ORIGINAL RESEARCH ARTICLE

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

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Running head: Dual actions of a novel compound to lower glucose

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Docosahexaenoic acid (DHA 22:6n-3) and salicylate (acetylsalicylic acid) 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 acted synergistically to reduce inflammation mediated via nuclear factor kappa B (NFκB) in cultured macrophages. Notably, oral administration of the bifunctional compound acted in two distinct ways to mitigate hyperglycemia in high-fat diet (HFD)-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.
KEYWORDS:

- Obesity
- Insulin-resistance
- Docosahexaenoic acid
- Salicylate
INTRODUCTION

Type 2 diabetes is a disease process characterized by hyperglycemia, insulin resistance, and pancreatic β-cell dysfunction, 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 also have substantial limitations (21).

Two existing drug strategies, 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 pro-inflammatory M1 macrophages 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 GLP-1 from the intestine in mice (22). Salicylate, a widely used plant medicinal, inhibits cyclo-oxygenases 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 pro-inflammatory 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 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 RAW264.7 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 pre-treated 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 uM with 1% BSA (Sigma Aldrich) in PBS solution. The BSA-EtOH-Sal-DHA solution was diluted with media (low 1 g/L glucose DMEM + 10% low endotoxin FBS + 1% Pen/Strep) to yield a pre-treatment solution with final concentrations of 0.1% BSA, 0.2% EtOH, and 100 uM Sal-DHA. For mRNA analysis, cells were pre-treated with the pre-treatment solution for 2 hours followed by stimulation with 10 ng/ml LPS, 10 ng/ml TNFα, or 10 ng/ml Pam3CSK4 for 6 hours. For western blot analysis, cells were pre-treated with pre-treatment solution for 2 hours followed by stimulation with 10 ng/ml and 100 ng/ml LPS for 15 minutes. Protein was isolated from cells after 0, 10, 20, 60, 120, and 180 minutes 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 ΔΔC_T 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 RAW264.7, THP-1, and intraperitoneal macrophage cells were subjected to western blot analyses according to standard techniques. The primary antibodies used were p-IKKb (Cell Signaling 2697), IKKb (Cell Signaling 2684), actin (Sigma A2066), p65 (Santa Cruz C-20), p-IkBa (Cell Signaling 4812), IkBa (Cell Signaling 4814), TAK1 (Cell Signaling 4505, rabbit polyclonal), p-TAK1 (Cell Signaling 4508), and HSP90 (Cell Signaling 4874). Anti-rabbit secondary antibody (Jackson ImmunoResearch Inc. 111-035-003) was used. After secondary antibody incubation and washing, the PDVF membrane was incubated in ECL solution (SuperSignal, Thermo Scientific) to visualize proteins via chemiluminescence.

**Luciferase reporter assay.** To evaluate the functional consequences of NFκB inhibition a cell-based NFκB reporter assay was developed. For this assay, the RAW264.7 cell line was stably transfected with a vector containing three NFκB response elements driving the transcription of a luciferase reporter gene. Treatment of RAW264.7-3X NFκB cells with LPS activates inflammatory signaling pathways primarily through the TLR4-NFκB signaling axis. For the assay, RAW264.7-3X NFκB 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 2h at 37°C. AlamarBlue reagent +/- LPS solution (final concentration of 200 ng/mL) was added to cells. 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 and emission=590 nm) on a plate reader. To assess NFκB 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.

Animals. C57/BL6 mice were purchased from Harlan Laboratories. Mice were fed either a normal chow (NC) or HFD (60% kcal from fat; D12492, Research Diets), and maintained on a 12h 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 months of age. For the gavage study, mice were first placed on the 60% HFD for 12 weeks 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 weeks. For the admixture study, Sal-DHA was added to the HFD, and mice received either the HFD-Sal-DHA admixture or HFD alone for 16 weeks. Body weight measurements were obtained weekly.

