Prevention of Diet-Induced Metabolic Dysregulation, Inflammation, and Atherosclerosis in *Ldlr*$^{-/-}$ Mice by Treatment With the ATP-Citrate Lyase Inhibitor Bempedoic Acid

Joshua P. Samsoondar, Amy C. Burke, Brian G. Sutherland, Dawn E. Telford, Cynthia G. Sawyez, Jane Y. Edwards, Stephen L. Pinkosky, Roger S. Newton, Murray W. Huff

Objective—Bempedoic acid (ETC-1002, 8-hydroxy-2,2,14,14-tetramethylpentadecanedioic acid) is a novel low-density lipoprotein cholesterol–lowering compound. In animals, bempedoic acid targets the liver where it inhibits cholesterol and fatty acid synthesis through inhibition of ATP-citrate lyase and through activation of AMP-activated protein kinase. In this study, we tested the hypothesis that bempedoic acid would prevent diet-induced metabolic dysregulation, inflammation, and atherosclerosis.

Approach and Results—*Ldlr*$^{-/-}$ mice were fed a high-fat, high-cholesterol diet (42% kcal fat, 0.2% cholesterol) supplemented with bempedoic acid at 0, 3, 10 and 30 mg/kg body weight/day. Treatment for 12 weeks dose-dependently attenuated diet-induced hypercholesterolemia, hypertriglyceridemia, hyperglycemia, hyperinsulinemia, fatty liver and obesity. Compared to high-fat, high-cholesterol alone, the addition of bempedoic acid decreased plasma triglyceride (up to 64%) and cholesterol (up to 50%) concentrations, and improved glucose tolerance. Adiposity was significantly reduced with treatment. In liver, bempedoic acid prevented cholesterol and triglyceride accumulation, which was associated with increased fatty acid oxidation and reduced fatty acid synthesis. Hepatic gene expression analysis revealed that treatment significantly increased expression of genes involved in fatty acid oxidation while suppressing inflammatory gene expression. In full-length aorta, bempedoic acid markedly suppressed cholesteryl ester accumulation, attenuated the expression of proinflammatory M1 genes and attenuated the *iNos*/*Arg1* ratio. Treatment robustly attenuated atherosclerotic lesion development in the aortic sinus by 44%, with beneficial changes in morphology, characteristic of earlier-stage lesions.

Conclusions—Bempedoic acid effectively prevents plasma and tissue lipid elevations and attenuates the onset of inflammation, leading to the prevention of atherosclerotic lesion development in a mouse model of metabolic dysregulation.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2017;37:647-656. DOI: 10.1161/ATVBAHA.116.308963.)**

Key Words: adenosine triphosphate citrate lyase ■ atherosclerosis ■ inflammation ■ lipids

Cardiovascular disease continues to be a major cause of death in developed countries, despite advances that have been made in lipid management therapy. Large-scale clinical trials have consistently shown that lowering plasma low-density lipoprotein (LDL) cholesterol (C) concentrations reduces cardiovascular disease mortality.[1] Statins have proven efficacy in lowering LDL-C and preventing cardiovascular disease events. Despite this, many at-risk patients are either unable to achieve their target LDL-C levels or are intolerant to statins because of side effects.[2,3] This leaves a large group of individuals with significant residual risk, indicating a great need for additional therapeutics including novel lipid-lowering medications.

ATP citrate lyase (ACLY), a cytoplasmic enzyme responsible for the generation of acetyl coenzyme A (acetyl-CoA) for the de novo synthesis of fatty acids (FAs) and cholesterol, has recently emerged as a promising therapeutic target for the reduction of plasma lipids.[4] The ACLY inhibitor bempedoic acid (ETC-1002, 8-hydroxy-2,2,14,14-tetramethylpentadecanedioic acid, BemA) was identified from the evaluation of a series of long hydrocarbon chain diacids for lipid-regulating activity.[5] Results from clinical trials up to Phase IIb have consistently shown as much as a 30% reduction in LDL-C and up to 40% lowering of high-sensitivity C-reactive protein.[4,6–9] In hypercholesterolemic patients on stable statin therapy, BemA treatment at 180 mg/d yielded 24% incremental reductions in LDL-C, indicating a complementary mechanism of action to statins.[10]

In rodents, BemA targets 2 distinct molecular pathways within liver: ACLY and AMP-activated protein kinase

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From the Molecular Medicine Research Laboratory, Robarts Research Institute (J.P.S., A.C.B., B.G.S., D.E.T., C.G.S., J.Y.E., M.W.H.), Department of Biochemistry (J.P.S., A.C.B., M.W.H.), and Department of Medicine (D.E.T., C.G.S., J.Y.E., M.W.H.), The University of Western Ontario, London, Canada; and Esperion Therapeutics Inc, Ann Arbor, MI (S.L.P., R.S.N.).

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Correspondence to Murray W. Huff, PhD, Robarts Research Institute, Room 4222, The University of Western Ontario, 1151 Richmond St N, London, Ontario, Canada N6A 5B7. E-mail mhuff@uwo.ca

Nonstandard Abbreviations and Acronyms	
ACC	acetyl-CoA carboxylase
ACLY	ATP citrate lyase
Acox1	Acyl-CoA oxidase 1
AMPK	AMP-activated protein kinase
Arg1	arginase 1
BemA	bempedoic acid
CE	cholesteryl ester
Cpt1a	carnitine palmitoyl transferase 1 α
CoA	coenzyme A
FA	fatty acid
FC	free cholesterol
Fasn	fatty acid synthase
HFHC	high-fat, high-cholesterol
LDL	low-density lipoprotein
Srebf	sterol regulatory element–binding factor
TC	total cholesterol
Tnf	tumor necrosis factor

(AMPK).[11] ACLY inhibition by BemA in rat hepatocytes rapidly reduces the levels of citrate-derived acetyl-CoA, the final common substrate for both FA and cholesterol synthesis.[11] ACLY is directly inhibited by a CoA thioester of BemA,[11] a reaction catalyzed by a liver-specific very long chain acyl-CoA synthetase-1 (ACSVL1).[12] In rodents, BemA-CoA activates AMPK in a liver kinase β1-dependent manner, without change in adenylate charge.[11,12] Activation of AMPK by BemA phosphorylates and inhibits acetyl-CoA carboxylase (ACC) and hydroxymethylglutaryl-CoA reductase, contributing to the inhibition of both FA and cholesterol synthesis.[11] ACC inhibition depletes the malonyl-CoA pool for FA synthesis and relieves inhibition of Cpt1α-mediated FA β-oxidation by malonyl-CoA.

In rats and hamsters, BemA treatment increased hepatic protein expression of peroxisome proliferator–activated receptor γ coactivator 1-α and elevated plasma levels of β-hydroxybutyrate, suggesting increased FA β-oxidation.[11] In high-fat–fed hamsters or mice, short-term BemA treatment decreased plasma cholesterol and triglycerides, plasma glucose and insulin, and hepatic fat content.[11] In murine macrophages and adipose tissue explants, BemA downregulated proinflammatory signaling pathways.[6,13] These results suggest BemA would be effective in the attenuation of metabolic dysregulation and inflammation, and prevent the development of atherosclerosis.

