Oxidative DNA damage and DNA repair enzyme expression are inversely related in murine models of fatty liver disease

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Gao, Daqing, Chiming Wei, Lei Chen, Jiawen Huang, Shiqi Yang, and Anna Mae Diehl. Oxidative DNA damage and DNA repair enzyme expression are inversely related in murine models of fatty liver disease. Am J Physiol Gastrointest Liver Physiol 287: G1070–G1077, 2004. First published July 1, 2004; doi:10.1152/ajpgi.00228.2004.—Mitochondrial generation of reactive oxygen species (ROS) is increased in mice with fatty livers induced by genetic obesity, chronic consumption of ethanol, or methionine/choline-deficient diet. Both nuclear and mitochondrial (mt) DNA are targets for ROS-induced damage and accumulate hydroxylated bases, such as 8-hydroxy-2'-deoxyguanosine (8-oxoG) and base substitution of adenine with 8-oxoG (A*8-oxoG), that introduce mutations that promote cancer as well as cell death. The mammalian homolog of the bacterial DNA mismatch repair enzyme MutY (MYH) removes A*8-oxoG from nuclear and mtDNA, reduces 8-oxoG accumulation, and restores genomic stability after ROS exposure. Cumulative damage to mtDNA occurs as fatty liver disease progresses. Therefore, differences in hepatic MYH activity may influence the severity of fatty liver disease. To evaluate this hypothesis, we compared mtH2O2 production, MYH activity, oxidative DNA damage, and hepatocyte death in healthy mice and different mouse models of fatty liver disease. The results show that diverse causes of steatohepatitis increase mtROS production, limit repair of mtDNA, and oxidatively damage DNA. However, there are important differences in the DNA repair response to oxidant stress among mouse models of fatty liver disease. Independent of the degree of mtROS generation, models with the least MYH exhibit the greatest accumulation of 8-oxoG and the most hepatocyte death. These findings raise the intriguing possibility that inherited or acquired differences in DNA repair enzyme activity may underlie some of the interindividual differences in fatty liver disease outcomes.

Mitochondrial DNA (mtDNA) is a circular, double-stranded molecule containing 16,569 base pairs. It encodes 22 transfer RNAs and 2 ribosomal RNAs. The remaining 13 genes encode subunits of seven enzymes in the respiratory chain. The enzymes responsible for mtDNA repair are encoded by the same genes as their nuclear counterparts. Intracellular localization is governed by mitochondrial-targeting sequences (18). In both mitochondria and nuclei, 8-oxoG is the most abundant type of oxidized guanine present in DNA (28). mtDNA has been shown to accumulate high levels of 8-hydroxy-2'-deoxyguanosine (8-oxoG), the product of hydroxylation of guanine at carbon 8 (3). 8-oxoG can mispair with 2'-deoxyctydine-5'-triphosphate or with 2'-deoxyadenosine triphosphate during DNA replication, forming C*8-oxoG and base substitution of adenine with 8-oxoG (A*8-oxoG) mismatches. The A*8-oxoG mismatches may result in deletions, C-G to A-T transversions (25). These mutations, in turn, can interfere with the transcription of mitochondrially encoded genes, thereby inhibiting electron transport and promoting further mitochondrial dysfunction and cell death (8).

For a long time, it was thought that mitochondria lacked DNA repair systems. In fact, however, DNA damage can be repaired in mitochondria. Base-excision repair (BER) in mitochondria is now well established. The enzymes responsible for mtDNA repair are encoded by the same genes as their nuclear counterparts. Intracellular localization is governed by mitochondrial-targeting sequences (18). In both mitochondria and nuclei, 8-oxoG is the most abundant type of oxidized guanine present in DNA (28). mtDNA has been shown to accumulate high levels of 8-hydroxy-2'-deoxyguanosine (8-oxoG), the product of hydroxylation of guanine at carbon 8 (3). 8-oxoG can mispair with 2'-deoxyctydine-5'-triphosphate or with 2'-deoxyadenosine triphosphate during DNA replication, forming C*8-oxoG and base substitution of adenine with 8-oxoG (A*8-oxoG) mismatches. The A*8-oxoG mismatches may result in deletions, C-G to A-T transversions (25). These mutations, in turn, can interfere with the transcription of mitochondrially encoded genes, thereby inhibiting electron transport and promoting further mitochondrial dysfunction and cell death (8).

