RESEARCH ARTICLE

Acrolein-induced dyslipidemia and acute-phase response are independent of HMG-CoA reductase

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Scope: Aldehydes are ubiquitous natural constituents of foods, water and beverages. Dietary intake represents the greatest source of exposure to acrolein and related aldehydes. Oral acrolein induces dyslipidemia acutely and chronically increases atherosclerosis in mice, yet the mechanisms are unknown. Because lipid synthesis and trafficking are largely under hepatic control, we examined hepatic genes in murine models of acute and chronic oral acrolein exposure.

Methods and results: Changes in hepatic gene expression were examined using a Steroltalk microarray. Acute acrolein feeding modified plasma and hepatic proteins and increased plasma triglycerides within 15 min. By 6 h, acrolein altered hepatic gene expression including *Insig1, Insig2* and *Hmgcr* genes and stimulated an acute-phase response (APR) with up-regulation of serum amyloid A genes (*Saa*) and systemic hypoalbuminemia. To test if decreased HMG-CoA reductase activity could modify acrolein-induced dyslipidemia or the APR, mice were pretreated with simvastatin. Statin treatment, however, did not alter acrolein-induced dyslipidemia or hypoalbuminemia associated with an APR. Few hepatic genes were dysregulated by chronic acrolein feeding in apoE-null mice. These studies confirmed that acute acrolein exposure altered expression of hepatic genes involved with lipid synthesis and trafficking and APR, and thus, indicated a hepatic locus of acrolein-induced dyslipidemia and APR that was independent of HMG CoA-reductase.

Conclusion: Dietary intake of acrolein could contribute to cardiovascular disease risk by disturbing hepatic function.

Keywords:
Acute-phase response / Aldehyde / Cholesterol / HMG-CoA reductase / Lipoproteins

1 Introduction

In addition to being constituents of combustion-derived air and industrial aqueous pollution, aldehydes, such as acrolein, are abundant in foods. More than 300 different aldehydes have been detected in various foods [1, 2]. In most foods and beverages, e.g. beer, rum, and bread, aldehydes are found at a concentration of 10–20 mg/kg; however, some foods and spices (e.g. cinnamon) contain high concentrations of specific aldehydes. The formation of acrolein in foods, especially cooking oils, is further increased by high-temperature cooking, frying and re-heating because frying or heating decreases the *cis*-double bond content of triglycerides and increases the formation of trans-unsaturated aldehydes [3]. Overall, we estimate that the maximal daily human consumption of unsaturated aldehydes is nearly 5 mg/kg and the total aldehyde (unsaturated and saturated) consumption is about 7 mg/kg [4].

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Abbreviations: APR, acute-phase response; **DE,** differentially expressed; **HMGCR,** HMG-CoA reductase; **MDS,** multidimensional scaling; **SAA,** serum amyloid A; **SPE,** serum protein electrophoresis; **SREBP,** sterol regulatory element-binding protein

Recently, we showed that acrolein feeding increases the circulating levels of cholesterol and triglycerides within 24 h in mice [5]. Similarly, we have found that chronic acrolein feeding leads to not only a sustained increase in cholesterol levels but that it also increases atherosclerotic lesion progression in apoE-null mice [6]. Because acrolein-induced dyslipidemia appears, in part, as a product of a coordinated acute-phase response (APR), whereby plasma lipoprotein levels increase following toxicant exposure or infection [7, 8], we sought to better understand how hepatic gene changes regulate this response. Moreover, we tested whether acute hepatic gene changes were sustained with chronic acrolein feeding in an atherosclerotic murine model. Finally, because acrolein feeding acutely depressed HMG-CoA reductase gene expression (*Hmgcr*), we tested whether suppression of HMGCR activity by statin treatment would alter either acrolein-induced dyslipidemia or the APR. Our results indicated that acute hepatic gene responses to acrolein were extensive and complex wherein APR genes were up-regulated, cholesterol synthesis genes were down-regulated and cholesterol transport genes were dysregulated. The APR response was sustained up to 24 h as evidenced by elevated plasma SAA level. Interestingly, acrolein also suppressed plasma leptin levels – a metabolic appetite suppressant. Although only a few hepatic gene changes were identified in the chronic acrolein-feeding model, collectively, these findings implicate food-derived aldehyde intake as a potentially important cardiovascular disease risk factor.

2 Materials and methods

2.1 Animals

Adult male C57BL/6 and apoE-null mice (8–16 wk-old; Jackson Laboratories, Bar Harbor, ME, USA) were housed and fed water and food ad libitum according to the animal care guidelines and UofL IACUC-approved protocols. Animals were fed autoclaved standard Rodent Diet 5010® (LabDiet, PMI Nutritional International).

2.2 Acrolein experiments

Because we previously published dose-dependent effects of acrolein (po) in mice, mice were gavage-fed water (control) or acrolein in water (treated) at 5 mg/kg in 100 μL water and euthanized 15 min, 30 min, 60 min, 6 h, 12 h and 24 h after gavage with sodium pentobarbital in distilled water (60 mg/kg i.p. in 0.1 mL). To examine chronic changes, apoE-null mice were gavaged water or acrolein (0.05–1 mg/kg) daily for 8 wk. Mice were allowed free access to food and water throughout the treatment except when a group was not provided food following gavage treatment (24 h fast). To test the beneficial effects of statins, simvastatin (40 or 80 mg/kg, ip; Calbiochem) was administered 1 h before acrolein gavage, and mice were euthanized 24 h post-treatment [5].

2.3 Analytical measurements

Blood was withdrawn from the right ventricle of pentobarbital-anesthetized mice using 0.2 M $Na_4 \cdot$ EDTA-coated syringes (25 Ga) and placed in EDTA pre-coated Eppendorf tubes (20 μL/mL blood). Plasma (50 μL) was shipped frozen on dry ice to AssayGate (Ijamsville, MD, USA) for analysis of plasma chemokines/cytokines. Total cholesterol, triglycerides and phospholipids in plasma were measured using the Cholesterol CII Enzymatic Kit (Wako 276-64909), the L-Type TG-H Kit (Wako 432–40201) and Phospholipids B Kit (Wako 990–54009), respectively. Lipids, total protein (Bradford) and albumin (bromocresol green) were measured using calibrated standards with a Cobas Mira Plus 5600 Autoanalyzer [9]. Lipogel and serum protein electrophoresis (SPE) gels were performed with 2 μL plasma as per manufacturers' instructions as previously published [5]. Plasma and liver protein–acrolein adducts were detected as previously published [10].

