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Journal of Nutritional Biochemistry 18 (2007) 127–133
Journal of Nutritional Biochemistry

Effects of vitamin E on oxidative stress and atherosclerosis in an obese hyperlipidemic mouse model☆

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Received 8 February 2006; accepted 30 March 2006

Abstract

Vitamin E is a natural antioxidant that has been used in animal and human studies to determine its potential in reducing cardiovascular risk; however, a detailed study in an established obese model of atherosclerosis has yet to be performed. In our current study, we show that obesity and hyperlipidemia cause a synergistic, age-related increase in urinary isoprostane levels in mice deficient in both leptin and low-density lipoprotein receptor (ob/ob;LDLR$^{-/-}$). Based upon this observation, we hypothesized that vitamin E supplementation would induce potent antiatherogenic effects in this model. Lean and obese LDLR$^{-/-}$ mice were provided vitamin E (2000 IU/kg) in a Western-type high-fat diet for 12 weeks. Plasma lipid parameters, such as total cholesterol (TC), triglyceride (TG) and free fatty acid, were significantly higher in obese mice compared to lean mice at baseline ($P<.001$). Western-type diet (WD) feeding caused an increase in TC levels in all groups ($P<.001$); however, TG ($P<.001$) and free fatty acid ($P<.01$) were elevated only in lean mice following WD feeding. Vitamin E supplementation neither influenced any of these parameters nor reduced urinary isoprostanes in lean or obese mice. Vitamin E supplementation in ob/ob;LDLR$^{-/-}$ mice resulted in a trend toward a reduction in atherosclerotic lesion area ($P=.10$), although no differences in lesion area were noted in lean LDLR$^{-/-}$ animals. These data provide evidence that vitamin E supplementation is not sufficient to reduce extreme elevations in systemic oxidative stress due to hyperlipidemia and obesity and, thus, may not be cardioprotective in this setting.

Keywords: Vitamin E; Isoprostanes; Atherosclerosis; Obesity; Hyperlipidemia

1. Introduction

Much research over the past decade has focused on the role of oxidative stress in cardiovascular disease. Because of the clear atherogenic potential of oxidized low-density lipoprotein (LDL), many animal [1–5] and clinical studies [6–10] have been performed to evaluate the potential of various antioxidants to reduce atherosclerotic and overall cardiovascular risks. Most [1–4], but not all [5], mouse studies have shown the beneficial results of vitamin E treatment on reducing atherosclerotic lesions (reviewed in Upston et al. [11]). Epidemiological studies in humans have shown an inverse correlation between estimated vitamin E intake and cardiovascular disease [12,13], providing the impetus for clinical trials to test the efficacy of vitamin E in reducing cardiovascular risk. Human studies have been equivocal, with results demonstrating beneficial effects [7], mixed effects [8], and lack of effects [9,10,14–16] of vitamin E.

It has recently been suggested that incongruous results of vitamin E treatment pertaining to cardiovascular risk are due to lack of consistent criteria for the selection of candidates who might benefit from antioxidant therapy [17]. It is possible that vitamin E treatment is of therapeutic value only in individuals with higher levels of oxidant stress. For example, urinary isoprostane levels are elevated in diabetics [18], obese individuals [19] and smokers [20].

☆ This work was supported by National Institutes of Health grants GM15431, DK48831, CA77839, ES13125 and RR00095. A.H. Hasty was also supported by the American Heart Association (0330011N) and the American Diabetes Association (1-04-JF-20).

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doi:10.1016/j.jnutbio.2006.03.012

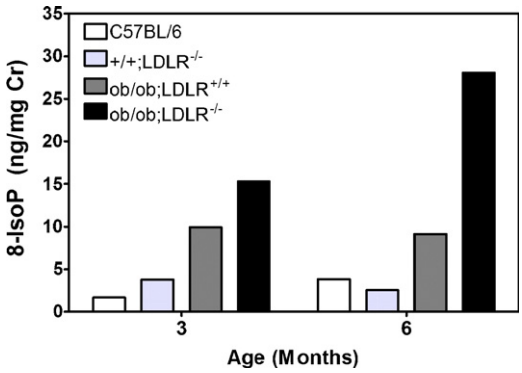

Fig. 1. Urinary isoprostanes in ob/ob;LDLR$^{-/-}$ mice. Urine was collected over a 24-h period from two male and two female mice in each group. IsoP was measured by gas chromatography–mass spectrometry and normalized to urinary Cr levels. The same animals were used for measurements at 3 and 6 months of age. C57BL/6, white bars; +/+;LDLR$^{-/-}$, light gray bars; ob/ob;LDLR$^{+/+}$, dark gray bars; ob/ob;LDLR$^{-/-}$, black bars.

