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Pretreatment with n-6 PUFA protects against subsequent high fat diet induced atherosclerosis-Potential role of oxidative stress-induced antioxidant defense

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Abstract

Objectives—Recent evidence suggests that oxidative stress can promote antioxidant defense and thus be athero-protective. n-6 Polyunsaturated fatty acids (n-6 PUFA) are more prone to oxidation, compared to monounsaturated fatty acids (MUFA) and yet have proven anti-atherosclerotic effects. In this study, we tested whether early exposure to a diet rich in n-6 PUFA, compared to a MUFA rich diet would reduce lesion burden, even with subsequent exposure to a high fat, high cholesterol diet (HF). Further, we tested to determine whether oxidative mechanisms are involved in such protection.

Methods and Results—Twenty four, 4 week old, male, LDL receptor knockout (LDL-R^{-/-}) mice were divided into two groups and fed either a n-6 PUFA rich or a MUFA rich diet for a period of 12 weeks. At this point, 4 mice from each group were euthanized and the remaining 8 mice from each group were fed a HF diet for four weeks. Atherosclerotic lesions, plasma lipids, autoantibodies to lipid peroxide modified proteins, isoprostanes and aortic catalase levels were measured. The n-6 PUFA diet reduced aortic lesions and plasma lipids compared to the MUFA diet and this reduction in lesions continued even after the mice were switched over to the HF diet, despite the fact that the plasma lipids were similar in both groups after the HF diet. n-6 PUFA fed mice had highest plasma isoprostane levels, indicating oxidative stress, but also had higher levels of aortic catalase. On the other hand, MUFA fed mice had comparatively lower levels of isoprostanes and their aortic catalase levels remained low. Finally, aortic lesions were negatively correlated with isoprostanes and catalase.

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Author's contributions: MP participated in carrying out the study, analyzing samples, data analysis and manuscript preparation. MS and NM assisted with surgery to dissect mouse aorta, digital photography and critical review of the manuscript. SP conceived and designed the study and methods and provided critical insight in drafting and revising the manuscript for important intellectual content.

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Conclusion—An initial exposure to a n-6 PUFA rich diet compared to a MUFA rich diet reduces atherosclerotic lesions and this protection probably involves oxidative stress induced by PUFA.

Keywords

Monounsaturated fatty acids; Atherosclerosis; Isoprostanes; Catalase

1. Background

Oxidative stress may be involved in the initiation and progression of experimental atherosclerosis^{1,2}. We have previously shown that exercise, a deterrent of CVD, promoted oxidative stress in the plasma but reduced atherosclerotic lesions in LDL receptor knockout (LDL-R^{-/-}) mice by up regulating the antioxidant enzymes catalase and endothelial nitric oxide synthase (eNOS) in the aorta³. This induction of antioxidant enzymes in the presence of oxidative stress has been demonstrated time and again by others both *in vivo* and *in vitro*⁴⁻⁷. Oxidized lipids also have been recognized as ligands for PPARs⁸ and thus may have effects that haven't been realized yet. Extrapolating from these studies we hypothesized that n-6 poly unsaturated fatty acids (n6-PUFA), which are more vulnerable to oxidation as compared to saturated fatty acids or monounsaturated fatty acids, might also act in a similar manner by generating sustained oxidative stress and antioxidant defense. Despite their vulnerability for oxidation, n-6 PUFA have been shown to reduce the risk of atherosclerosis in both humans⁹ and animals¹⁰. Since MUFA are resistant to oxidation, it has been hypothesized by some that MUFA rich diets would more likely reduce the risk of CVD as compared to diets rich in PUFA. The evidence for this is mixed¹¹. Epidemiologic studies in humans demonstrate that MUFA consumption is associated with lowering the risk of cardiovascular disease (CVD)^{12,13} while some animal studies demonstrate an increase^{14,15} and others demonstrate a decrease¹⁶ in atherosclerotic lesion burden with MUFA compared to PUFA rich diets. In this study we tested whether (1) an initial exposure during the post weaning period to a n-6 PUFA rich diet is more athero-protective (lessened lesion burden) compared to a MUFA rich diet, 2) if athero-protective effects of n-6 PUFA would persist when the animals are subsequently fed a HF diet and 3) if the athero-protection seen in the n-6 PUFA fed animals is associated with increased oxidative stress in the plasma and increased antioxidant defense in the aorta.