The objective of this study was to determine the impact of BemA on metabolic dysregulation, inflammation and atherosclerosis in a mouse model of the metabolic syndrome, namely the *Ldlr*$^{-/-}$ mouse fed a high-fat, cholesterol-containing diet. BemA treatment attenuated the elevation of plasma lipids, glucose, and insulin, and improved glucose tolerance. Reduced hepatic lipids were accompanied by suppressed inflammation. Lower aortic cholesteryl ester content and diminished aortic inflammation were associated with marked attenuation in atherosclerosis development.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Bempedoic Acid Attenuates Weight Gain and Adipose Tissue Accumulation in High-Fat, High-Cholesterol-Fed *Ldlr*$^{-/-}$ Mice

Eight-week-old male *Ldlr*$^{-/-}$ mice were fed chow or a high-fat, high-cholesterol–containing diet (HFHC; 42% kcal fat, 0.2% cholesterol) supplemented with daily doses of 0, 3, 10, or 30 mg of BemA per kg body weight (mg/kg), for 12 weeks. The HFHC diet increased weight gain compared with chow-fed mice, which was partially prevented by treatment with 30 mg/kg per day of BemA (Figure 1A). By 12 weeks, body weight was significantly less (−5%) than mice fed HFHC alone. At euthanization, the 4.5-fold increase in epididymal fat pad weight induced by the HFHC diet was significantly attenuated (−29%) by BemA at 30 mg/kg per day (Figure 1B). Micro-computed tomographic imaging revealed that total body fat significantly increased 3.1-fold in HFHC-fed mice compared with chow. BemA at 30 mg/kg per day decreased total adipose tissue content (−18%; Figure 1C; Figure IA in the online-only Data Supplement). There was no difference in caloric intake among mice receiving the HFHC diet, with or without BemA (Figure IB in the online-only Data Supplement). The 2 lower doses of BemA had no significant effect on body weight or adipose tissue mass.

Bempedoic Acid Decreases Diet-Induced Dyslipidemia

The HFHC diet induced significant hypertriglyceridemia and hypercholesterolemia by 4 weeks, which persisted throughout the study. By week 12, plasma cholesterol was significantly lower in BemA-treated mice at both 10 (−29%) and 30 mg/kg per day (−41%; Figure 1D). At week 12, plasma triglycerides were significantly lower in mice treated with 3 (−33%), 10 (−37%), and 30 mg/kg per day (−52%; Figure 1E). The reduced plasma cholesterol in BemA-treated mice (30 mg/kg per day) was because of significant reductions in very low–density lipoprotein cholesterol (VLDL-C; −58%) and LDL-C (−26%; Figure 1F). Plasma triglyceride reduction was confined to VLDL with a decrease of 49% at the 30 mg/kg per day dose (Figure 1G).

Bempedoic Acid Improves Glucose Metabolism and Insulin Sensitivity

The moderately elevated levels of blood glucose induced by the HFHC diet were significantly decreased by BemA at 30 mg/kg per day (−14%; Figure 1H). The hyperinsulinemia in HFHC-fed mice was markedly attenuated with BemA at 10 (−23%) and 30 mg/kg per day (−50%; Figure 1I). Glucose and insulin tolerance tests revealed a significant dose-dependent improvement in glucose tolerance at 10 and 30 mg/kg per day, whereas insulin tolerance was unaffected by HFHC or BemA (Figure IC and ID in the online-only Data Supplement).

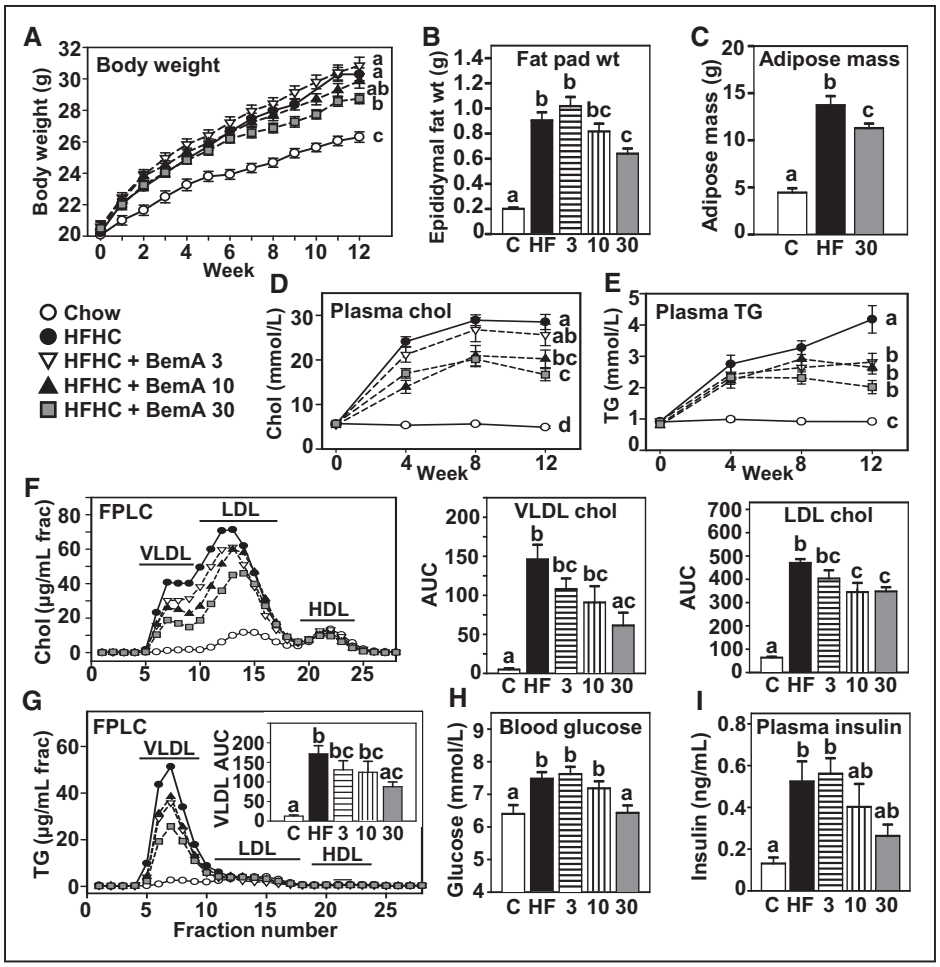

Figure 1. Bempedoic acid improves diet-induced dyslipidemia. *Ldlr*$^{-/-}$ mice were fed chow, or a high-fat, high-cholesterol (HFHC) diet supplemented with bempedoic acid (BemA, 0, 3, 10, or 30 mg/kg per d) for 12 wk. **A**, Body weight (n=22–24/group). **B**, Epididymal fat pad weight (n=22–24/group). **C**, Whole body adipose tissue mass assessed by micro-computed tomography (n=8/group). **D** and **E**, Plasma cholesterol (chol) and triglyceride (TG) concentrations at weeks 0, 4, 8, and 12 (n=12/group). **F** and **G**, Cholesterol and TG plasma concentrations (determined in eluted fractions of plasma separated by fast protein liquid chromatography [FPLC] at week 12 with area under the curve [AUC]) determinations (n=7–19/group). **H**, Fasting blood glucose concentrations and (**I**) fasting plasma insulin concentrations at 12 weeks (n=12/group). Data are presented as mean±SEM. Different letters indicate significant differences (P<0.05). Letters indicating statistical significance for **A**, **D**, and **E** apply only to the 12-week data. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; and VLDL, very low–density lipoprotein.

Effect of Bempedoic Acid on Diet-Induced Hepatic Steatosis, Gene Expression, and Lipid Metabolism

In liver, the HFHC diet induced significant increases in total cholesterol (TC, 4.3-fold), free cholesterol (FC; 1.5-fold) and cholesteryl ester (CE, 16-fold; Figure 2A). Liver TC, FC, and CE were significantly lower with BemA at 10 (−54%, −23%, and −65%) and 30 mg/kg per day (−60%, −33%, and −70%). The HFHC diet-induced increase in liver triglyceride content (2.2-fold) was significantly lower with BemA treatment at 10 (−40%) and 30 mg/kg per day (−52%; Figure 2B). This is consistent with histological analyses of liver sections, which revealed that treatment substantially decreased neutral lipid staining (Figure IIA in the online-only Data Supplement). Liver weight increased dose-dependently with BemA treatment (up to 1.25-fold at 30 mg/kg per day; Figure IIB and IIC in the online-only Data Supplement), and together with increased *Acox1* expression (see below) indicates modest peroxisome proliferation. Plasma concentrations of the liver enzymes aspartate aminotransferase and alanine aminotransferase were not different between groups (Figure IID in the online-only Data Supplement).