Metabolic studies. For glucose tolerance (GTT) or insulin tolerance (18) testing, mice were allowed to feed overnight and then fasted for 7 hours (GTT) or 4 hours (18). After collection of basal blood, animals were given intraperitoneal or oral dextrose (1 g/kg or 5 g/kg, Hospira, Inc.) for the GTT, or intraperitoneal 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 glucose and insulin concentrations obtained after 6 hours of food withdrawal, using the following formula: \([\text{fasting blood glucose (mg/dl)} \times \text{fasting insulin (µU/ml)}] \div 405\) (20). HbA1c levels were measured with whole blood samples of non-fasted mice 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 mid-scapular region and externalized. Five days after surgery, the clamp experiments began with the infusion of an equilibration solution of \([3-3^H]D\)-glucose (NEN, Boston) for 90 min at a constant rate of 5 µCi/h. Then, insulin was infused at 8 mU/kg/min while 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 or GDR) equals the sum of hepatic glucose production (HGP) and the glucose infusion rate (GIR). The insulin-stimulated GDR (IS-GDR) is equal to the total GDR minus the basal glucose turnover rate.

**Serum TNFa measurements.** Serum TNFa levels were measured using a TNFa ELISA kit (ALPCO). Mice were first pretreated with the Sal-DHA compound or vehicle (saline), then given an intraperitoneal 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 111-165-144) and Alexa-488 nm conjugated anti-guinea-pig (Molecular Probe 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., USA).

Lipomics analysis. Lipid measurements were conducted by Metabolon, Inc. 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 transesterified 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 readied for gas chromatography under nitrogen. Finally, fatty acid methyl esters were separated and quantified by capillary gas chromatography equipped with a 30 m DB-88MS capillary column and a flame-ionization detector.

Statistical analysis. All values are expressed as means ± SEM 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 of $<0.05$ were considered significant.
RESULTS

The 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) (Figure 1A). Sal-DHA was much more potent than equimolar amounts of either DHA or salicylate alone or non-linked DHA and salicylate in its ability to inhibit LPS-stimulated pro-inflammatory gene expression in RAW264.7 cells (Figure 1B), demonstrating that the linked compound constituents act synergistically to reduce TLR4-mediated inflammation in vitro. Sal-DHA also strongly inhibited pro-inflammatory gene expression in intraperitoneal murine macrophages (Figure 1C). We next investigated the expression of pro-inflammatory signaling proteins and found that Sal-DHA decreased LPS-induced IκKβ phosphorylation (Figure 1D), and IκBα degradation in THP1 cells (Figure 1E). Furthermore, the data suggested that Sal-DHA reduced TLR-4 mediated inflammation in vivo by decreasing serum TNFα in mice who received intraperitoneal administration of LPS following pre-treatment with the Sal-DHA compound (Figure 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 Figure 2, Sal-DHA significantly reduced pro-inflammatory gene expression, IκBα degradation, and IκKβ 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 weeks, body weights again did not differ between mice receiving the Sal-DHA admixture versus those receiving high fat diet alone (Figure 3A). Interestingly, Sal-DHA significantly reduced fed glucose levels measured throughout the day (Figure 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) (Figure 3C), reflecting lower average blood glucose levels over a 6 week period since the lifespan of the murine red blood cell is about 42d.

Although we did not detect differences in glucose excursion between groups during an intraperitoneal glucose tolerance test, concurrent plasma sampling revealed significantly lower insulin values, demonstrating improved insulin sensitivity in the Sal-DHA treated mice (Figure 3D and 3E). 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 (Figure 3F). In contrast, pancreatic islet area and size were markedly smaller in Sal-DHA treated mice (Figure 3F-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 weeks of treatment. The glucose infusion rate required for maintaining euglycemia was higher after Sal-DHA treatment, but this difference did not reach statistical significance (Figure 4A, p = 0.09). The insulin-stimulated glucose disposal rate, a measure of skeletal muscle insulin sensitivity, was also unchanged between groups (Figure 4B). However, although basal hepatic glucose production (HGP) was similar between groups (data not shown), HGP during the clamp was significantly lower in treated mice (Figure 4C) indicating much greater insulin-stimulated HGP suppression (Figure 4D, p = 0.001). This result reveals that enhanced liver insulin sensitivity accounts for the improved insulin resistance following Sal-DHA administration.

