Plasma lipid oxidation predicts atherosclerotic status better than cholesterol in diabetic apolipoprotein E deficient mice

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Abstract

Increased levels of oxidative stress have been suggested to play a detrimental role in the development of diabetes-related vascular complications. Here, we investigated whether the concentration of malondialdehyde, a marker of lipid oxidation correlated to the degree of aortic plaque lesions in a proatherogenic diabetic mouse model. Three groups of apolipoprotein E knockout mice were studied for 20 weeks, a control, a streptozotocin-induced diabetic, and a diabetic enalapril-treated group. Enalapril was hypothesized to lower oxidative stress level and thus the plaque burden. Both diabetic groups were significantly different from the control group as they had higher blood glucose, HbA_{1c}, total cholesterol, low-density lipoprotein, very low-density lipoprotein, together with a lower high-density lipoprotein concentration and body weight. Animals in the diabetic group had significantly higher plaque area and plasma malondialdehyde than controls. The two diabetic groups did not differ significantly in any measured characteristic. In summary, there was a positive correlation between plasma malondialdehyde concentration and aorta plaque area in apolipoprotein E knockout. Even though further investigation of the role of lipid oxidation in the development of atherosclerosis is warranted, these results suggest that biomarkers of lipid oxidation may be of value in the evaluation of cardiovascular risk.

Keywords: Diabetes, oxidative stress, lipid oxidation, atherosclerosis, APOE^(-/-) mice, malondialdehyde

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Introduction

Oxidative stress is an imbalance between antioxidants and reactive oxygen species that is accelerated by hyperglycemia and has been suggested to play a pivotal role in the development of diabetic late complications.¹ Macrovascular diabetic complications include cardiovascular diseases (CVD), which are the leading cause of mortality among diabetes patients. These include atherosclerosis, which is an important element in CVD, and the estimation of atherosclerotic status may help assess the risk of cardiovascular events. Low-density lipoprotein (LDL) is considered a predictive risk marker of cardiovascular events but recently, several outcome studies have shown that ratios between cholesterol fractions and apolipoprotein concentrations may provide even better prediction of cardiovascular risk.² This underlines the complexity of biomarker-based CVD risk assessment and the need for more predictive markers. Oxidative modifications increase the atherogenic potential of LDL,^{3–5} suggesting that assessment of oxidative stress status may improve predictivity of atherosclerosis.

The association between lipid oxidation and CVD has long been recognized (reviewed in Naito et al.⁶). Accordingly, malondialdehyde (MDA)—a biomarker of lipid oxidation—has been found to be associated with increased coronary intima-media thickness⁷ and atherogenic index ((total cholesterol – HDL cholesterol)/HDL cholesterol).^{8,9}

Lipid oxidation has also been investigated in animal models of diabetes and atherosclerosis, and studies have identified a relationship between increased lipid oxidation and atherosclerosis as well as arterial stiffening.^{10–12}

In the present study, we investigated the correlation between MDA and aorta plaque burden in long-term diabetic apolipoprotein E knockout mice. Furthermore, we wanted to test if lowering oxidative stress attenuates plaque burden. Here, the angiotensin-converting enzyme inhibitor, enalapril, was used as a pharmacological tool. Enalapril lowers hypertension by reducing the formation of angiotensin II and thereby aldosterone production, both of which have been shown to increase oxidative stress *in vitro* and *in vivo*.^{10,13} Thus, enalapril was expected to

decrease plaque formation in a cholesterol and glucose independent manner.

Methods

Animals

The experiment was approved by Danish Animal Experimentation Inspectorate (permission number: 2012-15-2934-00304). Five- to eight-week-old male apolipoprotein E knockout B6-129P2-*ApoE*^{tm1Unc}N11 (*APOE*^(-/-)) mice (Taconic, Ejby, Denmark) were housed under controlled conditions (temperature: $22 \pm 2^\circ\text{C}$; humidity: $50 \pm 20\%$; 12 h/12 h light/dark cycle). The animals were randomly divided into three groups: control (CTRL; $n=7$), diabetic (DIAB; $n=9$), and diabetic enalapril (ENAL; $n=10$) with free access to water and chow (Altromin 1324, GmbH, Lage, Germany).

Treatment protocols

Diabetes was induced in DIAB and ENAL animals by streptozotocin (STZ) (Sigma-Aldrich, Saint Louis, MO, USA) IP injections over a five-day period (55 mg/kg/day). No animals died from this treatment, but only animals with blood glucose > 15 mmol/L three weeks after STZ initiation were included in the study (the n values specified in "Animals" section are the final n values). From this time point onward, enalapril (Sigma-Aldrich, Saint Louis, MO, USA) was administered to ENAL animals for 20 weeks through the drinking water (38 mg/L) calculated based on water intake to result in a dose of ~ 10 mg/kg/day. During the study, samples for blood glucose (once a week—data are only shown from the last sample) and HbA_{1c} (two weeks after STZ injections and six and 20 weeks after initiation of enalapril treatment—data not shown) were collected from a tail vein.

