Dual actions of a novel bifunctional compound to lower glucose in mice with diet-induced insulin resistance

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Chen K, Jih A, Kavalier ST, Lagakos WS, Oh D, Watkins SM, Kim JJ. Dual actions of a novel bifunctional compound to lower glucose in mice with diet-induced insulin resistance. Am J Physiol Endocrinol Metab 309: E293–E301, 2015. First published June 9, 2015; doi:10.1152/ajpendo.00045.2015.—Docosahexaenoic acid (DHA) and salicylate are both known to exert anti-inflammatory effects. This study investigated the effects of a novel bifunctional drug compound consisting of DHA and salicylate linked together by a small molecule that is stable in plasma but hydrolyzed in the cytoplasm. The components of the bifunctional compound act synergistically to reduce inflammation mediated via nuclear factor κB in cultured macrophages. Notably, oral administration of the bifunctional compound acted in two distinct ways to mitigate hyperglycemia in high-fat diet-induced insulin resistance. In mice with diet-induced obesity, the compound lowered blood glucose by reducing hepatic insulin resistance. It also had an immediate glucose-lowering effect that was secondary to enhanced glucagon-like peptide-1 (GLP-1) secretion and abrogated by the administration of exendin(9–39), a GLP-1 receptor antagonist. These results suggest that the bifunctional compound could be an effective treatment for individuals with type 2 diabetes and insulin resistance. This strategy could also be employed in other disease conditions characterized by chronic inflammation.

obesity; insulin-resistance; docosahexaenoic acid; salicylate

TYPE 2 DIABETES IS A DISEASE PROCESS characterized by hyperglycemia, insulin resistance, and pancreatic β-cell dysfunction and whose prevalence is increasing rapidly worldwide. Existing drug strategies have been found to have positive effects in combating the hallmark symptoms of type 2 diabetes and its cardiovascular comorbidities, but they also have substantial limitations (21).

Two existing drug strategies, docosahexaenoic acid (DHA) and salicylate, have both been previously studied and shown to exert anti-inflammatory effects to attenuate adipose tissue inflammation and improve insulin resistance. DHA, a component of ω-3 fish oils, activates the GPR120 receptor to inhibit both Toll-like receptor (TLR) and tumor necrosis factor-α (TNFα) signaling pathways (25). In addition, DHA-derived resolvin D1 reduces proinflammatory M1 macrophage content, increases adiponectin production, and promotes adenosine monophosphate-activated protein kinase (AMPK) activation in adipose tissue (9). Intriguingly, DHA has been shown to lower glucose levels acutely by stimulating the endogenous secretion of glucagon-like peptide-1 (GLP-1) from the intestine in mice (22). Salicylates, a widely used plant medicinal, inhibits cyclooxygenases and prostanoid biosynthesis (11), as well as IκB kinase B (IKKβ) in the NF-κB pathway (33). Salicylate has also been shown to activate AMPK to increase fat utilization and lower plasma fatty acids (7). Oral salicylates, especially the salicylate diester salsalate, have been found to improve glycemic control in individuals with type 2 diabetes (6, 29).

Since chronic inflammation is a key feature of obesity-related insulin resistance, we postulated that combining the actions of DHA and salicylate would reduce proinflammatory immune activation to reduce insulin resistance and improve glucose metabolism. In the present study, we investigated a novel bifunctional compound, Sal-DHA, comprised of DHA and salicylate linked together by a small molecule. Sal-DHA is stable in plasma but is hydrolyzed in the cytoplasm. In vitro administration of Sal-DHA showed potent anti-inflammatory effects on NF-κB activation, demonstrating synergism between DHA and salicylate. In mice with diet-induced obesity (DIO), oral administration of Sal-DHA lowered blood glucose by two main effects: reduced hepatic insulin resistance and enhanced endogenous GLP-1 secretion. The data findings suggest a novel drug strategy to mitigate hyperglycemia, reduce insulin resistance, and increase insulin secretion.

