# Proteinuria increases oxylipid concentrations in VLDL and HDL but not LDL particles in the rat

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**Abstract** **We previously established that proteinuria alters the apolipoprotein content of lipoproteins. This study was conducted to establish whether proteinuria also alters the concentrations of oxidized lipids within lipoprotein density fractions. To this end, we induced passive Heymann nephritis in Sprague Dawley rats and measured an array of alkaline-stable oxylipids in VLDL, LDL, and HDL particles. Proteinuria increased the total oxylipid amounts in the HDL and VLDL fractions. More importantly, these levels were increased when expressed per unit lipoprotein protein, indicating that the oxidized lipid load per particle was increased. Epoxides and diols increased ~2-fold in HDL and ~5-fold in VLDL, whereas LDL showed ~2-fold decreases. The hydroxyeicosatetraenoic acids and hydroxyoctadecadienoic acids (HODEs) increased >4-fold in HDL and >20-fold in VLDL, whereas LDL showed ~2-fold decreases in the HODEs. Therefore, nephrotic syndrome alters the lipoprotein oxylipid composition independently of an increase in total lipoprotein levels. These proteinuria-induced changes may be associated with the cardiovascular risk of lipoprotein oxidation.**—Newman, J. W., G. A. Kaysen, B. D. Hammock, and G. C. Shearer. **Proteinuria increases oxylipid concentrations in VLDL and HDL but not LDL particles in the rat.** ***J. Lipid Res.*** **2007.** 48: **1792–1800.**

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Even small amounts of proteinuria are associated with cardiovascular disease. This association is found in patients with hypertension (1) and diabetes (2) and even in patients having neither of these cardiovascular disease risk factors (3). The prevalent hypothesis linking microalbuminuria to vascular disease is that proteinuria reports endothelial injury (4–7). However, even small amounts of urinary protein loss in the rat alter plasma lipid levels and the apolipoprotein content of lipoproteins (8). In addition to their role in mediating forward and reverse lipid transport, lipoproteins also influence inflammation, vascular tension (9), vascular permeability, and oxidative stress (10). Therefore, structural changes to lipoprotein particles may provide a causal link to, rather than simply a report of, vascular injury. However, it is unclear how or whether the changes in lipoprotein structure associated with urinary protein loss result in vascular injury. It is known that lipoproteins carry biologically active materials, vasoactive metabolites, and oxidants. But it remains to be seen whether the lipid composition of individual lipoprotein particles is altered in association with proteinuria, potentially altering their biological effects on endothelial surfaces in disease states. Clearly, oxidized LDL is associated with vascular injury, with both protein and lipid components being altered (11–13). Here we propose that proteinuria alters the lipid composition of lipoproteins, increasing the availability of extracellular vasoactive lipids to the vascular endothelium. Within the context of the recently reported lipase/peroxisome proliferator-activated receptor (PPAR) -dependent actions of lipoproteins (14–16), such changes would provide a mechanism for a lipoprotein-mediated vascular pathophysiology associated with VLDL clearance defects.

The oxidation of polyunsaturated fatty acids produces mediators of vascular homeostasis and inflammation. Although these compounds appear in the circulation as both free and esterified products (17), the role lipoproteins play in their transport, delivery, and excretion is poorly described. Moreover, recent studies exploring the ability of particle-associated oxylipids to influence physiological responses have focused on triglyceride-rich lipoprotein (TGRL) particles (14, 15, 18). However, it is likely that lipoproteins differentially target and transport oxylipids, as they do other lipids. If this is true, then an under-

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Abbreviations: apoA-I, apolipoprotein A-I; CUDA, 1-cyclohexylureido, 3-dodecanoic acid; EET, epoxyeicosatrienoic acid; EpOME, epoxyoctadecamonoenoic acid; DHET, dihydroxyeicosatrieneoic acid; DHOME, dihydroxyoctadecamonoenoic acid; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; HPLC/ESI-TOF, HPLC electrospray ionization time-of-flight; LpL, lipoprotein lipase; MS, mass spectroscopy; TGRL, triglyceride-rich lipoprotein; TG, triglyceride.

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standing of such transport phenomena may offer a different view of circulating oxylipids. Specifically, understanding lipase/oxylipid interactions may be necessary in order to decipher the functional impact of this circulating pool of bioactive agents. If the lipoprotein-dependent transport of oxidized lipids is a passive process, with nonspecific generation, loading, and/or unloading of oxylipids into and from various lipoproteins, a uniform distribution of oxylipids among particles could be expected. However, if these agents are carried in specific lipid classes, if they are differentially transported by various lipoprotein particles, or if they are actively transported by systems with substrate specificities, then the oxylipid content and composition among different lipoproteins would be expected to vary considerably. Regardless, a dyslipidemia disrupting lipoprotein metabolism and clearance would exaggerate these changes, and provide an excellent system in which to explore the complexity of these interactions.

Nephrotic syndrome is characterized by proteinuria, hypoalbuminemia, and decreased TGRL clearance (19–21). The proteinuria itself alters apolipoprotein distribution and lipoprotein clearance (19, 20), including effects on HDL in the nephrotic rat (22). Decreased TGRL clearance is associated with reductions in both TGRL apolipoprotein E (apoE) content (23) and lipoprotein lipase (LpL) binding to the vascular endothelium (24). The expression of clearance-mediating lipoprotein receptors (25, 26), and the activity of lecithin-cholesterol-acyltransferase can also influence dyslipidemias (27). Therefore, if the accumulation of oxidized lipids in lipoproteins is time dependent, an elevation in lipoprotein-associated oxylipids would be expected in the nephrotic syndrome. Moreover, measurement of oxylipid profiles could provide a means to correlate mechanisms of dyslipidemia with vascular risk. Here we test the hypothesis that proteinuria is associated with altered lipoprotein oxylipid composition, and demonstrate that oxylipid profiles of each lipoprotein density class are differentially altered in nephrotic rats.

