**Brief Communication**

**Salsalate reduces atherosclerosis through AMPKβ1 in mice**

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**ABSTRACT**

Objective: Salsalate is a prodrug of salicylate that lowers blood glucose in people with type 2 diabetes. AMP-activated protein kinase (AMPK) is an αβγ heterotrimer which inhibits macrophage inflammation and the synthesis of fatty acids and cholesterol in the liver through phosphorylation of acetyl-CoA carboxylase (ACC) and HMG-CoA reductase (HMGR), respectively. Salicylate binds to and activates AMPKβ1-containing heterotrimers that are highly expressed in both macrophages and liver, but the potential importance of AMPK and ability of salsalate to reduce atherosclerosis have not been evaluated.

Methods: ApoE−/− and LDLr−/− mice with or without (−/−) germline or bone marrow AMPKβ1, respectively, were treated with salsalate, and atherosclerotic plaque size was evaluated in serial sections of the aortic root. Studies examining the effects of salicylate on markers of inflammation, fatty acid and cholesterol synthesis and proliferation were conducted in bone marrow—derived macrophages (BMDMs) from wild-type mice or mice lacking AMPKβ1 or the key AMPK-inhibitory phosphorylation sites on ACC (ACC knock-in (KI)-ACC KI) or HMGR (HMGR-KI).

Results: Salsalate reduced atherosclerotic plaques in the aortic roots of ApoE−/− mice, but not ApoE−/− AMPKβ1−/− mice. Similarly, salsalate reduced atherosclerosis in LDLr−/− mice receiving wild-type but not AMPKβ1−/− bone marrow. Reductions in atherosclerosis by salsalate were associated with reduced macrophage proliferation, reduced plaque lipid content and reduced serum cholesterol. In BMDMs, this suppression of proliferation by salicylate required phosphorylation of HMGR and the suppression of cholesterol synthesis.

Conclusions: These data indicate that salsalate suppresses macrophage proliferation and atherosclerosis through an AMPKβ1-dependent pathway, which may involve HMGR phosphorylation and cholesterol synthesis. Since rapidly-proliferating macrophages are a hallmark of atherosclerosis, these data indicate further evaluation of salsalate as a potential therapeutic agent for treating atherosclerotic cardiovascular disease.

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**Keywords** Sterol synthesis; Lipogenesis; Macrophage; Proliferation; Salicylate; Aspirin

1. INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide [1]. Atherosclerosis, the underlying cause of CVD, results from the development of atherosclerotic plaques, which when disrupted can lead to thrombus formation, heart attack and/or stroke. Atherosclerosis results from a buildup of lipid-laden cells (foam cells), which are predominantly macrophages, within the intima of large blood vessels. These foam cells propagate a positive feedback loop of pro-inflammatory cytokine release, classical M1 macrophage polarization and the recruitment and local proliferation of monocytes, macrophages and T cells [2–6]. Dyslipidemia is the leading risk factor for CVD and atherosclerosis. Currently, statins are the most common treatment for CVD due to their lipid-lowering properties; however, recent evidence suggests statins may increase the risk of type 2 diabetes, which is a common comorbidity with CVD [7–9]. Additionally, a significant portion of the population (~25%) are statin intolerant, which precludes the optimal use of statins in achieving their LDL-lowering target [10,11]. While Proprotein convertase subtilisin/kexin type 9 (PCSK-9) inhibitors lower LDL cholesterol effectively, these therapies are not widely used for a variety of reasons [12,13]. In addition to LDL-lowering therapies, targeting vascular inflammation through anti-IL-1β antibodies has also been shown to reduce cardiovascular events in patients taking statins [14]. Therefore, atherosclerosis can be viewed as impaired lipid metabolism overlaid with maladaptive immune response [2], and therapies aimed at inhibiting these pathways are viewed as important therapeutic strategies.