MATERIALS AND METHODS

Cell culture. Maintenance of RAW 264.7 and THP-1 cells was performed as previously described (23, 27). Intraperitoneal (ip) macrophages were obtained from C57/BL6 mice and cultured as described previously (13). Cells were pretreated with Sal-DHA compound or vehicle before stimulation with LPS, TNFα, or Pam3CSK4. A 50 mM Sal-DHA solution was prepared in 200 proof ethanol and diluted to 50 μM with 1% BSA (Sigma-Aldrich) in PBS solution. The BSA-EtOH-Sal-DHA solution was diluted with medium (1 g/l low-glucose DMEM + 10% low-endotoxin FBS + 1% Pen-Strep) to yield a pretreatment solution with final concentrations of 0.1% BSA, 0.2% EtOH, and 100 μM Sal-DHA. For mRNA analysis, cells were pretreated with the pretreatment solution for 2 h followed by stimulation with 10 ng/ml LPS, 10 ng/ml TNFα, or 10 ng/ml Pam3CSK4 for 6 h. For Western blot analysis, cells were pretreated with pretreatment solution for 2 h followed by stimulation with 10 and 100 ng/ml LPS for 15 min. Protein was isolated from cells after 0, 10, 20, 60, and 180 min after LPS stimulation.

mRNA isolation, RT-PCR, qPCR, and microarray analysis. mRNA was isolated using the RNeasy Mini Kit (QIAGEN), followed by reverse transcription using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and real-time PCR using an ABI 7300. Each reaction was measured in duplicate under standard reaction conditions. SYBR Green oligonucleotides were used for detection and quantification of a given gene, expressed as mRNA level normalized to a standard housekeeping gene (GAPDH or β-actin) using the
ΔΔCq method. We performed separate control experiments to ensure that the efficiencies of target and reference amplification were equal. The specificity of the PCR amplification was verified by melting curve analysis of the final products using Opticon 3 software (Bio-Rad). Primer sequences are available upon request.

Western blot analysis. Protein isolated from RAW 264.7, THP-1, and ip macrophage cells were subjected to Western blot analyses according to standard techniques. The primary antibodies used were p-IKKβ (Cell Signaling, 2967), IKKβ (Cell Signaling, 2684), actin (Sigma, A2066), p65 (Santa Cruz Biotechnology, C-20), p-IκBα (Cell Signaling, 4812), IkBa (Cell Signaling, 4814), TGFβ-activated kinase-1 (TAK1; Cell Signaling, 4505, rabbit polyclonal), p-TAK1 (Cell Signaling, 4508), and HSP90 (Cell Signaling, 4874). Anti-rabbit secondary antibody (Jackson ImmunoResearch 111-035-003) was used. After secondary antibody incubation and washing, the PDVF membrane was incubated in ECL solution (SuperSignal, Thermo). After incubation, membranes were washed and exposed to radiographic film. Antibodies were detected using a chemiluminescence detection system.

Luciferase reporter assay. To evaluate the functional consequences of NF-κB inhibition, a cell-based NF-κB reporter assay was developed. For this assay, the RAW 264.7 cell line was stably transfected with a vector containing three NF-κB response elements driving the transcription of a luciferase reporter gene. Treatment of RAW 264.7-3X NF-κB cells with LPS activates inflammatory signaling pathways primarily through the TLR4-NF-κB signaling axis. For the assay, RAW 264.7-3X NF-κB cells were plated in 96-well culture plates at a density of 50,000 cells/well in column 2–12. The plates were incubated overnight at 37°C in a humidified CO2 chamber. Compound dilutions or FBS alone were added to the plates, and the plates were incubated for an additional 2 h at 37°C. AlamarBlue reagent ± LPS solution (final concentration of 200 ng/ml) was added to the plates. The plates were returned to the 37°C humidified CO2 chamber for 3 h. Cell viability was assessed by visual inspection of the cells by microscopy and also by measuring the AlamarBlue fluorescence (excitation 550 nm, emission 590 nm) on a plate reader. To assess NF-κB activity, the supernatant was removed from the cells from each well, and Britelite Plus luciferase reagent was added to each well. The plate was shaken briefly, and the luciferase signal was measured in counts per second (CPS) on a plate reader.