To understand the molecular mechanisms underlying the metabolic improvements from BemA treatment, select hepatic gene and protein expressions were examined. Activation of AMPK was assessed by immunoblot for phosphorylated (p) AMPK and its target ACC (Figure 2C and 2D). Both pAMPK/tAMPK and pACC/tACC were suppressed in HFHC-fed mice compared with chow. Treatment at 30 mg/kg per day significantly increased pAMPK/tAMPK (1.6-fold) and pACC/tACC (1.3-fold) relative to HFHC alone. Compared to HFHC-fed mice, total AMPK was decreased and total ACC was increased with treatment at 30 mg/kg per day (Figure 2C). At 30 mg/kg per day, BemA had no effect on *Ppara* mRNA, whereas *Cpt1a* and *Acox1* expression were significantly increased (1.3- and 3-fold, respectively) consistent with the induction of hepatic FA oxidation in both

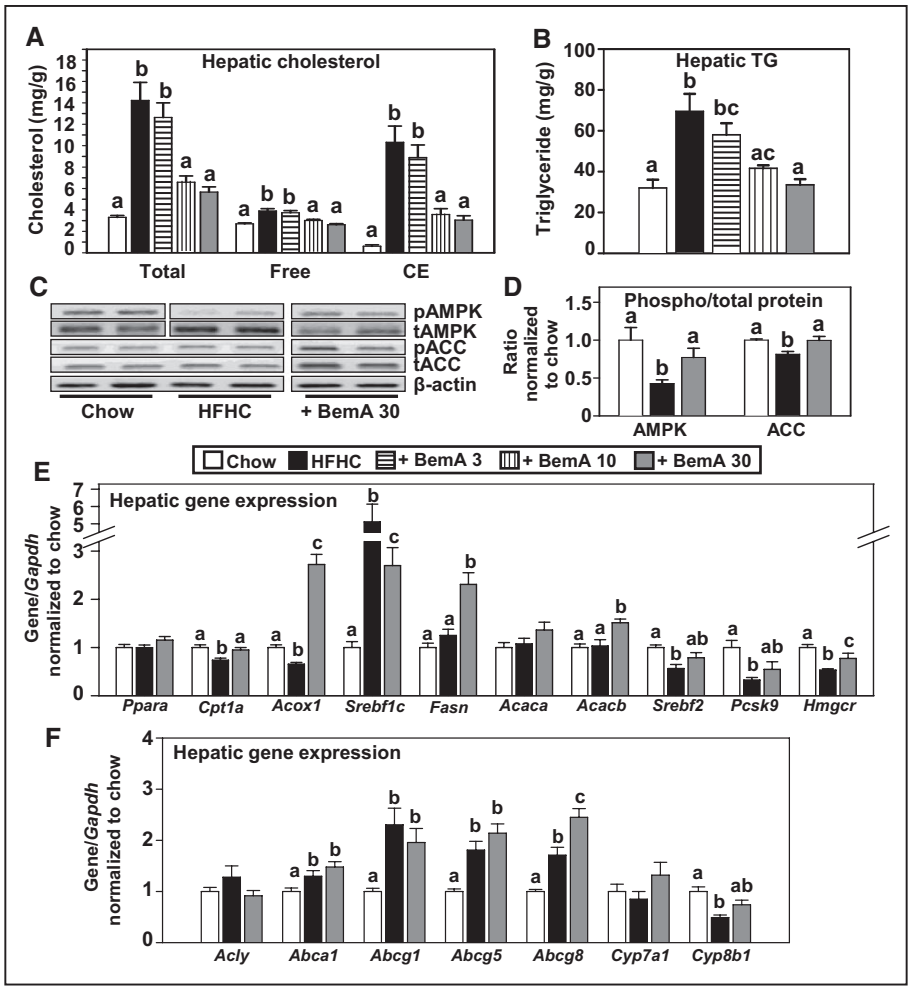

Figure 2. Bempedoic acid (BemA) attenuates hepatic steatosis, increases phosphorylation of hepatic AMP–activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC), and modulates lipid metabolism gene expression. *Ldlr*$^{-/-}$ mice were fed chow, or a high-fat, high-cholesterol (HFHC) diet supplemented with BemA (0, 3, 10, or 30 mg/kg per day) for 12 weeks. **A**, Hepatic total cholesterol, free cholesterol, and cholesteryl ester (CE) content (n=14/group). **B**, Hepatic triglyceride (TG; n=14/group). **C**, Representative immunoblots (2 mice/group, all from the same blot) of phosphorylated (p) and total (t) AMPK and ACC in freeze-clamped liver lysates with quantitation (**D**; n=7–8/group). Ratios are normalized to chow-fed mice from the same immunoblot. **E** and **F,** mRNA expression of genes involved in hepatic lipid metabolism, expressed relative to *Gapdh* and normalized to values in chow-fed mice (n=10–12/group). Data are presented as mean±SEM. Different letters indicate significant differences (P<0.05).

mitochondria and peroxisomes. The elevated hepatic expression of *Srebf1c* (5-fold) in HFHC-fed mice was significantly lower in BemA-treated mice (−50%; Figure 2E), which is most likely a response to the treatment-induced decrease in plasma insulin concentrations. Compared to the HFHC diet alone, hepatic expression of other lipogenic genes was generally increased with treatment at 30 mg/kg per day: *Fasn* (2-fold), *Acaca* (1.3-fold, trend), *Acacb* (1.4-fold), *Srebf2* (1.2-fold, trend), *Pcsk9* (1.7-fold, trend), and *Hmgcr*; (1.3-fold; Figure 2E). This is consistent with a compensatory feedback response resulting from the inhibition of ACLY and reduced intracellular lipids. Compared to HFHC diet alone, *Acly*, *Abca1*, and *Abcg1* expressions were unaffected by BemA treatment (Figure 2F). BemA did not affect *Cyp7a1* or *Cyp8b1* genes that regulate bile acid synthesis, whereas BemA increased the expression of genes coding for the half-transporters that promote hepatic cholesterol efflux into bile; *Abcg5* (1.2-fold, trend) and *Abcg8* (1.4-fold; Figure 2F).

BemA at 30 mg/kg per day significantly increased hepatic FA β-oxidation (1.5-fold), compared with the HFHC diet alone (Figure 3A). The increase in hepatic FA-synthesis induced by the HFHC diet (2.8-fold) was significantly reduced by 30% with the treatment (Figure 3B). Muscle FA-synthesis increased significantly with the HFHC diet (3.5-fold) and was not affected further by BemA (Figure III in the online-only Data Supplement). Hepatic synthesis of triglyceride and CE was significantly decreased by BemA at both 10 mg/kg per day (−42% and −48%) and 30 mg/kg per day (−52% and −65%), respectively (Figure 3C and 3D). Hepatic cholesterol synthesis was suppressed significantly by the HFHC diet (−85%), and was not further affected by BemA at 30 mg/kg per day (Figure 3E).