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Salicylate, an active metabolite of aspirin, has been used for thousands of years to relieve pain and inflammation (for review see [15,16]). Salsalate, a prodrug of salicylate, is rapidly cleaved into two salicylate molecules by carboxylesterases within the gastrointestinal tract (for review see [15,16]). But unlike aspirin, salsalate does not inhibit platelet function or cause gastrointestinal bleeding, and as a result can be taken at high doses (~4 g/day) that elicit serum salicylate concentrations of ~1 mM [17–20]. In addition to treating arthritis, salicylate has been evaluated in clinical trials in people with type 2 diabetes, where it has been shown to reduce circulating glucose and triglycerides [17–19]. Salsalate has also been tested in people with atherosclerosis taking statins, where it was reported to have no effect on LDL cholesterol, C-reactive protein or the progression of calcified atherosclerotic plaques (TINSAL-CVD, Clinical Trial Identifier: NCT00624923) [21]. However, it should be noted that this study had several important limitations. Firstly, the placebo group in this trial showed no atherosclerotic progression, so interpretation of these results is limited. Furthermore, the effect of salsalate in statin-naive patients has not been assessed. Additionally, unlike the Odyssey and Fournier trials [22,23] or the CANTOS trial [14], this trial did not use a minimum LDL cholesterol level or CRP level as inclusion criteria to repress an inhibitor of nuclear factor kappa B.

Mechanistically, salicylate has been shown to reduce blood glucose through a number of potentially interrelated mechanisms, including the repression of an inhibitor of nuclear factor kappa B kinase subunit (IκK-β) [24–27], mitochondrial uncoupling [28] and activation of the AMP-activated protein kinase (AMPK) [29]. AMPK is a heterotrimeric enzyme consisting of alpha catalytic and regulatory beta and gamma isoforms which have unique tissue-specific expression patterns. AMPK suppresses macrophage inflammation [30] as well as fatty acid and cholesterol synthesis in the liver through phosphorylation of acetyl-CoA carboxylase (ACC) and HMG-CoA reductase (HMGCR), respectively [30–32]. Previous studies have shown that salicylate directly binds to and activates AMPKβ1-containing heterotrimerers [29], which are predominantly expressed in hepatocytes and macrophages, and that this is important for increasing fatty acid oxidation and reducing fatty acid synthesis [30,33]. However, salicylate’s reduction of atherosclerosis and the role of AMPK in mediating these effects have not been evaluated. In the current study, we investigate the effects of salsalate using two independent mouse models of atherosclerosis (ApoE−/− and LDLr−/−) and find that salicylate reduces macrophage proliferation and atherosclerotic plaque development through a hematopoietic AMPKβ1-dependent pathway. Furthermore, salicylate suppresses macrophage cholesterol synthesis through phosphorylation of HMGCR, which is important for reducing macrophage proliferation.

2. MATERIAL AND METHODS

2.1. Animal ethics and models

All animal experiments were approved by the McMaster University Animal Research Ethics Board. The generation and characterization of AMPKβ1−/− [34], Apoe−/−/AMPKβ1−/− [35], HMGCR Ser871Ala knock-in (HMGKR KI) [36] and ACC1/ACC2 Ser79/212Ala Double Knock-in (ACC DKI) [32] mice have been described previously. Mice were group-housed at the conventional temperature (22–23 °C) on a 12-hour light–dark schedule with ad libitum access to food and water. ApoE−/− and ApoE−/−/AMPKβ1−/− mice were fed a western diet (TD.09821, Envigo) or western diet containing 2.5 g/kg salsalate (TD.130,258, Envigo, Salsalate from Cayman Chemicals) for 6 weeks, starting at 6 weeks of age. During week 5 of treatment, mice were fasted for 6 h (beginning at 7 am) and a glucose tolerance test was performed using intraperitoneal injection of 2 g/kg glucose. Blood glucose was measured at the indicated time points using an Accu-Chek Performa blood glucose meter. After 6 weeks, mice were anaesthetised with Ketamine (75 mg/kg) and Xylazine (10 mg/kg) before tissues were harvested. A subset of mice was injected with BrdU (Invitrogen) 2 h prior to sacrifice, as described [37].

2.2. Bone marrow transfer

Whole-body irradiation and bone marrow transfer were completed as previously described [30]. Briefly, bone marrow was collected from wild-type or AMPKβ1−/− mice. Recipient 8-week-old LDLr−/− mice purchased from JAX (stock: 002207) were irradiated using two doses of 550 Rads 3–4 h apart. Bone marrow was collected from femur and tibia of wild-type and AMPKβ1−/− mice through centrifugation. Cells were resuspended in PBS and intravenously injected in recipients at 5 × 106 cells per mouse at a volume of 200 μL. Mice were allowed to recover for 6 weeks, at which point engraftment has been shown to be established [30], followed by 8 weeks of western diet when they were assigned to either continue receiving western diet or western diet ±2.5 g/kg salsalate for another 8 weeks. Engraftment was confirmed using genomic DNA isolation from whole blood using the Fast Blood/Cell PCR Genotyping Kit (EZ Bioresearch, G1002-10) per manufacturer's instructions.