Values are reported as mean ± standard error (SEM). For statistical comparison between two groups, Student's unpaired *t*-test was used. For comparing more than two groups, one-way ANOVA and Bonferroni multiple testing correction was used. In both cases, the significance level $\alpha = 0.05$ was used.

2.4 Hepatic Steroltalk v2 microarray analyses

2.4.1 RNA isolation

Hepatic RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol, and RNA pellets in ethanol were additionally washed with 75% ethanol, dried and dissolved in 100 μL of RNase-free water. Nanodrop and Bioanalyzer (Agilent) analyses showed that RNA was of good quality and purity (ratio A260/280 and A260/230 > 1.8).

2.4.2 Steroltalk v2 microarray hybridization

Total RNA from each animal was labeled separately and hybridized with a reference sample. Reference sample was a mixture of all samples used in the experiment. Steroltalk indirect labeling protocol was used to prepare labeled cDNA for hybridizations [11]. Briefly, 20 μg of total RNA was reverse transcribed using aminoallyl-dUTPs. RNA was hydrolyzed and cDNA purified. Fluorescent dyes were chemically bound to the aminoallyl-cDNA and cDNA was again purified. Samples were labeled with Cy3 and reference with Cy5. Samples were hybridized to the Steroltalk v2

microarrays, in total 28 microarrays were used. Hybridization, washing and scanning of the Steroltalk slides were performed as described previously [12, 13].

2.4.3 Microarray data analysis

Microarray images were analyzed using the Array-Pro Analyzer 4.5 (Media Cybernetics, Bethesda, MD, USA). The median feature and local background intensities were extracted together with the estimates of their standard deviation. Raw data ware normalized using Orange [14] widget for normalization of focused microarrays. Data were first filtered to exclude spots of low quality: only features with foreground to background ratio higher than 1.5 and coefficient of variation (CV, ratio between standard deviation of the background and the median feature intensity) lower than 0.5 in both channels were considered. Log_2 ratios were normalized using LOWESS fit to spike in control RNAs according to their average intensity [15]. For normalization, the controls were spiked with custom-made Firefly luciferase and commercial Lucidea Universal Scorecard. Data from Lucidea Universal Scorecard ratio controls, for which RNAs were added to each sample RNAs in different concentrations, were adjusted prior to fit of normalization curve according to the ratios between the concentrations.

Pair-wise distances between normalized data of samples were calculated and multi-dimensional scaling (MDS) was used to project the samples into two-dimensional space. Differential expression (DE) of genes was assessed using R/Bioconductor/Limma package [16] by controlling the false discovery rate [17] at level $\alpha = 0.05$.

3 Results

3.1 Acrolein feeding rapidly modifies plasma and hepatic proteins

Previously, we showed that at 12 h post-acrolein oral treatment, plasma hepatic lipase activity (and *Lipc* mRNA) is strongly correlated with plasma hypoalbuminemia ($R^2 = 0.874$) indicating a relationship between acrolein-induced injury and regulation of dyslipidemia [5]. To better understand the rapidly evolving changes that follow acrolein feeding, time-dependent protein–acrolein adducts were measured in both plasma and liver within 15 min after gavage (Fig. 1A and B) and which declined in plasma by 1 h. There was a corresponding and significant increase in plasma triglycerides (2–3-fold above background) within 15 min, and this was confirmed by the presence of a very low-density lipoprotein (VLDL)-specific band on Lipogel (Fig. 1C and D). From these data, we infer that hepatic protein–acrolein adducts may be derived, in part, from delivery and uptake of plasma-derived protein–acrolein adducts. Subsequently, at 6 h (Fig. 2A and B) and 12 h (Fig. 2C and D) post-treatment, plasma samples from water- (control) and acrolein-treated (Acro) mice were electrophoretically separated for lipoprotein staining (i.e. Lipo-gels; Fig. 2A and C) and plasma protein staining (SPE-gels; Fig. 2B and D). Lipoproteins separated into diffuse high-density (HDL), low-density (LDL) and VLDL bands, and acrolein treatment of 6 h resulted in obvious modification of HDL and VLDL/LDL fractions (Fig. 2A and C). Plasma proteins separated into major albumin, α, β and γ protein bands (see Table 1 for quantification of major lipid and protein fractions), and acrolein treatment by 6 h resulted in an obvious decrease in albumin band, a shift in the β band with a more subtle modification of γ band (Fig. 2B and D). Modifications in HDL and LDL/VLDL due to acrolein treatment were associated with subtle changes in plasma lipids levels when compared with water controls (Table 1). Acrolein treatment significantly decreased cholesterol, phospholipids, albumin and total protein levels by 6 h post-treatment (Table 1). These data show that lipoprotein and protein modifications precede the peak of acrolein-induced hypercholesterolemia and hypertriglyceridemia by almost 18 h [5].

3.2 Hepatic sterol gene alterations in acrolein-treated mice

To gain a better understanding of the relationship between plasma modifications and hepatic sterol and lipoprotein biosynthesis/clearance following oral acrolein exposure, mouse liver RNA from acrolein- and water-treated mice (6 h and 12 h) was analyzed using the Steroltalk v2 microarrays. The MDS of microarray data exposed clear separation between acute acrolein-treated samples (Fig. 3, top) and all other (i.e. chronic acrolein-treated and control) samples (Fig. 3, bottom), which emphasizes the robust nature of rapid genomic changes induced by acrolein. DE analysis exposed 62 DE genes in the 6 h treatment (i.e. 35 up-regulated, 27 down-regulated) and 49 DE genes in the 12 h treatment (i.e. 25 up-regulated, 24 down-regulated) (Tables 2 and 3).