If unrepaired, further replication of A*8-oxoG mismatches results in C-G and A-T transversions and causes genomic instability (26). Consequently, polymorphisms that reduce MYH expression and/or activity have been associated with predisposition to certain cancers (10, 25, 30, 32, 42). With aging, the mitochondrial import of proteins needed for DNA repair declines, leaving a large fraction of DNA repair enzymes stuck to mitochondrial outer membranes in their precursor forms, despite higher total DNA repair enzyme activity in aging cells (37). As a result, aging increases levels of 8-oxoG in mtDNA but not nuclear DNA (15). Decreased ability to repair mtDNA damage directly increases cellular sensitivity to oxidative stress and promotes cell death (8). Premature oxidative aging of hepatic mtDNA has been reported in Wilson’s disease, an inherited type of steatohepatitis (21). All of this information demonstrates that repair of oxidative damage to DNA is a dynamic and highly regulated process. When nuclear
DNA repair is inhibited, mutations in oncogenes and tumor suppressor genes occur, promoting carcinogenesis. When mtDNA repair is blocked, cells age prematurely and become sensitive to ROS-induced death.

Fatty livers are unusually vulnerable to damage from oxidative stress (6, 7) and also exhibit an increased risk for hepatocellular carcinoma (1, 22, 24). Cumulative damage to mtDNA has been documented during liver disease progression in humans and animals with ethanol-induced fatty liver disease (reviewed in Ref. 2). However, very little is known about the status of nuclear or mtDNA repair mechanisms in this condition (23, 41). Even less has been reported about DNA repair in nonalcoholic fatty liver disease, despite strong evidence for mitochondrial dysfunction in humans with nonalcoholic steatohepatitis (27) and rodents with either obesity-related (44) or diet-induced fatty liver disease (17, 34). Hence, the purpose of this study was to compare mtH$_2$O$_2$ production, expression of the DNA repair enzyme MYH, oxidative DNA damage, and hepatocyte death in healthy mice and different mouse models of fatty liver disease. Our hypothesis is that there is an inverse relationship between MYH expression and hepatocyte oxidative DNA damage and cell death. Our results support this hypothesis and thus identify MYH activity as a genetically and environmentally regulated variable that influences the progression of fatty liver disease.

MATERIALS AND METHODS

Animal Feeding Protocols

Twenty-four wild-type and six genetically obese, ob/ob adult (aged 12 wk) male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). All experiments satisfied NIH and Johns Hopkins University standards for humane utilization of laboratory animals. Wild-type mice were divided into two groups (n = 12 mice/group) for feeding purposes. Half of group 1 (n = 6) was fed a high-fat, liquid diet. Our earlier studies demonstrate that such feeding protocol leads to steatohepatitis in the ethanol-fed group. Earlier work described (44). Mitochondrial protein content was quantified with BCA protein assay reagents (Pierce, Rockford, IL) based on the method of Smith et al. (33).

Evaluation of mtH$_2$O$_2$ Production

mtH$_2$O$_2$ production was quantified in a Berthold model LB9505 bioluminometer at 37°C using the chemiluminescence probe 5-amino-2,3-dihydro-1,4-phenalazineinedione (luminol; Sigma, St. Louis, MO), as we have described (44). The reaction mixture contained 0.1 mg of mitochondrial protein from each mouse in 1 ml of air-saturated respiration buffer plus succinate (6 mM) and horseradish peroxidase (10 µg/ml). The luminol-derived chemiluminescence was initiated by adding 10 µM luminol and was continuously monitored for 60 min. Results are normalized per milligram of mitochondrial protein. Mitochondrial protein was quantified by Bio-Rad assay (Pierce) as in Isolation of Hepatic Mitochondria.

