Oxidatively damaged DNA in aging dyslipidemic ApoE\(^{-/-}\) and wild-type mice

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The free radical theory of aging depicts an accumulation of cellular oxidatively damaged DNA. In this study, we investigated this theory in mice with knocked-out apolipoprotein E gene (ApoE\(^{-/-}\)), which develops atherosclerosis and wild-type counterparts. The level of oxidatively damaged DNA was investigated as strand breaks, endonuclease III- and formamidopyrimidine DNA glycosylase-sensitive sites by the comet assay. The level of DNA damage was mainly increased with age in the liver of ApoE\(^{-/-}\) mice, whereas no increase was observed in the aorta or lung of the mice. This suggests that the accumulation of oxidized DNA in the liver of dyslipidemic ApoE\(^{-/-}\) mice could be secondary to dysfunction of the lipid metabolism. Visually, the aortas of the ApoE\(^{-/-}\) mice were clearly atherosclerotic as indicated by rigid texture and yellowish in color. However, the unaltered levels of oxidized DNA in severely atherosclerotic aortas of old (\(-70\) weeks) ApoE\(^{-/-}\) mice indicate that oxidative stress may not be a generalized phenomenon, but rather related locally to the individual plaques. In conclusion, the results of this study suggest that dyslipidemic ApoE\(^{-/-}\) mice suffer from hepatic oxidative stress in terms of oxidized DNA, and this effect could be due to the dysfunction of lipid metabolism.

Introduction

Common diseases of the Western world such as cancer, coronary heart disease and Type 2 diabetes are associated with elevated levels of oxidized DNA, and oxidative stress is considered to be an important toxicological mechanism of action (1,2). The incidence of these diseases increases in an age-related manner, which may be due to a declining action of the antioxidant defense and repair systems that protect biomolecules against oxidation in a theory commonly referred to as the free radical theory of aging (3).

In rodents, the age-associated accumulation of oxidized DNA is best described in the liver. However, there is no consensus about the effect of age on the activity of endogenous antioxidant defense and DNA repair enzymes, and the age-associated accumulation of oxidized DNA is conflicting (3). Age-related accumulation of oxidized DNA in other organs has been investigated to a much lesser extent than the liver, which could be due to limitations in the tissue required to make reliable measurements. The alkaline single-cell gel electrophoresis (comet) assay can be used to assess oxidatively damaged DNA in samples with limited material (4). Using this sensitive assay, it was shown that the level of oxidized DNA was higher in the aorta of mice aged 26 months than in mice at 6 months of age (5). However, wild-type mice do not develop plaques that are the hallmark of the human atherosclerosis. In a study of cholesterol-fed rabbits that developed plaques, there were increased levels of strand breaks (SB) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) in the aorta compared to the animals living on a cholesterol-free diet (6). A different animal model of atherosclerosis has been the apolipoprotein E knockout (ApoE\(^{-/-}\)) mice. Three different groups have independently developed ApoE\(^{-/-}\) mice models that differ in the genetic background (7–9). In wild-type mice, apoE proteins are constituents of lipoproteins, except low-density lipoproteins, where they function as ligands for receptors that clear chylomicrons and very low-density lipoproteins from the circulation (10). A lack of the apoE protein is associated with elevated levels of plasma cholesterol even on a low-fat diet (7,8,11). The atherosclerotic process in dyslipidemic mouse models can be altered by dietary components and is influenced by interactions of sex, genetic background and immunological status (12). The hepatic expression level of ApoE is high, although it is also expressed in other tissues and cells such as monocytes and macrophages where it affects the T-lymphocyte activation (13). ApoE\(^{-/-}\) mice develop severe atherosclerosis that appears to be caused by hypercholesterolemia and altered apoE-mediated immunological tolerance that is hypothesized to contribute to plaque development and inflammation (7,8,11,14). However, the hepatic expression of ApoE clearly is pivotal for the atherosclerotic process as evidenced by a complete lack of plaques by somatic gene transfer of apoE to the liver in ApoE\(^{-/-}\) mice (15).

