Genetic differences in hepatic lipid peroxidation potential and iron levels in mice

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Abstract:

Oxidative damage to macromolecules, including lipids, has been hypothesized as a mechanism of aging. One end product of lipid peroxidation, malondialdehyde (MDA), is often quantified as a measure of oxidative damage to lipids. We used a commercial colorimetric assay for MDA (Bioxytech LPO-586, Oxis International, Portland, OR) to measure lipid peroxidation potential in liver tissue from young (2 month) male mice from recombinant inbred (RI) mouse strains from the C57BL/6J (B6) × DBA/2J (D2) series (BXD). The LPO-586 assay (LPO) reliably detected significant differences ($P < 0.0001$) in lipid peroxidation potential between the B6 and D2 parental strains, and yielded a more than two-fold variation across the BXD RI strains. In both B6 and D2 mice, LPO results were greater in old (23 month) mice, with a larger age-related increase in the D2 strain. As the level of iron can influence lipid peroxidation, we also measured hepatic non-heme iron levels in the same strains. Although iron level exhibited a slightly negative overall correlation ($r^2 = 0.119$) with LPO results among the entire group of BXD RI strains, a sub-group with lower LPO values were highly correlated ($r^2 = 0.704$). LPO results were also positively correlated with iron levels from a group of 8 other inbred mouse strains ($r^2 = 0.563$). The BXD RI LPO data were statistically analyzed to nominate quantitaive trait loci (QTL). A single marker, Zfp4, which maps to 55.2 cM on chromosome 8, achieved a significance level of $P < 0.0006$. At least two potentially relevant candidate genes reside close to this chromosomal position. Hepatic lipid peroxidation potential appears to be a strain related trait in mice that is amenable to QTL analysis.

Keywords: Lipid peroxidation; QTL; Iron; Recombinant inbred mice

Article:

1. Introduction

Various strains of mice differ in many biological traits, including life span (Gelman et al., 1988) and other age-related phenotypes (Ingram and Jucker, 1999; Jackson et al., 1999). This strain specific variation in aging is likely to be influenced by the genetic differences among strains. Poly-morphisms in specific genes may underlie such complex traits, and when genetically mapped are known as quantitative trait loci or QTL (Plomin and McClearn, 1993). Mice are an ideal species for a genetic analysis of age-related phenotypes in part because of the available biological resources, such as recombinant inbred (RI) strains (Plomin et al., 1991). RI strains are generated by initially interbreeding two inbred strains, such as the C57BL/6J (B6) and DBA/2J (D2) strains. The F1 animals are then mated to produce F2 animals. Prolonged brother-sister mating of the F2 generation to derive new inbred lines (BXD RI lines) produces mice that are homozygous at essentially all loci; however, each locus is derived from one or the other parental strain. Each inbred BXD RI line thus possesses a unique combination of parental B6 and D2 loci. By measuring a phenotypic characteristic that varies among strains, genetic markers mapped in each of the BXD RI strains may then be statistically correlated with the measured characteristic to nominate QTL (Bennett et al., 1994).

We have begun QTL studies in BXD RI mouse lines by analyzing lipid peroxidation potential as an age-related marker of oxidative stress. Significant increases in liver peroxidation have been observed in both aging rats (Sawada and Carlson, 1987) and mice (Uysal et al., 1989), which can be modulated by caloric restriction (Koizumi et al., 1987). Such increases may be due to a shift in fatty acid profile from saturated molecules to

more peroxidizable unsaturated chains (Laganiere and Yu, 1993) since incorporation of molecular oxygen into a polyunsaturated fatty acid to yield a lipid hydroperoxide is a primary peroxidation product. As the detection of lipid hydroperoxides is difficult, techniques have been used for the detection of the secondary or end-products of hydroperoxide decomposition, i.e. malondialdehyde (MDA) (Esterbauer, 1996). The thiobarbituric acid test (TBA) has been a singular assay for the measurement of MDA over the past several decades (Valenzuela, 1991).

