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Low-Molecular-Weight Peptides from Salmon Protein Prevent Obesity-Linked Glucose Intolerance, Inflammation, and Dyslipidemia in LDLR$^{-/-}$/ApoB$^{100/100}$ Mice[1–3]

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Abstract

Background: We previously reported that fish proteins can alleviate metabolic syndrome (MetS) in obese animals and human subjects.

Objectives: We tested whether a salmon peptide fraction (SPF) could improve MetS in mice and explored potential mechanisms of action.

Methods: ApoB^{100} only, LDL receptor knockout male mice (LDLR$^{-/-}$/ApoB$^{100/100}$) were fed a high-fat and -sucrose (HFS) diet (25 g/kg sucrose). Two groups were fed 10 g/kg casein hydrolysate (HFS), and 1 group was additionally fed 4.35 g/kg fish oil (FO; HFS+FO). Two other groups were fed 10 g SPF/kg (HFS+SPF), and 1 group was additionally fed 4.35 g FO/kg (HFS+SPF+FO). A fifth (reference) group was fed a standard feed pellet diet. We assessed the impact of dietary treatments on glucose tolerance, adipose tissue inflammation, lipid homeostasis, and hepatic insulin signaling. The effects of SPF on glucose uptake, hepatic glucose production, and inducible nitric oxide synthase activity were further studied in vitro with the use of L6 myocytes, FAO hepatocytes, and J774 macrophages.

Results: Mice fed HFS+SPF or HFS+SPF+FO diets had lower body weight (protein effect, $P = 0.024$), feed efficiency (protein effect, $P = 0.018$), and liver weight (protein effect, $P = 0.003$) as well as lower concentrations of adipose tissue cytokines and chemokines (protein effect, $P \leq 0.003$) compared with HFS and HFS+FO groups. They also had greater glucose tolerance (protein effect, $P < 0.001$), lower activation of the mammalian target of rapamycin complex 1/S6 kinase 1/insulin receptor substrate 1 (mTORC1/S6K1/IRS1) pathway, and increased insulin signaling in liver compared with the HFS and HFS+FO groups. The HFS+FO, HFS+SPF, and HFS+SPF+FO groups had lower plasma triglycerides (protein effect, $P = 0.003$; lipid effect, $P = 0.002$) than did the HFS group. SPF increased glucose uptake and decreased HGP and iNOS activation in vitro.

Conclusions: SPF reduces obesity-linked MetS features in LDLR$^{-/-}$/ApoB$^{100/100}$ mice. The anti-inflammatory and glucoregulatory properties of SPF were confirmed in L6 myocytes, FAO hepatocytes, and J774 macrophages. *J Nutr* 2015;145:1415–22.

Keywords: low-molecular-weight peptide, omega-3 fatty acids, glucose metabolism, insulin signaling, anti-inflammatory, metabolic syndrome, protein hydrolysate, amino acids

Introduction

Increased fish consumption has been suggested to prevent metabolic syndrome (MetS)[11] and to reduce the incidence of type 2 diabetes (T2D) and cardiovascular diseases (CVDs) in obese subjects (1, 2). However, this is still the subject of considerable debate (3, 4), and despite decades of research, the

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[3] Supplemental Tables 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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relation between ω-3 PUFA supplementation and the incidence of CVD and T2D remains controversial. This led us to propose many years ago that another component in fish, namely fish protein, could contribute to the beneficial effects of fish consumption on components of MetS. Indeed, we have shown that dietary cod protein influences glucose tolerance and insulin sensitivity in sucrose-fed rats (5) and that substituting casein for cod as the sole source of dietary protein reduced insulin resistance in rats fed a high-fat and -sucrose (HFS) diet through prevention of defective insulin signaling (6).

We also found that protein hydrolysates from salmon and other fish sources reduced inflammation in visceral adipose tissue (7). The effects of dietary fish protein have been validated in insulin-resistant human subjects in whom ~63% of their daily dietary proteins were replaced with cod protein. The cod protein diet was shown to improve insulin sensitivity and to reduce plasma C-reactive protein (8, 9), an inflammatory marker and an independent predictor of CVD events and T2D incidence (10).

We hypothesized that the bioactive peptides produced from gastric digestion are responsible for the beneficial effects of fish proteins on components of MetS. Indeed, it was previously shown that fish peptides exert profound physiologic effects in rodent models and in humans, particularly on the cardiovascular and immune systems [reviewed in (11–13)].

Methods

Production of the salmon peptide fraction. Frozen Atlantic salmon (*Salmo salar*) frames were provided courtesy of Cooke Aquaculture (Canada). Frames were thawed, mechanically deboned, and divided into 12.5-kg aliquots, which were mixed with 1.0 mol/L NaOH and homogenized. Salmon peptide fraction (SPF) was prepared as previously described (14).