2.3. Atherosclerotic measurements

The heart and aortic root were removed, formalin fixed and paraffin embedded. 4-μm thick sections of the aortic root were then collected and stained with H&E to measure plaque size at 80-μm intervals; subsequent sections were used for immunohistochemistry (IHC) as previously described [38,39]. Images were captured using a Nikon Eclipse microscope. Atherosclerotic plaque size was determined from 5 sections per mouse, and the area was calculated by manually outlining the plaques on ImageJ software by an individual who was blinded to the groups. Lesion area is reported as an average of the 5 sections per mouse. IHC was done using primary antibodies to Mac-3 (1:1000, BD-Pharmingen 553,322), Ki67 (1:100, Abcam, ab16667) and BrdU (1:500 Abcam ab1893) and biotinylated secondary antibodies (1:500, MJS BioLynx, VECTBA1000, VECTBA4001, VECTBA6000) and detected using streptavidin peroxidase and Nova-Chek Performa blood glucose meter. Whole-body irradiation and bone marrow transfer were completed as previously described [30]. Mice were then suspended in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic. After 5 h, L929 media (a source of M-CSF) was added and media was replaced with DMEM (10% FBS, 1% antibiotic-antimycotic) overnight. The following morning, media was replaced with DMEM +/− M-CSF (2 ng/mL) and + salsalate (1 mM) as indicated for 72 h. For add-back experiments, as previously described [40], 1% BSA (sterile, fatty acid free,
low endotoxin, Sigma A8806) + mevalonate 100 μM (79,849, Sigma) and oleate 50 μM (07501, Sigma) were added as indicated. After 72 h, cells were formalin fixed, stained with crystal violet, washed thoroughly, and allowed to dry. The dye was solubilized in NaH₂PO₄ and absorbance read at 570 nm. Data is expressed relative to M-CSF stimulated samples.

2.4.2. De novo lipogenesis
Cells were reseeded in 12-well plates at 2 × 10⁶ cells/mL and allowed to adhere overnight. The following day, media was replaced with serum-free DMEM with [3H]-acetate and salicylate (1 mM). Lipid extraction was performed as previously described [41,42], and a portion of the sterol and fatty acid fractions were counted for radioactivity.

2.4.3. In vitro cytokine analysis
Cells were seeded in 6-well culture dishes at 2 × 10⁶ cells/mL and allowed to adhere overnight. The following day, media was replaced and salicylate (1 mM) was added 30 min prior to the addition of LPS (100 ng/mL). After 6 h, media and/or cells were frozen at -80 °C until future analysis.

2.5. Analytical methods

2.5.1. ELISAs
Cytokines were measured in media using Duo-Set ELISA (all from R&D) for TNF-α (Catalogue: DY410), IL-1β (Catalogue: DY401), IL-6 (Catalogue: DY406) and IL-10 (Catalogue: DY:417) according to manufacturer protocols.

2.5.2. RT-q-PCR
RNA was isolated using RNEasy Columns (Qiagen) with on-column DNase treatment. RT-q-PCR was performed as previously described [41]. All Taqman primers were purchased from Invitrogen, and relative gene expression was calculated using (2^ΔΔCt) method. Values were normalized to housekeeping gene β-actin of the wild-type control.

2.5.3. Serum salicylate levels
 Serum salicylate levels were measured in blood (collected from a small nick in the tail vein at indicated time points) using Salicylates (RTU Forensic Kit (133,619, Neogen).

2.5.4. Plasma triglycerides and cholesterol
Plasma lipids were measured from mice fasted for 6 h prior to sacrifice. Blood was collected retro-orbitally through a heparinized capillary tube into an Eppendorf containing EDTA. Samples were diluted 10-fold and cholesterol was measured using the Infinity cholesterol kit (TR13421, Thermo Scientific) according to manufacturer’s instructions. Triglycerides were measured in samples diluted 2-fold using the Triglyceride Colorimetric Assay Kit (10010303, Cayman Chemicals) according to manufacturer’s instructions.