At termination, animals were anaesthetized using Hypnorm (VetaPharma Ltd, Leeds, United Kingdom)/Midazolam (Accord Healthcare, Copenhagen, Denmark) (0.6, 19, and 9 $\mu\text{g/g}$ of fentanyl/fluanisone/midazolam, respectively) and blood was collected by puncture of the orbital sinus. Abdomen and thorax were opened and animals were perfused with sterile isotonic saline (10 mL; 23 G cannula). The aortas were cut at the base of the heart and by the seventh rib and kept cold while cleansed (removal of fat), and opened for *en face*, where a picture was taken and analyzed for plaque content by morphometry (Visiomorph, Visiopharm A/S, Hørsholm, Denmark). Subsequently, aortas were stored at -80°C until homogenization in 150 μL RTL lysis buffer using TissueLyser II (Qiagen GmbH, Hilden, Germany).

Biochemical analyses

For determination of blood glucose, 5 μL whole blood collected in Na-heparinized capillary tubes (Vitrex Medical A/S, Herlev, Denmark) was diluted in 250 μL glucose/lactate system solution (EKF Diagnostics, Barleben, Germany) and analyzed in a Biosen S-line glucose analyzer (EKF Diagnostics, Barleben, Germany). HbA_{1c} was determined in 10 μL Na-heparinized whole blood stabilized with hemolyzing reagent using a Cobas 6000 (Roche/

Hitachi, Mannheim, Germany). Total plasma cholesterol and lipoprotein fractions were determined at Department of Pathology/Lipid Sciences, Wake Forrester University School of Medicine Winston-Salem, NC, USA by a method previously described.¹⁴ In short, total plasma cholesterol concentration was determined using the enzymatic Cholesterol/HP kit (Roche Diagnostics, Indianapolis, IN, USA). Cholesterol fractions were separated by fast protein liquid chromatography (FLPC) size exclusion following HPLC and subsequently measured colorimetrically using Infinite (Thermo Fischer Scientific, Waltham, MA, USA) and integrated using Chrom Perfect Spirit software (Justice Laboratory Software). The area percent distribution for each of the lipoprotein fractions was used to determine the cholesterol fraction concentration, by multiplying them with the total cholesterol concentration. Lipid oxidation was measured as MDA in plasma and aorta by HPLC as described previously.¹⁵ Aorta protein concentrations were determined by bicinchoninic acid assay (Merck, Darmstadt, Germany).

Statistical analysis

Statistical analysis was carried out by one-way ANOVA using Tukey's *post hoc* comparisons (SAS Enterprise 7.1, Cary, NC, USA). Stepwise multiple regression analysis was performed using total cholesterol, LDL, very low-density lipoprotein (vLDL), total cholesterol/high-density lipoprotein (HDL) ratio, and plasma MDA as explanatory variables and plaque area as dependent variable. All data are presented as mean $\pm$ SEM and p -values < 0.05 were considered significant.

Results

As expected, STZ-treated animals became diabetic (30.3 ± 1.4 mmol/L glucose versus 8.7 ± 0.3 for CTRL, $p < 0.001$). Animals remained hyperglycemic for the entire study period and no difference between the diabetic groups was observed (Figure 1). HbA_{1c} was 8.9 ± 0.4 and $9.3 \pm 0.6\%$ in DIAB and ENAL animals, respectively versus $4.0 \pm 0.1\%$ among CTRLs. Plasma concentrations of total cholesterol, LDL, and vLDL were increased in diabetic versus CTRLs, while HDL was higher in CTRLs versus diabetic groups ($p < 0.05$; Figure 1). Total cholesterol/HDL ratio was approximately threefold higher in the diabetic versus CTRL animals. None of the measures differed significantly between DIAB and ENAL.

En face evaluation showed increased aortic plaque lesion area ($p < 0.01$) and higher plasma MDA ($p < 0.05$) in DIAB versus CTRL animals. ENAL animals were not significantly different from CTRL or DIAB. Aorta MDA concentrations did not differ between groups (Figure 1). A positive correlation was found between aortic plaque lesion area and plasma MDA ($p = 0.0076$, $R^2 = 0.2713$). As aortic plaque lesion area was also correlated to total cholesterol, LDL, and total cholesterol/HDL ratio, a stepwise multiple regression analysis with backward elimination was performed identifying plasma MDA as the variable describing aortic plaque lesion area the best.