The aim of this study was to investigate age-related alterations in oxidized DNA in aorta, liver and lung from ApoE\(^{-/-}\) mice and wild-type counterparts. The size of atherosclerotic lesions rapidly increases in ApoE\(^{-/-}\) mice when passing the age of 10 weeks (16). We therefore investigated the level of DNA damage in the aorta, liver and lung of wild-type and ApoE\(^{-/-}\) mice ranging from approximately this age to \(70\) weeks of age. A full life span of the mice was disregarded because the ApoE\(^{-/-}\) mice develop diseases at advanced age (17). We find that the level of oxidized DNA increased age dependently only in the liver from ApoE\(^{-/-}\) mice, whereas no accumulation of DNA damage was found in plaque-rich aorta segments from ApoE\(^{-/-}\) mice, or in normal aorta or lung tissue.

Material and methods

Mice

Female wild-type C57BL/6J and apolipoprotein E-deficient mice (C57BL/6-ApoE\(^{\text{null}}\)) were obtained from Taconic (Ry, Denmark) and acclimatized for at least 1 week before

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entering the experiments. The mice were housed in a temperature- (22–24°C) and moisture- (40–70%) controlled room with a 12:12-h light–dark cycle. All mice were given free access to tap water and standard mouse chow (Standard Altromin no. 1314, Lage, Germany) during acclimatisation and housing/treatment periods. The mice were killed in groups at 11–13, 23–25, 31–32 and 67–82 weeks of age; the four groups are referred to by their mean age, i.e. 12, 24, 32 or 70 weeks of age. All animal procedures followed the guidelines for the care and handling of laboratory animals established by the Danish government, and The Animal Experiment Inspectorate, Ministry of Justice, approved the study (no. 2003/561-751).

**Measurement of oxidized DNA by the comet assay**

The mice were anaesthetized in an inhalation chamber with 4% isoflurane in 1:1 N2O/O2 and killed by cervical dislocation. Liver and lung tissues were snap frozen in liquid nitrogen and stored at −80°C. Aortas were perfused with 9 mg/ml NaCl and dissected free from fat and connective tissues in ice-cold Merchants buffer (0.14 M NaCl, 1.47 mM KH2PO4, 2.7 mM KCl, 8.1 mM Na2HPO4, 10 mM Na2EDTA, pH 7.4) using a dissecting microscope.

The level of oxidized DNA was determined by the comet assay. Single nuclei preparations of whole aorta (not frozen, opened longitudinally from the proximal part of the aorta to the abdominal bifurcation), lung and liver (both frozen) nuclei were obtained by placing the tissue into a stainless steel cylinder and using a plunger to force the tissue through a sieve in one end of the cylinder (0.5 cm in diameter, mesh size 0.4 mm), while the cylinder was submerged in 2 ml ice-cold Merchants buffer. The cell extract was filtered through a nylon mesh to remove cell debris, and the aorta extracts were centrifuged at 6000×g for 1 min. One-hundred microliters of the tissue extract was mixed with 800 μl 0.75% low melting point agarose [dissolved in phosphate-buffered saline (PBS)] and 120 μl was applied onto a GelBond® film (Cambrex, Medinova Scientific A/S, Hellerup, Denmark). The GelBonds were placed in lysis solution (1% Triton X-100, 2.5 mM NaCl, 100 mM Na2EDTA, pH 10) overnight. Afterward the GelBond films were washed 3 × 5 min in a buffer containing 40 mM HEPES, 0.1 M KCl, 0.5 mM Na2EDTA, 0.2 μg/ml BSA, pH 8 referred to as ENDO buffer. Sixty microliters of endonuclease III (ENDOIII, 1 mg/ml), formamidopyrimidine DNA glycosylase (FPG, 1 mg/ml) or ENDO buffer were applied on separate agarose gels and were incubated for 45 min at 37°C (the ENDOIII and FPG enzymes were dissolved in ENDO buffer). FPG breaks DNA at sites of oxidized purines and ENDOIII breaks DNA at oxidized pyrimidines. The ENDOIII and FPG enzymes were gifts from Prof. Andrew Collins, University of Oslo, Norway. After rinsing with water, the nuclei were immersed in an alkaline solution (300 mM NaOH, 1 mM Na2EDTA, pH > 13.0) for 40 min and electrophoresed 20 min in the same solution (25 V and 300 mA). The electrophoresis solution was recirculated at a rate of 3.6 l/h and maintained at 4°C. After electrophoresis, the nuclei were washed 3 × 5 min in Tris buffer (0.4 M Tris–HCl, pH 7.5), rinsed with water and placed in 96% ethanol for 1.5 h or overnight. The nuclei were visualized in an Olympus fluorescence microscope at ×40 magnification after staining with YOYO-1 in PBS buffer. ENDOIII- and FPG-sensitive sites were obtained as the difference in scores of parallel slides incubated with and without enzymes. The level of DNA damage was scored according to five classes of damage (0–4) in 100 randomly selected nuclei from each sample and two slides were examined for each sample. All samples were coded before scoring. The score was converted to sites per 10⁶ bp by use of an individual calibration factor (1 unit = 0.012 modifications/10⁶ bp) for the investigator who analyzed the slides as described previously (18).