Thus, the potential of vitamin E to improve cardiovascular outcomes in situations of oxidative challenge remains open for further investigation.

We have previously reported the development of an obese mouse model that is susceptible to developing atherosclerotic lesions [21]. Mice that are both leptin-deficient (ob/ob) and low-density-lipoprotein-receptor (LDLR)-deficient ($^{-/-}$) develop extreme hyperlipidemia and spontaneous atherosclerosis. Following our report, Mertens et al. [22] demonstrated increased LDL oxidation and decreased high-density lipoprotein (HDL) antioxidant activity in these same animals.

In our current study, we have supplemented lean LDLR$^{-/-}$ and obese ob/ob;LDLR$^{-/-}$ animals with vitamin E to determine the potential of antioxidants to reduce atherosclerotic lesion burden in situations of mild to extreme elevations in systemic oxidative stress. Ob/ob;LDLR$^{-/-}$ mice had dramatic age-dependent increases in urinary isoprostanes compared to lean and normolipidemic obese controls. Vitamin E supplementation did not influence body weight or plasma lipid levels in lean or obese animals. Despite supplementation with 2000 IU/kg food, urinary isoprostanes were not reduced and vitamin-E-treated mice were not protected against lesion formation. These data provide evidence that supplemental vitamin E may not be potent enough to reduce the oxidative stress that develops in a setting where obesity and hyperlipidemia promote extensive oxidative modification.

2. Materials and methods

2.1. Mice

All mice were originally purchased from Jackson Laboratories (Bar Harbor, ME, USA) and are on the C57BL/6 background. Mice heterozygous for leptin (ob/+) were successively crossed with LDLR$^{-/-}$ mice to obtain animals deficient in both LDLR and leptin (ob/ob;LDLR$^{-/-}$). Mice were fed ad libitum and were maintained on a 12-h light/dark cycle. All animal procedures were performed in accordance with institutional guidelines after approval from the Animal Care and Use Committee of Vanderbilt University.

2.2. Diets

Mice were maintained on a rodent chow diet (LabDiet 5001; 12% of calories from fat) until they were 3 months of age, at which time they were placed on a Western-type diet (WD) with or without vitamin E supplementation for 12 weeks. The WD (TD 88137; Harlan Teklad) contained

Table 1
Plasma lipid parameters in +/+;LDLR$^{-/-}$, ob/+;LDLR$^{-/-}$ and ob/ob;LDLR$^{-/-}$ mice at baseline and 12 weeks after WD feeding

	n	Body weight (g)	Cholesterol (mg/dl)	TG (mg/dl)	NEFA (mEq/ml)
+/+;LDLR$^{-/-}$					
Prediet	18	26.1±1.8	219±7	71±6	ND
Control diet	9	32.7±2.4*	858±57**	369±50***	ND
Vitamin E diet	9	32.4±1.2*	946±22**	358±35***	ND
ob/+;LDLR$^{-/-}$					
Prediet	22	27.7±1.2	198±11	70±7	0.71±0.04
Control diet	10	35±2.1*	937±59**	426±72***	1.36±0.1****
Vitamin E diet	12	35±1.5*	971±32**	438±52***	1.39±0.2****
ob/ob;LDLR$^{-/-}$					
Prediet	14	47±2.3*****	861±44*****	612±100*****	1.97±0.32*****
Control diet	6	63.5±1.6*	1750±66**	683±104	1.06±.08****
Vitamin E diet	8	65.8±2.2*	1682±60**	542±88	0.92±.07****

Body weight, TC, TG and NEFA were measured in +/+;LDLR$^{-/-}$, ob/+;LDLR$^{-/-}$ and ob/ob;LDLR$^{-/-}$ mice at baseline and 12 weeks after WD feeding, with and without vitamin E supplementation.
ND = not determined.
* $P<.001$ compared to baseline body weight for the same genotype.
** $P<.001$ compared to baseline TC for the same genotype.
*** $P<.001$ compared to baseline TG for the same genotype.
**** $P<.01$ compared to baseline NEFA for the same genotype.
***** $P<.001$ compared to baseline body weight, TC, TG and NEFA in lean mice.

