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

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REACTIVE OXYGEN SPECIES (ROS), including superoxide anion and hydrogen peroxide (H2O2), are byproducts of normal metabolism. Various types of cellular stress, including that induced by aging (37), overeating (20), alcohol consumption (5), and exposure to bacterial LPS (35), exacerbate mitochondrial (mt) ROS generation significantly. Given roles for each of these factors in human fatty liver disease pathogenesis (2, 27), 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.

levels of 8-hydroxy-2′-deoxyguanosine (8-oxoG), the product of hydroxylation of guanine at carbon 8 (3), 8-oxoG can mispair with 2′-deoxycytidine-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 deleterious C:G and A:T transversions (25). These mutations, in turn, can interfere with the transcription of mitochondrial DNA, thereby inhibiting electron transport and promoting further mitochondrial dysfunction, alterations of mitochondrial death regulators, and increased cell death (36).

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 modified DNA base generated when ROS attack DNA. 8-oxoG is removed from DNA by the specific glycosylase, OGG1. Additional postreplication repair is needed to correct the 8-oxoG/A mismatches that are produced by persistent 8-oxoG residues. The latter process occurs in stages. The mismatched adenine is removed by a homolog of the Escherichia coli DNA repair enzyme MutY (MYH) followed by preferential insertion of a cytosine leading to the formation of 8-oxoG/C pairs, which are then corrected by OGG1-mediated BER (12).

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 senescence 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 is damaged, the nuclear repair system is activated, whereas damaged mtDNA is repaired by mitochondrial DNA repair enzymes (16). This differential repair response may help explain why oxidative stress preferentially damages mtDNA, which is a major source of ROS (11). Oxidative damage to mtDNA is further exacerbated by the lack of repair mechanisms specific for mtDNA, the presence of single-stranded regions, and the limited number of DNA repair enzymes encoded by the mitochondrial genome (16).
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 hepato-cellular 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 mtH₂O₂ 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 in which 36% of calories were supplied by ethanol (Biopure, Thorofare, NJ). The remaining mice in that group (n = 6) were pair-fed an isocaloric volume of a similar control diet in which the ethanol calories were substituted by dextrin maltose. Both groups received their respective diets for 4 wk. Our earlier studies show that by 4 wk, this protocol leads to steatohepatitis in the ethanol-fed group. Half of group 2 (n = 6) was fed a commercial methionine/choline-deficient (MCD) diet. The remaining mice in that group (n = 6) were pair-fed isocaloric amounts of the respective control diet. Earlier work shows that mice that have been fed MCD diets for 4 wk have steatohepatitis. Concurrently with these feeding paradigms, the genetically obese mice (n = 6) were provided ad libitum access to control, high-fat liquid diet. Our earlier studies demonstrate that such treatment of ob/ob mice results in steatohepatitis. At the end of the feeding period, all mice were anesthetized and immediately killed to obtain liver tissue.

Isolation of Hepatic Mitochondria

Mitochondria were isolated from freshly harvested livers of 3 mice/group according to the method of Rickwood et al. (29), as we 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 mtH₂O₂ Production

mtH₂O₂ production was quantified in a Berthold model LB9505 bioluminometer at 37°C using the chemiluminescent probe 5-aminono-2,3-dihydro-1,4-phthalazinedione (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

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 reported 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% PBS was added for 1 h at room temperature to block nonspecific staining sites. Slides were then incubated with 3% H₂O₂ 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 20 h at 4°C. After endogenous peroxidase activity was quenched by exposing the slides to 0.3% H₂O₂ 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-aminio-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 mM 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 ApopTag 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) containing 30 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 Fishers least significant difference test of repeated measures when appropriate. Comparisons between groups were performed with factorial ANOVA followed by Fishers 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. 2. DNA damage product 8-hydroxy-2'-deoxyguanosine (8-oxoG) in mice with fatty livers. Photomicrographs of representative normal control mouse (A), ob/ob mouse (B), MCD-fed mouse (C), and ethanol (EtOH)-fed mouse (D). Final magnification, ×1,000. Arrows indicate representative 8-oxoG-positive cells. E: mean ± SE percentage of 8-oxoG-positive cells in livers of 3 mice/group.

*P < 0.05 vs. control group; †P < 0.05 vs. ob/ob mice.
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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Fig. 4. TUNEL staining in mice with fatty livers. Photomicrographs of a representative normal control mouse (A); ob/ob mouse (B); MCD-fed mouse (C); and EtOH-fed mouse (D). Arrows indicate representative TUNEL-positive cells. Final magnification, ×1,000. E: percentage of TUNEL-positive liver cells (means ± SE) in each treatment group (n = 3 mice/group). *P < 0.05 vs. control group; †P < 0.05 vs. ob/ob mice.
REFERENCES