**Statistics**

The results were analyzed by general linear models with the strain of mice (ApoE−/− or wild type) and age as categorical and continuous variables, respectively. Probability values <0.05 were considered statistically significant. The statistical analysis revealed an interaction between age and strain for the level of FPG sites in the liver and the post hoc analysis was carried out as linear regressions in the two different strains of mice. There were no interactions between the age and strain for the other end points and the subsequent analysis of single-factor effects of strain and age revealed only statistical significance of the latter. Hence, the P values and corresponding correlation coefficients (r values) reported represent the statistics related to the effects of age. All data are expressed as mean and standard error of the mean (SEM).

**Results**

The weight gain in the two strains of mice did not differ, whereas there was an age-dependent weight gain in both groups (P < 0.001, single-factor effect of age). The body weights were 21 ± 1, 24 ± 2, 25 ± 1 and 30 ± 4 g in the groups of wild-type mice aged 12, 24, 32 and 70 weeks, respectively. The corresponding groups of ApoE−/− mice weighted 20 ± 2, 25 ± 1, 24 ± 2 and 28 ± 1 g, respectively. The youngest ApoE−/− mice (12 weeks) had barely visible plaques, whereas the aortas of ApoE−/− mice in the oldest group (70 weeks) had visible fatty deposition as revealed by whitish yellow appearance and rigid flexibility. The aortas from the aged wild-type mice did not appear to be different compared to the young.

Figures 1–3 outline the level of oxidatively damaged DNA in the aorta (Figure 1), lung (Figure 2) and liver (Figure 3). There were no effects of age or strain in the aorta and lung, whereas the level of SB (P < 0.05) and ENDOIII sites (P < 0.001) increased with age in the liver. There was a more pronounced accumulation of FPG sites in the liver of ApoE−/− compared to the wild-type mice (P < 0.05 for interaction between age and strain; rmodel = 0.40). This interaction was clearly driven by the effect of age in the ApoE−/− as revealed by the corresponding regression coefficients: rage = 0.49 (P < 0.005) and rage = 0.09 (P = 0.69) for the ApoE−/− and wild-type mice, respectively. A visual inspection of the results in Figure 3 suggests that the liver of ApoE−/− mice had more SB and ENDOIII sites, although the statistical analysis of these modifications did not indicate an interaction between the strain and age. It also appears that the effect of the strain was most pronounced in the oldest mice. Analysis of the total level of DNA damage (SB, ENDOIII and FPG sites) indicated a similar interaction compared to that observed for the FPG sites alone (rmodel = 0.49, P < 0.05 for interaction between age and strain); this was mainly driven by the effect in the ApoE−/− mice (rage = 0.56, P < 0.005) and there was no effect of age in the wild-type mice (rage = 0.23, P < 0.23).
Discussion

In this investigation, we have found an age-related accumulation of DNA damage in the liver of ApoE<sup>−/−</sup> mice living on a standard animal feed diet. Altered levels of oxidized DNA were not observed in the aorta and lung of these mice. The oldest mice used in this study were 70 weeks of age. Although some strains of mice easily live to 2 years of age, ApoE<sup>−/−</sup> mice develop severe atherosclerosis and may die prematurely. The maximal age of the mice in this experiment was chosen to be 70 weeks because it has been reported that ApoE<sup>−/−</sup> mice about this age (>17 months) developed xanthomatos lesions in the brain (17). In addition, initial investigations indicated that ApoE<sup>−/−</sup> mice had foam cell-rich lesions in aortas at 3 months, which progressed to almost complete occlusion by 8 months (7).