Determination of DNA Damage in Liver Cells

Formalin-fixed, paraffin-embedded liver sections from the remaining three mice per group were evaluated for 8-oxoG with anti-8-oxoG antibody (Trevigen, Gaithersburg, MD) as we described previously (19). Briefly, the slides were fixed and washed with PBS, pH 7.4, and incubated for 40 min at 37°C. The DNA was denatured by soaking the slides in 4 N HCl for 7 min. After slides were incubated with 50 mM Tris base for 3 min at room temperature and two washings with PBS, 10% BSA was added for 1 h at room temperature to block nonspecific staining sites. Slides were then incubated with 3% H$_2$O$_2$ for 30 min at room temperature to block endogenous peroxidase. Thereafter, slides were incubated with primary anti-8-oxoG monoclonal antibody (diluted 1:100 in 10 mM Tris–HCl, pH 7.5, 10% serum) overnight at 4°C, rinsed twice with PBS, and incubated with secondary anti-mouse antibody (1:100) conjugated with 20 µg/ml streptavidin-horseradish peroxidase in 1 × PBS for 1 h at room temperature. After being stained with diaminobenzamide tetrahydrochloride (DAB; DAKO, Carpinteria, CA) and counterstained with methyl green, slides were examined under light microscopy. The percentage of cells staining positive for 8-oxoG was quantified as follows: number of positive hepatocytes/total number of hepatocytes times 100 equal percent 8-oxoG-positive cells.

Evaluation of MYH Expression

In the same mice studied for 8-oxoG, MYH expression was assessed by two different methods: immunocytochemistry and immunoblot analysis.

Immunohistochemistry. Immunohistochemical staining was performed as we previously described (19). Briefly, after slides were deparaffinized, sections were rinsed in 0.1 M PBS for 20 min and blocked in 2% normal horse serum for 2 h. Then sections were incubated with primary MYH antibody (1:100) in 10% normal horse serum or 10% normal rabbit serum and 0.3% Triton X-100 for 40 min at 37°C. After endogenous peroxidase activity was quenched by exposing the slides to 0.3% H$_2$O$_2$ and 10% methanol for 20 min, the slides were washed in PBS and incubated with secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences, Little Chalfont, UK). The final reaction was achieved by incubating the sections with freshly prepared reagent containing 3-aminon-9-ethylcarbazole (Sigma) dissolved in N,N-dimethylformamide and sodium acetate. The sections were counterstained with hematoxylin, mounted, and reviewed with an Olympus microscope. Two trained independent observers reviewed each section. Sample identities were concealed during scoring, and at least three samples were scored per group. Results are expressed as means ± SE for each group. For each sample, MYH expression was evaluated by scoring the percentage of positive staining on the entire liver section as we previously described (16, 39, 40). According to this scoring system, 0 = no staining; 1 = minimal staining (<10% section MYH positive); 2 = mild staining (10–30% section MYH positive); 3 = (31–50% MYH positive); and 4 = strong staining (>50% section MYH positive). The specificity of positive staining was further confirmed by substitution of normal rabbit serum for the primary antiserum.

Immunoblot analysis. Western blot analysis was performed as we described (19). Briefly, tissue samples were homogenized in a lysis buffer (0.1 M NaCl, 0.01 M Tris–HCl, pH 7.5, 1 mM EDTA, and 1 µg/ml aprotinin), and then the homogenates were centrifuged at 7,000 g for 15 min at 4°C. Supernatants were used as protein samples.
SDS-PAGE was performed in a 10% polyacrylamide gel. Protein samples were boiled at 100°C in 2.5% SDS and 5% β-mercaptoethanol, and lysates equivalent to 20 μg of protein from each sample were run on the gel for 90 min at 20 mA, together with a size marker (Invitrogen, Carlsbad, CA). The electrophoresis running buffer contained 25 mmol Tris base, 250 mM glycine, and 0.1% SDS. Proteins on the gel were transferred to a nitrocellulose membrane with a transfer buffer consisting of 48 mM Tris base, 39 mM glycine, 0.4% SDS, and 20% methanol. After being transferred, membranes were placed in 1% powdered milk in PBS to block nonspecific binding. After reaction with the primary and secondary antibodies, the membrane was subjected to the enhanced chemiluminescence analysis system from Amersham. Polyclonal antibody against MYH was obtained from Novus Biological (Littleton, CO). Monoclonal antibody against actin (Oncogene Research Products) was used to control for differences in protein loading. To ascertain specific binding of the anti-MYH antibody, another membrane was studied without this primary antibody.