2. Methods

Low density lipoprotein receptor knock out (LDL-R^{-/-}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). n-6 PUFA rich diet, monounsaturated fatty acid (MUFA) rich diet and the high fat, high cholesterol (HF) diet were purchased from Harlan Teklad (Madison, WI). Nutrient composition of these diets is given in table 1.

Study design

LDL-R^{-/-} male mice (n=24), at the age of 4 weeks, were randomly divided into two groups. One group was fed a n-6 PUFA rich diet (TD 01560) and the other group was fed a MUFA rich diet (TD 06427) for a period of 12 weeks. These diets contained 5% fat in the form of specific enriched PUFA or MUFA but contained only basal (0.005%) cholesterol (Table 1). At the end of the 12 week period four mice from each group were euthanized to obtain data on lesions, plasma lipids and markers of oxidative stress. The remaining 8 animals from each group were switched to a HF diet (TD 88137) containing 21% fat, mostly in the form of milk fat and 0.15% cholesterol for a period of 4 weeks. All the diets were stored at 4°C and mice were given a measured amount of food every week and the remaining food was weighed. Mice were weighed once a week. The Emory Animal Care Committee approved

all protocols and animals were treated in compliance with Emory University Animal Committee regulations.

Collection of plasma, preparation of arterial samples and quantification of aortic lesion areas

Blood was obtained from retro orbital puncture from mice under isoflurane anesthesia at the end of 12 weeks of PUFA or MUFA feeding and after the HF diet. Mice were sacrificed by CO₂ asphyxia and fasting blood was drawn in heparinized tubes from the inferior vena cava or by heart puncture. Red blood cells and plasma were immediately separated by centrifugation (2,000×g, 10 min) and then frozen at -80°C. The aortic trunk was washed with cold phosphate buffered saline (PBS) containing 10 mg/L aprotinin and 0.1 mmol/L PMSF through the left ventricle. The dissection of the aorta was performed under a stereomicroscope from the iliac bifurcation up to the heart, including the beginning of the carotid and subclavian arteries. The excess fat and connective tissue was carefully removed and the aorta was opened longitudinally and pinned up on black wax for *en face* observation¹⁷. In order to use the aortas for future Western blot and enzyme assay analysis, no staining was used to visualize the lesions. After capturing of different areas of the aorta, the lesions were circled on a printout under direct microscopic observation. Lesion areas were quantified using Adobe Photoshop 8.0®. Pixels were transformed to mm² by using a microscopic standard scale treated under the same condition as the aortas.

Quantification of plasma lipids

A 50µl aliquot of plasma was analyzed by Cardiovascular Specialty Labs, Inc (Atlanta, GA) to quantify the levels of plasma total cholesterol (TC), LDL cholesterol (LDLc), HDL cholesterol (HDLc) and triglycerides (TG).

Detection of autoantibodies against lipid peroxide-modified proteins (AUAB-LOOH-PRO) in plasma by ELISA

Ninety-six-well ELISA plates were coated overnight at 37°C with a solution of LOOH-RSA (5 µg/well). Every step of the following method was separated by 6 washes with 1×PBS using a plate washer. Plates were blocked for 2 hrs with 30 g/L bovine serum albumin in PBS and incubated with 100 µL of 1:50 diluted plasma (1 hr at 37°C). The wells were then incubated for 1 hr at 37°C with 100 µL of anti-mouse immunoglobulin G-conjugated alkaline phosphatase (1:10 000 dilution), and then incubated with the substrate (*p*-nitrophenyl phosphate) for 30 min. Plates were read at 405 nm using an ELISA plate reader. Results are expressed as optical density (OD) equivalent of *p*-nitrophenol formed.