## EXPERIMENTAL PROCEDURES

### Animals

Studies were approved by the Animal Review Committee of the University of California Davis. Rats were all male Sprague-Dawley, obtained from Simonson Farms (Hayward, CA). They were kept in 12 hr light/dark rooms and fed ad libitum. Nephrotic syndrome was induced at 5 weeks of age by intraperitoneal injection of FX1A antibody produced in the laboratory of Dr. William Couser (University of Washington, Department of Medicine, Seattle, WA) at 0.2 ml/100 g. Two weeks postinjection, 24 hr urines were collected for the assessment of urinary albumin excretion. Treated rats with albuminuria of >250 mg/day/100 g (n = 5) and untreated controls (n = 4) were anesthetized with an intraperitoneal injection of 0.75 g/kg (control) or 0.40 g/kg (nephrotic) sodium pentobarbital and exsanguinated.

### Clinical lipid analysis

Enzymatic kits were used for the determination of triglyceride (TG) (kit 2780-400H; Thermo DMA, Chatsworth, CA), cholesterol (kit TR12351; Thermo DMA), phospholipids (kit 990-54009; Wako Chemicals, Richmond, VA), and nonesterified fatty acids (kit 994-75409; Wako Chemicals). Protein was measured using the bicinchoninic acid assay (kit 23225; Pierce Chemicals, Milwaukee, WI). Serum HDL, LDL, and VLDL cholesterol were determined by the UC Davis Clinical Nutrition Research Unit. The oxylipid amounts measured in each density fraction subaliquot were expanded by the total fraction amount by multiplying by the total/subfraction cholesterol ratio. The sum of the HDL, LDL, and VLDL oxylipid content was then used to estimate the total plasma oxylipid concentration.

### Lipoprotein isolation and characterization

Plasma was collected in sodium ethylene diamine tetraacetate-coated syringes from the abdominal aorta of pentobarbital-anesthetized rats and handled using recommended methods (28). Lipoproteins were isolated by sequential flotation and purged with nitrogen (29). The lipid and protein content of each lipoprotein fraction was measured, and the remaining lipoprotein was stored under nitrogen at −20°C for oxylipid analysis. The pseudo-quantification of fractional apoE and apoA-I was accomplished by HPLC electrospray ionization time-of-flight (HPLC/ESI-TOF) mass spectral detection as previously described (8). Oxylipids were determined in a 250 μl aliquot of each density fraction. These were enriched with sodium ethylene diamine tetraacetate and the lipophilic antioxidant butylated hydroxytoluene, spiked with analytical surrogates, diluted with 250 μl methanol, and extracted twice with 500 μl chloroform. The isolated chloroform was combined and evaporated under nitrogen. Residues were immediately dissolved in 0.25 ml methanol, diluted with 0.25 ml 1 N sodium hydroxide, incubated at 4°C for 18 hr, and back-extracted twice with 0.5 ml ethyl acetate. The ethyl acetate was then evaporated under nitrogen, and the residue was dissolved in 50 μl of methanol containing 1-cyclohexylureido,3-dodecanoic acid (CUDA); (CAS 479413-68-8) used as an internal standard to quantify surrogate recoveries.

### Oxylipid analysis

A suite of alkaline-stable oxylipids, including epoxides, diols, and alcohols of linoleate and arachidonate, were liberated and quantified using HPLC with electrospray ionization and tandem quadrupole mass spectral detection as previously reported (30). Although hydroperoxy fatty acids are amenable to detection using HPLC mass spectroscopy (MS) techniques (31, 32), these analytes were not investigated in this study. Analytes in 20 μl extract aliquots were separated with a Waters 2790 HPLC on a 2.1 × 150, 5 μm Luna C18(2) (Phenomenex; Torrance, CA) and held at 40°C using the following gradient of solvent A (water + 0.1% v/v acetic acid) and solvent B (88:12 acetonitrile-methanol + 0.1% v/v/v acetic acid) at a flow rate of 350 μl/min: 0–0.5 min = 85% A; 2.0 min = 70% A; 8.0–13 min = 45% A; 33 min = 25% A; 34–38 min = 0% A; 38.1–40 min = 85% A. Samples were held at 10°C. Separated residues were detected by negative-mode electrospray ionization and multiple reaction monitoring on a Quattro Ultima tandem quadrupole mass spectrometer (Micromass; Manchester, UK) using the following operating parameters: capillary voltage = −3.2 kV; cone gas = 125 l/h; desolvation gas = 650 l/h; source temp = 100°C; desolvation temp = 400°C; collision gas pressure = 2.3 mTorr argon; photo multiplier voltage = 650. Analyte retention times, mass transitions, optimized cone and collision voltages, dwell times, and analytical surrogate associations for each analyte are shown in **Table 1**. Ion dwell times yielded a minimum of eight scans across the chromatographic peaks. Analytes were quantified with internal standard methods and 5 point calibration curves fit with 1/x weighted