We have used a commercial assay for MDA to measure lipid peroxidation potential in liver tissue from young mice of the BXD RI series. Since iron levels may affect lipid peroxidation (Minotti and Aust, 1992), we have also determined hepatic iron levels in BXD RI strains as well as several other inbred strains, and correlated these levels with lipid peroxidation potential. Distinct strain related differences are present in both lipid peroxidation potential and iron status that should facilitate further genetic analysis.

2. Methods

2. 1. Mice

Two male mice at 5–6 weeks of age from 20 BXD RI strains, B6, D2, and 6 other inbred strains, AKR/J (AKR), SWR/J (SWR), 129/J (129), SJL/J (SJL), C3H/J (C3H), and C57L/J (L), were obtained from The Jackson Laboratory (Bar Harbor, ME). Two 23-month-old male B6 and D2 mice were obtained from the National Institute on Aging rodent colony. Upon arrival, mice were housed individually and quarantined for one week in standard polycarbonate boxes with stain-less steel wire covers. Temperature was maintained at 22 °C with a 14-h light: 1 0-h dark cycle. Mice were allowed free access to demineralized water and standard lab chow. All mice were fasted overnight for a minimum of 10 h and a maximum of 13 h prior to sacrifice. Mice were euthanized by CO_2 inhalation and portions of liver harvested and frozen at − 80 °C until analysis.

2.2. Lipidperoxidation assay

The Bioxytech LPO-586 (Oxis International, Portland, OR) assay for MDA was performed as per the kit protocol. All other reagents were from Sigma Chemical (St. Louis, MO). A 20–30% (w/ v) homogenate of liver was prepared in 20 mM Tris buffer, pH 7.4, containing 5 mM butylated hydroxytoluene (BHT) to prevent sample oxidation. Experiments were also performed with the addition of EDTA (5 or 10 mM final concentration), deferoxamine mesylate (250 uM final concentration), and/or human catalase (400 U/ml final concentration) to the tissue homogenate. Following centrifugation at 3000 × *g* at 4 °C for 10 min, the LPO-586 R1 reagent, *N*-methyl-2- phenylindole in 25% methanol/75% acetonitrile, was added to the supernatants, followed by the addition of 12 N HCI with incubation at 45 °C for 60 min. Following centrifugation at 15 000 × *g* at 4 °C for 10 min, absorbances were read at 586 nm. In order to correct for intrinsic A_{586} absorbance from the tissue homogenate, a second reaction mix for each sample (sample blank) was prepared using a 25% methanol/75% acetonitrile mixture without the R1 reagent. A reaction mix omitting sample homogenate but containing all other reaction components (reagent blank) was also measured. A standard curve using 1,1,3,3,-tetra-methoxypropane (TMOP) that generates free MDA during the acid hydrolysis step was also prepared. The concentration of MDA was calculated using a molar extinction coefficient at 586 nm of 1 10 000 with the formula:

$$\frac{A_{586}\text{sample} - A_{586}\text{sample blank} - A_{586}\text{reagent blank}}{0.11}$$

Protein concentration was measured using the Bio-Rad Protein Assay (Hercules, CA) according to manufacturer's instructions. All statistical analyses were performed using Excel 5.0 (Micro-soft, Seattle, WA).

2.3. Hepatic iron determination

Non-heme iron concentrations were measured spectrophotometrically following digestion at 65 °C for 20 h prior to colorimetric analysis (Torrance and Bothwell, 1980).

2.4. QTL analysis

QTL analysis was performed using Map Man-ager QTb15ppc (http://mcbio.med.buffalo.edu/ mapmgr.html). Lecithin cholesterol acyltransferase (LCAT) and NAD(P)H:menadione oxidoreductase (NMO1) cDNA sequences were obtained from Genbank. Primers were designed to amplify fragments using the polymerase chain reaction (PCR) from coding region sequences. DNA was obtained (Qiagen, Germany) from liver tissue of B6 and D2 mice. PCR fragments were resolved for length differences by 3% agarose gel (FMC, ME) electrophoresis, gel isolated (Qiagen, Germany), cloned (Invitrogen, CA), and subject to automated sequencing (Perkin Elmer, CA).

