Supplemental Materials

Endogenous Cholesterol Ester Hydroperoxides Modulate Cholesterol Levels and Inhibit Cholesterol Uptake in Hepatocytes and Macrophages

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Supplemental Methods

Cholesterol efflux assay

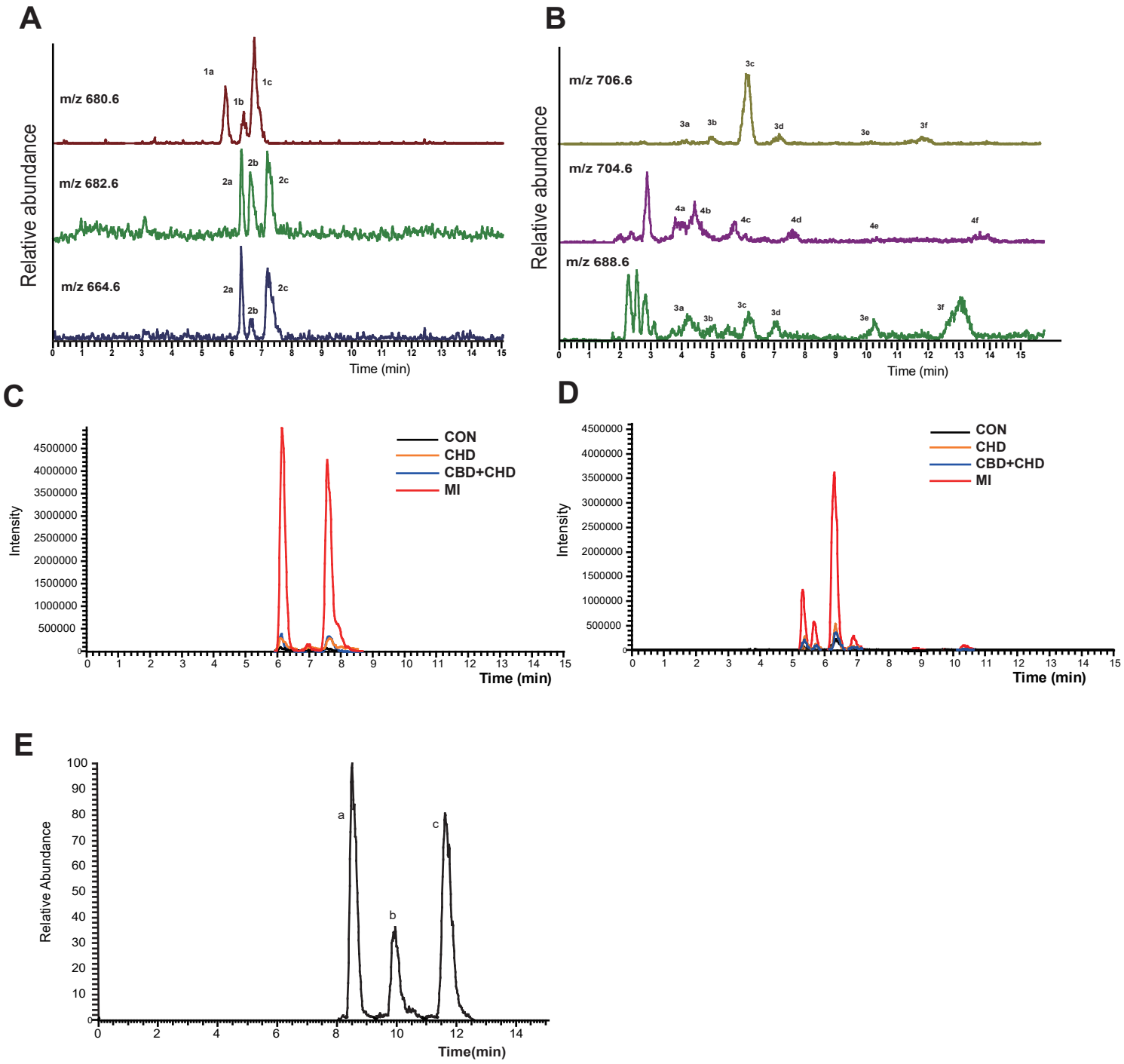
After cells were treated with 50μg/ml oxLDL and 15μg/ml chol-d7 for 24h, the cells were stimulated with control and 10μg/ml ch-13(*c,t*)-HpODE for 24h, then cholesterol efflux of the cells was induced by 50μg/ml HDL. After 24h, the total chol-d7 in media were measured for cholesterol efflux assay by GC-MS. And cells were collected for quantification. The extraction and detection method of media were the same as the method of cholesterol uptake assay. Final results were normalized to protein contents of cell lysates. The experiments were carried out by at least three independent experiments.