TABLE 1. HPLC/ESI MS/MS parameters

| Analyte | Retention time | Transition | Cone | Collision | Dwell time | Internal standards |
|---|---|---|---|---|---|---|
| | *min* | *m/z* | *V* | *V* | *s* | |
| CUDA | 13.35 | 340.3 > 214.1 | 60 | 20 | 0.4 | — |
| 10,11-DHHep | 13.81 | 301.2 > 283.2 | 60 | 24 | 0.4 | 10,11-DHN |
| 12,13-DHOME | 13.81 | 313.2 > 183.1 | 60 | 23 | 0.4 | 10,11-DHN |
| 9,10-DHOME | 14.50 | 313.2 > 201.1 | 60 | 23 | 0.4 | 10,11-DHN |
| 14,15-DHET | 15.42 | 337.2 > 207.1 | 60 | 17 | 0.6 | 10,11-DHN |
| 11,12-DHET | 16.65 | 337.2 > 167.1 | 60 | 20 | 0.6 | 10,11-DHN |
| 8,9-DHET | 17.78 | 337.2 > 127.1 | 60 | 20 | 0.4 | 10,11-DHN |
| 19-HETE | 18.10 | 319.2 > 275.2 | 60 | 16 | 0.4 | 10,11-DHN |
| 20-HETE | 18.44 | 319.2 > 275.2 | 60 | 16 | 0.4 | 10,11-DHN |
| 5,6-DHET | 19.27 | 337.2 > 145.1 | 60 | 17 | 0.4 | 10,11-DHN |
| 13-HODE | 21.04 | 295.2 > 195.2 | 55 | 17 | 0.4 | 10,11-DHN |
| 10,11-DHN | 21.10 | 329.2 > 311.2 | 55 | 26 | 0.4 | CUDA |
| 9-HODE | 21.27 | 295.2 > 171.1 | 55 | 17 | 0.4 | 10,11-DHN |
| 15-HETE | 22.23 | 319.2 > 219.1 | 55 | 13 | 0.4 | 10,11-DHN |
| 11-HETE | 23.23 | 319.2 > 167.1 | 55 | 14 | 0.35 | 10,11-DHN |
| 12-HETE | 23.91 | 319.2 > 179.1 | 55 | 14 | 0.35 | 10,11-DHN |
| 8-HETE | 23.97 | 319.2 > 155.1 | 55 | 14 | 0.35 | 10,11-DHN |
| 9-HETE | 24.59 | 319.2 > 123.1 | 55 | 14 | 0.35 | 10,11-DHN |
| 5-HETE | 25.27 | 319.2 > 115.1 | 55 | 14 | 0.35 | 10,11-DHN |
| 10(11)-EpHep | 26.18 | 283.2 > 185.1 | 55 | 20 | 0.5 | CUDA |
| 12(13)-EpOME | 26.25 | 295.2 > 195.1 | 55 | 17 | 0.5 | 10(11)-EpHep |
| 14(15)-EET | 26.75 | 319.2 > 219.1 | 55 | 13 | 0.5 | 10(11)-EpHep |
| 9(10)-EpOME | 26.79 | 295.2 > 171.1 | 55 | 17 | 0.5 | 10(11)-EpHep |
| 11(12)-EET | 28.25 | 319.2 > 208.1 | 55 | 13 | 0.5 | 10(11)-EpHep |
| 8(9)-EET | 28.87 | 319.2 > 155.1 | 55 | 13 | 0.5 | 10(11)-EpHep |
| 5(6)-EET | 29.33 | 319.2 > 191.1 | 55 | 10 | 0.5 | 10(11)-EpHep |

DHET, dihydroxyeicosatrieneoic acid; DHHep, dihydroxyheptadecanoic acid; DHN, dihydroxynonadecanoic acid; DHOME, dihydroxyoctadecamonoenoic acid; EET, epoxyeicosatrienoic acid; EpHep, epoxyheptadecanoic acid; EpOME, epoxyoctadecamonoenoic acid; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; HPLC/ESI MS/MS, HPLC electrospray ionization tandem mass spectroscopy. Analytes were separated under the conditions described. Collision-induced dissociation was performed with argon at a pressure of 2.3 mTorr. Dashed lines indicate separation between mass spectral multireaction monitoring functions. Analytes were corrected for recoveries of listed surrogates. 1-Cyclohexylureido,3-dodecanoic acid (CUDA) (CAS 479413-68-8) was introduced immediately prior to analysis and used to quantify surrogate recoveries.

quadratic curves ($r^2 \geq 0.997$). Calibrants and internal standards were either synthesized (10,11-dihydroxynonadecanoic acid; 10,11-dihydroxyheptadecanoic acid; 10,11-epoxyheptadecanoic acid; CUDA) or purchased from Cayman Chemical (Ann Arbor, MI) unless otherwise indicated. Surrogates associated with the reported analytes showed >70% throughout this study, and epoxide hydrolysis was maintained at <2% for all samples.

### Statistical analysis

Regression analysis was performed using GraphPad Prism v 4.01 for Windows (GraphPad Software, San Diego CA; www.graphpad.com). Mean differences were determined using two-tailed Student's *t*-test, and correlation was determined using the least-squares method. When specified, two-way ANOVA was performed using SigmaStat v 3.00.0 for Windows (Systat Software Inc., San Jose, CA; www.systat.com). All data are reported as mean ± SEM.

## RESULTS

### Clinical parameters

Five animals treated with FX1A antiserum had elevated urinary albumin excretion (194.8 ± 19.8 mg/100 g body weight), reduced plasma albumin (2.84 ± 0.08 g/dl vs. 3.60 ± 0.02 g/dl in control, $P < 0.0001$), and 4-fold increases in plasma TG (166.3 ± 18.8 mg/dl vs. 40.2 ± 7.0 mg/dl in control, $P < 0.0001$) and cholesterol (190.9 ± 19.9 vs. 50.3 ± 6.3 mg/dl in control, $P < 0.0001$).

### HDL apolipoproteins

Proteinuria has been reported to shift the balance of lipoprotein apolipoproteins, with the apoA-I to apoE ratio being a good reporter of dysfunctional lipoproteins in the nephrotic syndrome (8). Therefore, the influence of proteinuria on HDL apoA-I and apoE was assessed by HPLC/ESI-TOF MS to further characterize the experimental animals. As shown in **Fig. 1**, the relative abundance of pro-apoA-I decreased from 24 ± 1% in controls to 17 ± 2% in nephrotic animals (i.e., ~70% of controls; $P = 0.017$), whereas the ratio of apoE to total apoA-I decreased ($P < 0.001$) to 26 ± 7% of controls.