To further investigate the BemA-induced increase in hepatic FA-oxidation, whole body energy balance was assessed. Total energy expenditure was modestly, but significantly, higher (8%) in BemA-treated mice (30 mg/kg per

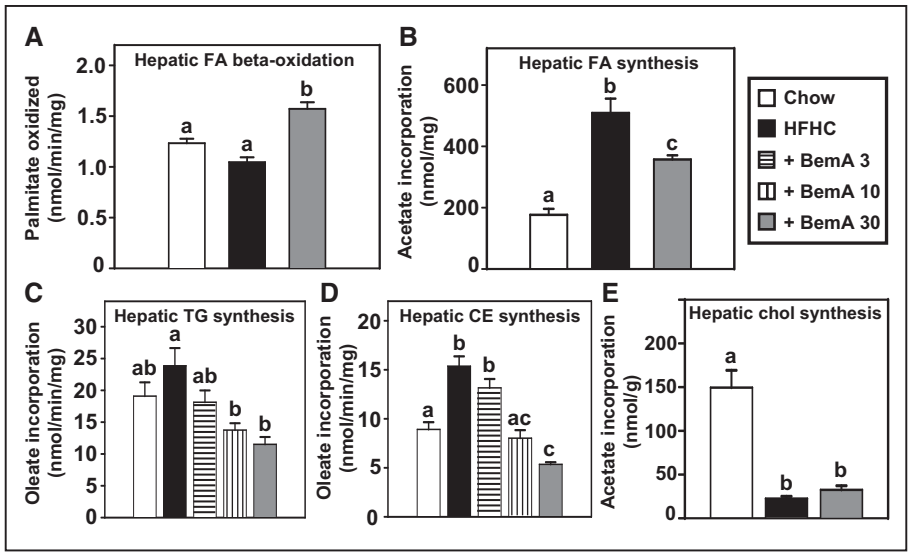

Figure 3. Bempedoic acid (BemA) increases hepatic fatty acid (FA) oxidation and decreases hepatic synthesis of FA, triglyceride and cholesteryl ester. *Ldlr*$^{-/-}$ mice were fed chow, or a high-fat, high-cholesterol (HFHC) diet supplemented with BemA (0, 3, 10, or 30 mg/kg per day) for 12 weeks. **A**, Hepatic FA β-oxidation in liver homogenates. **B**, FA synthesis in liver obtained 60 minutes postinjection (*IP*) with [^{14}C] acetate. Incorporation of [^{14}C]oleate into (**C**) triglyceride (TG) and (**D**) cholesteryl ester (CE) in liver homogenates. **E**, Cholesterol synthesis in liver obtained 60 minutes post injection (*IP*) with [^{14}C]acetate. Data are presented as mean±SEM, n=8/group. Different letters indicate significant differences (P<0.05).

day) compared with HFHC-fed mice (Figure IVA and IVB in the online-only Data Supplement). The respiratory exchange ratio, which reflects the relative utilization of carbohydrate (respiratory exchange ratio ≈1.0) versus fat (respiratory exchange ratio ≈0.7), was similar between groups, indicating that increased energy expenditure in treated mice was associated with an increase in both carbohydrate and fat utilization (Figure IVC in the online-only Data Supplement).

Bempedoic Acid Prevents Hepatic Inflammation

HFHC-induced hepatic inflammation and the effect of BemA were assessed by immunoblotting for mitogen-activated protein kinase and nuclear factor-κB signaling, 2 major inflammatory pathways linked to hepatic steatosis.[14,15] Hepatic p38 and extracellular signal–regulated kinase 1/2 phosphorylation revealed little or no effect of the HFHC diet, whereas BemA treatment at 30 mg/kg per day significantly decreased signaling through these kinases (−40% and −52%, respectively; Figure 4A and 4B). Inflammatory gene expression is often mediated through nuclear factor-κB activation, which is regulated by the inhibitor of nuclear factor-κBα and its kinase, inhibitor of nuclear factor-κBα-kinase. However, hepatic inhibitor of nuclear factor-κBα-kinase and Iκ-Bα phosphorylation were unaffected either by the HFHC diet or by BemA (Figure 4B).

Hepatic inflammation was further assessed by measuring the expression of genes known to be markers of a macrophage M1 (inflammatory) or M2 (anti-inflammatory) phenotype. In general, the significantly elevated M1 inflammatory gene expression in HFHC-fed mice trended lower with 30 mg/kg per day BemA treatment and was significantly lower for the genes *Ccl3* and *Nos2* (Figure 4C). Expression of the M2 gene *Arg1* was increased with treatment. The significantly elevated

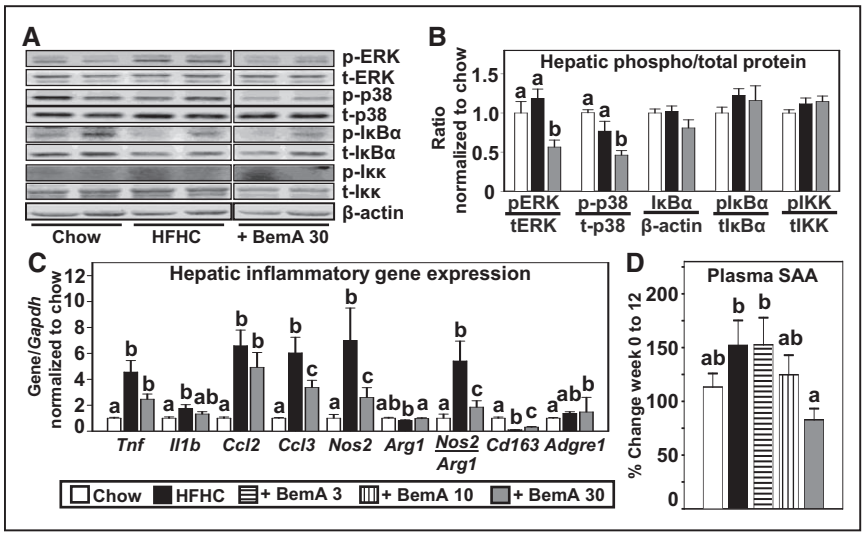

Figure 4. Bempedoic acid (BemA) attenuates hepatic inflammation. **A**, Representative immunoblots (2 mice/group, all from the same blot) of phosphorylated (p) and total (t) inflammatory signaling proteins extracellular signal–related kinase (ERK) 1, 2, p38, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα) and IκB kinase (IKK) in liver lysates from *Ldlr*$^{-/-}$ mice at 12 weeks with quantitation (**B**; n=7–8/group). Ratios are normalized to chow-fed mice from the same immunoblot. **C,** Liver mRNA abundances of *Tnf*, *Il1b Ccl2*, *Ccl3*, *Nos2*, *Arg1*, *Cd163*, and *Adgre1*, expressed relative to *Gapdh* and normalized to values in chow-fed mice (n=5–8/group). **D**, Percentage change in serum amyloid A (SAA) from week 0 to week 12 (n=10–16/group). Data are presented as mean±SEM. Different letters indicate significant differences (P<0.05).

ratio of *Nos2*/*Arg1* in HFHC-fed mice was significantly decreased by BemA, indicating the predominance of macrophages with an M2 phenotype (Figure 4C).

Chronic systemic inflammation was assessed by measuring the plasma levels of serum amyloid A (SAA). In response to inflammatory stimuli in obese mice, SAA expression increases in liver and adipose tissue, although only liver-derived SAA contributes to circulating SAA levels.[16] The HFHC-fed mice showed a 1.3-fold increase in plasma concentrations of SAA at 12 weeks compared with baseline, and this was prevented (−45%) in the 30 mg/kg per day BemA-treated mice (Figure 4D).

Bempedoic Acid Prevents Aortic Lipid Accumulation, Inflammation, and Atherosclerotic Lesion Development

The aortic sinus was sectioned for histological examination of atherosclerotic lesion size and morphology. Analysis of Oil Red-O–stained sections revealed small lesions in chow-fed mice and much larger lesions (16-fold) in HFHC-fed mice. BemA treatment at 30 mg/kg per day robustly attenuated lesion size by 44% (Figure 5A). Compared to the HFHC-diet alone, treated mice had significantly fewer lesion macrophages (−46%) as assessed by CD68 staining, but relative to total lesion area, the percent of macrophages was unchanged (Figure 5B). The percent of lesion area occupied by smooth muscle α-actin trended lower (−8%; Figure 5C) and that occupied by collagen as assessed by picrosirius red trended lower (−11%) with treatment, when compared with the HFHC-diet alone (Figure 5D). Levels of lesion apoptosis, which were determined by the number of cleaved caspase-3–positive nuclei as a percent of total nuclei, were significantly lower with treatment (0.6% versus 1.1%; Figure 5E). In whole aortae, the HFHC-diet increased TC (1.7-fold), FC (1.4-fold), CE (4.4-fold), and triglyceride (2.4-fold) (Figure 6A). BemA significantly decreased TC (−30%), FC (−22%), and CE (−50%) at both 10 and 30 mg/kg per day doses. Aortic triglyceride was significantly decreased by 51% at 30 mg/kg per