Animal experiments

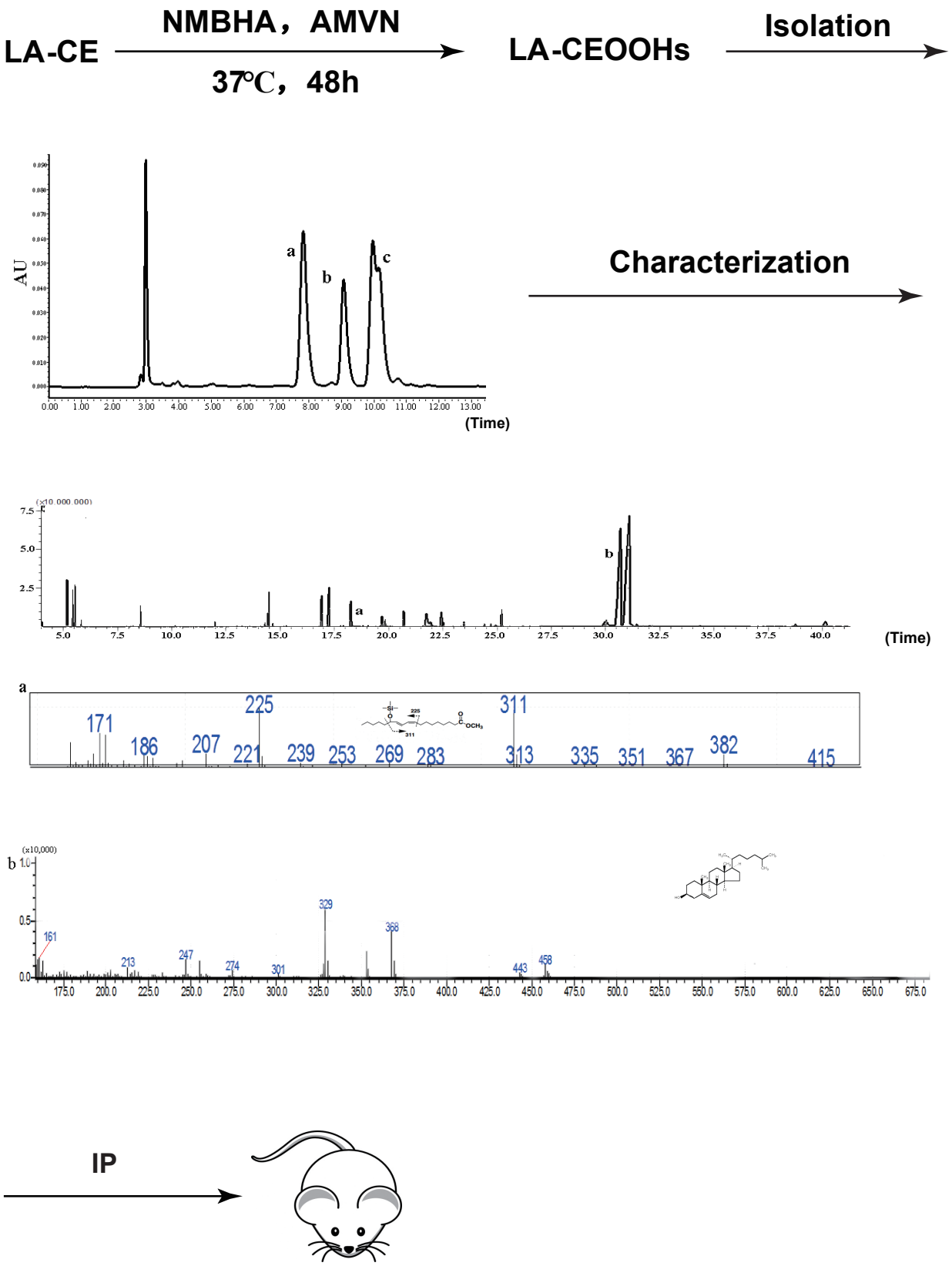
After treating mice with compounds, the plasma and livers were collected. For liver histological examination, the H&E staining was performed. And the parameters ALT or AST were detected following the manufacturers' instructions (ALT/AST detection reagent kit, China).