3. Results

3.1. Precision of the LPO assay

Due to the expense and limited availability of both aged mice and BXD RI strains, which restricted sample size, the precision of the LPO assay was first investigated in young B6 and D2 parental strains to determine the inherent variation of the assay. Six replicates from a single liver homogenate from a young B6 mouse and six replicates from a young D2 mouse produced coefficients of variation of 5 and 11%, respectively. Six replicates from an old (23 months) B6 mouse and six from an old (23 months) D2 mouse produced coefficients of variation of 12 and 13%, respectively. Assays performed on separate homogenates from either five young B6 or five young D2 mice produced coefficients of variation ranging from 16 to 29% in two separate experiments. These data are similar to previous studies using the TBA test (Rojas et al., 1993; Uysal et al., 1989) and indicate that a moderate but accept-able level of intra-and inter-sample variation is present in the LPO assay.

3.2. Effect of chelators and antioxidants

The widely used TBA test has been criticized for its lack of specificity (Janero and Burghardt, 1988) in part due to the effect of sample constituents, such as iron, on generating MDA during the assay procedure (Janero and Burghardt, 1989). BHT is often added to the assay in order to minimize these effects (Valenzuela, 1991); the LPO assay includes BHT in the homogenization buffer. We sought to determine whether metals such as iron present in the liver tissue were affecting LPO results by adding either EDTA or deferoximine mesylate, an iron specific chelator, to the homogenate prior to the LPO assay. No difference in LPO results was evident in the presence of 250 UM deferoximine mesylate or up to 10 mM EDTA (Fig. 1). As a positive control, the addition of 5 mM H_2O_2 and 200 UM $FeCl_2$ caused a more than 10 fold increase in LPO results that was almost completely suppressed by either BHT or deferoximine but not by 400 U/ml of catalase. These data suggest that BHT is a highly effective means of preventing peroxidative processes occur-ring during the LPO assay procedure and that iron and H_2O_2 can be potent inducers of MDA formation in vitro.

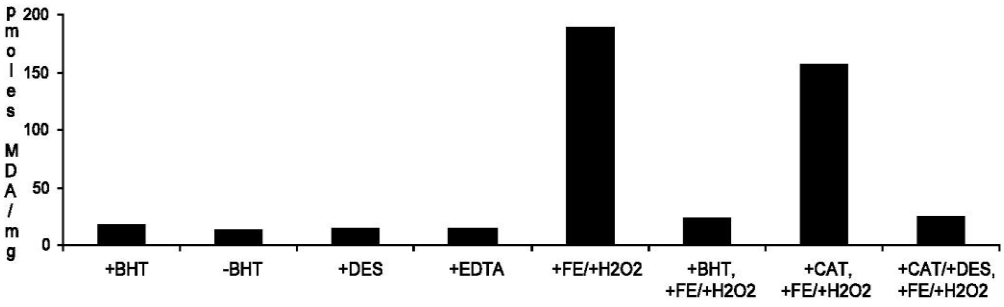

Fig. 1. Effects of antioxidants, chelators, and prooxidants on hepatic LPO results. The addition of Fe and H_2O_2 to the tissue homogenate increases LPO levels, while BHT or CAT/DES prevents the increase. CAT, catalase, DES, deferoximine.

3.3. Effect of age

Previous studies have shown an increase in TBA test reactivity with age in mouse liver (Uysal et al., 1989). We confirmed these observations by comparing hepatic LPO results obtained from 2-month-old mice with those from 23-month-old B6 and D2 mice (Fig. 2). LPO results increase by almost 50% in B6 mice and by about 400% in D2 mice. The approximate 50% inter-strain difference observed in young mice increases to about 400% at 23 months.