Fig. 1. Novel bifunctional compound synergistically decreases the inflammatory response in cultured macrophages via reduced NF-κB signaling. A: chemical structure of novel bifunctional compound. The compound consists of docosahexaenoic acid (DHA) bound to salicylate by a small linker molecule. B: application of the bifunctional compound Sal-DHA inhibits NF-κB driven reporter activity in RAW 264.7 cultured macrophages. The effect of the linked compound is much greater than the effect of DHA and salicylate administered separately. Data are presented as means ± SE. *P < 0.05 vs. control (n = 4 per group). C: Sal-DHA reduces inflammatory gene expression in intraperitoneal mouse macrophages and human THP-1 cultured macrophages. Intraperitoneal macrophages or THP-1 cells were incubated in the presence or absence of LPS for 4–6 h following pretreatment with vehicle (Veh) or Sal-DHA. Gene expression was measured by qPCR. *P < 0.05 vs. LPS vs. LPS/Sal-DHA (n = 3 control, n = 5 LPS, n = 3 LPS/Sal-DHA). D: Sal-DHA reduces LPS-stimulated IKKβ phosphorylation in macrophages. THP-1 cultured human macrophage cells were treated with DHA, salicylate, or Sal-DHA. LPS 200 ng/ml was administered following pretreatment of cells with Sal-DHA for 1 h. Westerns were immunoblotted with phosphospecific IKKβ, total IKKβ, or actin antibodies. E: Sal-DHA stabilizes IκBα protein. THP-1 cells were treated with DHA, salicylate, Sal-DHA, or an IKKβ inhibitor. LPS 200 ng/ml was administered following pretreatment of cells with Sal-DHA for 1 h. Westerns were immunoblotted with IκBα or actin antibodies. F: Sal-DHA reduces serum TNFα. Mice were administered LPS by ip injection 2 h after pretreatment with vehicle, dexamethasone, and Sal-DHA. Serum TNFα was measured. Data are presented as means ± SE. ***P < 0.001 (n = 10 per group).
DUAL ACTIONS OF A NOVEL COMPOUND TO LOWER GLUCOSE

Animals. C57/BL6 mice were purchased from Harlan Laboratories. Mice were fed either normal chow (NC) or high-fat diet (HFD; 60% kcal from fat, D12492, Research Diets) and maintained on a 12:12-h light-dark cycle with free access to food and water. Only male mice were used for this study. All animal procedures adhered to University of California, San Diego institutional guidelines for the ethical treatment of animals. The in vivo studies were initiated when mice were 3 mo of age. For the gavage study, mice were first placed on the 60% HFD for 12 wk to cause diet-induced obesity (DIO). They then continued on HFD, receiving either Sal-DHA (300 mg/kg: 100 mg/kg salicylate, 200 mg/kg DHA) or a placebo solution (vehicle alone) by oral gavage daily for 3 wk. For the admixure study, Sal-DHA was added to the HFD, and mice received either the HFD-Sal-DHA admixture or HFD alone for 16 wk. Body weight measurements were obtained weekly.

Metabolic studies. For glucose tolerance (GTT) or insulin tolerance (ITT) (18) testing, mice were allowed to feed overnight and were then fasted for 7 h (GTT) or 4 h (ITT). After collection of basal blood, animals were given intraperitoneal or oral dextrose (1 or 5 g/kg, Hospira) for the GTT or ip insulin (0.6 U/kg Novolin R, Novo Nordisk) for the ITT. Blood samples were drawn by tail nick at basal and indicated times, and glucose was measured using a One-Touch glucose meter (Lifescan). Plasma insulin was quantified using the Ultra Sensitive Mouse Insulin ELISA kit (ALPCO). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following formula: [fasting insulin (µU/ml) × fasting glucose (mg/dl)] ÷ 405 (20). HbA1c levels were measured with whole blood samples of nonfasted mice by using a Single-use device (A1CNow, Bayer Contour).