Supplemental Table S1 The primer information of qRT-PCR

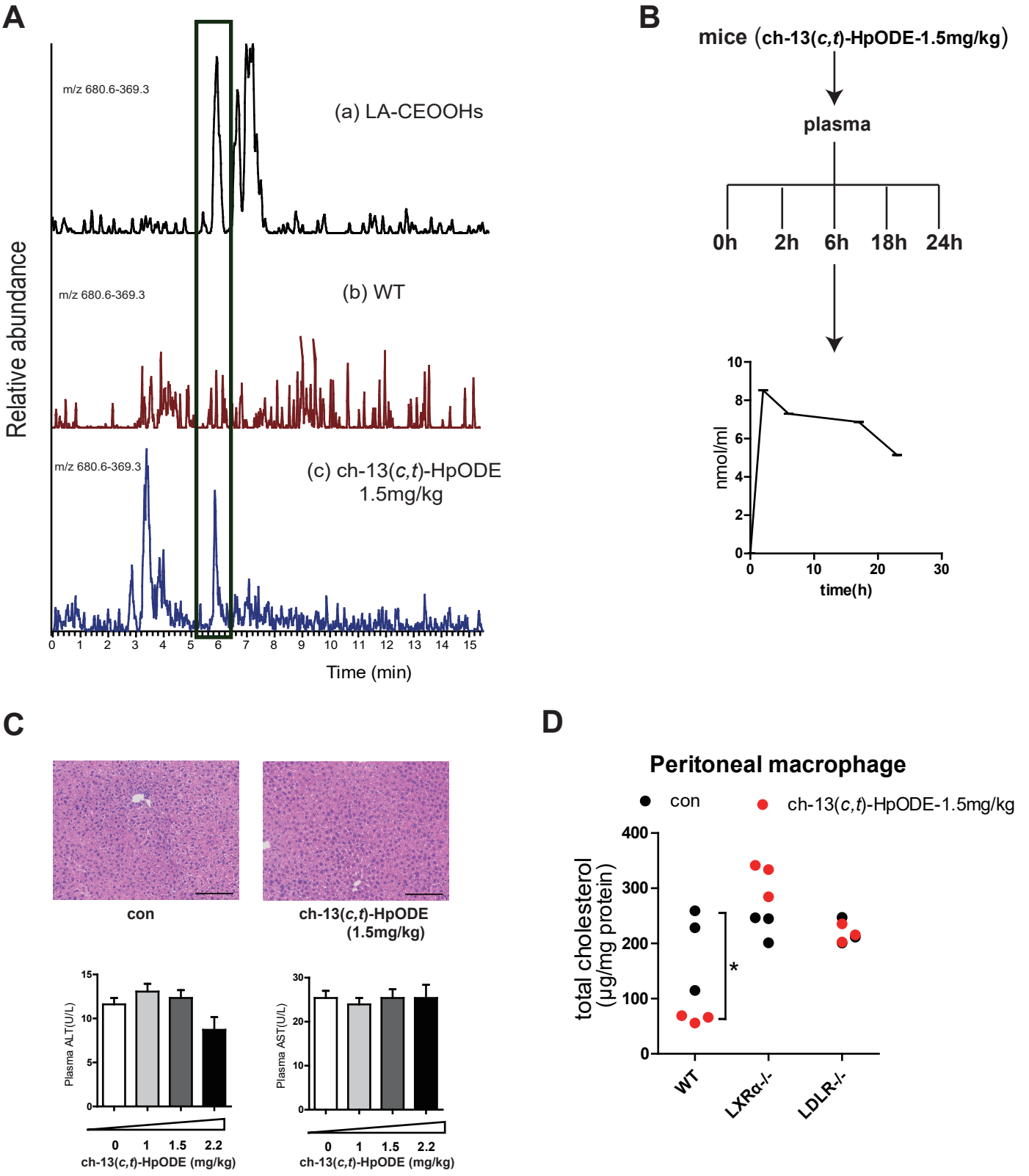
Gene (mouse)	Forward (5’-3’)	Reverse (5’-3’)
L32	TTAAGCGAA ACTGGCGGAAAC	TTGTTGCTCCCATAACCGATG
β-actin	CCACAGCTGAGAGGGAAATC	AAGGAAGGCTGGAAAAGAGC
Ldlr	TGACTCAGACGAACAAGGCTG	ATCTAGGCAATCTCGGTCTCC
Idol	AGCATGTCCAGCACGTCTAC	ATACTGCAGCTTGTGGGGAC