Figure 1 Effects of diabetes and enalapril treatment in APOE^{-/-} mice. (a) Blood glucose at the end of the experiment. Plasma total cholesterol (b), HDL (c), LDL (d), and vLDL (e) concentrations at the end of the experiment. (f) Plaque area in the aorta of the mice, determined by *en face*. MDA concentrations in plasma (g) and aorta homogenates (h) at the end of the experiment. (i) Correlation between MDA in plasma and plaque area in the aortas. Values are mean $\pm$ SEM. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control. HDL: high-density lipoprotein; LDL: low-density lipoprotein; MDA: malondialdehyde; T-Chol: total cholesterol; vLDL: very low-density lipoprotein

Discussion

In the present study, plasma lipid oxidation as measured by MDA was found to be a better predictor of atherosclerotic status than LDL and cholesterol in STZ-induced diabetic APOE^{-/-} mice.

Both diabetic groups had higher total cholesterol, LDL, and vLDL concentrations combined with a concurrent decrease in HDL. The cholesterol profile supports the face validity of the model as compared to the human situation. Moreover, the increases in the well-established human risk factors for CVD, i.e. total cholesterol, LDL, vLDL, and total cholesterol/HDL ratio, in both diabetic groups are assumed to contribute to the atherosclerosis development.¹⁶ Increased plaque formation has previously been shown in STZ-induced diabetic APOE^{-/-} mice without the simultaneous shift in cholesterol fraction composition.¹² In a study in diabetic rats, treatment with statins lowered plasma and aorta thiobarbituric acid reactive substances (TBARS) level (an unspecific marker of lipid oxidation) and arterial stiffness without the expected effect on lipid profile, presumably due to the low dose used.¹¹ Thus, these studies indicate that lipid oxidation *per se* is important in CVD pathogenesis in diabetic animals and may contribute to

the risk assessment in addition to the lipid profile. In humans, CVD events are not necessarily associated with high cholesterol concentrations, indeed supporting the need for a wider range of biomarkers for risk assessment.¹⁷

In long-term diabetic APOE^{-/-} mice (four months), increased plaque areas were accompanied by a concurrent rise in plasma TBARS level, a drop in erythrocyte reduced-to-oxidized glutathione ratio, and an upregulation of glutathione peroxidase gene expression in the aorta.¹² Interestingly, aorta and kidney concentrations of 4-hydroxynonenal (a lipid oxidation marker; 4-HNE) were increased in the latter study in contrast to aorta MDA in the present study. This could indicate that the extended time course of the present study results in different levels of lipid oxidation or that 4-HNE and MDA are accumulated and degraded differently in aortic tissue. Regardless, the observed increase in 4-HNE does support the involvement of lipid oxidation in the development of atherosclerosis in diabetes and hence the findings of the present study. It should be noted, however, that atherosclerotic lesions from the APOE^{-/-} mouse, rabbits, and humans have been shown to contain MDA-modified LDL and circulating auto-antibodies against MDA modified LDL have been found in

APOE^(-/-) mice.³⁻⁵ Moreover, degradation of MDA in these lesions can result in MDA conjugated breakdown products, potentially not measurable by the HPLC methodology used in the present study. Thus, as we only measured unbound MDA, LDL-bound MDA could well be present in higher quantities in the aortas from the diabetic groups that have increased plaque area.

Enalapril treatment decreased lipid oxidation and aortic plaque lesion area compared to DIAB animals as expected, albeit these changes did not reach statistical significance. Histological evaluation could perhaps have separated the groups, as morphological investigations typically offer more detailed and precise information on plaque severity compared to a two-dimensional qualitative analysis such as the *en face* method.

The present study suggests a relationship between MDA and atherosclerotic status in diabetic APOE^(-/-) mice. Whether MDA is a simple secondary biomarker or plays a pathological role has yet to be unraveled, although the latter could be implied by the data presented above. Furthermore, the biological half-life of MDA and its pathways of degradation and excretion have to be more clearly defined to make it a good candidate for the evaluation of atherosclerotic status in diabetes. Studies similar to the present but with additional and larger study groups euthanized at different time points assessing MDA in plasma and aorta, *en face* measurements, and histological examination of plaque morphology could help clarify if and how accurately measured MDA may be used to assess the level of atherosclerosis *in vivo*. Investigating known therapies of hyperglycemia and dyslipidemia could be useful as well to investigate the effect of dyslipidemia on atherosclerosis in this model. However, prospective cohort studies in large human populations of diabetic and non-diabetic patients with and without established atherosclerosis are needed to evaluate the value of plasma MDA as biomarker in human atherosclerosis.

Author contributions: The study was designed by all authors. The experiments were carried out by KEP and GR. The initial data analysis was performed by KEP followed by data interpretation by all authors. The draft manuscript was written by KEP and subsequently edited by all authors and all authors have approved the final version of the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: KR and GR are employees of Novo Nordisk A/S that produces insulin.

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