Hyperinsulinemic-euglycemic clamp studies were performed as previously described (19, 25). In brief, two jugular catheters were inserted under anesthesia, tunneled to the midscapular region, and externalized. Five days after surgery, the clamp experiments began with the infusion of an equilibration solution of [3-3H]glucose (NEN, Boston, MA) for 90 min at a constant rate of 5 µCi/h. Then, insulin was infused at 8 mU·kg⁻¹·min⁻¹, and the glucose infusion rate was adjusted as necessary until euglycemia was achieved (~120 mg/dl for >20 min). Blood samples were collected prior to insulin infusion and upon euglycemia to confirm steady state. At steady state, the rate of glucose disappearance [total glucose disposal rate (GDR)] equals the sum of hepatic glucose production (HGP) and the glucose infusion rate. The insulin-stimulated GDR (IS-GDR) is equal to the total GDR minus the basal glucose turnover rate.

Serum TNFα measurements. Serum TNFα levels were measured using a TNFα ELISA kit (ALPCO). Mice were first pretreated with the Sal-DHA compound or vehicle (saline) and then given an ip LPS injection. Blood samples were drawn by tail nick.

Histology. For islet morphometry, paraffin-embedded pancreatic tissues were first stained with rabbit anti-insulin (DAKO N1542) antibodies. Secondary antibodies used for immunofluorescence detection were Cy3-conjugated anti-rabbit (Jackson Labs, 111-165-144) and Alexa 488 nm-conjugated anti-guinea pig (Molecular Probes, S-40174A). Specimens were viewed on a Zeiss AxioObserver Z1 microscope, and 24-bit TIFF images were acquired with a Zeiss AxioCam digital camera driven by Zeiss AxioVision v. 3.1 software. Images were processed with Adobe Photoshop CS2 9.0. Morphometry was performed on Sal-DHA-treated mice (n = 6) and control mice (n = 5) using Image-Pro Plus v. 5.0.1 (Media Cybernetics). Relative pancreatic areas of β-cells were then calculated. Mean islet size and number were determined using NIH Image J software (Bethesda, MD).

Lipomics analysis. Lipid measurements were conducted by Metabolon, as described previously (25). The lipids were extracted from liver tissues in the presence of authentic internal standards using chloroform mixed with methanol (2:1 vol/vol), and individual lipid classes were separated by HPLC. Lipid class fractions were trans-esterified in 1% sulfuric acid (in methanol) in a sealed vial with nitrogen at 100°C for 45 min. Fatty acid methyl esters were extracted from the mixture with hexane containing 0.05% butylated hydroxytoluene and redried for gas chromatography under nitrogen. Finally, fatty acid methyl esters were separated and quantified by capillary gas chroma-

Fig. 2. The bifunctional compound inhibits NF-κB pathway activation via TLR2 and TNFα. Intraperitoneal macrophages were collected from mice that received prior injection with ip thiglycollate and cultured for 5 days. A: Sal-DHA reduces TLR2-mediated inflammatory gene expression in intraperitoneal macrophages. Cells were subjected to treatment with vehicle or 25 µM Sal-DHA for 2 h followed by stimulation by TLR2 ligand 1 ng/ml Pam3CSK4 for 6 h. Gene expression was measured by qPCR (n = 3 per group). B: Sal-DHA stabilizes IκBα protein in intraperitoneal macrophages following stimulation with PAM3. Westerns were immunoblotted with total IκBα and HSP90 antibodies. C: Sal-DHA reduces TNFα-stimulated inflammatory gene expression in intraperitoneal macrophages. Cells were subjected to treatment with either vehicle or Sal-DHA 25 µM for 2h, followed by stimulation by TNFα 10 ng/ml for 6 h. Gene expression was measured by qPCR (n = 3 per group). D: Sal-DHA stabilizes IκBα protein and reduces IKKβ phosphorylation in intraperitoneal macrophages following stimulation with TNFα. Westerns were immunoblotted with phospho-IKKβ, total IKKβ, phospho-IκBα, total IκBα, or HSP90 antibodies. Data are presented as the mean ± SE. *P < 0.05, **P < 0.01.
tography equipped with a 30-m DB-88MS capillary column and a flame ionization detector.