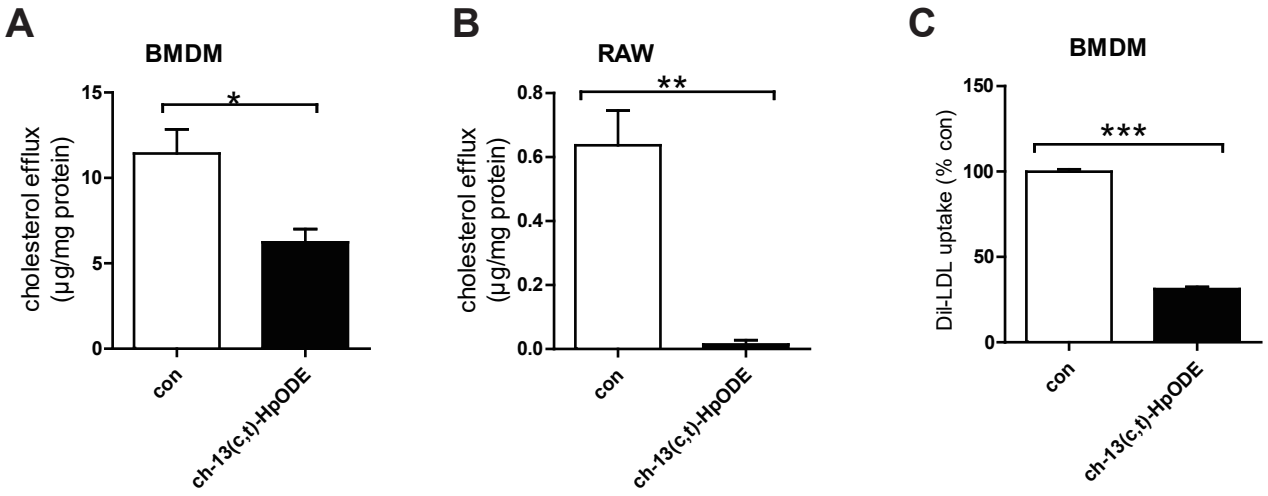
Supplemental Figure S1: Analysis of oxCEs in synthetic standard, human plasma and atherosclerotic plaque. A-B. MRM chromatogram of oxLA-CE (A) and oxAA-CE (B) in synthetic standards, the chemical structures and MS details were shown in Table 2. C. MRM chromatogram (m/z=682.6-369.3) of LA-CEOHs were analyzed in four groups of human plasma: CON, control; CHD, coronary heart disease; CBD: cerebrovascular disease; MI: myocardial infarction. These peaks represented ch-13(*c,t*)-HODE, ch-13(*t,t*)-HODE, ch-9-HODE (included two isomers). D. MRM chromatogram (m/z=688.6-369.3) of AA-CEOHs were measured in four groups of human plasma. These peaks represented ch-15, 12, 11, 9, 8, 5-HETE. E. MRM chromatogram (m/z=682.6-369.3) of LA-CEOHs detected in human atherosclerotic plaque. Peak (a-c) represented ch-13(*c,t*)-HODE, ch-13(*t,t*)-HODE, ch-9-HODE (included two isomers).