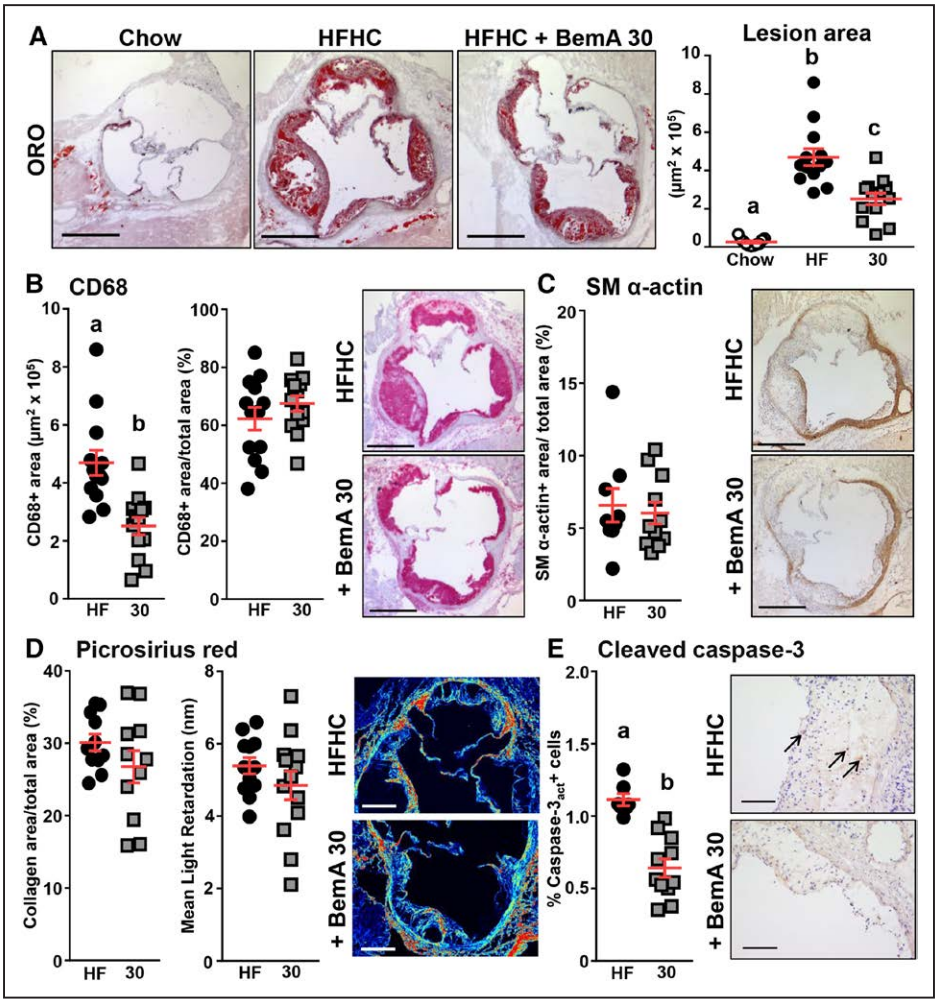

Figure 5. Addition of bempedoic acid (BemA; 30 mg/kg per day) to a high-fat, high-cholesterol (HFHC or HF) diet attenuates atherosclerosis. **A,** Representative photomicrographs of aortic sinus sections stained with Oil Red-O (ORO) and counterstained with hematoxylin (bar, 500 μm). **B–E**, Representative photomicrographs of aortic sinus sections stained for (**B**) macrophages with anti-CD68 antibody (bar, 500 μm), (**C**) smooth muscle (SM) cells with anti-SM α-actin antibody (bar, 500 μm), (**D**) collagen with picrosirius red (bar, 250 μm) and (**E**) apoptotic cells with anti-cleaved-caspase-3 antibody (bar, 100 μm). Arrows point to positive cells. Quantitation is shown beside each set of micrographs (n=8–13/group). Values from individual mice are represented by symbols and the mean is indicated by a single horizontal line and the SEM by the vertical lines. Different letters indicate significant differences ($P<0.05$).

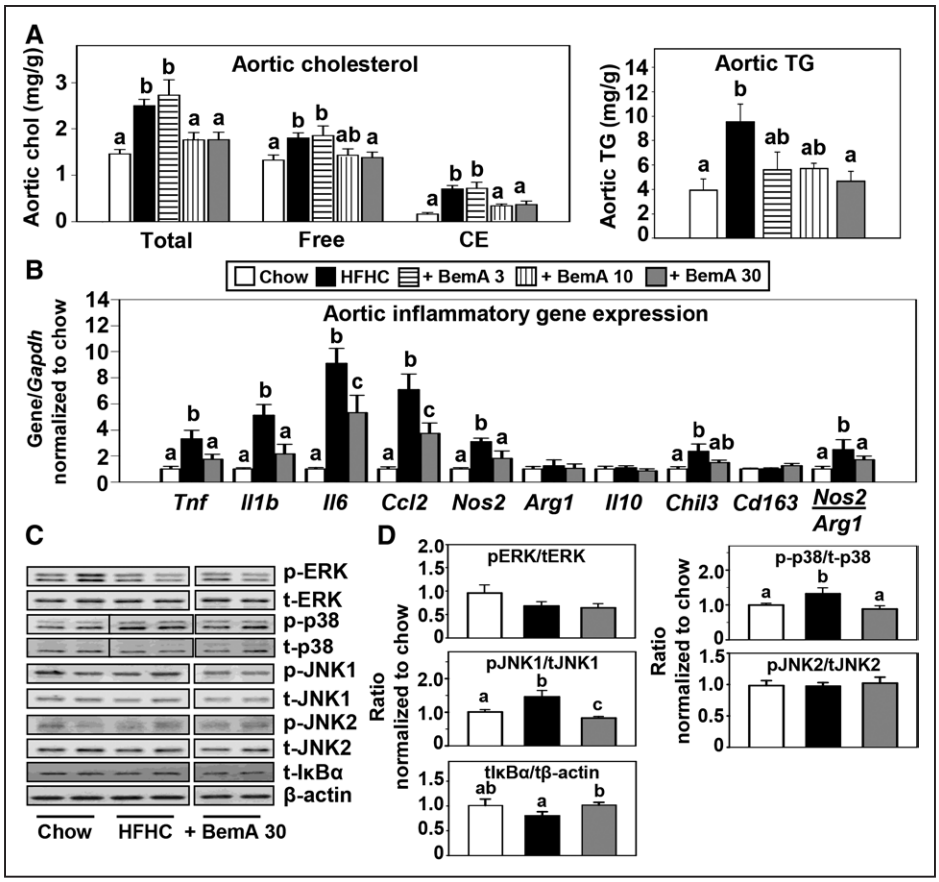

Figure 6. Bempedoic acid (BemA) attenuates lipid accumulation, inflammatory cytokine expression, and proinflammatory signaling in full-length aortae. **A,** Cholesterol (total cholesterol, free cholesterol, and cholesteryl ester [CE]) and triglyceride (TG) concentrations (n=11–12/group). **B**, mRNA abundance of the indicated proinflammatory M1 cytokines and anti-inflammatory M2 cytokines determined in full-length aortae and normalized to values in chow-fed mice (n=5–8/group). **C,** Representative immunoblots (2 mice/group, all from the same blot) of phosphorylated (p) and total (t) proteins extracellular signal–related kinase (ERK) 1,2, p38, c-Jun N-terminal kinase 1 (JNK1), JNK2, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα) and β-actin with quantitation (**D**; n=6–8/group). Ratios are normalized to chow-fed mice from the same immunoblot. Data are presented as mean±SEM. Different letters indicate significant differences, (P<0.05).

day. Collectively, these analyses indicate that BemA prevents lesion progression, resulting in smaller, lipid-depleted, less complex lesions.