Statistical analysis. All values are expressed as means ± SE unless otherwise noted. We used the two-tailed Student’s *t*-test or analysis of variance (ANOVA) to determine differences between groups and repeated-measures ANOVA testing for comparisons over time. *P* values < 0.05 were considered significant.

RESULTS

Constituents of the Sal-DHA bifunctional compound act synergistically to reduce inflammation in vitro. To generate the novel bifunctional compound, DHA and salicylate were joined by a small molecule linker that is stable in plasma but degraded by the enzyme fatty acid amide hydrolase, in the cytoplasm (4) (Fig. 1A). Sal-DHA was much more potent than equimolar amounts of either DHA or salicylate alone or nonlinked DHA and salicylate in its ability to inhibit LPS-stimulated proinflammatory gene expression in RAW 264.7 cells (Fig. 1B), demonstrating that the linked compound constituents act synergistically to reduce TLR4-mediated inflammation in vitro. Sal-DHA also strongly inhibited proinflammatory gene expression in intraperitoneal murine macrophages (Fig. 1C). We next investigated the expression of proinflammatory signaling proteins and found that Sal-DHA decreased LPS-induced IKKβ phosphorylation (Fig. 1D), and IkBα degradation in THP1 cells (Fig. 1E). Furthermore, the data suggested that Sal-DHA reduced TLR4-mediated inflammation in vivo by decreasing serum TNFα in mice that received intraperitoneal administration of LPS following pretreatment with the Sal-DHA compound (Fig. 1F).

Sal-DHA inhibits the activation of NF-κB via signaling mediated via TLR2, TLR4, and TNFα. Given the potent anti-inflammatory effect on NF-κB activation mediated via TLR4 as shown above, it was of interest to examine whether Sal-DHA also inhibited inflammatory signaling mediated via TLR2 or TNFα receptor signaling. As shown in Fig. 2, Sal-DHA significantly reduced proinflammatory gene expression, IkBα degradation, and IKKβ phosphorylation following stimulation with Pam3CSK4 (a TLR2 ligand) or TNFα in intraperitoneal macrophages. Thus, the Sal-DHA compound inhibits TLR2-, TLR4-, and TNFα-mediated inflammatory responses.

Sal-DHA improves insulin resistance in DIO mice. When Sal-DHA was delivered by adding the compound to 60% high-fat chow for 16 wk, body weights again did not differ between mice receiving the Sal-DHA admixture vs. those receiving high-fat diet alone (Fig. 3A). Interestingly, Sal-DHA significantly reduced fed glucose levels measured throughout the day (Fig. 3B). Consistent with this reduction in glucose,
HbA1c values were also lower in the DIO mice receiving Sal-DHA (Sal-DHA group: HbA1c 4.6 ± 0.13% vs. control group: 5.2 ± 0.15%, \( P < 0.01 \); Fig. 3C), reflecting lower average blood glucose levels over a 6-wk period, since the lifespan of the murine red blood cell is about 42 days.

Although we did not detect differences in glucose excursion between groups during an IGTT, concurrent plasma sampling revealed significantly lower insulin values, demonstrating improved insulin sensitivity in the Sal-DHA treated mice (Fig. 3, D and E). When insulin resistance initially develops in obese states, insulin secretion increases to maintain relatively normal glucose levels. This compensatory hyperinsulinemia is accompanied by an increase in pancreatic islet area, which we observed in untreated DIO mice (Fig. 3F). In contrast, pancreatic islet area and size were markedly smaller in Sal-DHA-treated mice (Fig. 3, F–H), consistent with improved insulin sensitivity and the observed reduction in insulin secretion after glucose challenge.