2.5.5. Serum cytokine analysis
Serum cytokines were assessed by Bio-Plex Pro Mouse Cytokine 23-plex Assay (Bio-Rad laboratories), according to manufacturer’s instructions, using a 4-fold dilution of serum.

2.5.6. Cytotoxicity assay
Cytotoxicity was assessed using CytoTox 96® Non-Radioactive Cytotoxicity Assay (G1780, Promega) according to manufacturer instructions. Briefly, fresh supernatants were collected from BMDMs treated with indicated doses of salicylate for 6 h and mixed 1:1 with assay reagent for 30 min at room temperature, at which point stop solution was added. Absorbance was read at 490 nm. Data is expressed as a percent of the completely lysed well.

2.6. Statistical analysis
All statistical analysis was performed using Graphpad Prism version 8. Data was tested for normality using the Shapiro–Wilk test. Unless otherwise noted, data were normally distributed and analysed with a two-way ANOVA, with post-hoc analysis performed with Sidak’s multiple comparison correction. Non-normal data were analysed with a non-parametric One-way ANOVA, non-parametric t-test or Kruskal–Wallis test with Dunn’s multiple comparison as appropriate (indicated in the figure legends). All data is presented as mean ± S.E.M. Statistical outliers were determined using two standard deviations from the mean.

3. RESULTS

3.1. Salsalate reduces atherosclerosis in ApoE⁻/⁻ but not ApoE⁻/⁻/AMPKβ¹⁻/⁻ mice
For 6 weeks starting at 6 weeks of age, ApoE⁻/⁻ and ApoE⁻/⁻/AMPKβ¹⁻/⁻ mice were fed a western diet (control) or western diet supplemented with salsalate. The dose of salsalate (2.5 g salsalate/kg diet) was selected to generate a sustained serum salicylate concentration of ~900 μM (Figure 1A) throughout the day, consistent with previous rodent studies [28], and is aligned with serum concentrations of salicylate in clinical trials of patients with T2D or CVD treated with 3–4 g/day of salsalate [17–20]. Food intake between groups was comparable (Figure 1B) and there were no differences in body mass (Figure 1C) or glucose tolerance between treatments or genotypes (Figure 1D,E). Despite similar body mass, and glucose homeostasis, salsalate reduced atherosclerotic plaques in ApoE⁻/⁻ and ApoE⁻/⁻/AMPKβ¹⁻/⁻ mice by approximately 30%, an effect which was not observed in ApoE⁻/⁻/AMPKβ¹⁻/⁻/ mice (Figure 1F,G). Necrotic area within the atherosclerotic plaque is an important marker of plaque stability that can contribute to poor outcomes clinically. In this model, we saw very little plaque necrosis (~2%), and there were no differences between groups (Figure 1H). Total plaque lipid area, which includes necrotic areas, cholesterol crystals and intracellular lipid droplets, was dramatically reduced in ApoE⁻/⁻ but not ApoE⁻/⁻/AMPKβ¹⁻/⁻ mice treated with salsalate (Figure 1I). This effect was discordant with plasma triglycerides, which were elevated in ApoE⁻/⁻/AMPKβ¹⁻/⁻ mice but not altered by salsalate in either genotype (Figure 1J). However, mirroring reductions in plaque size and plaque lipid content, salsalate lowered plasma cholesterol in ApoE⁻/⁻ but not ApoE⁻/⁻/AMPKβ¹⁻/⁻ mice (Figure 1K). Plasma cholesterol was not different between genotypes of mice (Figure 1K). These data indicate that salsalate reduces atherosclerosis through a mechanism requiring AMPKβ¹.