**Evaluation of Liver Cell Death**

To quantify the relative numbers of liver cells with DNA fragmentation TUNEL assay was performed in liver sections from each mouse according to our previously reported method (19) using an ApoTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD). Briefly, after deparaffinizing the sections, tissue nuclei were stripped of proteins by incubation with 20 μg/ml proteinase K for 10 min. After treatment with 0.3% H2O2 in distilled water for 5 min, the sections were incubated with terminal deoxynucleotidyl transferase buffer (TdT; Boehringer-Mannheim, Indianapolis, IN), 30 mM Tris, pH 7.2, 140 mM sodium cacodylate, and 1 mM cobalt chloride containing TdT enzyme (0.5 U/ml, Boehringer-Mannheim) and biotin-16-dUTP (0.04 mM; Boehringer-Mannheim, Indianapolis, IN), 30 mM Tris, 1 mM cobalt chloride in a humidified chamber at 37°C for 120 min. The reaction was terminated by incubating with 300 mM NaCl and 30 mM sodium citrate for 15 min at 25°C. After being washed with 50 mM Tris-HCl, pH 7.7, sections were stained with DAB/H2O2 solution and counterstained with hematoxylin. After being washed three times in Tris-HCl, pH 7.7, sections were dehydrated in ascending ethanol series and immersed in xylene, and coverslips were mounted with Permount. To determine the percentage of dead cells, TUNEL-positive and TUNEL-negative liver cells were counted. Results are expressed as number of TUNEL-positive cells per total number of cells per field magnification (×100).

**Statistical Analysis**

Results of all quantitative studies are expressed as means ± SE. Statistical comparisons within each group were performed with ANOVA for repeated measures followed by Fisher’s least significant difference test of repeated measures when appropriate. Comparisons between groups were performed with factorial ANOVA followed by Fisher’s least significant difference test of repeated measures. Statistical significance was accepted at a P value of < 0.05.

**RESULTS**

In all mice with fatty livers, mtH2O2 production (Fig. 1) and 8-oxoG accumulation are increased (Fig. 2). In general, these results support the concept that increased mtROS contribute to the pathogenesis of fatty liver disease. However, closer scrutiny of these data demonstrate that the degree of DNA damage (reflected by 8-oxoG accumulation) does not correlate particularly well with the level of H2O2 generation. For example, among the three fatty liver disease models, H2O2 release is intermediate in ob/ob mice, but this group exhibits the fewest cells with 8-oxoG. Similarly, mitochondria from MCD-fed mice release more than twice as much H2O2 as mitochondria from ethanol-fed mice, and yet hepatocyte accumulation of 8-oxoG is virtually identical in these two groups.

These findings suggested to us that in the liver, net oxidative DNA damage might be governed more by the relative efficiency of mtDNA repair mechanisms than absolute ROS production. To evaluate this possibility, we used immunohistochemistry to compare the expression of MYH, an important BER enzyme, among the various groups (Fig. 3, A–E). In all groups, MYH protein was much more evident in the extranuclear compartment. The punctate distribution of MYH within the cytosol is consistent with published evidence that isoforms of this DNA repair enzyme are targeted to mitochondria normally (18). Compared with healthy control mice, ob/ob mice exhibited a slight (~30–40%), albeit statistically significant, decrease in MYH protein expression. In contrast, MYH levels are drastically reduced in ethanol-fed and MCD-fed mice. In both of the latter groups, MYH expression is decreased by ~60–80%. These differences in MYH were confirmed by immunoblot analysis of MYH protein in liver extracts from the various groups (Fig. 3, F and G). Therefore, fatty liver disease generally reduces hepatic MYH expression and increases liver DNA damage. Moreover, the highest levels of DNA damage occur in livers with the lowest expression of the DNA repair enzyme, MYH.

In other types of cells, oxidative mtDNA damage has been associated with the release of factors that promote lethal fragmentation of nuclear DNA by both caspase-dependent (8) and caspase-independent mechanisms (38). We performed TUNEL staining to determine whether a similar relationship exists in fatty livers. TUNEL-positive cells are increased in all animals with fatty liver disease (Fig. 4). Of the three fatty liver models, ob/ob mice have the fewest TUNEL-positive cells. Compared with ob/ob livers, the livers of ethanol-fed mice and MCD-fed mice contain 50–100% more TUNEL-positive cells. The results of our studies suggest that liver cell death is more likely in situations that limit mtDNA repair, permitting the accumulation of oxidatively damaged mtDNA, than in settings at which mtDNA damage is limited by relatively intact DNA repair mechanisms.
DISCUSSION