**Hepatic insulin resistance is reduced in DIO mice treated with Sal-DHA.** To assess tissue-specific insulin action more definitively, we conducted hyperinsulinemic-euglycemic clamp experiments after 16 wk of treatment. The glucose infusion rate required for maintaining euglycemia was higher after Sal-DHA treatment, but this difference did not reach statistical significance (\( P = 0.09 \); Fig. 4A). The insulin-stimulated glucose disposal rate, a measure of skeletal muscle insulin sensitivity, was also unchanged between groups (Fig. 4B). However, although basal HGP was similar between groups (data not

![Fig. 4. Sal-DHA improves hepatic insulin sensitivity in DIO mice. A: glucose infusion rate (GIR) is higher in mice receiving HFD-Sal-DHA admixture. B: insulin-stimulated glucose disposal rate is higher in mice receiving HFD-Sal-DHA admixture. C: hepatic glucose production at the clamped state is significantly lower in mice receiving HFD-Sal-DHA admixture. D: hepatic glucose production suppression is significantly higher in mice receiving HFD-Sal-DHA admixture. Data are presented as means ± SE. *\( P < 0.05 \), **\( P < 0.01 \), ***P < 0.001 (n = 10 per group). E: TAK-1 phosphorylation is decreased in mice treated with HFD admixture. Whole liver lysates were immunoblotted with anti-phospho-TAK1 and total TAK1 antibodies, as well as \( \alpha \)-tubulin for protein loading control. F: Liver cholesterol is decreased in mice receiving HFD-Sal-DHA admixture. Lipid classes in the liver were measured by lipidomic analysis. G: hepatic genes that regulate cholesterol synthesis are significantly decreased in mice receiving HFD-Sal-DHA admixture. H: total EPA, DPA, and DHA n3 fatty acids are increased in liver of mice receiving HFD-Sal-DHA admixture. Data are presented as means ± SE. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.0001 \) (n = 7 for HFD group, n = 6 for HFD-Sal-DHA group).
pared n3 fatty acids between groups and found that doco-
cholesterol levels were significantly lower in treated mice (Fig.
were unchanged with Sal-DHA treatment (data not shown), total
composition in that liver has been linked to hepatic inflammation
reduced liver inflammation was associated with the improved
hepatic insulin sensitivity. Since altered cholesterol and fatty acid
composition in that liver has been linked to hepatic inflammation
and insulin resistance (25, 32, 34), we performed a lipidomic
analysis of liver tissues. Although hepatic triacylglycerol levels
were unchanged following Sal-DHA administration.

We observed a significant reduction in TAK1 activation in mice
that activates NF-
reduced inflammation in vitro, we next investigated whether liver
inflammation was decreased in Sal-DHA-treated mice. TAK1 is
an intermediate signaling protein in the proinflammatory pathway
that activates NF-
resulting in decreased TAK1 activity, which was supported by the
reduction in NF-

In mice treated with the bifunctional compound (Fig. 4E) that suggested
reduced liver inflammation was associated with the improved
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composition in that liver has been linked to hepatic inflammation
and insulin resistance (25, 32, 34), we performed a lipidomic
analysis of liver tissues. Although hepatic triacylglycerol levels
were unchanged following Sal-DHA treatment (data not shown), total
cholesterol levels were significantly lower in treated mice (Fig.
The reduced cholesterol levels were accompanied by reduced
expression of hepatic genes known to regulate cholesterol synthe-
sis, including HMG CoA-synthase, HMG CoA-reductase, farne-
syl diphosphate, and squalene synthase (Fig. 4G). We also com-
pared n3 fatty acids between groups and found that doco-
saptaenoic acid (DPA), eicosapentaenoic acid (EPA), and
DHA levels were increased in the liver of Sal-DHA-treated mice (Fig. 4H).

Single-dose Sal-DHA acutely lowers glucose via an incretin-
based effect. Long-chain free fatty acids are known to stimulate
the release of gut incretins such as GLP-1 (12). Although DHA
has been shown to lower glucose levels acutely by stimulating the
endogenous secretion of GLP-1 in mice, it must be admin-
istered directly into the colon to achieve this effect (22). In
contrast, we found substantial reductions in blood glucose in
weight-matched (Fig. 5A) drug-naïve DIO mice occurring 4 h
after receiving a single gavage dose of the compound (Fig. 5B).
The experiment was repeated, and blood glucose levels were
measured every 2 h to identify the timeline of the compound’s
effectiveness showing glucose-lowering for 2 to 6 h after dose
administration (Fig. 5C).