Various statistical models yielded slightly different results of the age effect in terms of hepatic DNA damage in the two strains of mice. A collective interpretation indicates that the level of oxidized DNA was increased more with age in ApoE<sup>−/−</sup> mice compared to the wild-type counterparts. This could be due to oxidative stress caused by an altered hepatic lipid metabolism in the ApoE<sup>−/−</sup> mice. For example, it has been shown that dietary supplementation of saturated fat-rich palm and coconut oil increased the level of lipid peroxidation in the liver of ApoE<sup>−/−</sup> mice (20). These measurements represent the whole-body generation of lipid peroxidation products, but major sources are likely to be the liver and lipoproteins. The hypercholesterolemia in ApoE<sup>−/−</sup> mice may enforce a pro-oxidant effect on hepatocytes that could explain the accumulation of oxidized DNA in this strain. Elevated hepatic contents of cholesterol and triacylglycerol have been reported in a different strain of ApoE<sup>−/−</sup> mice fed a normal chow diet (21). This hepatic steatosis appears to be due to accumulation of lipids in microsomes, and the reduced secretion of very low-density lipoproteins of ApoE<sup>−/−</sup> can be considered as functional defect in the hepatic lipid metabolism (22). In the original publication of the ApoE<sup>−/−</sup> mice we used, it was reported that the knockout mice had lipid deposits in the liver, but not in the lung (7). In this respect, it is interesting that we found unaltered levels of oxidized DNA damage in the lung of ApoE<sup>−/−</sup> mice. Considering the liver as the primary organ of lipid metabolism, our data indicate that ApoE<sup>−/−</sup> mice suffer from hepatic oxidative stress, which is associated with a dysfunction of the lipid metabolism. A recent investigation indicates that the hepatic accumulation of lipids is modulated by the dietary content of fatty acids (23). This probably means that the level of hepatic oxidative stress is also influenced by components in the diet.

Most of the studies on the effect of age in the liver in terms of oxidized DNA have focussed on 8-oxodG. A comparative analysis done by the European Standards Committee on Oxidative DNA damage reported that estimates of the level of 8-oxodG in pig liver was ~10 modifications per 10<sup>6</sup> dG, which was slightly higher than the level of 8-oxodG measured in cultured cells and mononuclear blood cells, and probably represents a degree of spurious oxidation (24). Some of the studies have reported levels of hepatic 8-oxodG that are very high and probably should be interpreted with caution because of possible spurious oxidation of the samples during analysis (25–28). However, there are reports with acceptable level of 8-oxodG indicating an age-associated accumulation of 8-oxodG in the liver. Using HPLC measurements, Hamilton et al. (29) found age-related increases in hepatic 8-oxodG of 6, 18 and 26 weeks wild-type mice. Other studies measuring hepatic 8-oxodG by HPLC on dichotomized age groups have indicated accumulation (30) or no effect (31,32). Detection of 8-oxodG by immunohistochemistry also indicates an age-associated accumulation (33,34). Various types of modifications are recognized by the FPG enzyme, including 8-oxodG and ring-opened purine bases. This measurement generally yields ~10-fold lower levels of oxidatively damaged DNA compared to the HPLC measurement of 8-oxodG, assuming that all sites
detected by the FPG enzyme are 8-oxodG (35). The findings of our study is in a sense similar to the findings by Osterod et al. (36) who observed no accumulation of FPG sites in wild-type mice, whereas these modifications increased with age in repair-deficient Ogg1/C0/C0 mice. This suggests that FPG sites may only accumulate in mice during conditions of oxidative stress (e.g. altered hepatic lipid metabolism) or decreased repair capacity. However, it should also be noted that it has been shown recently that the FPG enzyme may detect DNA modifications generated by alkylating agents (37,38). The FPG protein does not excise N7-methylguanine, and some other primary alkylated bases, from DNA (39). It was suggested that the modifications detected by the FPG enzyme are alkylated ring-opened purine derivates that are similar to the formamidopyrimidine modifications, which the FPG enzyme also recognizes (37,38). The existence of these modifications in tissues of rodents is unclear since the methylated formamidopyrimidine modifications usually are generated in vitro by converting the primary alkylated bases to ring-opened derivates in strong alkaline solution (40). Clearly, this is not a physiological
environment and furthermore we do not know whether or not methylated formamidopyrimidine modifications accumulate with age. Above all, the most important collective interpretation of these studies reporting effects of aging in terms of 8-oxodG is that the accumulation has been observed in three different assays, i.e. HPLC, antibody-based and enzymic (FPG) methods. This strongly indicates that 8-oxodG increases with age in the liver of rodents and the discrepancies between studies may be attributed to strain-specific susceptibility to oxidative stress, sex and diets.