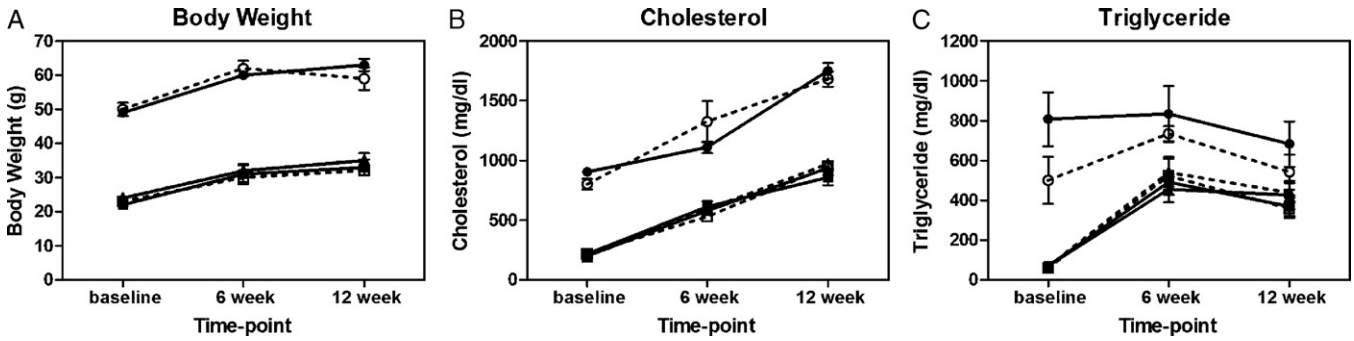

Fig. 2. Body weight, TC levels and TG levels at baseline, and 6 and 12 weeks after WD feeding. Three-month-old mice were placed on a WD with and without vitamin E supplementation for 12 weeks. Body weight (A), TC (B) and TG (C) were measured at baseline, and 6 and 12 weeks postdiet in +/+;LDLR$^{-/-}$ (squares), ob/+;LDLR$^{-/-}$ (triangles) and ob/ob;LDLR$^{-/-}$ (circles) mice. Closed symbols represent mice receiving the control diet, and open symbols represent mice supplemented with vitamin E.

42% of calories from milk fat and 0.15% cholesterol. Both diets contained a 1% vitamin mix (40060) and ethoxyquin (0.004%) as antioxidant. The vitamin E diet was supplemented with 2000 IU/kg vitamin E.

2.3. Measurement of urinary isoprostanes

Isoprostanes in urine were quantified by a highly precise mass spectrometric assay, as previously reported [23].

2.4. Plasma lipid measurements

Blood was collected from 5-h-fasted mice via retro-orbital venous plexus puncture. Glucose was measured on whole blood using OneTouch glucometer (Johnson&Johnson). Plasma was isolated by centrifugation and frozen at −80°C for future analysis of metabolic parameters. Total cholesterol (TC) and triglyceride (TG) levels were measured using kits from Raichem (according to the manufacturer's instructions) that were adapted to microtiter plate assay. Nonesterified fatty acids (NEFAs) were measured using NEFA-C kit (Wako).

2.5. Lipoprotein profile analyses

Plasma lipoproteins were separated on a Superose 6 column, as previously described [24–26]. Briefly, 100-μl plasma samples were separated into forty 500-μl aliquots in a buffer containing 0.15 M NaCl, 0.01 M Na_2HPO_4 and 1 mM EDTA. Each aliquot was assayed for cholesterol content using a kit from Raichem. Fractions 15–20 contained very-low-density lipoproteins (VLDL), fractions 21–26 contained LDL and fractions 27–33 contained HDL.