3.2. Salsalate reduces atherosclerosis in LDLrWT but not LDLr¹KO mice
ApoE⁻/⁻/AMPKβ¹⁻/⁻ mice show large reductions in AMPK activity in both liver and macrophages [35,41], both of which could contribute to reductions in atherosclerosis. Salicylate elicits anti-inflammatory effects in macrophages [43,44], so we hypothesized that AMPKβ¹ may be critical for mediating reductions in atherosclerosis in macrophages. To directly examine this hypothesis, we conducted bone marrow transfers from WT and whole-body AMPKβ¹⁻/⁻ mice into LDLr⁻/⁻ mice to generate LDLrWT and LDLr¹KO mice. Genotyping of whole-blood genomic DNA confirmed successful reconstitution of the LDLr⁻/⁻ mice with WT or β1KO bone marrow (Figure 2A). There were
no differences in body mass between groups (Figure 2B). Consistent
with observations in the ApoE<sup>−/−</sup> mice, salsalate reduced athero-
sclerotic lesions by approximately 30% in LDLr<sup>WT</sup> but not LDLr<sup>1KO</sup>
mice. However, complicating the interpretation of this finding,
atherosclerotic lesions were smaller in LDLr<sup>1KO</sup> compared to LDLr<sup>WT</sup>
mice (Figure 2C,D), a finding consistent with a previous study that
showed that irradiated mice reconstituted with bone marrow from
AMPK<sub>1</sub> KO mice (the primary α subunit in hematopoietic cells) mice also
show reductions in atherosclerosis [45]. Similarly, percent necrotic
area of the plaques was smaller in LDLr<sup>1KO</sup> plaques than LDLr<sup>WT</sup>
mice, and salsalate reduced necrotic area in LDLr<sup>WT</sup> but not LDLr<sup>1KO</sup>
plaques (Figure 2E). The same trend (p = 0.062) was seen in lipid area
of the plaques, which includes necrotic area, cholesterol crystals and
intracellular lipids (Figure 2F).

Importantly, in contrast to the ApoE<sup>−/−</sup> mice but consistent with normal levels of AMPK<sub>1</sub> in the liver, serum
cholesterol levels were reduced by salsalate in both LDLr<sup>WT</sup> and
LDLr<sup>1KO</sup> mice (Figure 2G). Further, salsalate tended to reduce serum triglycerides in both LDLr<sup>WT</sup> and LDLr<sup>1KO</sup>
mice (Figure 2H). In contrast to the ApoE<sup>−/−</sup> model, LDLr<sup>1KO</sup> mice did not have increased serum triglycerides relative to the LDLr<sup>WT</sup> mice (Figure 2H). These data indicate that salsalate reduces atherosclerosis and that haematopoietic AMPK<sub>1</sub> is an important factor contributing to the mechanism.

3.3. Salsalate reduces Ki67 and BrdU incorporation in plaques of
ApoE<sup>−/−</sup> but not ApoE<sup>−/−</sup> AMPK<sub>1</sub><sup>−/−</sup> mice
While multiple haematopoietic cell types can contribute to the devel-
opment of atherosclerosis, macrophage accumulation in the artery wall
is well documented underlying the pathogenesis of disease develop-
ment [4–6]. Given the important role of macrophage inflammation in
the development of atherosclerosis [46], we examined plasma in-
fammatory markers IL-1α, IL-1β, IL-12 (p40), IL-12 (p70), IL-13, G-CSF, IFNγ, RANTES, TNFα and MCP-1 and found that, surprisingly,
salsalate had no effect (Supplemental Figs. 1A–J). As salsalate has
been demonstrated to reduce inflammation in macrophages [43,44],
we hypothesized that there may be subtle changes in this cell type that
are not represented by levels in the plasma. To directly evaluate this
hypothesis, we cultured bone marrow-derived macrophages (BMDMs) with LPS (100 ng/mL) or LPS + salicylate (1 mM, approxi-
mate serum concentration achieved in both mouse and human studies). Consistent with our in vivo data, salicylate had no effect on
the levels of IL-1β, TNFα, IL6 or anti-inflammatory IL-10 transcript or
secretion following LPS stimulation (Supplementary Figs. 2A–H).
Additionally, we confirmed that this dose of salsalate did not induce
cytotoxicity (Supplementary Fig. 2). These data suggest that when
treated with a therapeutically achievable concentration of salicylate,
LPS-induced inflammation is not suppressed in mouse primary
macrophages.