Apparently, the effect of aging on levels of oxidized DNA in the lungs appears to have been subjected to remarkably lesser investigation as compared to the liver and brain, where accumulation of oxidized products of DNA and lipids are investigated to a greater extent (3). This is somewhat surprising since the lungs are the primary target tissue for a number of inhaled oxidants. It has been shown that susceptibility to ozone-induced 8-oxodG in the lungs was highest in adult rats (6 months) as compared to both immature (3 weeks) and aged (20 months) rats (41). However, our results support this investigation, which reported similar levels of 8-oxodG in the lungs of unexposed rats at the age of 6 and 20 months, whereas the immature rats had lower 8-oxodG compared to the older rats (41). To the best of our knowledge, our investigation is the first to report effects of aging in lungs of mice in terms of oxidized DNA. We have previously reported that exposure to ionizing radiation increases the level of FPG sites in mice lungs (42) and elevated numbers of FPG sites are detected in the lungs of repair-deficient Ogg1−/− mice (43). A cautious interpretation suggests that age-related accumulation of oxidized DNA is a tissue-specific phenomenon, and the lungs appear to have less accumulation of oxidized DNA compared to the liver. Taking into account the few studies that have investigated age-related accumulation of pulmonary oxidized DNA, this interpretation warrants further investigation before firm conclusions should be reached. Especially, the use of transgenic experimental models of aging may help further elucidation of the effect of aging in the lung.

An innovative study published a decade ago revealed an association between the aortic level of 8-oxodG in humans and known risk factors of coronary artery diseases such as elevated blood triglycerides and high blood pressure (44). In that study, the level of 8-oxodG was very high; the median level of 8-oxodG exceeded 300 modifications per 106 dG. A more recent study using immunohistochemistry revealed higher levels of 8-oxodG (1600 modifications per 106 dG) in plaques of carotid arteries as compared to mammary arteries (30 modifications/106 dG) that served as control vessels (45). In keeping with the recent awareness of the true nuclear level of 8-oxodG, we should be skeptical about these reports. Our data in ApoE−/− mice aortas with atherosclerosis revealed no difference in the level of oxidized DNA compared to the wild-type mice. It can be hypothesized that the unaltered levels of oxidized DNA in aorta from wild-type and ApoE−/− mice is because the assessment of DNA damage from the whole aorta, including the endothelium and smooth muscle cells, diluted the effect observed in the plaques. In preliminary studies, we attempted to isolate endothelial cell by scraping off the endothelium with a glass slide or by enzymatic treatment of the aorta. Both procedures yielded very high levels of SB and enzyme-sensitive sites could not be measured reliably (data not shown). The current procedure had the advantage of being fast and it was possible to keep a low temperature of the aorta, which diminishes the generation of DNA damage. The variation observed in this study was within the expected range considering that it is determination of oxidized DNA modifications in tissues. For example, the coefficients of variation of aorta SB and FPG sites in the 12-week wild-type mice were 34 and 40%, respectively. The corresponding coefficients of variation in the liver were 56 and 32%, respectively. The variance of the aorta and liver samples thus is similar, and they do not appear to be larger than the variation of SB in lymphocytes in biomonitoring studies, which has been reported to be 36% (95% confidence interval: 27–46%) (46). It may be speculated that the lack of effect in the aorta is because the mice were not sufficiently old, although this does not explain the lack of effect between the strains. Interestingly, Martinet et al. (6) found a regression in the level of SB subsequent to withdrawal of dietary cholesterol even though the cell composition and size of the plaques remained unaltered. This suggests that it may not be the number or size of plaques per se that increases the level of DNA damage, but rather the activity in the plaque such as ongoing inflammatory reactions.

In summary, in this study we have shown that dyslipidemic ApoE−/− mice suffer from hepatic oxidative stress in terms of oxidized DNA, whereas unaltered levels of oxidized DNA were observed in the lungs and aorta. The susceptibility of ApoE−/− mice toward oxidative stress could be due to dysfunction of the lipid metabolism.

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References


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