3.2.1 Cholesterol homeostasis

In the 6 h treatment, we observed down-regulation of cholesterol biosynthesis genes, which diminished by 12 h. We observed down-regulation of *Insig1* at both time points, yet up-regulation of *Insig2* – both *Insig* proteins are regulators of active sterol regulatory element-binding protein (SREBP) protein. Genes regulating the exchange of cholesterol between liver and plasma were altered. Both mRNA of LDL uptake (*Ldlr*, *Lip1*) and cholesterol efflux *via* VLDL (*Acat2*) were down-regulated, while *apolipoprotein B* mRNA was up-regulated. The HDL receptor (*Scarb1*), apolipoprotein-A1 (*Apoa1*) and ATP-binding cassette, sub-family A (ABC1), member 1 (*Abca1*) essential for HDL cholesterol trafficking were up-regulated. At 12 h, an mRNA for a gene involved in cholesterol excretion to the bile was down-regulated (*Abcg5/*

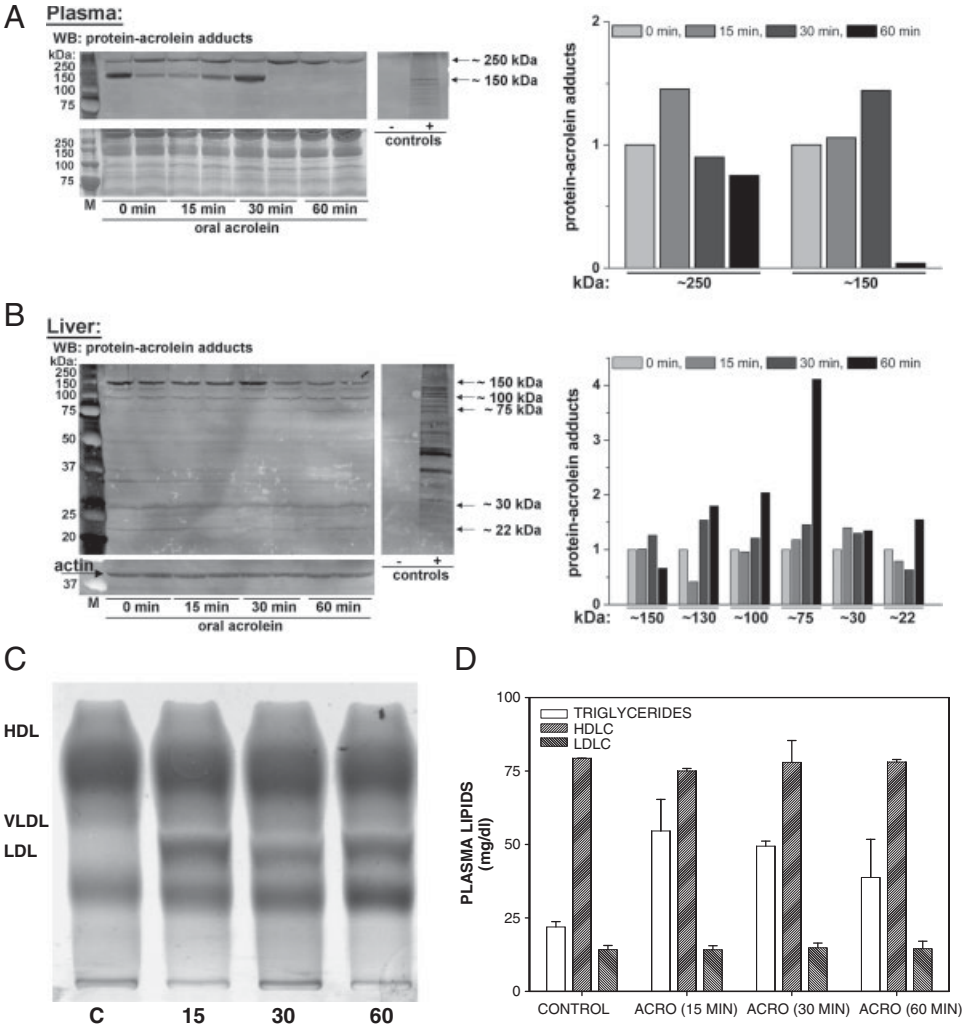

Figure 1. Acrolein-induced protein and lipoprotein modifications in vivo. At 15, 30 and 60 min post-treatment, plasma and organ samples from water (control; 30 min only; $n = 2$) and acrolein- (ACRO; $n = 2$) treated mice were separated electrophoretically by SDS-PAGE. Western blots were developed using anti-protein–acrolein antibodies (A, B). Alternatively, the plasma proteins were separated on Lipo-gels and stained for cholesterol (C). Plasma lipids were quantified at each time point (D). Lipoproteins separated into diffuse bands corresponding to HDL, LDL and VLDL.

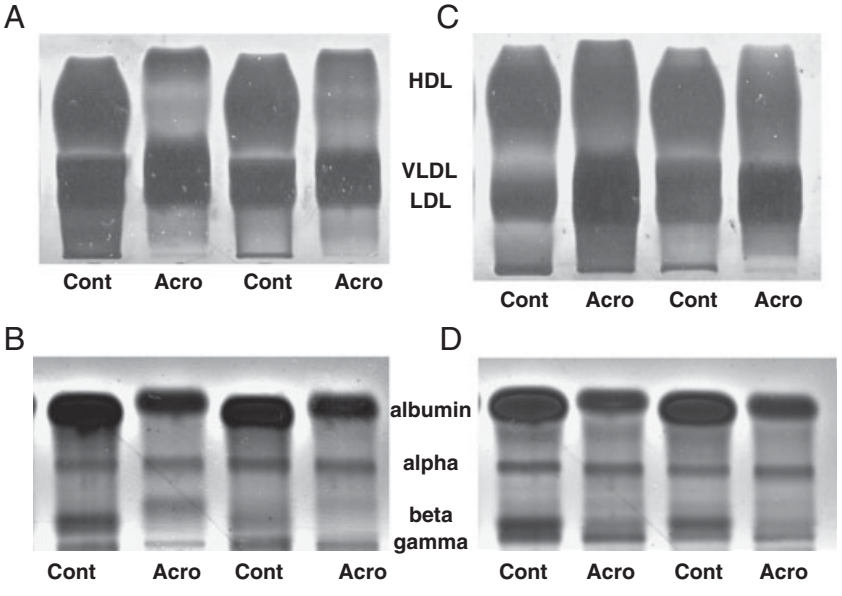

Figure 2. Acrolein-induced delayed protein and lipoprotein modifications in vivo. At 6 h (A, B) and 12 h (C, D) post-treatment, plasma samples from water (control) and acrolein- (Acro) treated mice were electrophoretically separated for measuring changes in lipoprotein (i.e. Lipo-gels; A, C) and plasma protein (SPE-gels; B, D). Lipoproteins separated into diffuse bands corresponding to HDL, LDL and VLDL. Plasma proteins separated into major bands that correspond to albumin, α, β and γ protein bands (see Table 1 for quantification of major lipid and protein fractions; $n = 3$–4).