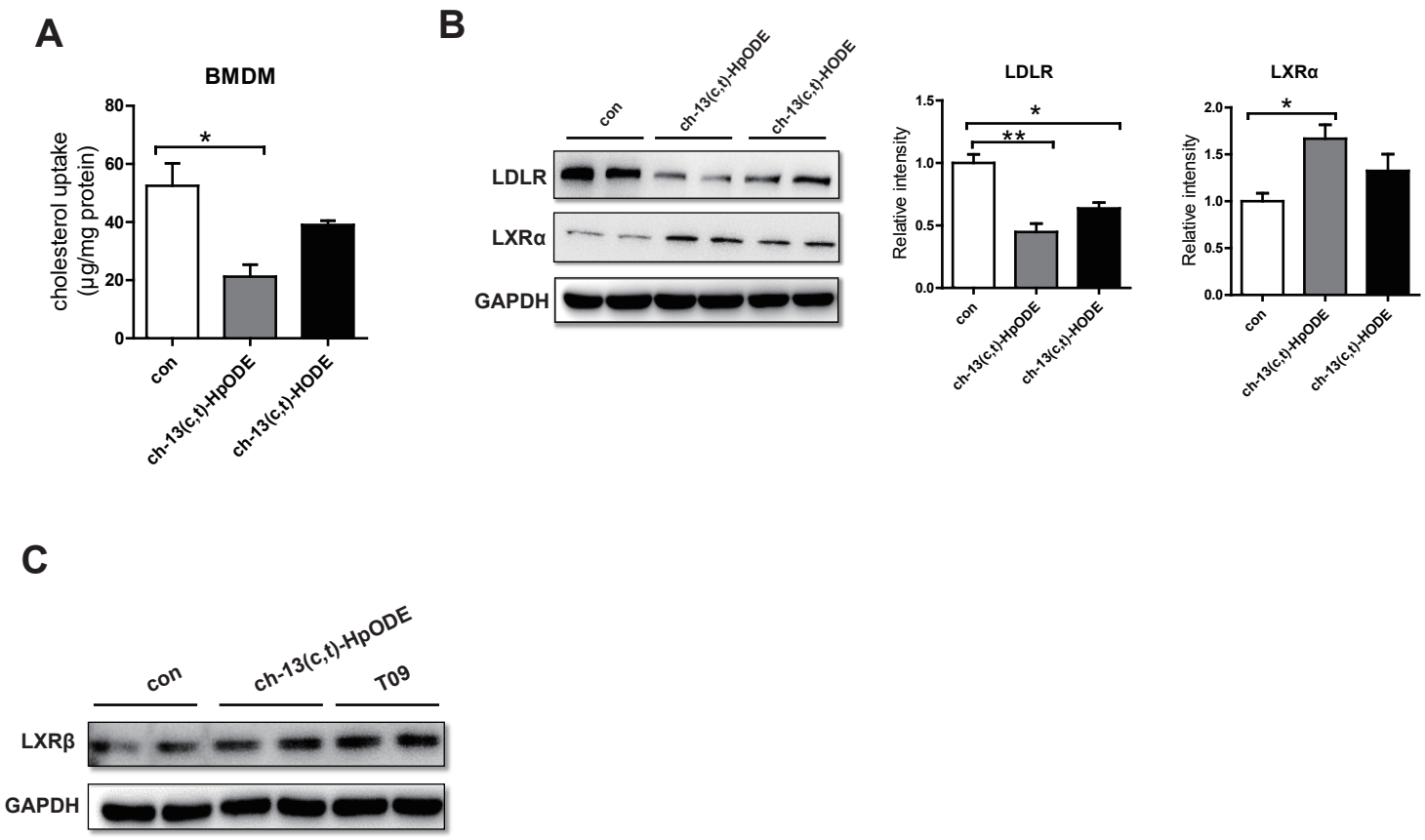
Supplemental Figure S2：**Formation, isolation and characterization of ch-13(*c,t*)-HpODE.** Formation (the first part): NMBHA was used as hydrogen atom donor in the oxidation and AMVN was used as free radical initiator. Isolation (the second part): chromatograms of oxLA-CE by HPLC, these peaks (a-c) were ch-13(*c,t*)-HpODE, ch-13(*t,t*)-HpODE, ch-9-HpODE (included two isomers) respectively. Characterization (the third part): Peak (a, in the middle panel) represented TMS-derivatized HODE methyl ester (RT=18.2 min, m/z 225, 311), peak (b, the bottom panel) represented cholesterol (RT=30.5 min, m/z 329, 368, 458). In the animal experiments, mice were injected with purified ch-13(*c,t*)-HpODE.