### Lipoprotein neutral and phospholipids

Measurements of total plasma cholesterol and the amount of cholesterol in each lipoprotein fraction were

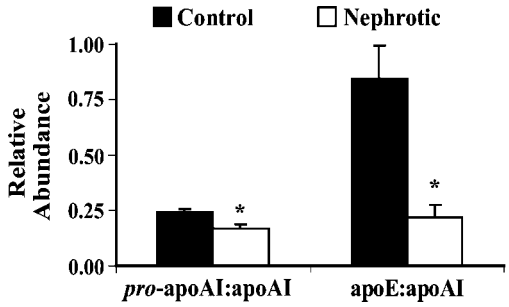


**Fig. 1.** The apolipoproteins of HDL were differentially altered in nephrotic rats. The abundance of apolipoprotein A-I (apoA-I; 27.4 kDa), pro-apoA-I (28.3 kDa), and apoE (33.8 kDa) were measured in control and nephrotic HDL by HPLC electrospray ionization time-of-flight mass spectroscopy (n = 4 per group). Results were expressed relative to the abundance of the 27.4 kDa protein identified as apoA-I. The HDL from proteinuric animals is relatively depleted in pro-apoA-I and apoE, showing levels of ~70% and ~25% control, respectively. Results are means ± SEM with differences indicated (*$P < 0.05$).

used to estimate the sizes of each lipoprotein compartment in the whole plasma as described above in Clinical lipid analysis. When expressed per unit volume of plasma, the nephrotic animals showed expansion of various lipid compartments, including HDL cholesterol, VLDL NEFAs, and all LDL components except NEFA; however, only the LDL fraction was characterized by a significant change in protein content (**Table 2**). The protein to lipid ratio in nephrotic rats was 53, 50, and 56% of control in VLDL, LDL, and HDL, respectively ($P < 0.05$). When expressed per unit fractional protein, phospholipid levels remained unchanged in all but the LDL fraction, TGs were elevated ~2-fold in the VLDL fraction, and cholesterol was increased >2-fold in each fraction (Table 2). Although the cholesterol concentration of both HDL and LDL fractions was expanded, the HDL:LDL cholesterol ratio was decreased from 1.5 ± 0.2 mg/mg in controls to 0.40 ± 0.01 in nephrotic animals ($P = 0.001$).

### Lipoprotein oxylipids

The quantitative distribution of each oxylipid among the VLDL, LDL, and HDL of the plasma is shown in **Table 3**, and the plasma distribution of oxylipid classes is shown on a fractional protein basis in **Fig. 2**. The oxylipid pools in VLDL and HDL were increased 26- and 7-fold, respectively, ($P < 0.05$) in nephrotic animals, whereas LDL levels were marginally decreased. Because VLDL and HDL protein content is unchanged in nephrotic animals (Table 2), these data suggest that the oxidized lipid load in these lipoprotein particles has increased. The relative concentration of oxylipid classes showed the following pattern in all fractions: mid-chain alcohols of arachidonate [hydroxyeicosatetraenoic acids (HETEs)] and linoleate [hydroxyoctadecadienoic acids (HODEs)] >> epoxides [epoxyeicosatrienoic acids (EETs) and epoxyoctadecamonoenoic acids (EpOMEs)] > diols [dihydroxyeicosatrieneoic acids (DHETs) and dihydroxyoctadecamonoenoic acids (DHOMEs)]. The substantial levels of 9-HETE found in all fractions (Table 3), and the elevation of this compound in lipoproteins isolated from nephrotic animals suggest that the lipids within the lipoprotein fractions were either subject to direct autooxidation (33) or were a vehicle for the transport of oxidized lipids from their site of production.

The HETEs and HODEs were differentially distributed among the lipoprotein species, and proteinuria influenced this distribution. Plasma oxylipids were distributed among the lipoprotein density fractions such that the concentrations found in the VLDL < LDL < HDL in controls, but LDL < VLDL < HDL in proteinuric animals. Expressing the oxylipid concentrations as a function of the protein amount within each lipoprotein fraction suggests that proteinuria increased the concentration of oxylipids within each particle (Fig. 2). In addition, in the LDL and HDL of nephrotic animals, the alcohol patterns were positively correlated (**Table 4**), with HODEs lower than HETEs and HETE isomers increasing in their relative abundance as the site of oxidation moved toward the carboxyl terminal. In contrast, VLDL showed HODEs > HETEs in controls,

TABLE 2. Clinical measures of lipoprotein density fractions expressed per unit of plasma volume or density fraction protein