Table 1. Early time course of acrolein (ACRO)-induced dyslipidemia and hypoalbuminemia

Parameter	6 h		12 h	
	Control (3–4)	ACRO (4)	Control (4)	ACRO (4)
Cholesterol[1]	71.8±1.0	52.6±5.9*	66.1±3.7	84.4±4.6*
Phospholipids[1]	142.4±1.7	76.0±8.1*	123.9±6.5	150.1±18.7*
Triglycerides[1]	62.6±29.0	52.2±9.0	35.9±10.5	167.9±56.2*
Albumin[2]	29.1±1.1	13.6±1.3*	30.1±0.5	12.9±0.5*
Protein[2]	43.2±1.0	23.0±2.0*	45.1±1.0	23.6±0.5*

Adult C57BL/6 mice were gavage fed either water (control) or acrolein (ACRO; 5 mg/kg) and changes in the indicated plasma constituents were measured at either 6 h or 12 h post-treatment. Values = mean±SE; Units: 1 = (mg/dL), 2 = (g/L); (*n*), number of mice per group; *significant difference between ACRO treatment and respective control values ($p<0.05$).

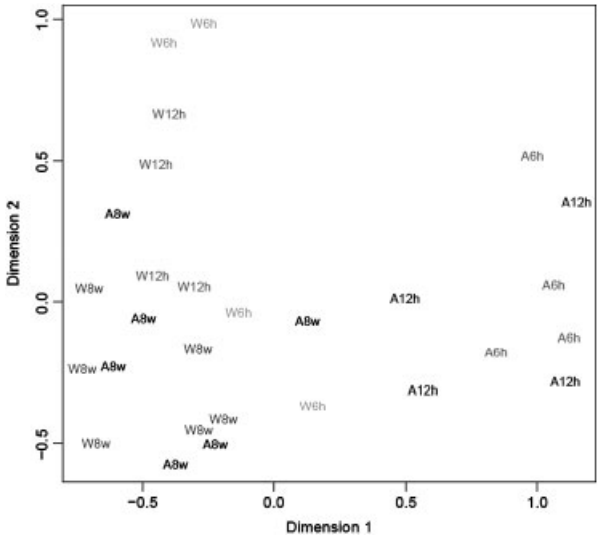

Figure 3. MDS analysis of microarray results. MDS was used to project the samples into two-dimensional space using top 80 probes. The 8-wk acrolein treatment (A8w) had minimal effect on hepatic gene expression as detected by the Steroltalk v2 microarray. However, both 6 h (A6 h) and 12 h (A12 h) acrolein-treated samples were grouped away from respective water-treated (W) samples consistent with statistically significant alterations in over 50 differentially expressed genes (see Tables 2 and 3).

8), while paraoxonase 2 (*Pon2*) mRNA was up-regulated. Bile acid synthesis mRNAs were also disturbed. The classical pathway (*Cyp7a1, Cyp8b1*) was down-regulated at 6 h, while an alternative pathway using *Cyp39a1* was up-regulated, and yet *Cyp7b1* was down-regulated. Several genes for bile acid transport from plasma to liver (*Slc10a1, Slc01b2* and *Slco1a1*) and liver to bile (*Abcb11*) were down-regulated. Many of these genes are regulated by *Fxr* (*Nr1h4*), which was also down-regulated at 6 h. After 12 h, the *Cyp17a1* gene of steroid biosynthesis was up-regulated.

3.2.2 Xenobiotic metabolism

Our analyses showed that acrolein up-regulated the *Cyp2b* family at 6 h and 12 h yet down-regulated several other members of the xenobiotic-metabolizing *Cyp* families (e.g. *Cyp2a4, Cyp1a2, Cyp2c40, Cyp2j5* and *Cyp3a25*). Modest down-regulation of nuclear receptor constitutive androstane receptor, *Car* (*Nr1i3*), gene at 6 h as well as several other nuclear receptor family members was observed. The transporter gene *Abcc1* (*Mrp1*) was modestly up-regulated at both 6 h and 12 h.

3.2.3 Acute-phase response genes

The major up-regulated APR genes were the serum amyloid A genes (*Saa*), but also up-regulated were *Orrm1, Fgb, Hpxn, Apcs* and *C4bp*. The adiponectin receptor was down-regulated at 6 h, whereas adiponectin gene expression (*Adipoq*) increased at 12 h. Heme synthesis gene (*Alas1*) was down-regulated at 6 h. Some circadian rhythm genes were up-regulated (*Clock, Cry1, Crem*) at 6 h and down-regulated (*Nr1d2*) at 12 h.

3.2.4 Glucose and fatty acid regulatory genes

Glucose homeostasis genes were changed; namely genes of gluconeogenesis (*Pck1*) and transport of glucose (*Slc2a4–Glut4*) were up-regulated. Other genes involved in glucose regulation (*Foxo1, Ppp1r3c, Crem, Sirt1*) were up-regulated at 6 h. The fatty acid synthesis gene (*Fasn*) was down-regulated, but a gene (*Scd1*) of the unsaturated fatty acid pathway was up-regulated at 6 h.

3.3 Inflammation markers

At 24 h post-oral acrolein, various plasmatic factors including APR reactants (CRP, fibrinogen and SAA), cytokines (IL-1ß, IL-6, IL-10, TNF-α) and chemokines (ICAM-1, VEGF, leptin) were measured by commercial vendor (Table 4). As expected, SAA level was significantly increased by acrolein treatment as were VEGF and IL-6 levels. Surprisingly, fibrinogen (not significantly), leptin and sICAM-1 levels were lower in acrolein-treated mice than in water controls.