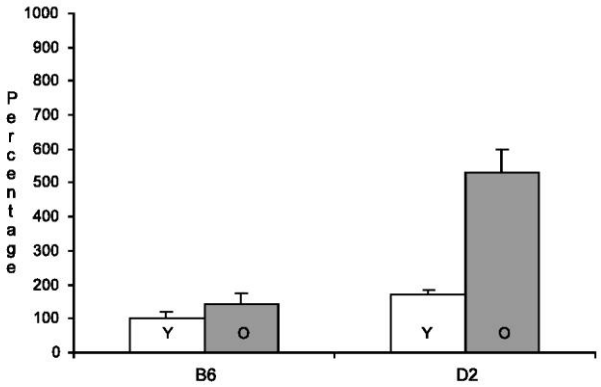

Fig. 2. Hepatic LPO results for 2 month (Y, open bars) and 23 month old (O, solid bars) B6 and D2 mice. Values are expressed as a percentage of the young B6 result. LPO levels in young D2 mice are about 50% higher than in young B6 mice, which increases to about 400% in old mice.

3.4. LPO assay in BXD RI strains

After confirming that the LPO assay could detect differences in the B6 and D2 parental strains, as well as age-related increases in each strain, we then analyzed 20 BXD RI strains (Fig. 3). The strain distribution pattern places the B6 parental strain at the low extreme of the group and the D2 parental strain close to mid range. Since each RI strain is a unique set of B6 and D2 alleles, strains with phenotypes that fall outside of the range of the parents' indicate that certain combinations of B6 and D2 alleles are more favorable to generating LPO assay reactivity than either the all B6 or all D2 allelic configuration. These results are also reflective of a continuously distributed trait, consistent with the contribution of multiple loci to the phenotype.

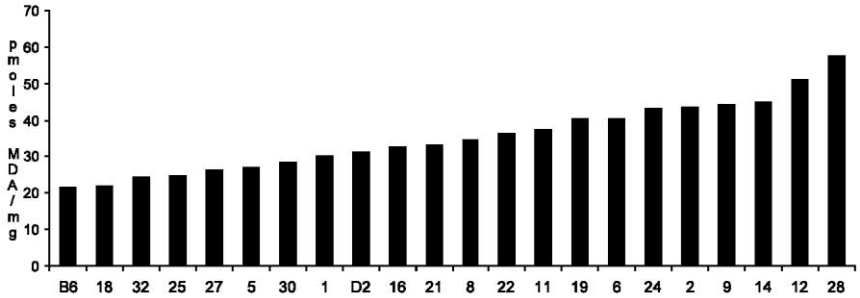

Fig. 3. Strain distribution pattern of hepatic LPO results for 20 BXD RI strains and parental B6 and D2 strains. BXD RI strain numbers are listed under each bar. LPO results are continuously distributed across the RI strains, consistent with a multigenic complex trait.

3.5. LPO assay-longevity correlation

Longevity data has been published for 18 of the BXD RI strains on which LPO results were deter-mined (Gelman et al., 1988), and for the B6 and D2 parental strains (Turturro et al., 1999). A low correlation (r^2 = 0. 196) was present between the reported mean life spans and the LPO results presented here (data not shown), although major methodological differences, including gender, diet, and husbandry conditions, complicate this analysis. Future studies using mice of the same gender, consuming the same diet, and housed under specific pathogen free conditions will be required to adequately assess the relationship between hepatic LPO results and life span.

3.6. LPO assay-iron correlation

Since iron has been reported to influence the results of TBA assays, we measured non-heme iron levels in the livers of the BXD RI strains, including parental strains and F1 animals and correlated those levels with the LPO results (Fig. 4). When analyzed as a group, a slight negative correlation was present (r^2 = 0. 119). However, closer inspection of the data revealed a linear clustering of strains with LPO results below approximately 33 pmoles MDA/mg. Above this value, no clear trend could be discerned. When treated as a sub-group, the low LPO strains were highly correlated with iron level (r^2 = 0.704), while the remainder of the strains were not

correlated (r^2 = 0.008). Within the allelic constraints of the B6 and D2 strains, iron level appears to have a significant influence on LPO results at low levels of lipid peroxidation potential, but does not have an effect when higher levels of lipid peroxidation potential are present.