Determination of plasma 8-iso-PGF_{2α}

Plasma 8-iso-PGF_{2α} levels were determined by a competitive immunoassay kit purchased from Assay Designs Inc (Ann Arbor, MI). The assay was carried out according to the manufacturer's protocol and the optical density of the finished reaction was measured at 405nm. This optical density is used to quantify the amount of 8-iso-PGF_{2α} in samples from a standard curve generated according to the protocol.

Western blot of aortic catalase

Approximately 25 µg of protein extract from the aorta was separated by 10% SDS-PAGE. The gel was transblotted onto a nitrocellulose membrane, blocked with 5% milk powder in phosphate-buffered saline with 0.1% Tween (PBS-T) overnight (4°C), incubated with rabbit polyclonal anti-human catalase antibody (1:100) for 1 hr at room temperature, washed with PBS-T and incubated with secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase, 1:5000) for 1 hr at room temperature. After 5 washes with PBS-T,

the signal was detected with a chemiluminescence kit (Amersham ECL kit). The membrane was blocked again with 5% milk-PBS-T overnight at 4°C and re-probed with anti β -tubulin antibody (1:100) at room temperature for 1hr. After 3 washes with PBS-T the membrane was incubated with anti-mouse IgG HRP in 5% milk PBS-T for 45 min at room temperature. After 3 washes with PBS-T, the signal was detected with a chemiluminescence kit (Amersham ECL kit).

Aortic catalase activity

Aortas were homogenized and sonicated in 50mmol/L KH_2PO_4 containing 0.01% deoxycholic acid and 0.1mM PMSF on ice. After centrifugation (13,000 rpm for 10 min at 4°C) an aliquot of the supernatant was saved for protein estimation and the assay for catalase activity was performed on the remaining supernatant. The initial rate of disappearance of H_2O_2 was recorded at a wavelength of 240 nm during 1 min (ΔA_{240} nm/min), according to the method of Aebi ¹⁸.

Statistical analyses

All data was assessed for normality. For parametric data, differences between group means (PUFA, MUFA, PUFA to HF and MUFA to HF) were assessed with one-way ANOVA followed by Gabriel's post hoc tests. For non-parametric data, differences between group means were assessed with the Kruskal-Wallis test followed by the Mann Whitney test for post-hoc comparisons. Correlation coefficients were determined by the non-parametric Spearman-rho test. Statistical significance was set at $p = 0.05$.

3. Results

$\text{LDL-R}^{-/-}$ mice were fed MUFA or PUFA diet for 12 weeks. At the end of 12 wks, mice were switched over to a HF diet. Mice that were fed a n-6 PUFA rich diet had significantly smaller aortic lesions as compared to mice fed a MUFA rich diet (Table 2). The greater amount of atherosclerosis in MUFA group is interesting as both the MUFA and PUFA diets contained only 5% fat in the form of added fatty acids and contained very low levels of cholesterol. Thus the atherosclerotic susceptibility of $\text{LDL-R}^{-/-}$ animals was accentuated by MUFA as compared to PUFA. The MUFA fed animals also had increased plasma and LDL cholesterol as compared to the PUFA fed animals although there was also a small but significant increase in HDL values (Table 2).

When the animals were switched to high fat diet containing cholesterol, there were increases in atherosclerosis in both the groups but the MUFA group of animals showed far greater lesion areas as compared to the PUFA group (MUFA to HF versus PUFA to HF; mean $\pm$ SEM = 2.819 ± 0.43 versus 0.087 ± 0.02 mm²). There was practically no difference in total or LDL or HDL cholesterol levels between the two groups after the switch to HF diet (Table 2), suggesting, that plasma lipid levels alone couldn't have been responsible for such a big difference in lesion areas. In other words, conversion to a HF diet from an n-6 PUFA rich diet resulted in increased lesion area. Additionally, the lesions were still significantly smaller in the PUFA+HF group as compared to the MUFA fed mice, even before they were switched to a HF diet.