Table 2. Up-regulated differentially expressed hepatic genes in acrolein-treated mice using the Steroltalk v2 microarray

Gene symbol	Gene description	Log_2 ratio 6 h	Log_2 ratio 12 h	GenBank accession
Xenobiotic metabolism				
Cyp2b10	Cytochrome P450, family 2, subfamily b, polypeptide 10	2.26	1.57	AK028103
Cyp2b9	Cytochrome P450, family 2, subfamily b, polypeptide 9	2.25	1.60	NM_010000
Cyp2b13	Cytochrome P450, family 2, subfamily b, polypeptide 13	2.14	1.55	NM_007813
Abcc1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	0.50	0.49	NM_008576
Cholesterol, bile acid and steroid homeostasis				
Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1	NO	1.01	NM_007809
Insig2	Insulin induced gene 2	2.47	1.50	BC023874
Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	1.27	1.38	NM_013454
Cyp39a1	Cytochrome P450, family 39, subfamily a, polypeptide 1	0.92	0.62	AF237981
Apoa1	Apolipopoprotein A-I	0.73	0.86	NM_009692
Scarb1	Scavenger receptor class B, member 1	0.60	NO	NM_016741
Apob	Apolipoprotein B	0.49	NO	XM_137955
Pon2	Paraoxonase 2	0.36	NO	NM_183308
Acute-phase Response and Serum proteins				
Saa2	Serum amyloid A 2	5.32	5.90	BC024606
Saa1	Serum amyloid A 1	5.22	5.82	BC087933
Saa3	Serum amyloid A 3	4.70	5.35	BC055885
Orm1	Orosomucoid 1	1.35	1.20	BC012725
Fgb	Fibrinogen, B β polypeptide	1.22	1.85	NM_181849
Hpxn	Hemopexin	0.95	0.86	BC019901
Apcs	Serum amyloid P-component	NO	2.27	BC061125
C4bp	Complement component 4 binding protein	NO	1.32	NM_007576
Cell signaling				
Egr-1	Early growth response 1	2.62	2.44	M20157
Foxo1	Forkhead box O1	1.88	NO	NM_019739
Ppp1r3c	Protein phosphatase 1, regulatory (inhibitor) subunit 3C	1.71	NO	NM_016854
Stat3	Signal transducer and activator of transcription 3	1.12	1.06	BC003806
Hnf4a	Hepatic nuclear factor 4, α	0.90	NO	NM_008261
Fos	FBJ osteosarcoma oncogene	0.83	NO	NM_010234
Crem	CAMP responsive element modulator	0.82	NO	M60285
Sp1	Trans-acting transcription factor 1, Specificity protein 1	0.60	NO	AF062566
Clock	Circadian locomoter output cycles kaput	0.50	NO	AF000998
Sirt1	Sirtuin 1, silent mating type information regulation 2, homolog 1	0.46	NO	NM_019812
Cry1	Cryptochrome 1 (photolyase-like)	0.44	NO	NM_007771
Ppargc1a	Peroxisome proliferative activated receptor, gamma, coactivator 1 α	0.41	NO	NM_008904
Mapk8	Mitogen activated protein kinase 8	0.27	NO	BC053027
Adipoq	Adiponectin, C1Q and collagen domain containing	NO	0.57	BC028770
P53	Transformation related protein 53	NO	0.41	BC005448
Other metabolic pathways				
Scd1	Stearoyl-Coenzyme A desaturase 1	0.97	NO	BC007474
Actb	Actin, β, cytoplasmic	0.67	0.67	NM_007393
Actb	Actin, β, cytoplasmic	0.63	0.59	NM_007393
Pck1	Phosphoenolpyruvate carboxykinase 1, cytosolic	0.55	NO	BC037629
Slc2a4	Solute carrier family 2 (facilitated glucose transporter), member 4	0.46	NO	BC014282
Uap1	UDP-*N*-acetylglucosamine pyrophosphorylase 1	NO	0.77	BC016406
TUBB2B	Tubulin, β 2B	NO	1.04	NM_001069

Probability of type I error $\alpha = 0.05$. NO – no differential expression was observed.

These data confirm that, at least for SAA, hepatic gene changes at 6 and 12 h (Table 2) are positively correlated with systemic protein changes evincing the predictive utility of the Steroltalk v2 microarray.

3.4 Effects of simvastatin

Because statins are effective inhibitors of HMGCR activity and have multiple beneficial effects in extra-hepatic tissues

Table 3. Down-regulated differentially expressed hepatic genes in acrolein-treated mice using the Steroltalk v2 microarray

Gene symbol xenobiotic metabolism	Gene description	Log_2 ratio 6 h	Log_2 ratio 12 h	GenBank accession
Cyp2a4	Cytochrome P450, family 2, subfamily a, polypeptide 5	−0.97	−1.36	BC011233
Cyp1a2	Cytochrome P450, family 1, subfamily a, polypeptide 2	NO	−0.80	NM_009993
Cyp2c40	Cytochrome P450, family 2, subfamily c, polypeptide 40	NO	−0.71	NM_010004
Cyp2j5	Cytochrome P450, family 2, subfamily j, polypeptide 5	NO	−0.52	NM_010007
Cyp3a25	Cytochrome P450, family 3, subfamily a, polypeptide 25	NO	−0.42	BC028855
Cholesterol biosynthesis and regulation				
Hmgcr	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	−1.49	NO	NM_008255
Insig1	Insulin induced gene 1	−1.16	−1.27	NM_153526
Mvd	Mevalonate (diphospho) decarboxylase	−1.14	NO	NM_138656
Fdft1	Farnesyl diphosphate farnesyl transferase 1, Squalene synthase	−1.10	NO	NM_010191
Acas2	Acyl-CoA synthetase short-chain family member 2	−1.03	−1.45	NM_019811
Sc4mol	Sterol-C4-methyl oxidase-like	−1.02	NO	NM_025436
Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	−0.83	NO	NM_145942
Sc5d	Sterol-C5-desaturase homolog	−0.59	−0.60	BC024132
Cholesterol, bile acid, steroid homeostasis				
Cyp8b1	Cytochrome P450, family 8, subfamily b, polypeptide 1	−1.51	−1.49	NM_010012
Ldlr	Low density lipoprotein receptor	−1.25	−0.63	BC019207
SLC10A1	Solute carrier family 10 (sodium/bile acid cotransporter family), member 1	−0.96	−0.75	BC094023
Slco1b2	Solute carrier organic anion transporter family, member 1b2	−0.88	−0.80	NM_020495
Cyp7a1	Cytochrome P450, family 7, subfamily a, polypeptide 1	−0.71	NO	NM_007824
Slco1a1	Solute carrier organic anion transporter family, member 1a1	−0.70	−0.66	AY195868
Lip1	Lysosomal acid lipase 1	−0.60	NO	NM_021460
Acat2	Acetyl-Coenzyme A acetyltransferase 2	−0.54	−0.81	NM_009338
Abcb11	ATP-binding cassette, sub-family B (MDR/TAP), member 11	−0.32	−0.53	NM_021022
Abcg5	ATP-binding cassette, sub-family G (WHITE), member 5	NO	−0.57	NM_031884
Abcg8	ATP-binding cassette, sub-family G (WHITE), member 8	NO	−0.43	NM_026180
Cyp7b1	Cytochrome P450, family 7, subfamily b, polypeptide 1	NO	−1.06	BC038810
Cell signaling				
Nr1i3	Nuclear receptor subfamily 1, group I, member 3	−0.93	NO	NM_009803
Nr1h4	Nuclear receptor subfamily 1, group H, member 4	−0.89	NO	NM_009108
Cebpa	CCAAT/enhancer binding protein (C/EBP), α	−0.83	−0.63	BC028890
Adipor2	Adiponectin receptor 2	−0.77	NO	NM_197985
Nr2f2	Nuclear receptor subfamily 2, group F, member 2	−0.77	−0.42	NM_009697
Pcaf	P300/CBP-associated factor	−0.49	−0.62	BC082581
Nr1d2	Nuclear receptor subfamily 1, group D, member 2	NO	−0.63	NM_011584
Prkaa1	Protein kinase, AMP-activated, α1 catalytic subunit	NO	−0.27	NM_001013367
Other metabolic pathways				
Fasn	Fatty acid synthase	−1.46	NO	BC046513
Alas1	Aminolevulinic acid synthase 1	−1.02	NO	NM_020559
Ogt	O-linked *N*-acetylglucosamine (GlcNAc) transferase (UDP-*N*-acetylglucosamine:polypeptide-*N*-acetylglucosaminyl transferase)	−0.65	−0.57	BC057319