2.6. Atherosclerotic lesion quantification

Hearts were collected from animals perfused with phosphate-buffered saline after 12 weeks of WD feeding. Hearts were embedded in optimal cutting temperature (Tissue-Tek) and frozen on dry ice. Cryosections of 10 μm thickness were collected starting at the aortic root and proceeding to 300 μm, according to the method of Paigen et al. [27]. Sections were stained with Oil Red O according to standard procedures, and lesion area was quantified on images captured with a Q-Imaging Micropublisher camera mounted on an Olympus upright microscope using Kinetic Histometrix 6 imaging and analysis software (Kinetic Imaging, Inc.). En face analyses were performed as previously described [28,29].

2.7. Statistical analyses

Comparisons of body weight, plasma lipids, glucose and lesion area between control and vitamin-E-supplemented

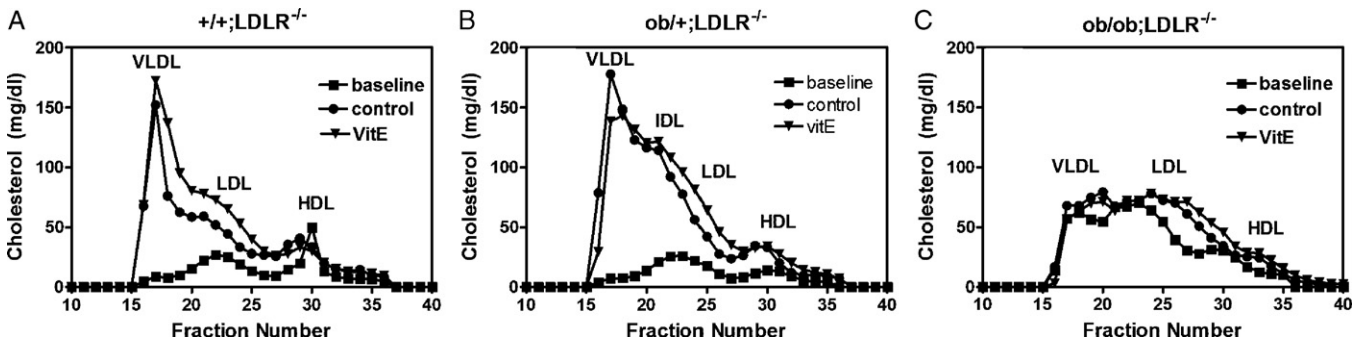

Fig. 3. Plasma lipoprotein profiles at baseline and 12 weeks after WD feeding. Plasma lipoproteins from +/+;LDLR$^{-/-}$ (A), ob/+;LDLR$^{-/-}$ (B) and ob/ob;LDLR$^{-/-}$ (C) mice collected at baseline and 12 weeks after WD feeding were separated by gel filtration chromatography, as described in Materials and Methods. Fractions 15–20 contain VLDL, fractions 21–26 contain LDL and fractions 27–33 contain HDL. Cholesterol measured in fractions 20 and 21 from ob/+/LDLR$^{-/-}$ after WD are designated as IDL. Baseline plots, squares; 12 weeks after control diet, circles; 12 weeks after vitamin E diet, triangles.