Given that there were no changes in markers of inflammation, we
examined alternative mechanisms by which salsalate-induced acti-
vation of AMPK<sub>1</sub> complexes might reduce atherosclerosis. Recent
studies have demonstrated that local macrophage proliferation is a
critical driver of disease development [3]; therefore, we examined
proliferating cells within atherosclerotic plaques to determine if this
was altered by salsalate treatment. In ApoE<sup>−/−</sup> but not ApoE<sup>−/−</sup>
AMPK\textsuperscript{1/1} mice, salsalate reduced staining of the proliferation marker Ki67 within macrophage-rich areas of the plaques (Mac-3 positive areas) (Figure 3A,B). Given that Ki67 is expressed in a cell cycle--dependent manner, and that cell cycle arrest could alter Ki67 levels, we examined a separate cohort of mice injected with BrdU, thus allowing for accurate measurements of in situ macrophage proliferation [57]. Importantly, mice were sacrificed 2 h after BrdU injection to eliminate effects from bone marrow niche or progenitor subsets in this analysis. As with Ki67, we found that salsalate reduced BrdU incorporation in ApoE\textsuperscript{1/1} but not ApoE\textsuperscript{1/1} AMPK\textsuperscript{1/1} mice, although there was a trend for decreased BrdU staining in the ApoE\textsuperscript{1/1} AMPK\textsuperscript{1/1} relative to ApoE\textsuperscript{1/1} (Figure 3A,C). These data suggest that salsalate reduces atherosclerosis by suppressing local macrophage proliferation and that this effect requires AMPK\textsuperscript{1/1}.

3.4. Salicylate suppresses proliferation of bone marrow--derived macrophages through phosphorylation of HMGCoA reductase

To directly test the effect of salicylate on macrophage proliferation, studies were conducted in BMDMs from WT and AMPK\textsuperscript{1/1} mice. In agreement with previous studies [30,33,41], macrophages from AMPK\textsuperscript{1/1} mice had no detectable AMPK alpha expression (Figure 4A) and dramatic reductions in the phosphorylation of acetyl-CoA carboxylase (a marker of cellular AMPK activity which takes into account allosteric activation elicited through direct \textsuperscript{1/1} activators like salicylate) compared to WT mice. Importantly, salsalate increased ACC phosphorylation in macrophages from WT but not AMPK\textsuperscript{1/1} mice (Figure 4A,B). Macrophages proliferate very slowly in the absence of stimulation, and in vitro and in vivo, M-CSF is critical for stimulating macrophage proliferation [47,48]. Therefore, we measured in vitro macrophage proliferation over 72 h when stimulated with M-CSF (2 ng/mL) + salicylate. Consistent with the activation of AMPK, we found that salicylate suppressed the proliferation of macrophages from WT but not AMPK\textsuperscript{1/1} mice (Figure 4C). AMPK exerts effects on multiple pathways critical for regulating cell growth and proliferation [49]; however, cell division and proliferation are ultimately dependent on the generation of new cellular membranes, which requires de novo synthesis of fatty acids and cholesterol. This regulation of fatty acid and cholesterol synthesis has been shown to be important for dividing cancer cells [50,51] but also many immune cell types, including macrophages, T cells and platelets [52–54]. Previous studies conducted in cancer cells have shown that salicylate-induced suppression of cellular proliferation requires the inhibition of fatty acid and cholesterol synthesis [40]; therefore, we hypothesized that a similar mechanism of action may be important for mediating the anti-proliferative actions of salicylate in macrophages. To examine the dependency of macrophage proliferation on cholesterol or fatty acid synthesis, the culture media was supplemented with mevalonate or oleate, respectively. We found that supplementation with mevalonate but not oleate blocked the effects of salicylate and reduced proliferation (Figure 4D), which is consistent with the hypothesis that salicylate induces AMPK\textsuperscript{1/1}-dependent suppression of cholesterol synthesis, resulting in reduced macrophage proliferation. AMPK inhibits fatty acid and cholesterol synthesis through the phosphorylation of ACC and HMGCR, respectively. Therefore, to directly test the hypothesis that salicylate activation of AMPK suppresses cholesterol synthesis, we generated BMDMs (in addition to AMPK\textsuperscript{1/1}
macrophages) from knock-in (KI) mouse models, where the AMPK phosphorylation site(s) on HMGCR (HMGCR-KI) [36] and ACC (ACC-DKI) [32] are mutated from a serine to an alanine, blocking the inhibitory effects of AMPK on cholesterol and fatty acid synthesis, respectively. As anticipated, salicylate induced suppression of cholesterol synthesis in wild-type BMDM, but this effect was impaired in AMPKβ1−/− and HMGCR KI but not ACC DKI macrophages (Figure 4E). In contrast, but consistent with previous studies [28], salicylate suppressed fatty acid synthesis in wild-type, AMPKβ1−/−, HMGCR KI and ACC DKI macrophages (Figure 4F). These data indicate that salicylate-induced suppression of cholesterol synthesis is mediated through HMGCR, but that the suppression of fatty acid synthesis is mediated through AMPK-ACC-independent pathways. Consistent with these findings, we found that the ability of salicylate to reduce proliferation was blunted in macrophages from the HMGCR KI but not ACC DKI mice (Figure 4G). These data indicate that AMPKβ1-dependent phosphorylation of HMGCR and suppression of the mevalonate pathway, which ultimately leads to cholesterol synthesis, is required for salicylate-induced reductions in macrophage proliferation.