Probability of type I error $\alpha = 0.05$. NO – no differential expression was observed.

(e.g. pleiotropic effects), we tested the potential benefits of statin pretreatment on plasma lipids, the APR and organ injury. Previously, we had shown that simvastatin-pretreated mice are not protected from acrolein-induced dyslipidemia at 24 h post-exposure [5]. In the current study, using two different statin doses (40 and 80 mg/kg), simvastatin pretreatment neither affected acrolein-induced dyslipidemia nor hypoalbuminemia (Tables 5 and 6). Moreover, simvastatin failed to protect against acrolein-induced increase in stomach weight/body weight ratio (data not shown) indicating that the effects of acrolein do not depend upon HMGCR activity in either hepatic or extra-hepatic tissues further supporting that genetic targets of acrolein other than *Hmgcr* are likely more important (e.g. *Lipc*).

Table 4. Inflammation markers in water- (control) or acrolein-treated (5 mg/kg) mice

Parameter	Control	ACRO	organ/source
CRP[1]	448.7 ± 13.3	438.0 ± 19.1	Liver
SAA[1]	< 1.0	353.1 ± 166.8*	Liver
Fibrinogen[2]	2,317 ± 39	1,310 ± 542	Liver
Leptin[3]	74.9 ± 8.3	21.9 ± 8.0*	Adipose
ICAM-1[1]	30.7 ± 1.3	20.6 ± 2.7*	Vascular
VEGF[3]	2.1 ± 0.2	3.4 ± 0.3*	Vascular
IL-1ß[3]	19.6 ± 4.4	22.9 ± 5.6	Inflammatory cells
IL-6[3]	5.9 ± 1.3	20.6 ± 4.2*	Liver, inflammatory cells
IL-10[3]	8.7 ± 4.8	3.9 ± 0.1	Inflammatory cells
TNF-α[3]	9.8 ± 3.4	11.1 ± 1.6	Inflammatory cells

Values = mean ± SE; Units: 1 = (ng/mL); 2 = (μg/mL); 3 = (pg/mL); $n = 3$ mice per group. *$p < 0.05$ treatment versus control.

3.5 Effect of fasting on acrolein-induced dyslipidemia

Because acrolein treatment decreased leptin levels (appetite suppressant) and increased stomach weight, we tested the importance of food intake as a modifier of acrolein action. Previously, we showed that post-treatment fasting did not significantly alter acrolein-induced dyslipidemia (water+fasting: cholesterol, 83 ± 4 mg/dL; triglycerides, 40 ± 4 mg/dL; ACRO+fasting: cholesterol, 103 ± 6 mg/dL; triglycerides, 132 ± 25 mg/dL; $n = 5$, 5, $p < 0.05$) [5], yet we did not quantify whether fasting altered specific cholesterol fraction, peripheral organ injury or hypoalbuminemia. Post-treatment fasting significantly decreased overall stomach weight compared with ad libitum-fed mice (data not shown), but empty stomach weight was significantly greater in acrolein-fed mice (+149 ± 9% controls; $n = 5$, 5) compared with controls. Similarly, fasting did not prevent hypoalbuminemia (albumin: control: 31.88 ± 0.08 g/L; acrolein: 19.26 ± 0.05 g/L; $p < 0.05$). Under fasting conditions, acrolein significantly increased LDL cholesterol (control: 12.96 ± 0.64 mg/dL; acrolein: 35.46 ± 2.87 mg/dL; $p < 0.05$) but not HDL cholesterol (control: 61.06 ± 3.30 mg/dL; acrolein: 56.92 ± 2.64 mg/dL). These data suggest a complex interplay between direct protein–acrolein adduct formation, systemic injury and the coordinated hepatic-driven responses that are dependent on metabolic state and feeding behavior.

3.6 Chronic acrolein treatment of apoE-null mice – 8 wk study

In the chronic experiment, analysis of hepatic mRNA from apoE-null mice fed acrolein (2.5 mg/kg/d for 8 wk) yielded only two modestly up-regulated genes (i.e. *Apoc1, Abcb1a*) (data not shown). This result was in accordance with the MDS analysis, wherein the 8-wk-treated samples were grouped together and were located closer to the water control samples from both chronic and acute experiments than to the acute acrolein samples (see Fig. 3). We recently published that an 8-wk acrolein (2.5 mg/kg/d) treatment accelerated atherosclerosis, most likely via platelet activation and/or dyslipidemia [6]. We measured plasma lipids levels in apoE-null mice treated with lower doses of acrolein (0.05, 0.1, 0.5, 1 mg/kg, p.o.). Acrolein feeding significantly increased the levels of cholesterol, phospholipids and triglycerides in mice treated with 0.1 or 0.5 mg/kg doses (Fig. 4), however, no dose dependence, was observed.