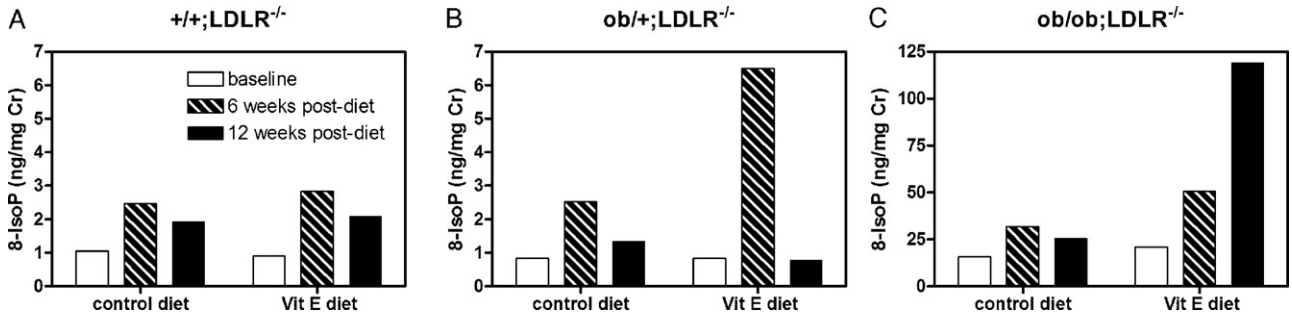

Fig. 4. Urinary isoprostanes at baseline, and 6 and 12 weeks after WD feeding. Urinary IsoP levels were measured at baseline (white bars), and 6 weeks (hatched bars) and 12 weeks (black bars) after WD from 24-h pooled samples of three to four male mice in each group. (A) +/+;LDLR$^{-/-}$; (B) ob/+;LDLR$^{-/-}$; (C) ob/ob;LDLR$^{-/-}$. IsoP levels were normalized to Cr levels.

mice within each genotype were performed using unpaired Student's *t* test. $P<.05$ was considered significant.

3. Results

3.1. Urinary isoprostanes in obese hyperlipidemic mice

Increased adiposity and elevated plasma lipoprotein levels are often accompanied by systemic elevations in oxidative stress and inflammation. The generation and secretion of 8-iso-prostaglandin $F_{2\alpha}$ (hereafter referred to as IsoP) into the urine are reliable markers of oxidative stress. To determine whether obesity and/or hyperlipidemia increases oxidative stress in mice, we measured IsoP levels in urine samples collected over a 24-h period from pooled groups of C57BL/6, LDLR$^{-/-}$, ob/ob and ob/ob;LDLR$^{-/-}$ mice at 3 and 6 months of age (Fig. 1). IsoP levels were at a concentration of 1.65 ng/mg Cr in 3-month-old C57BL/6 mice. Lean LDLR$^{-/-}$ mice were mildly hyperlipidemic [30] and demonstrated a 2.3-fold increase in urinary IsoP levels up to 3.76 ng/mg Cr. Obese ob/ob mice showed a marked sixfold increase in urinary IsoP levels compared to lean C57BL/6 mice (9.94 ng/mg Cr). Obesity and hyperlipidemia together resulted in synergistic effects on systemic oxidative stress, as ob/ob;LDLR$^{-/-}$ mice had IsoP levels 9.3-fold above those of C57BL/6 mice. Moreover, IsoP levels continued to rise in ob/ob;LDLR$^{-/-}$ mice as they aged, such that 24-h urine from 6-month-old mice contained 28 ng IsoP/mg Cr. Based upon the observation of increased oxidative stress in ob/ob;LDLR$^{-/-}$ mice, we sought to determine whether vitamin E