Chronic tissue inflammation is recognized as a key cause of insulin resistance in obese states. Since Sal-DHA markedly reduced inflammation in vitro, we next investigated whether liver inflammation was decreased in Sal-DHA-treated mice. TGF-β–activated kinase 1 (TAK1) is an intermediate signaling protein in the proinflammatory pathway that activates NF-κB via TLR2, TLR4 and TNFα receptors (14). We observed a significant reduction in TAK1 activation in mice treated with the bifunctional compound (Figure 4E) that suggested 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 with Sal-DHA treatment (data not shown), total cholesterol levels were
significantly lower in treated mice (Figure 4F). The reduced cholesterol levels were accompanied by reduced expression of hepatic genes known to regulate cholesterol synthesis including HMG CoA synthase, HMG CoA reductase, farnesyl diphosphate, and squalene synthase (Figure 4G). We also compared n3 fatty acids between groups and found that DPA, EPA and DHA levels were increased in the liver of Sal-DHA treated mice (Figure 4H).

**Single dose Sal-DHA acutely lowers glucose via an incretin-based effect**

Long chain FFAs 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 administered directly into the colon to achieve this effect (22). In contrast, we found substantial reductions in blood glucose in weight-matched (Figure 5A) drug-naïve DIO mice occurring 4 hours after receiving a single gavage dose of the compound (Figure 5B). The experiment was repeated and blood glucose levels were measured every two hours to identify the timeline of the compound’s effectiveness showing glucose-lowering for 2 to 6h after dose administration (Figure 5C).

A single dose of Sal-DHA also significantly improved glucose tolerance in these mice, increasing circulating GLP-1 levels by 10 minutes after glucose was administered. Interestingly, the effect of the compound on GLP-1 secretion was greater after oral versus intraperitoneal glucose challenge (Figures 5D-I). To determine whether a single dose 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 (Figure 5J), the percentage decrease in
blood glucose from baseline was unchanged (Figure 6K), indicating that insulin sensitivity was not improved.

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 glucose tolerance testing (OGTT) (Figures 6A-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 (Figure 6D).
Despite significant advances in 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 pro-inflammatory 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 potently diminish inflammation in vitro by inhibiting NFκB 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 when compared to 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 who 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 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 None.

REFERENCES


FIGURE LEGENDS

Figure 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 DHA bound to salicylate using a small linker molecule.

B) Application of the bifunctional compound Sal-DHA inhibits NF-κB driven reporter activity in RAW264.7 cultured macrophages. The effect of the linked compound is much greater than the effect of DHA and salicylate administered either separately or together. Data are represented as the mean ± 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 pre-treatment with vehicle or Sal-DHA. Gene expression was measured by qPCR. *p<0.05 control vs. LPS, #p<0.05 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 pre-treatment of cells with Sal-DHA for 1h. Westerns were immunoblotted with phospho-specific 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 pre-treatment 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 through intraperitoneal injection 2 hours after pre-treatment with vehicle, dexamethasone, and Sal-DHA. Serum TNFα was measured. Data are represented as the mean ± SE. ***p<0.001 (n=10 per group).

Figure 2. The bifunctional compound inhibits NFκB pathway activation via TLR2 and TNFα

Intraperitoneal macrophages were collected from mice that received prior injection with I.P. thioglycollate and cultured for 5 days.

A) Sal-DHA reduces TLR2-mediated inflammatory gene expression in intraperitoneal macrophages. Cells were subjected to treatment with either vehicle or Sal-DHA 25 uM for 2h, followed by stimulation by the TLR2 ligand, Pam3CSK4, 1 ng/ml for 6h. 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 uM 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-IKKα/β, total IKKβ, phospho-IκBα, total IκBα, or HSP90 antibodies.

Data are represented as the mean ± SE. *p<0.05, **p<0.01.
C57BL/6N mice were fed a 60% high-fat diet (HFD) or a 60% HFD admixture to which Sal-DHA (7.5g/kg chow) was added for 16 weeks.

**A)** Body weights do not differ between mice fed HFD alone vs. the HFD-Sal-DHA admixture.

**B)** Blood glucose levels are significantly lower during ad lib feeding in mice receiving the HFD-Sal-DHA admixture.

**C)** HbA1c measurement following 7w of HFD-Sal-DHA admixture. The murine RBC half-life is approximately 42 days. The HbA1c measurement reflects chronic glucose lowering over this time period.

**D-E)** Insulin sensitivity is improved in mice receiving the HFD-Sal-DHA admixture. Glucose tolerance was measured following intraperitoneal glucose challenge (D). Although glucose values were similar between groups, concurrent insulin levels were significantly lower in the Sal-DHA-treated mice (E).