Mean plasma 8-iso-PGF_{2 α} , levels were 4 times higher in the n-6 PUFA fed mice compared to the MUFA fed mice, but after conversion to the HF diet these levels came down and were similar to the levels in the MUFA fed animals (Figure 1a). There was no significant change in plasma 8-iso-PGF_{2 α} in the MUFA fed animals with the HF diet (Figure 1a). Another marker of oxidative stress, AUAB-LOOH-PRO were not different between the n-6 PUFA and MUFA fed mice. However, switching over to the HF diet increased the level of AUAB-

LOOH-PRO in both groups (Figure 1b). Thus, they seem to reflect plasma lipid levels rather than FA saturation or lesion areas.

Mean catalase protein levels were the highest in the n-6 PUFA fed mice compared to the MUFA fed mice, and levels decreased in both groups with the HF diet (Figure 2a). Mean aortic catalase activity was not significantly different between the n-6 PUFA and MUFA groups but tended to increase after the switch to the HF diet only in the PUFA group (Figure 2b). Finally, correlation analysis (Table 3) showed that there was a moderate negative association ($r=-0.428$; $p=0.047$) between lesion area and aortic catalase protein; a moderate direct association ($r=0.510$; $p=0.01$) between lesion area and AUAB-LOOH-PRO; and a moderate negative association ($r=-0.424$; $p=0.056$) between lesion area and plasma isoprostanes.

4. Discussion

This study supports previous findings that a n-6 PUFA rich diet is more athero-protective than a MUFA rich diet. Studies by Rudel in primates^{15, 19} and in mice¹⁴ have demonstrated that compared to a PUFA rich diet, a MUFA rich diet promotes atherosclerosis. n-6 PUFA fed mice had significantly lower plasma lipids and aortic lesions compared to MUFA fed mice. However, despite low and comparable amounts of fat (5%) and cholesterol (0.005%) in the diet, the MUFA fed animals had much higher levels of plasma and LDL cholesterol as well as triglycerides suggesting that n-6 PUFA might act to prevent lipid accumulation by additional mechanisms. An additional finding in this current study is that even with the HF diet, lesion size in the PUFA animals was smaller than that seen in MUFA animals before exposure to a HF diet. This dramatic finding emphasizes the benefits of a diet rich in PUFA. Among n-6 PUFA fed mice the lesser lesion burden was not associated with plasma lipid levels as the plasma TC, LDLc, HDLc and TG levels were similar in both groups after the switch to HF diet. This suggests that factors in addition to plasma lipids might contribute to athero-protection. The finding that high plasma cholesterol levels do not always correlate with lesion area has been demonstrated by others^{20, 21}. Our earlier studies²² have suggested that oxidized linoleic acid together with a high fat diet contributes to an increase in lesions despite comparable plasma lipids.

We hypothesized that feeding young mice a diet rich in n-6 PUFA would cause oxidative stress in the plasma and would then promote an adaptive response by increasing antioxidant defense in the aorta. As predicted, mice fed n-6 PUFA diet had five times more *8-iso-PGF_{2α}* levels in the plasma, a marker of non-enzymatic oxidation of arachidonic acid as compared to MUFA fed mice. Even though elevated isoprostane levels are generally accepted as a marker of oxidative stress and seem to be positively correlated with atherosclerosis in both humans and animals²³, some studies show no correlation²⁴. Paradoxically, in our study, plasma *8-iso-PGF_{2α}* levels were *negatively* associated with aortic lesions ($r=-0.424$), suggesting, that the greater the n-6 PUFA mediated oxidation, the lower the lesion development. The decrease in plasma isoprostanes after the switch to the HF diet suggests that the lack of PUFA in the HF diet probably reduced cellular levels of arachidonic acid markedly, as delta-6 desaturase, a key enzyme in arachidonic acid synthesis is known to be severely affected by saturated fat and cholesterol^{25, 26}.