Compared to chow-fed mice, the HFHC diet significantly increased aortic mRNA expression of macrophage M1 genes, *Tnf*, *Il1b*, *Il6*, *Ccl2*, and *Nos2*, all of which were significantly decreased by BemA at 30 mg/kg per day (Figure 6B). The HFHC diet had little effect on the expression of the M2 macrophage genes *Arg1*, *Il10*, and *Cd163* and were not further affected by treatment. The M2 gene *Chil3* was increased with the HFHC diet and decreased (trend) with treatment. The HFHC diet increased the ratio of *Nos2/Arg1* (2.4-fold), whereas BemA completely reversed this expression pattern (−32%). This suggests that BemA treatment decreased aortic proinflammatory macrophages and increased the proportion of macrophages with M2 polarization.

Cell signaling cascades known to regulate the macrophage inflammatory response were assessed in full-length aortae (Figure 6C and 6D). Immunoblotting for extracellular signal–regulated kinase 1/2 phosphorylation revealed little effect of the HFHC diet and was not affected further by BemA at 30 mg/kg per day. Phosphorylation of p38 (p-p38/t-p38) and c-Jun N-terminal kinase 1 (JNK1 [pJNK1/tJNK1]) were significantly elevated by the HFHC diet, whereas BemA at 30 mg/kg per day significantly decreased signaling through these kinases (−30% and −45%, respectively). pJNK2/tJNK2 was unaffected by HFHC or BemA. A signal for pIκBα in the aorta was not detected, although tIκBα was increased by BemA (Figure 6C and 6D). Collectively, these data suggest that BemA diminishes aortic inflammation, in part, by attenuating diet-induced activation of inflammatory signaling cascades.

Discussion

Therapeutic strategies to alleviate atherosclerosis associated with the metabolic syndrome remain sparse. The results of this study demonstrate that ACLY inhibition together with AMPK activation by BemA attenuate the progression of several key metabolic disturbances that underlie the development of atherosclerosis. When *Ldlr*$^{-/-}$ mice were fed the HFHC diet, they exhibited obesity, dyslipidemia, hepatic steatosis, hepatic and aortic inflammation, and increased atherosclerosis. Addition of BemA at 3 mg/kg per day appeared to exhibit little effect and for most parameters; results were similar to

the HFHC diet alone. However, the addition of BemA to the HFHC diet at 30 mg/kg per day effectively prevented or strongly attenuated hyperlipidemia, hepatic steatosis, adiposity, glucose intolerance and the rate of lesion development. The 10 mg/kg per day dose often showed similar potency to 30 mg/kg per day. BemA at 30 mg/kg per day also alleviated diet-induced inflammatory responses in liver, plasma and the aorta, thereby contributing to the attenuation of atherogenesis. Furthermore, BemA was associated with beneficial changes in lesion composition, including less lipid, fewer smooth muscle cells, more M2 cells and less apoptotic cells, all of which are characteristic of less mature lesions with a more stable phenotype.

The current experiments, together with previous studies, indicate that BemA primarily targets the liver.[11] This is based on the observations that ACSVL1—the enzyme that converts bempedoic acid to its active CoA derivative—is liver specific and BemA activates AMPK only through the β1-isoform, which is rodent liver specific.[12] In this study, BemA had a marked effect on hepatic lipid concentrations. In mice treated at 30 mg/kg per day, liver triglyceride was normalized and CE markedly decreased. This was attributed to increased FA β-oxidation and decreased de novo FA- and triglyceride-synthesis. Enhanced β-oxidation is consistent with the increase in hepatic expression of both *Cpt1a* and *Acox1*, which encode key enzymes in mitochondrial and peroxisomal FA metabolism, respectively. Malonyl-CoA normally inhibits Cpt1α. Thus, the expected decrease in malonyl-CoA in response to both ACLY inhibition and AMPK activation would relieve this inhibition. Reduced FA-synthesis was also an expected consequence of depleted hepatic malonyl-CoA. Increased hepatic expression of lipogenesis genes *Fasn* and *Acacb* may represent a compensatory response to the depletion of malonyl-CoA. The increase in *Acacb* mRNA and protein is likely secondary to the upregulation of ACC by citrate. Accumulation of hepatic citrate has been reported in rats in response to single-dose BemA treatment.[11] Despite this, the upregulation of gene expression must have been insufficient to overcome the greater inhibition of ACLY and the phosphorylation of ACC with BemA treatment because net FA-synthesis was markedly decreased. Collectively, these data are consistent with hepatic ACLY inhibition and AMPK activation by BemA. In an article published following the completion of this study, it was reported that in *Apoe*$^{-/-}$ mice fed a high-fat diet, BemA treatment improved parameters of lipid metabolism, including hepatic steatosis and plasma LDL-C independently of hepatic AMPK, implying that inhibition of ACLY was the primary mechanism for lipid regulation.[12]

The modest increase in liver weight in BemA-treated mice was most likely because of peroxisome proliferation, as evidenced by the increase in the expression of the peroxisome-specific gene, *Acox1*. The increase in hepatic *Ppara* mRNA expression, typically observed with peroxisome proliferation in mice,[17] was not evident, suggesting that peroxisome proliferation was modest. *Acox1* is the first enzyme of peroxisome FA β-oxidation, the pathway, responsible for the conversion of very long chain FAs to medium chain FAs, which are subsequently shuttled into mitochondria for complete β-oxidation. Therefore, it is possible that peroxisome β-oxidation contributes to the BemA-induced increase in overall hepatic FA-oxidation.

Hepatic expression of *Srebf1c* is markedly increased in this mouse model, consistent with the hyperinsulinemia, as insulin is a potent transcriptional activator of *Srebf1c*.[18] BemA decreased plasma insulin and hepatic *Srebf1c* expression to a similar extent. Although this is consistent with the decreased FA synthesis, it does not fit with the upregulation of one of its target genes, *Fasn*. It is possible that the compensatory increase in *Fasn* expression induced by ACLY inhibition partially attenuated the decrease in *Srebf1c*.

In the *Ldlr*$^{-/-}$ mouse, plasma clearance of apoB-containing lipoproteins is impaired, leading to increased levels of VLDL and LDL. The HFHC diet-induced hepatic steatosis contributes significantly to hepatic overproduction of VLDL, increased cholesterol in both plasma VLDL and LDL fractions, and increased lesion formation.[18,19] BemA attenuates atherosclerosis development in the aortic sinus, which was associated with a substantial reduction in plasma VLDL-C and LDL-C. Although not measured in this study, this was most likely because of the attenuation of hepatic VLDL overproduction consequent to the marked BemA-induced diminution of hepatic triglyceride and cholesterol content. The decreased size of aortic sinus lesions and marked reductions of aortic CE, TC, and triglyceride in treated mice are consistent with a study in which VLDL-C concentrations were a stronger predictor of aortic sinus atherosclerosis than that of LDL-C in *Ldlr*$^{-/-}$ mice fed a high-fat diet.[20] Thus, the marked reduction of VLDL-C along with the reduction in LDL-C contributes to the robust attenuation of lesion progression in BemA-treated mice.

BemA decreased hepatic cholesterol resulting in lower cholesterol concentrations in plasma and aortae. The mechanism(s) underlying this decrease are not entirely clear. Cholesterol absorption seems to be unaffected. Although BemA markedly suppressed hepatic cholesterol esterification, diminished hepatic cholesterol content is unlikely because of decreased cholesterol synthesis, as the suppressed rate of cholesterol synthesis in HFHC-fed mice was not further affected by BemA treatment. BemA may increase hepatic bile acid synthesis and enhance fecal acidic sterol excretion, although no increase in hepatic *Cyp7a1* or *Cyp8b1* expression was observed. Furthermore, BemA may increase fecal neutral sterol excretion via the bile or trans intestinal cholesterol efflux, as recently reported in statin-treated mice.[21] The latter is supported by increased hepatic expression of *Abcg5* and *Abcg8* and may explain the lower hepatic cholesterol and the increased hepatic expression of *Hmgcr* and the trends to increased *Srebf2*, *Pcsk9*, and cholesterol synthesis in BemA-treated mice. Future studies will be required to test these possibilities.