4 Discussion

In this report, we present a refined time course relationship between acute oral exposure to acrolein, showing rapid modification of plasma and hepatic proteins, and subsequent alterations in hepatic regulatory genes that orchestrate acrolein-induced dyslipidemia and APR in mice. We found that acrolein down-regulates *Hmgcr* by 6 h likely via complex changes in SREBP, *Insig1* and *Insig2*, indicating that acrolein action likely begins with transcriptional changes in sterol regulatory factors. These findings highlight the complexity of acrolein action and demonstrate that exposure to food-derived aldehydes could be an important contributor to cardiovascular disease risk.

4.1 Role of plasma protein modifications

In our previous study, we found that acrolein-induced lipoprotein modifications occurs over 6–24 h of exposure [5], whereas herein we show that there are immediate acrolein-induced modifications in plasma and hepatic proteins

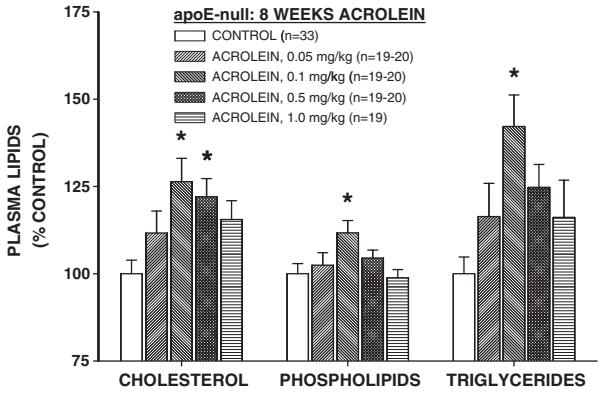

Figure 4. Effects of chronic acrolein on plasma lipids in apoE-null mice. Plasma lipids were measured in water- and acrolein- (0.05, 0.1, 0.5, 1 mg/kg/day, po) treated apoE-null mice after 8 wk of treatment. *Significant difference between control and acrolein treatments ($p < 0.05$; one-way ANOVA with Bonferroni correction).

Table 5. Effect of simvastatin (SIM; 40 mg/kg) on acrolein (ACRO) toxicity

Parameter	Control (4)	SIM (5)	ACRO (4)	SIM+ACRO (5)
Cholesterol[1]	68.8±13.2	82.5±6.9	90.7±6.0*	91.3±1.5*
Phospholipids[1]	188.6±20.9	192.0±11.7	241.4±20.8*	235.3±9.0*
Triglycerides[1]	37.4±7.9	44.6±5.8	214.0±44.0*	125.6±27.0*
Albumin[2]	29.0±2.2	32.8±1.1	20.6±2.0*	24.6±1.1*
Protein[2]	56.0±5.8	57.1±2.3	38.8±3.0*	46.6±1.7*

Adult C57BL/6 mice were gavage fed either water (control) or acrolein (ACRO; 5 mg/kg) and changes in the indicated plasma constituents were measured 24 h after treatment. Values = mean±SE; Units: 1 = (mg/dL), 2 = (g/L); (*n*), number of mice; *significant difference between ACRO or SIM+ACRO treatment and control values.

Table 6. Effect of simvastatin (SIM; 80 mg/kg) on acrolein (ACRO) toxicity

Parameter	Control (5)	SIM (6)	ACRO (6)	SIM+ACRO (6)
Cholesterol[1]	90.9±16.8	92.5±2.8	116.7±7.5*	140.9±1.7*
Phospholipids[1]	160.2±29.5	163.8±5.1	208.6±13.0*	238.4±5.4*
Triglycerides[1]	39.2±8.4	38.6±11.8	123.5±36.0*	161.6±20.6*
Albumin[2]	27.9±0.5	29.6±0.2	22.0±0.9*	19.1±1.2*
Protein[2]	39.8±0.3	41.6±1.4	34.1±1.8*	31.8±1.7*

Adult C57BL/6 mice were gavage fed either water (control) or acrolein (ACRO; 5 mg/kg) and changes in the indicated plasma constituents were measured 24 h after treatment. Values = mean±SE; Units: 1 = (mg/dL), 2 = (g/L); (*n*), number of mice; *significant difference between ACRO or SIM+ACRO treatment and control values.

(along with an abrupt yet modest increase in triglycerides). Because of a brief lag period between the peaks of plasma and hepatic protein modifications, we reason that acrolein-modified proteins could be distributed to hepatic sites. Similarly, Li et al., [18] reported the appearance of acrolein-modified plasma proteins of 100, 31, and 29 kDa at 1 h after oral acrolein exposure in Sprague–Dawley rats (9.2 mg/kg) [18]. Although we also showed that modified lipoproteins circulate for almost three days in the plasma [5], acrolein-modified proteins are rapidly removed (decreasing by 1 h) from the circulation and perhaps these are removed by the liver (e.g. one major M_r band, 150 kDa, is present in both the plasma and the liver) as well as at other sites. In a previous study, we identified an acrolein-modified ≈150 kDa protein as Complement Factor H using 2-D electrophoresis and MALDI-TOF analysis [19]. Whether or not these modified proteins are mechanistically linked to subsequent hepatic gene changes at 6 and 12 h or dyslipidemia is unknown and will serve as a focus of follow-up studies.