| | VLDL | | | | LDL | | | | HDL | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | Nephrotic | Fold control | *P* | Control | Nephrotic | Fold control | *P* | Control | Nephrotic | Fold control | *P* |
| Plasma | | | | | | *mg/ml* | | | | | | |
| Cholesterol | 0.058 ± 0.01 | 0.077 ± 0.003 | — | | 0.08 ± 0.01 | 0.92 ± 0.04 | 12 | [b] | 0.12 ± 0.01 | 0.37 ± 0.02 | 2.4 | [b] |
| NEFA | 0.02 ± 0.003 | 0.001 ± 0.002 | 0.45 | [a] | 0.034 ± 0.01 | 0.0068 ± 0.001 | 0.2 | [b] | 0.011 ± 0.003 | 0.0092 ± 0.001 | — | |
| Phospholipids | 0.16 ± 0.03 | 0.14 ± 0.01 | — | | 0.11 ± 0.02 | 0.85 ± 0.1 | 7.5 | [b] | 0.13 ± 0.02 | 0.2 ± 0.04 | — | |
| TG | 0.35 ± 0.1 | 0.5 ± 0.05 | — | | 0.068 ± 0.01 | 0.17 ± 0.04 | 2.5 | 0.06 | 0.27 ± 0.1 | 0.12 ± 0.02 | — | |
| Protein | 0.28 ± 0.04 | 0.18 ± 0.03 | — | | 0.16 ± 0.01 | 0.54 ± 0.04 | 3.3 | [b] | 0.23 ± 0.05 | 0.3 ± 0.04 | — | |
| Fraction | | | | | | *mg/mg protein* | | | | | | |
| Cholesterol | 0.21 ± 0.005 | 0.49 ± 0.2 | 2.3 | [a] | 0.49 ± 0.08 | 1.7 ± 0.2 | 3.5 | [b] | 0.56 ± 0.2 | 1.3 ± 0.4 | 2.3 | [a] |
| NEFA | 0.072 ± 0.02 | 0.052 ± 0.02 | — | | 0.2 ± 0.08 | 0.013 ± 0.007 | 0.062 | [a] | 0.046 ± 0.02 | 0.032 ± 0.01 | — | |
| Phospholipids | 0.58 ± 0.2 | 0.85 ± 0.2 | — | | 0.69 ± 0.2 | 1.6 ± 0.3 | 2.3 | [b] | 0.57 ± 0.1 | 0.65 ± 0.08 | — | |
| TG | 1.2 ± 0.3 | 3.1 ± 1 | 2.5 | [a] | 0.42 ± 0.1 | 0.3 ± 0.1 | — | | NA | NA | — | |

NA, not analyzed; TG, triglyceride; —, insignificant changes. All results are shown as the mean ± SEM of control (n = 4) and nephrotic (n = 4) measurement. Significant differences in group means were determined by 2-tailed *t*-tests.

[a] $P < 0.05$ unless otherwise indicated.

[b] $P < 0.01$ unless otherwise indicated.

TABLE 3. Plasma oxylipid concentrations (pM) in lipoprotein fractions from control (n = 4) and nephrotic (n = 4) rats

| | VLDL | | | | LDL | | | | HDL | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | Nephrotic | Fold control | *P* | Control | Nephrotic | Fold control | *P* | Control | Nephrotic | Fold control | *P* |
| Epoxides | | | | | | | | | | | | |
| 12(13)-EpOME | 28 ± 4 | 150 ± 20 | 5.4 | [a] | 20 ± 6 | 5.7 ± 2 | 0.29 | [a] | 23 ± 4 | 21 ± 1 | — | |
| 9(10)-EpOME | 32 ± 6 | 170 ± 7 | 5.3 | [b] | 23 ± 6 | 7.4 ± 2 | 0.32 | [a] | 29 ± 2 | 37 ± 1 | 1.3 | [a] |
| 14(15)-EET | 24 ± 10 | 220 ± 20 | 9.0 | [b] | 34 ± 10 | 21 ± 6 | — | | 41 ± 2 | 72 ± 9 | 1.7 | [a] |
| 11(12)-EET | 46 ± 20 | 280 ± 30 | 6.0 | [b] | 48 ± 30 | 22 ± 9 | — | | 66 ± 20 | 120 ± 10 | 1.8 | [a] |
| 8(9)-EET | 44 ± 30 | 150 ± 20 | 3.4 | [a] | 30 ± 10 | 23 ± 9 | — | | 35 ± 6 | 88 ± 9 | 2.5 | [b] |
| 5(6)-EET[c] | 16 ± 3 | 200 ± 30 | 13 | [a] | 16 ± 5 | 11 ± 5 | — | | 38 ± 10 | 56 ± 10 | — | |
| Diols | | | | | | | | | | | | |
| 12,13-DHOME | 27 ± 6 | 100 ± 20 | 3.9 | [b] | 19 ± 2 | 8.5 ± 1 | 0.46 | [b] | 13 ± 2 | 12 ± 3 | — | |
| 9,10-DHOME | 6.4 ± 2 | 69 ± 10 | 11 | [b] | 16 ± 3 | 6.8 ± 0.7 | 0.43 | [a] | 10 ± 2 | 12 ± 2 | — | |
| 14,15-DHET | 5.3 ± 0.6 | 37 ± 9 | 7.0 | [a] | 5 ± 0.8 | 2.5 ± 0.7 | 0.5 | [a] | 6.7 ± 0.5 | 14 ± 0.9 | 2 | [b] |
| 11,12-DHET | 4.9 ± 0.4 | 24 ± 6 | 5.0 | [a] | 2.6 ± 0.2 | 1.7 ± 0.5 | — | | 5.1 ± 1 | 11 ± 1 | 2.2 | [a] |
| 8,9-DHET | 6.3 ± 2 | 13 ± 0.2 | 2.1 | [b] | 3.5 ± 0.3 | 2 ± 0.6 | 0.56 | [a] | 7.4 ± 2 | 17 ± 2 | 2.3 | [b] |
| 5,6-DHET | 4.8 ± 0.6 | 310 ± 50 | 65 | [b] | 23 ± 2 | 41 ± 5 | 1.8 | [a] | 95 ± 20 | 240 ± 30 | 2.5 | [b] |
| Alcohols | | | | | | | | | | | | |
| 13-HODE | 550 ± 400 | 11,000 ± 4,000 | 20 | [a] | 1,100 ± 200 | 440 ± 70 | 0.41 | [a] | 3,900 ± 2,000 | 17,000 ± 1,000 | 4.3 | [b] |
| 9-HODE | 610 ± 500 | 12,000 ± 4,000 | 20 | [a] | 1,400 ± 200 | 550 ± 70 | 0.39 | [a] | 3,600 ± 2,000 | 16,000 ± 1,000 | 4.3 | [b] |
| 15-HETE | 370 ± 300 | 13,000 ± 4,000 | 35 | [a] | 2,300 ± 500 | 1,500 ± 300 | — | | 6,400 ± 4,000 | 49,000 ± 3,000 | 7.7 | [b] |
| 12-HETE | 370 ± 300 | 11,000 ± 4,000 | 31 | [a] | 2,500 ± 500 | 1,500 ± 300 | — | | 4,700 ± 3,000 | 35,000 ± 2,000 | 7.5 | [b] |
| 11-HETE | 310 ± 300 | 10,000 ± 3,000 | 33 | [a] | 2,000 ± 400 | 1,200 ± 300 | — | | 4,100 ± 3,000 | 32,000 ± 2,000 | 7.8 | [b] |
| 9-HETE | 350 ± 300 | 11,000 ± 4,000 | 32 | [a] | 2,700 ± 500 | 1,600 ± 300 | — | | 4,800 ± 3,000 | 39,000 ± 3,000 | 8.1 | [b] |
| 8-HETE | 390 ± 300 | 13,000 ± 4,000 | 32 | [a] | 2,700 ± 500 | 1,500 ± 300 | 0.58 | [a] | 3,700 ± 2,000 | 26,000 ± 2,000 | 7.1 | [b] |
| 5-HETE | 440 ± 400 | 14,000 ± 5,000 | 32 | [a] | 5,200 ± 900 | 6,000 ± 700 | — | | 14,000 ± 7,000 | 95,000 ± 9,000 | 6.9 | [b] |