The HFHC diet-induced inflammatory response in liver and aortae is consistent with previous reports.[15,22–24] In this study, we did not distinguish which cell type, Kupffer cells, or infiltrating macrophages, contributed to hepatic inflammation. The *Adgre1* (*F4/80*) expression data suggested that the number of hepatic immune cells was not increased by the HFHC diet and the cytokine expression pattern indicated that they were

predominantly an M1, proinflammatory phenotype. Although BemA treatment did not further affect *Adgre1* expression in liver, suggesting no decrease in inflammatory cells, signaling through the mitogen-activated protein kinase^{erk} pathway was attenuated, resulting in significantly reduced inflammatory responses in both liver and aortae. Furthermore, the gene expression pattern indicated a shift to a more anti-inflammatory, M2 phenotype. These findings extend a previous report that BemA decreased inflammatory cytokine expression in cultured macrophages and attenuated interleukin-6 release by inflamed adipose tissue ex vivo.[13]

Mechanisms by which the added dietary cholesterol promote hepatic and aortic inflammation include increased cellular FC concentrations leading to mitochondrial dysfunction, stimulation of mitogen-activated protein kinase- and nuclear factor-κB-signaling, and activation of NLRP3 inflammasomes.[25,26] The ability of BemA to diminish hepatic and aortic cholesterol may alleviate the FC-induced inflammatory stimulus in both tissues. Dietary saturated fat also promotes tissue inflammation. Incubation of macrophages with saturated FAs or VLDL containing saturated FAs enhances inflammatory cytokine secretion.[27,28] Furthermore, diets rich in saturated fat induce macrophage infiltration into adipose tissue, liver, and aortae.[15,23,24,29] Increased hepatic FA-oxidation in high-fat–fed transgenic mice overexpressing hepatic *Cpt1a* attenuated the expression of *Tnfa*, *Il6*, *Il1b*, and *Ccl3* in liver and adipose tissue.[30] Moreover, in cultured macrophages exposed to excess palmitate, activation of AMPK increased FA-oxidation and suppressed inflammation.[31] Collectively, these results suggest that BemA's anti-inflammatory properties in the aortae are secondary to decreased exposure to lipoprotein-derived lipids, and in liver are because of its ability to stimulate FA-oxidation and inhibit FA-synthesis. However, a direct effect of BemA on inflammatory signaling pathways in these tissues cannot be ruled out.

BemA treatment robustly attenuated lesion size in the aortic sinus by 44%. A report published after completion of this study revealed that in *Apoe*$^{-/-}$ mice, BemA treatment (same dose, time, and diet as in this study) resulted in a small reduction in the size of aortic sinus lesions (−21%).[12] This was likely reflective of the modest reduction in total plasma cholesterol (−18%), and no effect on the high concentrations of chylomicron- and VLDL-remnant cholesterol, which are the primary atherogenic lipoproteins in this model. Lesion morphology was not reported. In this study, the aortic sinus lesions of HFHC-fed mice were characterized by infiltration of both neutral lipid-rich macrophages and smooth muscle cells. Lesions also displayed collagen deposition consistent with a fibrillar plaque phenotype. In contrast, BemA treatment significantly decreased lesion size and lesions were less advanced as evidenced by a similar proportion of macrophages that were predominantly a less-inflammatory M2 phenotype, a trend to fewer smooth muscle cells and collagen, and a significant reduction in the number of apoptotic cells. These data demonstrate that BemA attenuates the development and progression of atherosclerosis in *Ldlr*$^{-/-}$ mice induced by an HFHC diet.

In conclusion, these in vivo studies demonstrate that BemA ameliorates the dyslipidemia, hyperinsulinemia, and obesity in HFHC diet–fed *Ldlr*$^{-/-}$ mice, resulting in marked attenuation of atherosclerosis. Although other mechanisms may have contributed, BemA mediates its anti-atherogenic and anti-inflammatory effects through potent lipid lowering. These observations also suggest that the ability of BemA to decrease plasma lipids and high-sensitivity C-reactive protein in humans may also translate into the suppression of atherosclerosis.

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References

1. Boekholdt SM, Hovingh GK, Mora S, et al. Very low levels of atherogenic lipoproteins and the risk for cardiovascular events: a meta-analysis of statin trials. *J Am Coll Cardiol*. 2014;64:485–494. doi: 10.1016/j.jacc.2014.02.615.
2. Hegele RA, Gidding SS, Ginsberg HN, McPherson R, Raal FJ, Rader DJ, Robinson JG, Welty FK. Nonstatin low-density lipoprotein-lowering therapy and cardiovascular risk reduction-statement from ATVB Council. *Arterioscler Thromb Vasc Biol*. 2015;35:2269–2280. doi: 10.1161/ATVBAHA.115.306442.
3. Stroes ES, Thompson PD, Corsini A, et al; European Atherosclerosis Society Consensus Panel. Statin-associated muscle symptoms: impact on statin therapy-European Atherosclerosis Society Consensus Panel Statement on Assessment, Aetiology and Management. *Eur Heart J*. 2015;36:1012–1022. doi: 10.1093/eurheartj/ehv043.
4. Burke AC, Huff MW. ATP-citrate lyase: genetics, molecular biology and therapeutic target for dyslipidemia [published online ahead of print January 5, 2017]. *Curr Opin Lipidol*. 2017. doi: 10.1097/MOL.0000000000000390.
5. Cramer CT, Goetz B, Hopson KL, Fici GJ, Ackermann RM, Brown SC, Bisgaier CL, Rajeswaran WG, Oniciu DC, Pape ME. Effects of a novel dual lipid synthesis inhibitor and its potential utility in treating dyslipidemia and metabolic syndrome. *J Lipid Res*. 2004;45:1289–1301. doi: 10.1194/jlr.M400018-JLR200.
6. Filippov S, Pinkosky SL, Newton RS. LDL-cholesterol reduction in patients with hypercholesterolemia by modulation of adenosine triphosphate-citrate lyase and adenosine monophosphate-activated protein kinase. *Curr Opin Lipidol*. 2014;25:309–315. doi: 10.1097/MOL.0000000000000091.
7. Ballantyne CM, Davidson MH, Macdougall DE, Bays HE, Dicarlo LA, Rosenberg NL, Margulies J, Newton RS. Efficacy and safety of a novel dual modulator of adenosine triphosphate-citrate lyase and adenosine monophosphate-activated protein kinase in patients with hypercholesterolemia: results of a multicenter, randomized, double-blind, placebo-controlled, parallel-group trial. *J Am Coll Cardiol*. 2013;62:1154–1162. doi: 10.1016/j.jacc.2013.05.050.
8. Gutierrez MJ, Rosenberg NL, Macdougall DE, Hanselman JC, Margulies JR, Strange P, Milad MA, McBride SJ, Newton RS. Efficacy and safety of ETC-1002, a novel investigational low-density lipoprotein-cholesterol-lowering therapy for the treatment of patients with hypercholesterolemia and type 2 diabetes mellitus. *Arterioscler Thromb Vasc Biol*. 2014;34:676–683. doi: 10.1161/ATVBAHA.113.302677.
9. Thompson PD, Rubino J, Janik MJ, MacDougall DE, McBride SJ, Margulies JR, Newton RS. Use of ETC-1002 to treat hypercholesterolemia