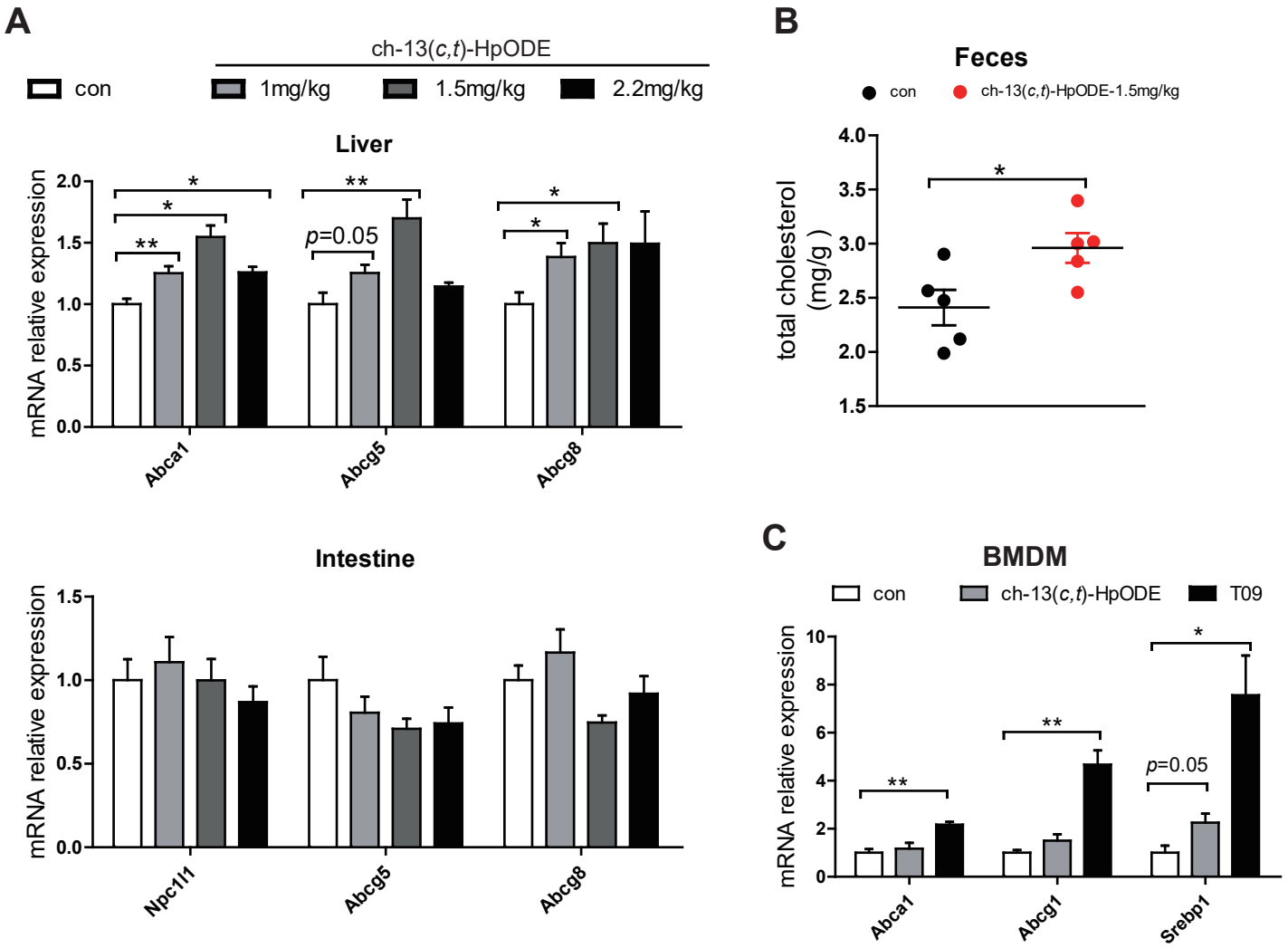
Supplemental Figure S3: **Analysis of ch-13(*c,t*)-HpODE in mice plasma and the effects of ch-13(*c,t*)-HpODE on liver and peritoneal macrophages.** A. MRM chromatogram of oxLA-CE in mice plasma. Panel (a): LA-CEOOHs in synthetic standard , the three peaks represented ch-13(*c,t*)-HpODE, ch-13(*t,t*)-HpODE, ch-9-HpODE (included two isomers). Panel (b): the analysis of LA-CEOOHs in plasma from wide type C57BL/6 mice. Panel (c): the analysis of LA-CEOOHs in plasma from 1.5mg/kg ch-13(*c,t*)-HpODE-treated mice. The peak of ch-13(*c,t*)-HpODE was shown in a green frame at m/z=680.6-369.3 (RT=5.8min). B. Experimental design: after treating mice (n=3) with 1.5mg/kg ch-13(*c,t*)-HpODE, the plasma was collected and pooled at different time before the levels of ch-13(*c,t*)-HpODE were detected. C. Histological staining (H&E staining, bar=50μm) of mouse liver after treating mice with 1.5mg/kg ch-13(*c,t*)-HpODE and biochemical analysis (ALT and AST) in plasma (n=5, duplicates). D. Total cholesterol was detected in peritoneal macrophages of WT, LXRα-/- and LDLR-/- mice (3 independent experiment) after treating with ch-13(*c,t*)-HpODE. Con:control. Statistical analyses, one or two-way ANOVA and unpaired t test. * $p < 0.05$.