—, Insignificant changes. All results are shown as the mean ± SEM of group measurement. Significant differences in group means were determined by 2-tailed *t*-tests.

[a] $P < 0.1$.

[b] $P < 0.01$.

[c] Recoveries of the 5(6)-EET are ~25%.

but a uniform abundance of these alcohols in the animals with the nephrotic syndrome (Table 3). The linoleate-derived alcohols, and epoxides, increased less than the corresponding arachidonate-derived components in the VLDL and HDL proteinuric animals. The measured PUFA alcohols tracked 13-HODE abundance within each density fraction ($r = 1.0$–$0.86$; n = 8; $P < 0.01$), with the exception of LDL 5-HETE ($r = 0.17$). By normalizing to the auto-oxidation marker 9-HETE, this differential behavior can be highlighted (**Fig. 3**). Specifically, rats with proteinuria showed a reduction in the relative abundance of 13-HODE in VLDL and LDL, but a relative increase in the 5-HETE in LDL and depletion in HDL. The 5-HETE:13-HODE ratio was increased in both VLDL and LDL, but not in HDL, despite HDL derived from nephrotic animals having the highest levels of both compounds (Table 3).

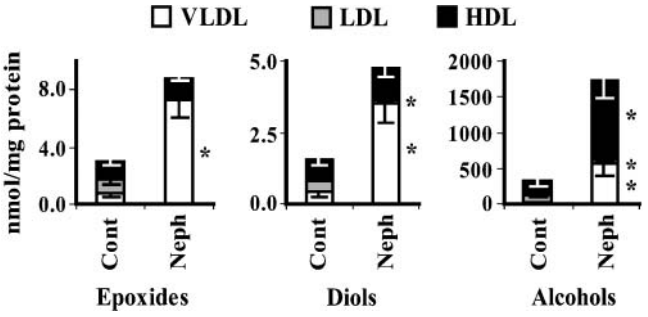


**Fig. 2.** Lipoprotein density fraction oxylipid distribution in control and nephrotic rats. When expressed as a function of the lipoprotein fraction protein content, Heymann nephritic increased all oxylipids in VLDL, decreased alcohols and diols in the LDL, and increased the alcohol levels in the HDL. Diol compositions were unchanged, whereas the relative abundance of specific epoxides and alcohols in lipoprotein fractions differed among experimental animals (see Table 3). Concentrations are means ± SEM reported per milligram of total protein within each lipoprotein density fraction. Significant differences from controls are indicated (*$P < 0.05$).

The epoxide concentrations of VLDL and LDL were nearly equivalent, and less than that of HDL from controls, whereas the epoxide concentrations in LDL < HDL < VLDL and subtle changes in isomer abundance were observed in proteinuric animals (**Fig. 4**). Moreover, with proteinuria, the LDL and HDL fractions showed lower fractional abundances of the linoleate epoxides as compared with the VLDL ($P < 0.05$). Conversely, the pattern of diols correlated between all nephrotic lipoprotein fractions (Table 4), with the 5,6-DHET and the DHOMEs comprising the majority of the residues (Fig. 4). Nearly equivalent diol concentrations were observed in all control fractions, but expanded in the nephrotic syndrome in VLDL and HDL, and contracted in the LDL. Similarly, in LDL, EpOMEs and HODEs declined significantly, whereas EETs and HETEs generally did not. Levels and changes in diols appeared more unique among isomers. In particular, the 5,6-DHET was the only diol found to increase in the nephrotic LDL, and this component showed a substantially greater elevation in the VLDL fraction. It should be noted that the changes in lipoprotein oxylipid composition associated with the nephrotic state are inconsistent with the shifts observed in the more routinely measured lipids shown in Table 2.

TABLE 4. Relationship between lipoprotein fraction oxylipid compositions in either control (n = 4) or nephrotic (n = 5) rats

| | VLDL vs. LDL | | VLDL vs. HDL | | LDL vs. HDL | |
|---|---|---|---|---|---|---|
| | Control | Nephrotic | Control | Nephrotic | Control | Nephrotic |
| Alcohols | −0.01 | −0.03 | −0.23 | −0.02 | 0.02 | 0.94[a] |
| Diols | 0.30 | 0.94[a] | 0.25 | 0.74[a] | 0.02 | 0.90[a] |
| Epoxides | 0.10 | 0.26 | −0.18 | −0.08 | 0.25 | 0.16 |

Results are Pearson correlation coefficient. Comparisons of all oxylipids in one analysis were not made because the combined data were not normally distributed.