in patients with statin intolerance. *J Clin Lipidol*. 2015;9:295–304. doi: 10.1016/j.jacl.2015.03.003.
10. Ballantyne CM, McKenney JM, MacDougall DE, Margulies JR, Robinson PL, Hanselman JC, Lalwani ND. Effect of ETC-1002 on serum low-density lipoprotein cholesterol in hypercholesterolemic patients receiving statin therapy. *Am J Cardiol*. 2016;117:1928–1933. doi: 10.1016/j.amjcard.2016.03.043.
11. Pinkosky SL, Filippov S, Srivastava RA, Hanselman JC, Bradshaw CD, Hurley TR, Cramer CT, Spahr MA, Brant AF, Houghton JL, Baker C, Naples M, Adeli K, Newton RS. AMP-activated protein kinase and ATP-citrate lyase are two distinct molecular targets for ETC-1002, a novel small molecule regulator of lipid and carbohydrate metabolism. *J Lipid Res*. 2013;54:134–151. doi: 10.1194/jlr.M030528.
12. Pinkosky SL, Newton RS, Day EA, Ford RJ, Lhotak S, Austin RC, Birch CM, Smith BK, Filippov S, Groot PH, Steinberg GR, Lalwani ND. Liver-specific ATP-citrate lyase inhibition by bempedoic acid decreases LDL-C and attenuates atherosclerosis. *Nat Commun*. 2016;7:13457. doi: 10.1038/ncomms13457.
13. Filippov S, Pinkosky SL, Lister RJ, Pawloski C, Hanselman JC, Cramer CT, Srivastava RA, Hurley TR, Bradshaw CD, Spahr MA, Newton RS. ETC-1002 regulates immune response, leukocyte homing, and adipose tissue inflammation via LKB1-dependent activation of macrophage AMPK. *J Lipid Res*. 2013;54:2095–2108. doi: 10.1194/jlr.M035212.
14. Hummasti S, Hotamisligil GS. Endoplasmic reticulum stress and inflammation in obesity and diabetes. *Circ Res*. 2010;107:579–591. doi: 10.1161/CIRCRESAHA.110.225698.
15. Bojic LA, Telford DE, Fullerton MD, Ford RJ, Sutherland BG, Edwards JY, Sawyez CG, Gros R, Kemp BE, Steinberg GR, Huff MW. PPARδ activation attenuates hepatic steatosis in Ldlr-/- mice by enhanced fat oxidation, reduced lipogenesis, and improved insulin sensitivity. *J Lipid Res*. 2014;55:1254–1266. doi: 10.1194/jlr.M046037.
16. Chiba T, Han CY, Vaisar T, Shimokado K, Kargi A, Chen MH, Wang S, McDonald TO, O'Brien KD, Heinecke JW, Chait A. Serum amyloid A3 does not contribute to circulating SAA levels. *J Lipid Res*. 2009;50:1353–1362. doi: 10.1194/jlr.M900089-JLR200.
17. Lee SS, Pineau T, Drago J, Lee EJ, Owens JW, Kroetz DL, Fernandez-Salguero PM, Westphal H, Gonzalez FJ. Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Mol Cell Biol*. 1995;15:3012–3022.
18. Mulvihill EE, Allister EM, Sutherland BG, Telford DE, Sawyez CG, Edwards JY, Markle JM, Hegele RA, Huff MW. Naringenin prevents dyslipidemia, apolipoprotein B overproduction, and hyperinsulinemia in LDL receptor-null mice with diet-induced insulin resistance. *Diabetes*. 2009;58:2198–2210. doi: 10.2337/db09-0634.
19. Mulvihill EE, Assini JM, Lee JK, Allister EM, Sutherland BG, Koppes JB, Sawyez CG, Edwards JY, Telford DE, Charbonneau A, St-Pierre P, Marette A, Huff MW. Nobiletin attenuates VLDL overproduction, dyslipidemia, and atherosclerosis in mice with diet-induced insulin resistance. *Diabetes*. 2011;60:1446–1457. doi: 10.2337/db10-0589.
20. VanderLaan PA, Reardon CA, Thisted RA, Getz GS. VLDL best predicts aortic root atherosclerosis in LDL receptor deficient mice. *J Lipid Res*. 2009;50:376–385. doi: 10.1194/jlr.M800284-JLR200.
21. Schonewille M, de Boer JF, Mele L, Wolters H, Bloks VW, Wolters JC, Kuivenhoven JA, Tietge UJ, Brufau G, Groen AK. Statins increase hepatic cholesterol synthesis and stimulate fecal cholesterol elimination in mice. *J Lipid Res*. 2016;57:1455–1464. doi: 10.1194/jlr.M067488.
22. Subramanian S, Goodspeed L, Wang S, Kim J, Zeng L, Ioannou GN, Haigh WG, Yeh MM, Kowdley KV, O'Brien KD, Pennathur S, Chait A. Dietary cholesterol exacerbates hepatic steatosis and inflammation in obese LDL receptor-deficient mice. *J Lipid Res*. 2011;52:1626–1635. doi: 10.1194/jlr.M016246.
23. Assini JM, Mulvihill EE, Sutherland BG, Telford DE, Sawyez CG, Felder SL, Chhoker S, Edwards JY, Gros R, Huff MW. Naringenin prevents cholesterol-induced systemic inflammation, metabolic dysregulation, and atherosclerosis in Ldlr$^{-/-}$ mice. *J Lipid Res*. 2013;54:711–724. doi: 10.1194/jlr.M032631.
24. Bojic LA, Burke AC, Chhoker SS, Telford DE, Sutherland BG, Edwards JY, Sawyez CG, Tirona RG, Yin H, Pickering JG, Huff MW. Peroxisome proliferator-activated receptor δ agonist GW1516 attenuates diet-induced aortic inflammation, insulin resistance, and atherosclerosis in low-density lipoprotein receptor knockout mice. *Arterioscler Thromb Vasc Biol*. 2014;34:52–60. doi: 10.1161/ATVBAHA.113.301830.
25. Ioannou GN. The role of cholesterol in the pathogenesis of NASH. *Trends Endocrinol Metab*. 2016;27:84–95. doi: 10.1016/j.tem.2015.11.008.
26. Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG, Abela GS, Franchi L, Nunez G, Schnurr M, Espevik T, Lien E, Fitzgerald KA, Rock KL, Moore KJ, et al. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature*. 2010;464:1357–1361.
27. Anderson EK, Hill AA, Hasty AH. Stearic acid accumulation in macrophages induces toll-like receptor 4/2-independent inflammation leading to endoplasmic reticulum stress-mediated apoptosis. *Arterioscler Thromb Vasc Biol*. 2012;32:1687–1695. doi: 10.1161/ATVBAHA.112.250142.
28. Bojic LA, Sawyez CG, Telford DE, Edwards JY, Hegele RA, Huff MW. Activation of peroxisome proliferator-activated receptor δ inhibits human macrophage foam cell formation and the inflammatory response induced by very low-density lipoprotein. *Arterioscler Thromb Vasc Biol*. 2012;32:2919–2928. doi: 10.1161/ATVBAHA.112.255208.
29. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest*. 2003;112:1821–1830. doi: 10.1172/JCI19451.
30. Orellana-Gavaldà JM, Herrero L, Malandrino MI, Pañeda A, Sol Rodríguez-Peña M, Petry H, Asins G, Van Deventer S, Hegardt FG, Serra D. Molecular therapy for obesity and diabetes based on a long-term increase in hepatic fatty-acid oxidation. *Hepatology*. 2011;53:821–832. doi: 10.1002/hep.24140.
31. Galic S, Fullerton MD, Schertzer JD, Sikkema S, Marcinko K, Walkley CR, Izon D, Honeyman J, Chen ZP, van Denderen BJ, Kemp BE, Steinberg GR. Hematopoietic AMPK β1 reduces mouse adipose tissue macrophage inflammation and insulin resistance in obesity. *J Clin Invest*. 2011;121:4903–4915. doi: 10.1172/JCI58577.

Highlights

- Bempedoic acid is a novel inhibitor of the enzyme ATP citrate lyase.
- In *Ldlr*$^{-/-}$ mice fed a high-fat, cholesterol-containing diet, bempedoic acid prevented or strongly attenuated hyperlipidemia, hepatic steatosis, adiposity, glucose intolerance, and the rate of atherosclerotic lesion development.
- Bempedoic acid alleviated the diet-induced inflammatory response in liver, plasma, and the aorta.
- Treatment was associated with beneficial changes in lesion composition: less lipid, decreased smooth muscle cells, more M2 macrophages, and fewer apoptotic cells, all of which are characteristic of less mature lesions with a more stable phenotype.