During normal intermediary metabolism and oxidative phosphorylation, mitochondria produce ROS. However, healthy cells use multiple mechanisms to limit ROS production. Thus ROS release at various sites in the electron transport chain is negligible for most endogenous substrates, except certain fatty acids. Nonetheless, efficient mechanisms to repair oxidative DNA damage are required to maintain liver cell viability, as evidenced by mice genetically deficient in ERCC1, an enzyme that is involved in BER. ERCC1-null mice are severely runted and die before weaning from liver failure with accelerated hepatocyte polyploidy that is reminiscent of premature aging disorders (31). Therefore, in healthy livers, effective mechanisms to constrain mtROS production combine with efficient repair mechanisms so that there is little accumulation of damaged DNA. Our observation that <0.5% of hepatocytes contain appreciable 8-oxoG is consistent with other evidence that steady-state levels of oxidative mtDNA modifications are low under physiological conditions (14).

Various stresses, including several that have been implicated in the pathogenesis of fatty liver disease, increase mtROS production (5, 20, 35, 37). Hence, it is not surprising that we observed increased mtROS in our mouse models of steatohepatitis. Situations that increase oxidant stress also generally induce net increases in DNA repair enzyme activity, at least transiently (4, 9, 23). However, it is not clear whether different subcellular compartments always benefit equally from this response. During aging, for example, mtDNA repair is compromised despite an overall increase in DNA repair enzyme activity, because an age-related defect inhibits the mitochondrial import of DNA repair enzymes (37). Furthermore, the initial increase in DNA repair enzyme activity is not always sustained. Reduced expression of some DNA repair enzymes has been noted during chronic alcohol exposure (23). Consequently, progressive accumulation of mtDNA damage occurs in chronic alcoholic liver disease (2). Premature aging of mtDNA has been observed in adults with Wilson’s disease, a congenital cause of chronic steatohepatitis (21). Here we demonstrate, for the first time, similar findings in mice with obesity-related and diet-induced fatty liver disease. Therefore, diverse causes of steatohepatitis are characterized by increased
Fig. 3. Expression of DNA mismatch repair enzyme MYH in mice with fatty livers. Photomicrographs of representative normal control mouse (A); ob/ob mouse (B); MCD-fed mouse (C); EtOH-fed mouse (D). Final magnification, ×1,000. E: Staining density scores (means ± SE) in each group (n = 3 mice/group). *P < 0.05 vs. control group, †P < 0.05 vs. ob/ob mice. F: Western blot analysis of MYH expression in liver extracts of representative mice from each group (top) and actin expression in the same samples (bottom). PF, pair-fed. G: Graphic representation of 3 Western blot analyses *P < 0.05 for each fatty liver group vs. controls, †P < 0.05 for MCD or EtOH-groups vs. ob/ob group.
mtROS production, limited repair of mtDNA, and the accumulation of oxidatively damaged DNA.

However, our studies also demonstrate important differences in the DNA repair response to oxidant stress among our animal models of fatty liver disease. Expression of the DNA repair enzyme MYH is least inhibited in the fatty livers of ob/ob mice and most suppressed in mice with ethanol- or MCD diet-induced liver damage. These model-related differences in DNA repair enzyme expression are not easily explained by differences in the degree of mtROS generation. However, they predict the extent of oxidative DNA damage and liver cell death, both of which are much higher in the latter two groups than in the ob/ob mice. The relatively benign course of fatty liver disease in ob/ob mice has been widely attributed to their genetic deficiency of leptin (13). The present work identifies a specific mechanism, i.e., relative preservation of MYH-mediated DNA repair, that may contribute to the indolent course of nonalcoholic fatty liver disease in leptin-deficient animals.

Fatty liver disease is also indolent in most obese, hyperleptinemic humans, but can be progressive in others, leading to cirrhosis and hepatocellular carcinoma (11). Our findings raise the intriguing possibility that inherited or acquired differences in DNA repair enzyme activity may underlie some of these interindividual differences in fatty liver disease outcomes. To our knowledge, this has not been tested yet in patients with alcoholic or nonalcoholic fatty liver disease but merits consideration. Given the emerging relationship between MYH inactivation and other epithelial malignancies (25), scrutiny of MYH activity in individuals who develop hepatocellular carcinoma during fatty liver disease is also justified.

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REFERENCES