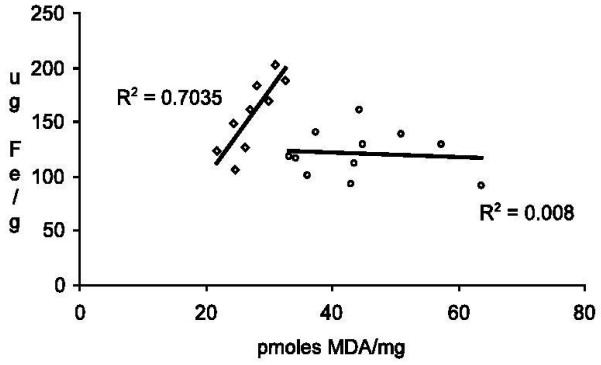

Fig. 4. Correlation of LPO results with hepatic iron levels for BXD RI strains. At lower LPO levels, one group of RI strains can be correlated with hepatic iron levels, while those manifesting higher LPO levels are not correlated with hepatic iron.

Several mouse strains have been reported to differ in levels of hepatic iron (Clothier et al., 2000; Morse et al., 1999). We also measured LPO and iron levels in six other commonly used and genotypically diverse inbred strains (Fig. 5). A moderate positive correlation (r^2 = 0.563) was present among the 8 inbred strains (including B6 and D2 mice) analyzed.

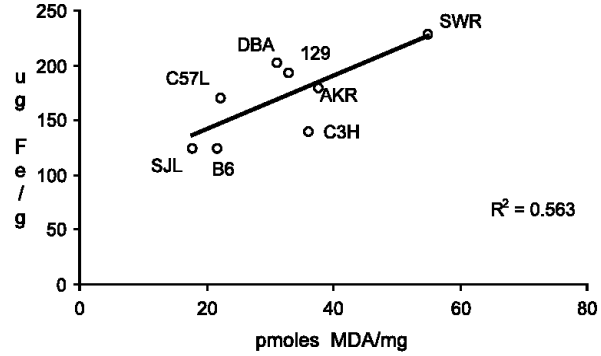

Fig. 5. Relationship of hepatic iron level to LPO results for eight inbred mouse strains. LPO results are positively correlated with hepatic iron level in this group of inbred strains.

3.7. Statistical genetic analysis

The data in Fig. 3 was analyzed with MapManager and a provisional QTL was identified at the $P < 0.001$ level using an additive regression model with no control for other QTLs and 5 to 20 informative progeny (Table 1). At the $P<0.01$ level, other provisional QTL were nominated on chromosomes 1, 2, 4, 6, 9, 11, 13, and 15 (data not shown).

Table 1
Provisional LPO QTL on chromosome 8

Locus	{chr}	Stat	%	*P*	Add	cM
Zfp4	{8}	11.8	41	0.00060	−6.74	55.2

A search of the Mouse Genome Informatics database (http://www.informatics.jax.org) for genes that mapped to within 5 cM of the position of the Zfp4 marker on chromosome 8 identified several potentially relevant candidate genes. A gene that is expressed in liver and involved in lipid metabolism is lecithin cholesterol acyltransferase (LCAT) (Warden et al., 1989) and a gene that is expressed in liver and involved in oxidative stress metabolism is Dia4 or NAD(P)H:menadione oxidoreductase (Nmo1) (Vasiliou et al., 1994). Both of these genes have been reported to underlie inter-strain variation in mice (Dueland et al., 1997; Kumaki et al., 1977), including the B6 and/or D2 strains. Sequence analysis of several regions of the Nmo1 gene and most of the coding region of the LCAT gene did not reveal any significant differences between B6 and D2 sequences.