A single dose of Sal-DHA also significantly improved glucose
tolerance in these mice, increasing circulating GLP-1 levels by 10 min after glucose was administered. Interestingly,
the effect of the compound on GLP-1 secretion was greater
after oral vs. intraperitoneal glucose challenge (Fig. 5, D–I). To
determine whether a single dose of Sal-DHA also affected
insulin resistance, we performed insulin tolerance testing and
found that although blood glucose values were lower overall in
the compound-treated group (Fig. 5J), the percent decrease in
blood glucose from baseline was unchanged (Fig. 5K), indicating
that insulin sensitivity was not improved.

Fig. 5. The bifunctional compound acutely low-
ers BG in mice through an incretin-based effect. Animals were placed on 60% high fat for 12 wk
prior to testing. A: mice receiving HFD alone and
HFD-Sal-DHA admixture were weight matched.
B and C: single-dose Sal-DHA has an immediate
glucose-lowering effect with significantly de-
creased BG by 2 h after administration. B: whole
BG was measured after a 4-h fast. Mice were
given a single dose of compound by gavage,
followed by BG measurement 4 h later. C: mice
were fasted at 8 AM (t = 0 h) followed by
administration of a single dose at 10 AM. BG
was measured at 2-h intervals following gavage.

Data are presented as means ± SE. (n = 12 per
group). D–I: the compound produces greater
glucose lowering following oral vs. ip glucose
administration during glucose tolerance testing.

BG in HFD-fed mice following ip dextrose (D)
or oral dextrose (E). Area under the curve (AUC)
glucose during IPGTT (F) or OGTT (G). DHA
mimetic or vehicle was gavaged 2 h prior to
GTG. (n = 7 per group). H and F: DHA mimetic
augments glucose-stimulated secretion of glu-
cagon-like peptide-1 (GLP-1). Total serum GLP-1
levels at 10 min following ip dextrose (H) or oral
dextrose (I). Sal-DHA or vehicle was gavaged 2
h prior to administration of 1 g/kg dextrose (n = 7
per group). J and K: single-dose Sal-DHA does
not improve insulin sensitivity. BG levels follow-
ing 0.35 U/kg ip insulin (J) and % decrease in BG
(K). Although BG values are lower overall in the
compound-treated group, the % decrease in BG
from baseline is unchanged, indicating no
improvement in insulin sensitivity. Mice were fasted
for 4 h prior to ITT. Compound or vehicle was
administered by gavage 2 h prior to ITT (n = 8
per group). Data are presented as means ± SE.
*p < 0.05, **p < 0.01, ***p < 0.001.
The acute glucose-lowering effect of Sal-DHA is mediated via GLP-1R signaling. To further probe whether the acute glucose-lowering effect of Sal-DHA was mediated via GLP-1 signaling, we conducted additional experiments in lean mice. Sal-DHA was equally effective in lowering glucose and increasing GLP-1 in these mice during oral GTT (Fig. 6, A–C). Notably, this effect was abrogated by the administration of exendin(9–39), a GLP-1 receptor antagonist, indicating that the GLP-1 signaling plays an important role in the ability of Sal-DHA to acutely reduce glucose (Fig. 6D).

DISCUSSION

Despite significant advances in our knowledge, the prevalence of type 2 diabetes and its comorbidities continues to rise, and alternative strategies to treat this disease are needed. We have shown that Sal-DHA, a novel bifunctional compound consisting of salicylate linked to DHA 22:6n-3, effectively lowers glucose in mice with diet-induced obesity, a well-established model of insulin resistance and diabetes. Our data indicate that the Sal-DHA compound acts as an insulin sensitizer that reduces hepatic glucose production without a significant effect on peripheral glucose disposal. In addition, Sal-DHA enhances GLP-1 secretion to acutely lower glucose even in drug-naïve animals.