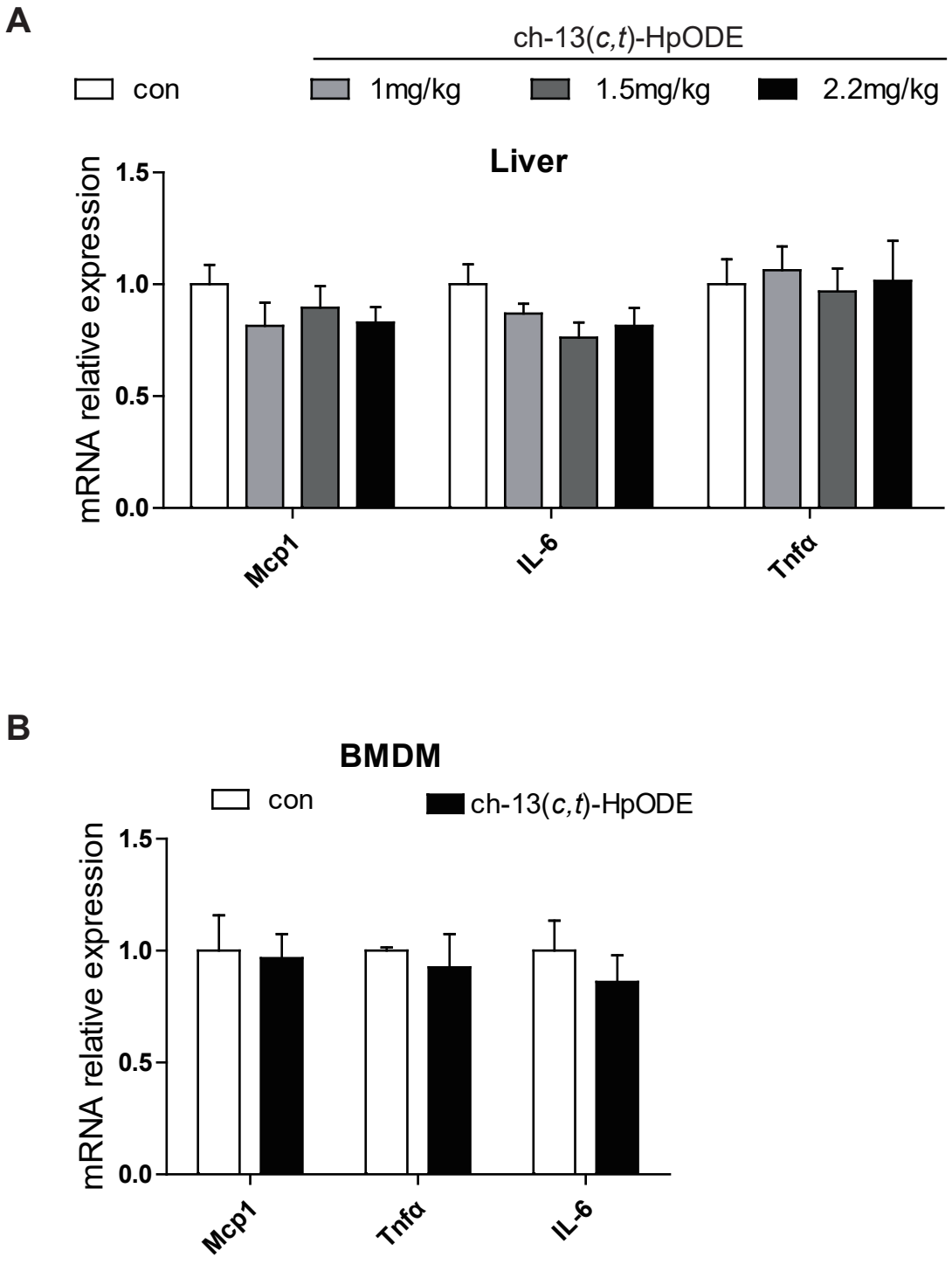
Supplemental Figure S4: **c h-13(*c,t*)-HpODE decreased cholesterol efflux and LDL uptake in BMDMs.** A-B. cholesterol efflux assay: after treating 50μg/ml oxLDL and 15μg/ml chol-d7 for 24h, BMDMs(A) and RAW264.7(B) were treated with con or 10μg/ml ch-13(*c,t*)-HpODE for 24h, then 50μg/ml HDL induced cholesterol efflux and chol-d7 in medium was quantified by GC-MS. C. BMDMs was cultured with con or 10μg/ml ch-13(*c,t*)-HpODE for 30min, then 20μg/ml Dil-LDL treating another 6h, and cells were washed and collected for Dil-LDL uptake assay. Data are expressed as the mean±SEM. and representative of ≥3 independent experiments. Statistical analyses, unpaired t test. * $p < 0.05$, ** $p < 0.01$, *** $p<0.001$.