[a] $P < 0.05$.

## DISCUSSION

Oxidized lipids are natural components of the circulation, with potent regulatory effects on cellular homeostasis and inflammation. However, the ability of circulating oxylipids to elicit cellular responses in vivo is poorly understood. As with neutral lipids, concentrations of oxylipid free fatty acids are low, with >95% of these lipids found associated with lipoprotein particles in both control and proteinuric animals (data not shown). Under conditions of oxidative stress, the circulating concentration of oxidized lipids can increase in association with lipoprotein oxidation, a process associated with an elevated risk of vascular disease (33). Therefore, considering that tissue-lipoprotein interactions are tightly regulated by enzyme- and receptor-mediated processes, we believe that an exploration of lipoprotein metabolism will be required to elucidate associations between circulating oxylipids and physiological responses. Here we have investigated the impact of Heymann nephritis-induced proteinuria on the concentrations and distributions of oxylipids among the lipoproteins of rats as a first step in this process.

In both healthy and proteinuric rats, the patterns of oxylipids differed among density fractions. Regardless of particle density, the HETEs and HODEs showed the greatest changes in the proteinuric state, with epoxides and diols tracking these changes at lower concentrations. These changes were generally followed by the autooxidation marker 9-HETE, arguing for a nonenzymatic source for the elevated lipoprotein-associated oxylipids (33). The primary exceptions were the behavior of 5-HETE and the HODEs (Fig. 4). The relative enrichment of LDL in 5-HETE while being depleted in HODEs would argue for selectivity in either the production or the transport of these lipids. Notably, it has been reported that 5-lipoxygenase activity and 5-HETE levels are elevated in nephrotic and proteinuric human kidneys (34, 35), and that 5-lipoxygenase inhibitors can ameliorate proteinuria (35–38). Regardless, HDL carried the greatest oxidized lipid load in both control and nephrotic rats, whereas VLDL displayed the greatest proteinuria-induced changes in lipoprotein oxylipids.

In the nephrotic rat, the PUFA alcohols increased 20- to 30-fold in VLDL, in contrast to other lipid species measured in this fraction, which were unchanged (Table 2).

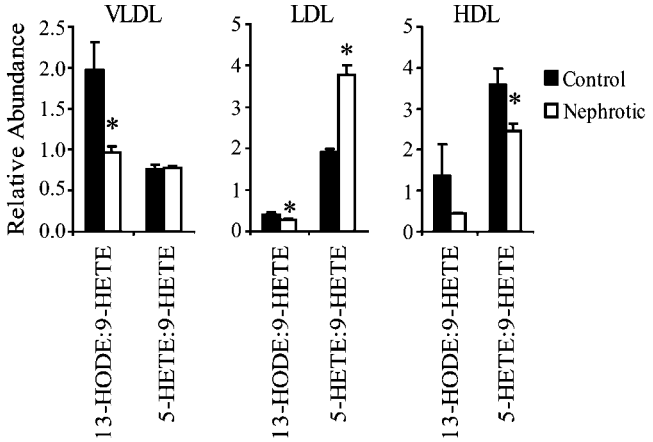


**Fig. 3.** Normalization of oxylipids to the auto-oxidation marker 9-hydroxyeicosatetraenoic acid (9-HETE) highlights oxylipid profile shifts in lipoprotein fractions of proteinuric rats. In animals with induced nephritis, 13-hydroxyoctadecadienoic acid decreased in VLDL and LDL fractions, while 5-HETE increased in the LDL and decreased in the HDL, relative to the lipid oxidation marker. All results are means ± SEM. The significance of differences in mean ratios were tested by 2-tailed *t*-tests and are indicated (*$P \leq 0.05$).

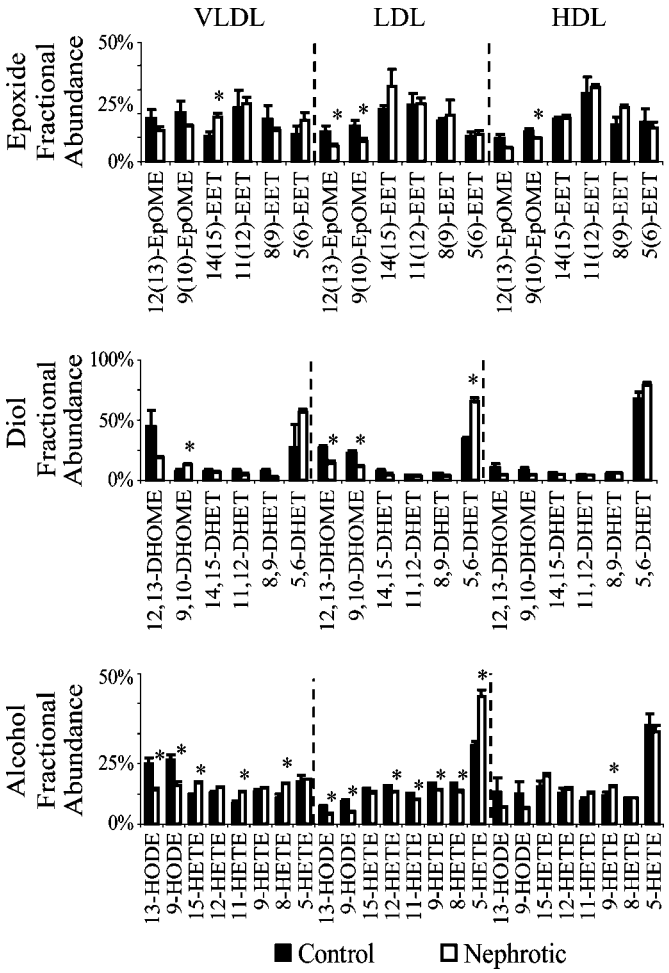


**Fig. 4.** Fractional abundance of each measured oxylipid class within each isolated lipoprotein density fraction. The sum of displayed lipids within each class for each lipoprotein density fraction is equal to 100%. The fatty acid epoxide, diol, and alcohol mean ± SEM percent compositions are shown. Differences between control and nephrotic animals are indicated (*$P < 0.05$).