We also looked at another marker of oxidative stress, AUAB-LOOH-PRO which indicate the presence of immune complexes generated against lipid peroxide modified proteins (LOOH-RSA). We anticipated that the PUFA fed mice would have higher levels of these AUAB-LOOH-PRO if PUFA are more vulnerable to oxidation as compared to MUFA. However, we found our results to be exactly the opposite. This can be explained by evidence suggesting that the greater the levels of circulating oxidized lipids, the greater the clearance

of endogenous lipids along with increased clearance of the immune complexes^{27, 28}. In our study, plasma LDL and AUAB-LOOH-PRO were lower in PUFA fed mice as compared to MUFA fed mice, suggesting that the greater propensity of PUFA to undergo oxidation could have enhanced the clearance of oxidized lipid containing immune complexes. Converting to the HF diet increased mean autoantibody levels in both groups, suggesting that a diet high in saturated fat interferes with this clearance. Alexander and associates demonstrated that dietary lard increases plasma levels of immune complexes compared to corn oil or fish oil in immune compromised mice²⁹. The modest *positive* correlation ($r=0.51$) between AUAB-LOOH-PRO and lesions that we report in this study is similar to the results reported by Tsimikas and associates³⁰. They found that a change in the titer of MDA-LDL-IgM was positively associated ($r=0.48$) with atherosclerotic surface area in LDL-R^{-/-} mice.

To determine if an increase in plasma oxidative stress would induce antioxidant defense, we measured the amount and activity of aortic catalase. The induction of several antioxidant enzymes including MnSOD⁶, catalase³, hemoxygenase⁵ and glutathione synthesis⁴, in the presence of oxidative stress has been demonstrated previously. Even though antioxidant enzyme systems (superoxide dismutase, nitric oxide synthase, etc.) work cooperatively in reducing oxidative stress, we chose to measure only aortic catalase because this antioxidant enzyme is sensitive to diet induced peroxide mediated oxidative stress³¹. Our previous work has demonstrated that lipid peroxides induce catalase gene expression in cultured cells³² and lipid peroxides are generated from oxidized n-6 PUFA. In addition, previous studies in apoE^{-/-} mice have shown that over expression of catalase, and not Cu/Zn superoxide dismutase (Cu/Zn SOD), decreased atherosclerotic lesions promoted by a high fat diet³¹. Furthermore, these authors speculate that H₂O₂ mediated oxidative stress was more likely the cause of the lesions and not superoxide. In a similar fashion, in our study the PUFA rich diet is more likely to propagate peroxide mediated oxidative stress. Aortic catalase protein was highest in n-6 PUFA fed mice even though it was not statistically different from the MUFA fed mice. However, the fact that switching over to a HF diet significantly reduced catalase levels in MUFA fed mice, and that there was a negative correlation between aortic catalase and lesion area demonstrates that the reduced lesion burden is associated with greater levels of aortic catalase. The two fold increase in catalase activity in PUFA fed animals after exposure to the HF diet, although not statistically significant, along with the negative correlation with lesions hints at the biological possibility, that it is at least partially responsible for the reduction in lesions.

Conclusions

These results indicate that a n-6 PUFA rich diet maintains high levels of oxidative stress (isoprostanes) along with aortic catalase. However, subsequent exposure to a high fat/cholesterol diet decreases oxidative stress and aortic catalase levels. Simply stated, our results suggest that increasing n-6 PUFA with a concomitant *decrease* in total fat might be better at reducing lesion burden. More importantly, the early exposure to MUFA not only increased plasma lipids and early lesions but also caused a decrease (although not significant) in aortic catalase. These results, together with literature findings would suggest that early intake of n-6 PUFA rich diet not only reduces lesion development but if sustained, might also reduce the severity of atherosclerosis.

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Figure 1a

Figure 1b

Figure 1.

Effect of a PUFA or MUFA rich diet on plasma *8-iso-PGF_{2α}* (1a) and AUAB-LOOH-PRO (1b) before (PUFA=white bar; MUFA= light grey bar) and after (PUFA= dark grey bar; MUFA=black bar) switching to a HF diet in LDL *r*^{-/-} mice. Bars represent mean ± SEM. Group means were compared by the Kruskal Wallis test and pair wise comparisons were made by Mann Whitney U test. Statistical significance was set at p 0.05. Means with “†” are significantly different as compared to PUFA. Means with “#” are significantly different as compared to PUFA and MUFA.