4.2 Hepatic gene changes orchestrate acrolein-induced dyslipidemia

Our previous analyses of limited hepatic genes changes implicated a locus of acrolein action via down-regulation of both hepatic lipase (*Lipc*) and *Ldlr* gene mRNAs at 6 h post-treatment [5]. Our current study complements and extends those measurements to implicate an important role of the upstream sterol synthesis regulators, insulin-induced genes 1 and 2 (*Insig1* and *Insig2*), that control cholesterol biosynthetic and trafficking pathways via interactions with SREBP. Acrolein feeding down-regulates *Insig1* but up-regulates *Insig2* at both 6 and 12 h time points (see Tables 2 and 3). *Insig* proteins anchor SREBP and SREBP cleavage activating protein (SCAP) in the ER in a cholesterol-dependent manner, and when cholesterol is low, INSIG is degraded and SREBP translocates to the nucleus to activate transcription [20]. It is known that SREBP promotes *Hmgcr* and *Insig1* transcription, yet both these genes are down-regulated by acrolein. However, *Insig2* is believed to play a more important role than *Insig1* in the liver [20]. As cholesterol is the primary negative feedback regulator of SREBP by binding to SCAP [21], these gene data are consistent with our previous finding that acrolein significantly increases hepatic cholesterol level at 6 h post-treatment [5], and thus, initiates a negative feedback response. Similarly, rats fed thermally oxidized sunflower oil (~18 mL/kg; 6d; significantly elevated carbonyl content of ~32-fold) had significantly lower hepatic *Hmgcr* and *Ldlr* mRNA levels compared with rats fed fresh sunflower oil [22]. Thus, carbonyl-containing compounds, such as aldehydes, can dramatically alter the hepatic transcriptome perhaps through inducing ER stress as demonstrated for acrolein in other tissues [23].

It is not clear how acrolein increases hepatic cholesterol while down-regulating *Hmgcr* and *Insig1* mRNAs, but it appears this is not HMGCR activity-dependent. We tested two doses of simvastatin (40 and 80 mg/kg) and statin treatment neither blocked acrolein-induced dyslipidemia

nor acrolein-induced gastric injury nor hypoalbuminemia. Collectively, these data indicate that acrolein feeding induces a coordinated transcriptionally regulated decrease in cholesterol biosynthesis genes. Thus, we think that aldehydes in foods and beverages [24] could, in general, suppress hepatic cholesterol biosynthesis and uptake genes via a common transcriptional mechanism dependent on negative feedback. This idea is partially supported by the modifying effect of fasting on acrolein-induced dyslipidemia, which led to an increase in plasma cholesterol in the LDL but not the VLDL fraction [5].

4.3 Hepatic *Cyp* genes and xenobiotic metabolism

Not surprisingly, numerous *Cyp* genes were dysregulated by acrolein treatment at 6 h and 12 h, but the 2b group of *Cyp* genes is consistently up-regulated at both 6 and 12 h. These genes are classified as xenobiotic metabolism genes and we showed previously that indeed CYP2B and 3A family member proteins catalyze unsaturated aldehyde oxidation to carboxylic acid [25]. The *Cyp2b* family genes are induced by compounds that activate the constitutive androstane receptor (CAR), and obesity is known to ameliorate expression of these genes [26]. Similarly, other *Cyp2* and *Cyp3* family genes are down-regulated by interleukins and inflammation [27, 28], and thus, these results suggest that acrolein feeding regulates these genes in a manner similar to the effects of inflammation or obesity. The role of AP1 proteins in down-regulation of these genes has been suggested by Blouin and co-workers [26, 27]. Because these proteins, in general, are likely part of a larger contingent of protective enzymes that are up-regulated in liver following xenobiotic exposure, amelioration of their expression may enhance liver cell toxicity. Paradoxically, the suppression of CAR expression is in contrast to the increase in *Cyp2b* family observed and different from the observations of Blouin and coworkers who observed suppression of *Cyp2b* family member expression in the obese mouse [26, 27]. Induction of *Cyp2b* in murine liver by agents like phenobarbital results in increased mRNA and protein levels, so the contradiction suggests that another mode of regulation of this gene also occurs, such as phosphorylation by protein kinases, such as AMP kinase [29]. Because of the specific focus of the Steroltalk microarray on sterol metabolism genes, we did not measure many phase II xenobiotic metabolism genes (e.g. GSTs, aldehyde reductases), which could be up-regulated in apoE-null mice by chronic acrolein feeding, and thus, could have diminished the sterol signal for these acute gene changes.

Similarly, many of the CYPs involved in bile acid synthesis are suppressed during inflammation [30]. Specifically, LPS suppresses bile acid formation by specifically decreasing expression of *Cyp7a* and *Cyp27a*, the rate limiting enzymes in conversion of cholesterol to bile acids. TNF-α and Il-1 have been shown to decrease *Cyp27a* levels in HepG2 cells demonstrating direct regulation of these genes in mice and humans. This decrease is also observed in our study of acrolein action, which further supports that acrolein feeding activates an inflammatory process.

4.4 Hepatic gene changes reflect an APR

Acrolein induces an APR as evidenced by elevated SAA and sustained hypoalbuminema up to 24 h. These changes appear to be a consequence of up-regulated hepatic *Saa* genes. Other APR genes were also up-regulated including fibrinogen (*Fgb*) and serum amyloid P (*Apcs*), although the former change did not result in increased plasma fibrinogen (measured at 24 h), and unexpectedly, plasma fibrinogen is significantly lower in acrolein-treated mice compared with water controls. This discrepancy could be explained because acrolein exposure (feeding or inhalation) increases platelet activation, and thus, an increased clotting (perhaps in stomach wall) could effectively lower the plasma level of fibrinogen due to platelet–fibrinogen interactions [31]. Additional evidence for a vascular inflammatory event includes significant changes in plasma sICAM-1, VEGF and IL-6 (see Table 4), which could also provide additional stimuli for hepatic gene changes.

4.5 Concluding remarks

Exposure to acrolein and related aldehydes is a major health concern [32, 33]. Environmental sources of acrolein include industrial emissions, automobile exhaust, combustion sources and consumables (foods and beverages) [34]. Aldehydes such as acrolein, crotonaldehyde and hexenal are natural constituents of several foods and spices and their concentration in food and drink is increased upon oxidation, brewing, heating, cooking and frying (see summary in [5]) [19]. Moreover, aldehyde exposure via ingestion, including acrolein, is likely to exceed that due to inhalation alone [4]. Hence our observation that oral intake of acrolein induces dyslipidemia in mice raises important issues regarding pollutants and constituents of diet and cardiovascular disease risk. Although the human risk due to acrolein exposure remains to be assessed, the results of the current study suggest the possibility that exposure to acrolein or similarly reactive constituents of foods could contribute to sustained dyslipidemia and inflammation that increase CVD risk.

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