4. Discussion

Lipid peroxidation occurs when molecular oxy-gen is incorporated into lipids possessing double bonds (Porter et al., 1995). Oxygen cannot directly react with such lipids due to incompatible spin chemistry, but the initial formation of lipid alkyl radicals allows for the addition of oxygen and hydrogen to form lipid hydroperoxides (Minotti and Aust, 1992). As reactive oxygen species can initiate lipid peroxidation, and have been proposed as a mechanism for aging, lipid peroxidation has been extensively studied in aging (Rikans and Hornbrook, 1997). Increased lipid peroxidation potential, as measured by MDA levels, has frequently been reported, although tissue and species specificity is apparent. Few studies have attempted to exploit inter-strain variation as a model to study lipid peroxidation (Cheeseman et al., 1985). We have used the results of an assay for MDA in liver as a phenotype for the application of QTL analysis in mice.

An important aspect of this approach is the nature of the phenotype. The assay commonly used to measure MDA is based upon the thiobarbituric acid (TBA) test, in which two molecules of TBA react stoichiometrically with one molecule of MDA at low pH and high temperature to form a pink pigment that has an absorption maximum at 532 to 535 nm (Valenzuela, 1991). Although some of the MDA detected in the TBA test is formed during in vivo lipid peroxidation, most is generated by decomposition of lipid peroxides during the acid-heating stage of the test, a process that is accelerated by transition metal ions, such as iron. The formation of metal stimulated peroxy radicals during the test may be prevented with the addition of an anti-oxidant such as butylated hydroxyl-toluene (BHT) (Jentzsch et al., 1996), but this does not prevent the decomposition of previously formed peroxides to MDA. Indeed, the peroxidation process beginning in the reaction mixture is effectively amplified in the assay itself, increasing its sensitivity (Gutteridge and Halliwell, 1990).

MDA formed during in vivo lipid peroxidation may also be metabolized (Mahmoodi et al., 1995), including enzymatic degradation (Valenzuela, 1991) and excretion in the urine (Siu and Draper, 1982). MDA may also react with macromolecules such as proteins, where it has been implicated in the formation of lipofuscins, or age pigments, in which it may condense with amino groups of proteins to form fluorescent species (Tsuchida et al., 1987). MDA may also react with DNA to form adducts that may be mutagenic (Marnett, 1999).

Despite the potential disadvantages, particularly in regard to its specificity, the TBA test for MDA has remained a widely used, simple, inexpensive, and easily quantifiable chemical measure of lipid peroxidation potential that can be assayed with high throughput. The commercial LPO assay for MDA used here employs a different chromogenic reagent than TBA, with a correspondingly different absorption wavelength, and a blank for each sample to mitigate the effects of interfering substances. A much lower reaction temperature also prevents the liberation of MDA bound to proteins via Schiff base and may decreases the formation of other aldehyde-chromophore complexes that could cause interference (Oxis International, 1997). The LPO assay thus appears to possess several advantages over the TBA test.

Although BHT may prevent assay induced per-oxidation, a potentially important pre-assay variable is iron, which can both initiate lipid peroxidation and can cause the decomposition of lipid hydroperoxides (Gutteridge and Halliwell, 1990). Although, the mechanisms involved in initiating or promoting oxidative damage are not completely known, iron may catalyze the conversion of hydrogen peroxide to the highly reactive hydroxyl radical via the Haber–Weiss reaction and/or may reductively cleave pre-formed lipid hydroperoxides (Minotti and Aust, 1992). Such in vitro evidence is supported by in vivo studies in which dietary iron administration in

rodents in-creased markers of lipid peroxidation (Ibrahim et al., 1997) and in human patients with transfusional iron overload, where hepatic iron levels were correlated with TBA test results (Linpisarn et al., 1991). We found a good correlation between non-heme hepatic iron levels and LPO results among 8 genetically diverse inbred mouse strains, as well as an excellent correlation within a sub-group of the genetically restricted BXD RI strains with low LPO results. The absence of a relationship in the high LPO BXD RI strains does not exclude iron as a contributor to lipid peroxidation, but may indicate that another factor(s) may have a greater effect.