Figure 2.

Effect of a PUFA or MUFA rich diet on aortic catalase protein (2a) or aortic catalase activity (2b) before (PUFA=white bar; MUFA= light grey bar) and after (PUFA= dark grey bar; MUFA=black bar) switching to a HF diet in LDL $r^{-/-}$ mice. Bars represent mean $\pm$ SEM. Group means were compared by the Kruskal Wallis test and pair wise comparisons were made by Mann Whitney U test . Statistical significance was set at p 0.05. Means with “†” are significantly different as compared to PUFA.

Table 1
Nutrient composition of diets fed to LDL $r^{-/-}$ mice

	PUFA rich diet	MUFA rich diet	High fat diet
		g/Kg	
Total fat	50	50	210
Total cholesterol	0.04-0.05	0.04-0.05	1.5
Anhydrous milk fat	0	0	210
High oleic sunflower oil	0	50	0
Safflower oil, linoleic	50	0	0
Casein	200	200	195
DL-Methionine	0	0	3
L-Cystine	3	3	0
Sucrose	120	120	341.46
Cellulose (fiber)	50	50	50
Maltodextrin	132	132	0
Corn Starch	397.49	397.49	0
Mineral mix	35	35	35
Vitamin mix	10	10	10
Fatty acid profile % of total fat (% of energy)			
Saturated	9 (1.2)	10 (1.32)	64 (27)
Polyunsaturated	78 (10.3)	7 (0.92)	4.3 (1.5)
Monounsaturated	9 (1.2)	83 (10.95)	28.4 (12)

Table 2
Aortic atherosclerotic lesion area and plasma lipid profile (mean $\pm$ SEM) in LDL- R^{-/-} mice

Parameter	PUFA	MUFA	PUFA to HF	MUFA to HF
Lesion area (mm ²)	0.00625 $\pm$ 0.006 [†]	0.198 $\pm$ 0.05 [†]	0.087 $\pm$ 0.02 [†]	2.819 $\pm$ 0.43 [†]
Plasma TC (mmol/L)	6.9 $\pm$ 0.54 [†]	21.51 $\pm$ 1.51 [†]	30.9 $\pm$ 0.88 ^{†, #}	31.5 $\pm$ 1.42 [†]
Plasma LDLc (mmol/L)	2.97 $\pm$ 0.4 [†]	14.6 $\pm$ 1.21 [†]	22.86 $\pm$ 0.71 ^{†, #}	22.91 $\pm$ 1.1 [†]
Plasma HDLc (mmol/L)	3.21 $\pm$ 0.16 [†]	4.96 $\pm$ 0.26 [†]	7.11 $\pm$ 0.21 ^{†, #}	7.27 $\pm$ 0.32 [†]
Plasma TG (mmol/L)	1.55 $\pm$ 0.21 [†]	4.14 $\pm$ 0.28 [†]	1.97 $\pm$ 0.27 ^{†, #}	2.84 $\pm$ 0.53 [†]

PUFA=Polyunsaturated fatty acid diet; MUFA=Monounsaturated fatty acid diet; HF=High fat high cholesterol diet. Statistical significance was set at P 0.05. Means with the superscript

[†] within a row are significantly different as compared to each other. Means with a superscript

[#] are not significantly different as compared to “MUFA to HF” group.

Table 3

Correlation between aortic lesions, markers of oxidative stress and aortic catalase.

Parameter 1	Parameter 2	Correlation coefficient (Spearman's rho)	p value ^a
Lesion	Aortic catalase protein	−0.428	0.047
Lesion	Autoantibodies to lipid peroxide modified proteins (AUAB-LOOH-PRO)	0.510	0.011
Lesion	Isoprostanes	−0.424	0.056

^aStatistical significance of the Spearman's rho correlation coefficients was determined by a two-tailed test