NQO2 in knockout mice did not affect the development and viability of mice. This was also observed with NQO1−/− mice (19). We did observe a very mild hepatic damage in some of the female NQO2−/− mice (data not shown). This was not observed in male NQO2−/− mice, and an analysis of additional female mice did not provide sufficient evidence
linking the hepatic damage to loss of NQO2 gene expression. However, most of the female mice analyzed showed slight anisocytosis (varying size of nuclei, suggesting regenerating liver cells), a small group of inflammatory cells and polymorphonuclear leukocytes in portal spaces (data not shown).

The disruption of the NQO2 gene in mice led to myeloid hyperplasia of bone marrow and an increase in granulocytes including neutrophils, basophils, and eosinophils in the peripheral blood and platelets. This presumably was caused by significant decreases in the apoptosis of myeloid cells in the bone marrow and granulocytes in the peripheral blood. However, the role of altered growth and differentiation of myeloid cells cannot be ruled out. Analysis of blood cells did not reveal transformed phenotypes of granulocytes (data not shown), and spontaneous development of myelogenous leukemia has not been observed to date. NQO2/H11002 mice also showed significant decreases in WBCs and lymphocytes. This presumably was caused by significant decreases in the apoptosis of myeloid cells in the bone marrow and granulocytes in the peripheral blood.

It is noteworthy that NQO1−/− mice also showed myeloid hyperplasia of bone marrow and an increase in granulocytes in blood (19). Further analysis of the bone marrow from NQO1−/− mice revealed that loss of NQO1 alters the intracellular redox status because of accumulation of NADPH, cofactors for NQO1. This causes a reduction in the levels of pyridine nucleotides and tumor suppressor proteins p53 and p73 and a decrease in apoptosis. The decrease in apoptosis causes myelogenous hyperplasia in NQO1-null mice. This is significant because 2–4% of human individuals without known abnormalities and greater than 25% of individuals with benzene poisoning and acute myelogenous leukemia are homozygous for a mutant allele (P187S) of NQO1 and lack NQO1 protein/activity (9–11). It is possible that the bone marrow hyperplasia in NQO2−/− mice is also the result of altered redox status caused by accumulation of NRH and changes in proteins related to cell growth and differentiation. The NQO2−/− and NQO1−/− mice studies combined indicate that NQO2 and NQO1 proteins may act as endogenous factors against myelogenous hyperplasia. These studies also provide sufficient evidence to test whether NQO2 and NQO1 proteins also protect against myelogenous leukemia.

Information on the role of NQO2 in quinone detoxification/activity is limited compared with NQO1. NQO1 activity has been shown to protect cells against redox cycling, oxidative stress, and other toxic effects caused by exposure to quinones and their derivatives (1–5). NQO1 activity prevents the formation of highly reactive quinone metabolites (36), detoxifies benzo(a)pyrene quinones (37), and reduces chromium(VI) toxicity (38). NQO1−/− mice treated with menadione showed increased sensitivity to hepatic damage and release of liver marker enzymes in blood (19). This indicated that NQO1 protected the hepatic toxicity of menadione. Interestingly, the treatment of NQO2−/− mice with menadione showed an effect opposite that observed with NQO1−/− mice. The NQO2−/− mice showed significantly lower menadione toxicity compared with wild-type mice. This indicated that NQO2 activated menadione, leading to hepatic damage. This role of NQO2 was highly significant in the presence of NRH, the cofactor for NQO2. All of the wild-type mice died because of treatment with 20 mg of menadione with NRH/kg of body weight. On the other hand, only 50% deaths were observed with a similar dose of menadione and NRH administered to NQO2−/− mice. In addition, the liver marker enzymes alanine aminotransferase and aspartate aminotransferase were found significantly elevated in the serum of wild-type mice with 10 mg of menadione with NRH/kg of body weight compared with marginal increases with a similar dose of menadione and NRH in NQO2−/− mice. In addition, the 50% of NQO2−/− mice that survived 20 mg dose of menadione and NRH/kg also showed a small increase in liver-specific enzymes in the serum. These results indicated that NQO2 activated menadione, which led to hepatic toxicity in wild-type mice, especially in the presence of NRH, whereas NQO2−/− mice were protected against the hepatic toxicity of menadione.

In conclusion, a NQO2-null mutant mouse was produced which develops normally and is completely viable and fertile. However, NQO2−/− mice exhibit myeloid hyperplasia of bone marrow and significantly decreased sensitivities to menadione toxicity compared with wild-type mice. The generation and establishment of NQO2−/− mice provide a very important tool to determine the in vivo role of NQO2 in protection against myelogenous hyperplasia and metabolic activation of quinones. In addition, the NQO2−/− mice will be an invaluable tool to study the role of NQO2 in activation of bioreductive drugs including anti-tumor drugs such as CB1954 (35, 39), mitomycin C, and indoloquinone. Finally, it will be of interest to determine whether mice lacking NQO2 have altered metabolism because of an accumulation of NRH, develop myeloid leukemia when exposed to chemicals, and/or have life spans that differ from wild-type mice because NQO2 has been shown to protect against myelogenous hyperplasia.

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