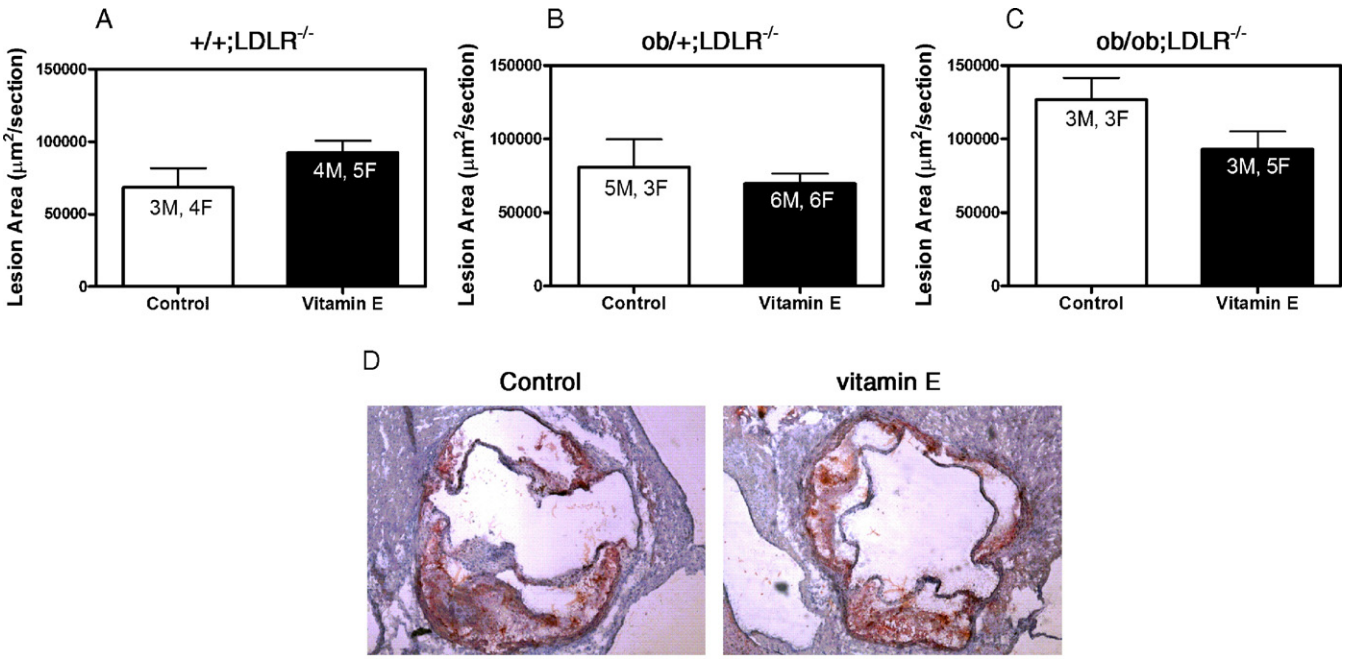

Fig. 5. Atherosclerotic lesion area in vitamin-E-supplemented mice. Mice were sacrificed after 12 weeks of feeding with WD, with or without vitamin E supplementation. The atherosclerotic lesion area was quantified in Oil-Red-O-stained sections from the aortic root by computer-assisted imaging analysis. +/+;LDLR$^{-/-}$ (A), ob/+;LDLR$^{-/-}$ (B) and ob/ob;LDLR$^{-/-}$ (C). Representative images of Oil-Red-O-stained lesions from control and vitamin-E-supplemented ob/ob;LDLR$^{-/-}$ mice are shown in (D). $P=.10$ between control and vitamin-E-supplemented ob/ob;LDLR$^{-/-}$ mice. The number of male and female mice in each group is designated within the bars on the graph (M, male; F, female).

supplementation could exert antioxidative and antiatherogenic protective effects in these animals.

3.2. Plasma lipids in WD-fed LDLR$^{-/-}$ mice supplemented with vitamin E

Three-month-old LDLR$^{-/-}$ mice with either zero, one or two copies of leptin-deficient ob alleles (+/+;LDLR$^{-/-}$, ob/+;LDLR$^{-/-}$ and ob/ob;LDLR$^{-/-}$, respectively) were place on a WD supplemented with 2000 IU/kg vitamin E for 12 weeks. Mice were bled at baseline, and 6 and 12 weeks postdiet for the analysis of TC, TG and NEFAs (Table 1, Fig. 2). As previously reported by our group and others [21,22,31,32], ob/ob;LDLR$^{-/-}$ mice displayed elevated levels of TC and TG compared to nonobese LDLR$^{-/-}$ groups ($P<.001$). Body weight was also elevated in ob/ob;LDLR$^{-/-}$ mice compared to other groups ($P<.001$). By 6 weeks after the feeding of high-fat diet, both lean LDLR$^{-/-}$ groups had significant increases in body weight, TC levels and TG levels, with TC levels becoming twofold elevated at 6 weeks and threefold elevated at 12 weeks postdiet ($P<.001$). After 12 weeks of WD feeding, TC levels continued to rise in all groups, while body weight and TG levels remained similar to the 6-week time point (Fig. 2). TC levels in ob/ob;LDLR$^{-/-}$ mice also rose during the WD feeding and were twofold elevated after 12 weeks; however, in contrast to the lean groups, TG levels were not significantly different after WD feeding. The presence of vitamin E in the diet did not influence these parameters in any of the groups.