Iron may also play a role in the increase in lipid peroxidation during aging since iron levels in-crease with age in several mammalian species, including mice (Massie et al., 1983; Sohal et al., 1999). Total body iron levels are primarily regulated at the level of intestinal absorption; eliminition of iron under normal conditions occurs primarily through normal cell loss from epithelial surfaces of the GI tract and skin, and blood loss (Oates et al., 2000). The apparent inefficiency of removal results in a slow accumulation of iron stores with age. Dietary restriction has been re-ported to suppress both age-related increases in iron levels and lipid peroxidation in rat liver, suggesting that the modulation of iron content may be a component of dietary restriction's effects (Cook and Yu, 1998). However, in mice, although iron concentrations in liver increased with age in ad libitum fed animals, they were not correlated with increased concentrations of lipid peroxides, and were further increased with caloric restriction (Sohal et al., 1999).

Factors known to inhibit lipid peroxidation may be relevant in the low LPO BXD RI strains including antioxidants, such as the superoxide dismutases (SOD) and catalase, which have been shown to affect lipid peroxidation in vitro by both metabolizing oxygen radicals and by altering iron reduction/oxidation state (Janero and Burghardt, 1989; Miller et al., 1993). In vivo, age-related decreases in several antioxidant enzymes, including superoxide dismutase and catalase, have been correlated with a concomitant increase in lipid peroxidation potential in rodents, and caloric restriction ameliorated those decreases (Xia et al., 1995). Strain variation in the levels of SOD1, SOD2, and catalase in mice have been reported (Schisler and Singh, 1985, 1991).

A previous study (Cheeseman et al., 1985) re-ported that the rate of in vitro lipid peroxidation stimulated by NADPH/ADP-iron in hepatic microsomes was lower in D2 mice than in B6, AKR, or BALB/c mice. Lipid peroxidation stimulated by three other methods, fatty acid saturation profile, and two microsomal enzymes were not different among the four strains. While these results contrast with the LPO results reported here, the studies are not exactly comparable. In vitro stimulated lipid peroxidation is not equivalent with the LPO assay, which measures pre-formed MDA and lipid hydroperoxides. In addition, the ages of the mice were not provided in the previous study and the levels of endogenous iron were not measured. Since LPO results appear to be correlated with in vivo iron level, and the ratio of ferrous to ferric iron is important for in vitro stimulated lipid peroxidation (Minotti and Aust, 1992), the higher level of tissue iron in the D2 mice may have played a role in the iron stimulated in vitro assay.

An advantage in using RI strains is the ready availability of genotypic data, which we used to nominate a provisional QTL. Although further studies will be needed to confirm the chromosome 8 QTL nominated by RI strains, such as a similar analysis of a genotyped F2 population derived from B6 and D2 mice, two interesting candidate genes whose chromosomal positions reside close to this QTL have potentially relevant strain related differences. LCAT is a plasma enzyme that is produced in the liver that circulates in association with the high density lipoproteins (HDL) and is responsible for the synthesis of most of the cholesteryl esters present in plasma (Warden et al., 1989). LCAT has also been shown to play a role in the detoxification of polar phophoti-dylcholines generated during lipoprotein oxidation (Goyal et al., 1997). NAD(P)H menadione oxi-doreductase (NMO1; Dia4) is an enzyme (EC 1.6.99.2) that catalyzes reduction of various quinones and redox dyes, protecting cells and tissues against oxidative stress and redox cycling (Vasiliou et al., 1994). B6 and D2 strains vary in inducibility of liver cytosolic NMO by 3-methyl-cholanthrene and TCDD (Kumaki et al., 1977). Our limited sequence analysis did not yield any significant differences for either gene, although only cDNA sequence information was available. As more mouse genomic sequence data is generated, further analysis of

these and other candidate genes will be possible. The identification of specific genes whose variation influences the results of the LPO assay may shed light on the genetic factors influencing age-related lipid peroxidation.

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