**F-H)** Compensatory β cell hyperplasia is reduced in mice receiving the HFD-Sal-DHA admixture. Pancreatic islet area and size are reduced in mice receiving the HFD-Sal-DHA admixture, suggesting decreased insulin resistance.

Data are represented as the mean ± SE. *p<0.05, **p<0.01 (n=10 per group).

**Figure 4. Sal-DHA improves hepatic insulin sensitivity in DIO mice**

**A)** Glucose infusion rate is higher in mice receiving the HFD-Sal-DHA admixture.

**B)** Insulin-stimulated glucose disposal rate is higher in mice receiving the HFD-Sal-DHA admixture.
C) Hepatic glucose production at the clamped state is significantly lower in mice receiving the HFD-Sal-DHA admixture.

D) Hepatic glucose production suppression is significantly higher in mice receiving the HFD-Sal-DHA admixture.

Data are represented as the mean ± SE. *p<0.05, **p<0.01, ***p<0.001 (n=10 per group).

E) Tak-1 phosphorylation is decreased in mice treated with the HFD admixture. Whole liver lysates were immunoblotted with anti-phosphoTak1 and total Tak1 antibodies, as well as alpha-tubulin for protein loading control.

F) Liver cholesterol is decreased in mice receiving the 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 the HFD-Sal-DHA admixture.

H) Total EPA, DPA, and DHA n3 fatty acids are increased in the liver of mice receiving the HFD-Sal-DHA admixture.

Data are represented as the mean ± SE. *p<0.05, **p<0.01, ****p<0.0001 (n=7 for HFD group, n=6 for HFD-Sal-DHA group).

Figure 5. The bifunctional compound acutely lowers blood glucose in mice through an incretin-based effect

Animals were placed on 60% high fat for 12 weeks prior to testing.

A) Mice receiving the HFD alone and HFD-Sal-DHA admixture were weight matched.

B-C) Single dose Sal-DHA has an immediate glucose lowering effect with significantly decreased blood glucose by 2h after administration. Whole blood glucose (BG) was measured
after a 4h fast (B). Mice were given a single dose of compound by gavage, followed by BG measurement 4h later. Mice were fasted at 8 AM (T = 0h), followed by administration of a single dose at 10 AM. BG was measured at 2-hour intervals following gavage (C). Data are represented as the mean ± SE (C). (n=12 per group).

D-G) The compound produces greater glucose lowering following oral vs. intraperitoneal 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 2h prior to GTT. (n=7 per group).

H-I) The DHA mimetic augments glucose-stimulated secretion of GLP-1. Total serum GLP-1 levels at 10 minutes following IP dextrose (H) or oral dextrose (I). Sal-DHA or vehicle was gavaged 2h prior to administration of dextrose 1g/kg. (n=7 per group).

J-K) Single dose Sal-DHA does not improve insulin sensitivity. Blood glucose levels following IP insulin 0.35 U/kg (J) and % decrease in blood glucose (K). Although blood glucose 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 4h prior to ITT. Compound or vehicle was administered by gavage 2h prior to ITT. (n= 8 per group).

Data are represented as the mean ± SE. *p<0.05, **p<0.01, ***p<0.001

Figure 6. The acute effects of Sal-DHA are mediated in part via GLP-1R signaling.

A-C) Single dose Sal-DHA improves glucose tolerance and GLP-1 secretion in lean mice. NC-fed mice following oral dextrose 5g/kg (A). AUC glucose during OGTT (B). Total serum GLP-1 levels at 10 minutes following dextrose (C). Compound or vehicle was gavaged 2h prior to OGTT. *p<0.05, **p<0.01 compared to vehicle-treated control (n=7 per group).
GLP-1R signaling is required for the acute glucose-lowering effect. Exendin(9-39) is a known inhibitor of the GLP-1 receptor. Fasting mice were injected with either IP Ex(9-39) or saline, then given the compound or vehicle by gavage 2h prior to OGTT with oral dextrose 5g/kg. *p<0.05 compound/Ex(9-39) vs. compound/saline, #p<0.05 vehicle/Ex(9-39) vs. compound/saline groups (n=5 per group).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6