Baseline lipoprotein profiles of +/+;LDLR$^{-/-}$ and ob/+;LDLR$^{-/-}$ mice were similar, containing LDL and HDL particles (Fig. 3A and B). After 12 weeks of WD feeding, +/+;LDLR$^{-/-}$ mice showed increased levels of VLDL and LDL, while the plasma from ob/+;LDLR$^{-/-}$ mice contained primarily VLDL and intermediate-density lipoproteins (IDL) (Fig. 3B). Baseline lipoprotein profiles of ob/ob;LDLR$^{-/-}$ mice reflected their increased TC levels, containing dramatically increased VLDL, IDL and LDL levels compared to those in lean controls (Fig. 3C). Surprisingly, the increased plasma cholesterol in WD-fed ob/ob;LDLR$^{-/-}$ mice was carried primarily on small LDL-sized particles with modest increases in VLDL cholesterol. Vitamin E supplementation did not alter postdiet profiles in any of the groups.

3.3. Impact of WD and vitamin E supplementation on urinary isoprostanes

Urinary isoprostanes in pooled samples from three to four male mice collected over a 24-h period were measured at baseline, and 6 and 12 weeks post-diet to determine the overall systemic oxidative stress. In +/+;LDLR$^{-/-}$ mice, urinary IsoP levels were equally increased at both 6 and 12 weeks in the presence and absence of vitamin E (Fig. 4A). Urinary isoprostanes were also increased in ob/+;LDLR$^{-/-}$ mice upon WD feeding. There was a surprising increase in IsoP levels after 6 weeks of vitamin E supplementation; however, levels were normalized by 12 weeks (Fig. 4B). In accordance with the data shown in Fig. 1, ob/ob;LDLR$^{-/-}$ mice had baseline urinary IsoP levels that were significantly higher than those of lean controls (Fig. 4C). Feeding with the WD for 6 and 12 weeks resulted in a 1.6- to 2-fold increase in urinary isoprostanes. Contrary to expectations, vitamin E supplementation resulted in as much as a sixfold increase in urinary isoprostanes in ob/ob;LDLR$^{-/-}$ mice.

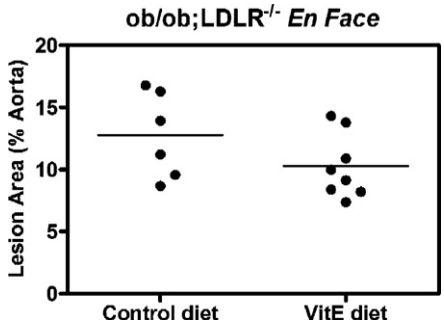

Fig. 6. En face lesion area in ob/ob;LDLR$^{-/-}$ mice. Whole aortae were collected from ob/ob;LDLR$^{-/-}$ mice, pinned out and stained with Sudan IV. Lesion area was quantified by computer-assisted imaging analysis. Data are presented as lesion area per entire aortic surface area. $P=.14$ between control and vitamin-E-supplemented groups.

3.4. Atherosclerotic lesion area in vitamin-E-supplemented mice

Atherosclerotic lesion area in the aortic root was measured in all animals after 12 weeks of WD feeding (Fig. 5). There was a trend toward larger lesions in ob/ob;LDLR$^{-/-}$ groups compared to lean animals. Vitamin E treatment did not significantly reduce lesion area in any of the groups, although the obese mice supplemented with vitamin E showed a trend toward reduced lesion area in both the aortic root ($P=.10$) and the whole aorta en face ($P=.14$) (Fig. 6).

4. Discussion

In this study, we demonstrated that obesity and hyperlipidemia synergistically promote systemic oxidative stress, as evidenced by the urinary output of IsoP. We hypothesized that these obese hyperlipidemic ob/ob;LDLR$^{-/-}$ mice would be an ideal model to study the potential of vitamin E supplementation to reduce atherosclerotic lesion area due to their elevated oxidative status. Contrary to our expectations, vitamin E supplementation did not reduce urinary IsoP in lean or obese mice. Atherosclerotic lesion area was not different between control and vitamin-E-fed lean animals, although a trend toward a decrease in lesion area was noted in ob/ob;LDLR$^{-/-}$ mice.