Similarly the HDL alcohols increased 4- to 8-fold in HDL, whereas only ~2-fold increases in HDL cholesterol were observed without changes in other measured lipid classes (Table 2). In contrast, the LDL oxylipids were constant or reduced ~50%, despite a ~2-fold expansion of TG and ~10-fold expansions of phospholipid and cholesterol. Considering that LDL particles constitute TG-depleted VLDL, the observed shifts in lipoprotein structure suggest that these lipids are differentially distributed in the TG, cholesteryl ester, and phospholipid pools of particles of different density. Regardless, the current data would argue that the LDL fraction contained within 1 ml of plasma from a proteinuric rat carries fewer oxylipids than do the control counterparts. Moreover, the maintenance of these trends, when expressed on a fractional protein basis, further argues that the individual particles carry lower amounts of oxidized lipids. Because the LDL fraction in the rat is simply a VLDL remnant pool, it is possible that particles with lower levels of oxidation are preferentially metabolized in these animals, thus effectively diluting the oxidized lipid load in this pool. However, such an explanation remains to be evaluated. In contrast, the VLDL and HDL particles are greatly enriched in the hydroxylated lipids, although the differential abundance of the specific alcohols in each fraction argues that these profiles are altered by different processes. Specifically, VLDL particles show a general increase in all oxylipids within a class, whereas the higher density particles are either selectively enriched or depleted in specific oxylipids (Table 3, Fig. 2).

If we consider these results within the context of changes in lipoprotein metabolism associated with the nephrotic syndrome, a new perspective on the potential impact of lipoprotein oxidation emerges. The combined reduction in endothelial LpL activity and the HDL-induced disruption of VLDL-apoE content (20, 24) are critical to producing nephrotic dyslipidemia. The resulting delay in lipid clearance is an attractive explanation for the massive increase in VLDL-oxylipids observed in the proteinuric rats. Specifically, if VLDL-oxylipids are dependent upon LpL for hydrolytic release from TGs, then VLDL-oxylipids should be elevated at the expense of LDL-oxylipids. This scenario is consistent with the observed LDL data, and the enzymatic release of HODEs from TGs has been reported (39). In the case of HDL, clearance of apoA-I is also reduced in the rat, and the decreased expression of scavenger receptor class B type 1 and increased expression of ABCA1 in Heymann nephritis (40) could play a role in the HDL-oxylipid balance. Thus, if HDLs naturally accumulate oxylipids over time, the reduced clearance of these particles provides a potential mechanism for the observed increases in oxylipids in these fractions (41). Moreover, the disparate nature of the changes in the HDL and VLDL fractions argues for the presence of an active process of oxylipid incorporation into HDL. It is interesting to hypothesize here on the existence of an HDL-dependent oxylipid transport system providing a mechanism for the body to control the elimination of these biologically active substances. With the embrace of this hypothesis, the question of VLDL-dependent forward transport is also raised. Ultimately, because each lipoprotein has unique targets, it seems likely that the exposure of tissues to lipoprotein oxylipids is likewise unique, determined not only by endogenous oxylipid production but also by lipoprotein exposure and tissue lipolytic activity.

The ramifications of the observed changes in lipoprotein oxylipid levels and composition are as yet unknown. However, the role that oxylipids play as regulators of cellular homeostasis and stress responses argues that pathological changes in their levels could impact health. Within this study, the largest changes between control and nephrotic particles were the shifts in 5-HETE and 13-HODE. Although 5-HETE is a growth stimulator (42), 13-HODE, being a PPAR-γ activator, would be expected to behave as a growth inhibitor (43). Regardless, 13-HODE can also induce cellular adhesion molecule expression (44), and possesses both PPAR-γ-dependent (45) and -independent (46) antiinflammatory activity. Nephropathies are generally associated with proinflammatory LDL particles (47, 48), making it intriguing to postulate that the ratio of pro- to antiinflammatory oxylipids within lipoprotein particles may translate inflammatory state signals. This is not to say that nonlipid components do not participate in such inflammatory stimuli, but rather that the oxylipids may add an as yet unappreciated level of complexity to the situation. Because the 5-HETE levels of the LDL did not change, while the 13-HODE levels declined, a net proinflammatory signal could result. However, although the HDL fractions showed a dramatic increase in oxylipids, the 5-HETE:13-HODE ratio did not change. This would argue against a pure relative abundance trigger, because the shift of HDL from an anti- to a proinflammatory state is well known in many inflammatory conditions.

In conclusion, we suggest, first, that lipoprotein oxylipid distribution is a potentially important biological phenomenon on the basis that *1*) oxylipids are actively and differentially distributed throughout lipoproteins, whose different metabolic fates would expose different tissues to their oxylipid content, and *2*) that pathology known to disrupt lipoprotein metabolism disrupts lipoprotein-oxylipid content in patterns consistent with the known metabolic deficits of the pathology. And second, we hypothesize that the disruption in VLDL oxylipid content is a consequence of the nephropathy-associated LpL deficit, highlighting novel trafficking functions for lipases. Further study is clearly warranted to establish a role for LpL and other vascular lipases in the regulation of oxylipid trafficking and transport. The elucidation of these dynamic biochemical processes may provide unique insight into the role of nephrotic dyslipidemia in nephropathy, and may identify unexpected pharmacological targets.

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