4. DISCUSSION

We found that there were no differences in the size of atherosclerotic lesions between Apoe−/− and Apoe−/−AMPKβ1−/− (Figure 1), consistent with our previous studies using this mouse line [35, 41]. In contrast, in LDLr−/− mice, reconstitution of bone marrow from AMPKβ1−/− mice to generate LDLr+MIX mice resulted in smaller plaques compared to LDLrWT mice (Figure 2). This finding is consistent with a previous study that showed that irradiated mice reconstituted with bone marrow from AMPKα1−/− (the primary α subunit in hematopoietic cells) mice also show reductions in atherosclerosis [45]. One limitation of this study is that atherosclerosis was only assessed at one site (aortic sinus), where lesions develop first. Site-specific differences in atherosclerosis, while uncommon, could also present in these models [55]. The mechanisms contributing to this reduction in atherosclerosis in the absence of hematopoietic AMPKβ1 but not germline Apoe−/−-AMPKβ1−/− mice are not fully understood, but may be related to differences between Apoe and LDLr null mice. Alternatively, they may involve compensatory adaptations present in the AMPKβ1 germline bone marrow niche that were not developed in the more acute setting following bone marrow transfer. Another possibility is that comparable plaque sizes in Apoe−/− and Apoe−/−-AMPKβ1−/− may be due to the loss of AMPKβ1 in other tissues, such as the liver, which may drive atherosclerotic plaque development and mask the effects of AMPKβ1 deletion in hematopoietic cells. We found that salicylate reduced atherosclerosis in two distinct mouse models (Apoe−/− and LDLr−/−). In Apoe−/− mice, this reduction in atherosclerosis required AMPKβ1 and was associated with reductions in plasma cholesterol, plaque lipid content and macrophage proliferation. Atherosclerosis involves interactions with multiple cell types and cellular pathways, but most notably hepatocytes and macrophages, tissues which predominately express the AMPKβ1 isoform in mice. To investigate the importance of macrophages in potentially mediating the beneficial effects of salicylate, experiments were then conducted in LDLr−/− mice reconstituted with bone marrow from WT mouse (LDLrWT) or AMPKβ1−/− mice. Again, we observed reductions in plaque size, an effect that was eliminated in LDLrWT mice. Importantly, in contrast to Apoe−/−-AMPKβ1−/− mice, this occurred despite comparable reductions in serum cholesterol in LDLrWT mice, suggesting that hematopoietic AMPKβ1 is important for reducing atherosclerosis. To further investigate the mechanisms contributing to reductions in plaque size in separate experiments, Apoe−/− and Apoe−/−-AMPKβ1−/− mice were injected...
with BrdU. Consistent with reduced plaque size and staining of the proliferative marker Ki67, ApoE<sup>−/−</sup> mice had reduced incorporation of BrdU, indicating reduced proliferation of macrophages within the atherosclerotic plaque. These data indicate that salaslate reduces macrophage proliferation and that this effect requires AMPKβ1. Here, we focused on the effects of salaslate on macrophage proliferation within the plaque as a mechanism for reduced atherosclerosis, as local proliferation has been shown to account for over 85% of macrophage content [3]; however, there could also be effects on hematopoietic stem cell (HSC) and monocyte proliferation, seen with other AMPK activators [56]. Reductions in HSC proliferation would be consistent with reduced white blood cell, lymphocyte, monocyte and neutrophil counts seen clinically with salaslate treatment [21]; however, this would not explain the reduced number of BrdU-positive cells within the plaques 2 h after BrdU injection. Therefore, our data suggest that in situ macrophage proliferation is reduced with salaslate via AMPKβ1, but potential effects on HSCs and monocytes cannot be excluded and warrant further investigation.