There are numerous assays used to measure lipid oxidation in vitro; however, they are not accurate for measuring in vivo oxidative stress from plasma and urine samples [33]. Based upon the "Biomarkers of Oxidative Stress Study" sponsored by the National Institutes of Health,

F_2 isoprostanes are considered the best index of systemic oxidative stress [34]. Thus, we used urinary IsoP levels as a biomarker for systemic oxidative stress in our study. Elevated plasma lipid levels have been shown to influence urinary isoprostanes in both mice [4] and humans [35,36]. In agreement with this, our data also show an increase in urinary IsoP in mildly hyperlipidemic LDLR$^{-/-}$ mice compared to wild-type controls at 3 months of age (Fig. 1). More dramatic is the effect of obesity on urinary isoprostane formation, where ob/ob mice have up to a sixfold increase in 24-h IsoP compared with that in controls. While it has been established that urinary IsoP correlates with body weight and is an important measure of systemic oxidative stress in humans [37,38], ours is the first report of this association in obese mice. Finally, we have made the novel observation that obesity and hyperlipidemia have synergistic effects on oxidative stress, with 6-month-old ob/ob;LDLR$^{-/-}$ mice excreting threefold more isoprostanes than their normolipidemic ob/ob controls. These data are consistent with the observations of Hansel et al. [39] that individuals with metabolic syndrome have elevations in systemic oxidative stress compared to nonobese normolipidemic controls, although conflicting results in humans with metabolic syndrome have also been reported [40].

In many animal studies wherein vitamin E has been shown to be atheroprotective, its administration was shown to have hypolipidemic effects [41–44], which could have accounted for the decreased atherosclerotic lesion formation. Yet, in other reports showing the atheroprotective effects of vitamin E, plasma lipids were unchanged [2,4] or even increased [1]. In our current study, we did not detect any impact of vitamin E on plasma lipid levels in lean or obese mice (Fig. 2, Table 1). It is possible that plasma cholesterol levels in our WD-fed mice were excessive (up to 970 mg/dl in lean animals and up to 1750 mg/dl in obese animals) for vitamin E supplementation to have any impact on lipoprotein metabolism. Along those lines, it has been suggested that severe hyperlipidemia can prevail over the therapeutic benefits of antioxidant supplementation [45]. Alternatively, the consumption of a high-cholesterol/high-fat diet may, in and of itself, reduce the efficacy of vitamin E. Thus, the absence of improvement in urinary IsoP levels and atherosclerotic lesion area in our vitamin-E-supplemented mice may be due to their high-fat diet consumption and/or their extreme elevations in plasma lipid levels.

One important finding of our study was the effect of high-fat diet on the three different genotypes of mice. +/+;LDLR$^{-/-}$ mice demonstrated a well-established increase in VLDL and LDL particles after 12 weeks of high-fat diet. Although +/+ and ob/+ mice are often used interchangeably in studies, our results show a clear difference in the lipoprotein profiles of ob/+;LDLR$^{-/-}$ mice, with a dramatic increase in IDL particles that was not seen in +/+;LDLR$^{-/-}$ mice. The presence or absence of vitamin E in the diets did not impact these phenotypes. We and others [21,22,31,32] have previously reported that ob/ob;LDLR$^{-/-}$ mice are hyperlipidemic, even on a chow diet. In this study, we demonstrate for the first time that WD feeding of ob/ob;LDLR$^{-/-}$ animals results in only modest increases in VLDL, with most of the additional cholesterol carried on small LDL particles.

In conclusion, our current data demonstrate that obesity and hyperlipidemia have synergistic effects on promoting systemic oxidative stress, as evidenced by increased excretion of IsoP into the urine. Dietary supplementation with vitamin E was insufficient to reduce urinary IsoP levels or plasma lipids, and resulted in only a trend toward a reduction in atherosclerotic lesion area in ob/ob;LDLR$^{-/-}$ mice. These studies point to the need for the development of more potent antioxidant therapies for the treatment of extreme oxidative stress that occurs in individuals with combined obesity and hyperlipidemia.

Acknowledgments

We would like to thank Shan Zhou for her technical assistance in preparing en face aortas.

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