Supplemental Figure S5: **The effect on cholesterol uptake and the protein expressions of ch-13(*c,t*)-HpODE and ch-13(*c,t*)-HODE in macrophages.** A. Total chol-d7 was detected in BMDMs after treating with control, 10 μg/ml ch-13(*c,t*)-HpODE and 10 μg/ml ch-13(*c,t*)-HODE for 24h (3 independent experiments). B. After treating with control, 10 μg/ml ch-13(*c,t*)-HpODE and 10 μg/ml ch-13(*c,t*)-HODE, cell lysates were analyzed by Western blot. C. The protein expression of LXRβ was measured in BMDMs. ch-13(*c,t*)-HpODE (10 μg/ml), T09 (5μM). Data are expressed as the mean±SEM. and representative of ≥3 independent experiments. Statistical analyses, one-way ANOVA and unpaired t test. * $p < 0.05$, ** $p < 0.01$.



Supplemental Figure S6: The gene expression of liver, intestine, BMDMs and the cholesterol level in feces after treating with ch-13(*c,t*)-HpODE. A. The gene expression in liver and intestine after treating mice with ch-13(*c,t*)-HpODE. B. The total cholesterol content in feces after treating mice with ch-13(*c,t*)-HpODE (n=5, triplicates). C. The gene expression in BMDMs after treating with ch-13(*c,t*)-HpODE. Data are expressed as the mean±SEM. and representative of ≥3 independent experiments in duplicate. Statistical analyses, one-way ANOVA and unpaired t test. * $p < 0.05$, ** $p < 0.01$.



Supplemental Figure S7: The inflammatory gene expressions in liver and BMDMs after ch-13(*c,t*)-HpODE treatment. A-B. The inflammatory gene expressions in liver (A, n=5) and BMDMs (B) after treating with ch-13(*c,t*)-HpODE. Data are expressed as the mean±SEM. and representative of ≥3 independent experiments in duplicate. Statistical analyses, one-way ANOVA or unpaired t test.