When administered in a diet admixture, Sal-DHA achieved metabolic efficacy at lower DHA or salicylate doses than reported in other previous studies where each component was administered alone (7, 25, 28, 33). DHA and salicylate have each been shown to attenuate inflammation (31, 33). We hypothesize that this chronic effect of the Sal-DHA compound on glucose metabolism may result from reduced inflammation. Many lines of evidence show that the activation of proinflammatory pathways within insulin target tissues can impair insulin signaling, and chronic, low-level tissue inflammation is now recognized as an important cause of systemic insulin resistance (26). Several studies have shown that macrophages, a key component of the innate immune defense against pathogens, are critical effector cells in this process. In our study, we were able to corroborate that Sal-DHA attenuated inflammation in vivo by decreasing LPS-induced secretion of TNFα. Moreover, the compound components appeared to act synergistically to potentially diminish inflammation in vitro by inhibiting NF-κB activation mediated by TLR2, TLR4, and TNFα signaling pathways in macrophage cells, indicating that the compound likely inhibits inflammatory signaling where these signaling pathways converge.

A direct comparison of salicylate or DHA alone with Sal-DHA in vivo has yet to be tested. However, as the Sal-DHA compound is lipid soluble and delivered in a lipid carrier, the compound is taken up by enterocytes and packaged into chylomicrons that are then delivered via the lymphatic system to target tissues. We hypothesize that the compound is transported through the cell membrane either passively or by fatty acid binding proteins and is not activated until it is within the cytoplasm of the target tissue. Activation of the compound occurs after the linker is cleaved by fatty acid amide hydrolase. The intracellular activation of the compound within target tissues may contribute to its greater efficacy compared with salicylate or DHA alone. We observed decreased phosphorylation of TAK1 in the liver of admixture-treated mice, suggesting that Sal-DHA may improve hepatic insulin sensitivity by reducing liver inflammation. The results also showed increased n3 fatty acids in liver tissues of compound-treated mice. Several recent studies have demonstrated metabolic improvement with the administration of DHA, often accompanied with the resolution of inflammation (3, 24). In addition, we noted...
reduced hepatic cholesterol with reduced expression of genes that regulate cholesterol synthesis. Inflammation has been shown to promote cholesterol accumulation in liver by increased uptake and de novo synthesis (34). However, hepatic lipogenesis and insulin resistance can be dissociated from inflammatory changes (2). Therefore, further work is needed to clarify the mechanisms by which the Sal-DHA compound improves hepatic insulin sensitivity in DIO mice.

Notably, single-dose Sal-DHA enhanced the secretion of GLP-1 to produce immediate glucose lowering. This effect was almost completely abolished by administration of a GLP-1 receptor antagonist. GLP-1 is released by L-cells of the intestinal epithelium upon food ingestion and plays an important role in promoting glucose-dependent insulin secretion, slowing gastric emptying, inducing satiety, and inhibiting postprandial glucagon secretion (5). Although single-dose Sal-DHA significantly improved oral glucose tolerance, it also improved glucose tolerance following intraperitoneal administration of glucose. Moreover, it lowered glucose and enhanced the secretion of active GLP-1 even in fasting mice that did not receive oral glucose, indicating that intestinal glucose exposure was not required for its incretin effect. Prior studies have shown that DHA and EPA can each lower glucose and augment GLP-1 secretion in the absence of an oral glucose challenge. However, they must be delivered directly to the colon by catheter (22). In our study, the effect of Sal-DHA on GLP-1 secretion is likely mediated via its DHA component and can be achieved by oral administration. Long-chain fatty acids activate GPR120 receptors that are abundantly expressed in the gut to promote the secretion of GLP-1 from intestinal L-cells (12). Further study is needed to determine whether Sal-DHA directly binds and activates GPR120 to increase GLP-1 secretion. An emerging literature also shows that interactions between incretins and inflammation may alter blood glucose (8, 15, 30). Although beyond the scope of our present study, it would be of interest to investigate the potential effect of Sal-DHA-mediated GLP-1 release on inflammatory signaling in future experiments.

Salicylate has been shown to improve glycemic control in humans with type 2 diabetes (6). In contrast, DHA lowers serum triglycerides in human subjects (17), but it has not been shown to affect fasting glucose or insulin resistance either alone or in combination with EPA (10, 16). Our present results indicate that the bifunctional compound combining DHA and salicylate potently lowers glucose and improves insulin resistance, presenting a novel strategy to improve glucose metabolism in individuals with type 2 diabetes.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


REFERENCES


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