As reductions in serum cholesterol are important for driving proliferation of macrophages, we subsequently conducted experiments in cultured BMDMs so that this variable could be controlled. Using a clinically relevant concentration of salaslate that did not affect viability, we observed reduced proliferation in BMDMs from WT but not AMPKβ1<sup>−/−</sup> mice. Subsequent experiments established that these anti-proliferative effects of salaslate on BMDMs were blunted in media supplemented with mevalonate or in mice lacking inhibitory AMPK phosphorylation sites on HMGCR. While it remains feasible that reductions in atherosclerosis mediated by salaslate in vivo involve reductions in serum cholesterol, the alignment of the in vivo reductions in macrophage proliferation observed in vivo support a direct effect of salaslate in suppressing local macrophage proliferation. The demonstration of the anti-proliferative effects of salaslate involves suppression of HMGCR and cholesterol synthesis and provides potential insight into why salaslate had no effect on atherosclerotic plaque development in the TINSAL-CVD trial, as the inclusion criteria was a stable dose of statins [21].

While the inhibition of HMGCR in the liver and the subsequent reduction of serum cholesterol are critical for plaque regression in APOE*3Leiden.Cholesteryl ester transfer protein (CETP) mice, which model human-like lipid changes in response to oral statin treatment [57]. These novel data, with respect to the mechanisms of statins in combination with our observations with salaslate, support a common mechanism mediating reductions in atherosclerosis, giving a potential reason for why no beneficial effects of salaslate were observed in the TINSAL-CVD trial.

Figure 4: AMPK phosphorylation of HMGCR is required for the decrease in macrophage proliferation with salaslate. A, B) Representative western blots and quantification of wild-type and AMPKβ1<sup>−/−</sup> bone marrow-derived macrophages were treated with 1 mM salaslate for 90 min (Statistical test: One-Way ANOVA). C) Proliferation relative to control (MCSF only) of wild-type and AMPKβ1<sup>−/−</sup>, simultaneously treated with M-CSF (2 ng/mL) in mice with reductions in macrophage proliferation observed 4 h. D) Proliferation relative to control (MCSF only) of wild-type, ACC DKI and HMGCR KI bone marrow-derived macrophages simultaneously treated with M-CSF (2 ng/mL) and salaslate (1 mM) for 72 h (Statistical Test, Kruskal-Wallis) E-F) Incorporation of radiolabelled acetate into sterol and fatty acid fractions in wild-type, AMPKβ1<sup>−/−</sup> ACC DKI and HMGCR KI bone marrow-derived macrophages over 4 h. G) Proliferation relative to control (MCSF only) of wild-type, AMPKβ1<sup>−/−</sup> ACC DKI and HMGCR KI bone marrow-derived macrophages simultaneously treated with M-CSF (2 ng/mL) and salaslate (1 mM) for 72 h (Statistical Test, Kruskal-Wallis with Dunn’s multiple comparison). Data is expressed as mean ± S.E.M. from 3 to 6 individual experiments. *p < 0.05 for comparison indicated.
We have demonstrated for the first time that salsalate reduces atherosclerosis, requiring AMPKβ1, while also describing a novel mechanism by which salicylate and AMPK inhibit macrophage proliferation. However, it should be noted that like many effective type 2 diabetes medications that exert cardioprotective effects, such as metformin, SGLT2 inhibitors and GLP1R agonists, multiple overlapping mechanisms may be involved in the beneficial effects of salsalate on atherosclerosis, including effects on the liver, serum lipids and macrophage metabolism. Future studies examining these mechanisms and whether salsalate is a useful therapeutic option for patients with impaired fasting glucose, at high risk of new incident diabetes with statin therapy or with an inability to tolerate effective statin doses due to statin intolerance are warranted.

AUTHOR CONTRIBUTIONS

E.A.D., R.J.F., B.K.S., V.P.H., S.R., S.S., L. and M.D.F. performed the experiments and analysis. E.A.D. and G.R.S. wrote the manuscript. All authors edited the manuscript and provided comments. G.R.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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CONFLICT OF INTEREST

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APPENDIX A. SUPPLEMENTARY DATA

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REFERENCES