The amino acid (AA) determination (**Supplemental Table 1**) of the SPF and casein hydrolysate (CH), used as a control in the dietary studies, was performed by reversed-phase HPLC (Alliance Waters e2695 system; Waters Corporation) with fluorescence detection (Waters 2495 Multi λ Fluorescence Detector). After 23 h acid hydrolysis carried out at 110°C, AAs were subjected to derivatization by using AccQ Tag Amino Acid Analysis kit (Waters Corporation) before being injected and quantified by HPLC. The SPF had notably higher amounts of alanine, arginine, aspartic acid, histidine, and glycine, whereas its glutamic acid, proline, and BCAA (isoleucine, leucine, and valine) content was lower than in CH.

Animals. On day 1, at 8–9 wk of age, male LDLR$^{-/-}$/ApoB$^{100/100}$ mice from our in-house colony were weighed and separated into individual, ventilated cages and randomly assigned to dietary groups, as described below. The animal protocols met the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of Laval University.

Diets. Mice were fed for 12 wk with either the standard nonpurified feed pellet diet [standard chow (SC); ~4.1 kcal/g] or 1 of 4 isocaloric HFS diets (~6.3 kcal/g). The HFS diet contained SPF or CH with or without ω-3 PUFAs [fish oil (FO)]. Briefly, the protein component of the HFS diets included 50% casein and 50% SPF or CH to test for the effect of protein hydrolysis per se. For the lipid component, 4.35 g/kg corn oil was replaced with FO (MEG-3; courtesy of Ocean Nutrition Canada) containing EPA (45%) and DHA (22%). Complete diet composition for the 4 dietary intervention groups—HFS, HFS+FO, HFS+SPF, and HFS +SPF+FO—is given in **Supplemental Table 2**. Food and water were available ad libitum. The SC diet (Harlan Laboratories, Teklad Global 18% Protein Rodent diet no. 2018) contained 18.6% (wt:wt) protein, 6.2% (wt:wt) fat, and 44.2% (wt:wt) carbohydrates. The HFS diets were prepared in our laboratory with a food-grade mixer.

Intraperitoneal glucose tolerance test. After 12 wk of being fed the indicated diet, mice were feed-deprived for 6 h and then received a 20% glucose solution by intraperitoneal injection (0.75 g/kg body weight). Blood samples were taken from the saphenous vein before (time = 0 min) and 15, 30, 60, 90, and 120 min after the glucose injection. The blood samples were kept on ice, and then plasma was separated by centrifugation at 2400 × *g* for 10 min at 4°C and stored at −80°C until further analysis. Blood glucose was measured immediately upon collection by using a One Touch Mini Ultra Glucometer (LifeScan).

Biochemical analyses. Mice were feed-deprived for 6 h and then anesthetized with isoflurane. An acute tail vein injection of 1.9 U/kg intravenous insulin (Novolin; Novo Nordisk) or saline control was then performed, followed by cardiac puncture 5 min later. Blood was collected into EDTA-coated tubes (Sarstedt) and placed on ice. Mice were then killed by cervical dislocation. The inguinal, epididymal, brown, and retroperitoneal adipose tissues and liver were immediately harvested, weighed, and snap-frozen in liquid nitrogen. The blood was then centrifuged, and plasma was stored at −80°C until further analysis. For Western blotting, liver was homogenized and processed as previously described (15). Equivalent amounts of proteins were loaded onto an acrylamide gel, subjected to SDS-PAGE, and then electrophoretically transferred onto nitrocellulose membranes overnight. Membranes were blocked, probed with antibodies, phosphorylated protein kinase B serine 473 (p-Akt S473), protein kinase B (Akt), eukaryotic elongation factor 2 (eEf2), actin, phosphorylated S6 serine 240/244 (p-S6 S240/244), phosphorylated S6 serine 235/236 (p-S6 S235/236), ribosomal protein s6 (S6), phosphorylated insulin receptor substrate 1 serine 1101 (p-IRS1 S1101), and insulin receptor substrate 1 (IRS1) (Cell Signaling Technology), and then detected by enhanced chemiluminescence (EMD Millipore). A liver protein standard was conducted on every gel for comparison of samples from different immunoblots.

Analytical methods. Plasma insulin concentrations were analyzed by using the Ultrasensitive mouse ELISA (EMD Millipore). Standard colorimetric kits were used for TGs (TR210), cholesterol (CH202; Randox Life Sciences), nonesterified FAs (NEFAs) [HF-Series NEFA-HR (2); Wako Diagnostics], and glycerol (Sigma-Aldrich). Chemokines and cytokines were assessed in epididymal adipose tissue lysates (500 μg/mL protein in PBS containing 1% NP-40 and 0.01% protease inhibitor cocktail) by using a Bio-plex 200 system and Pro Mouse Cytokine kit (Bio-Rad Laboratories). Liver TGs were analyzed by using an adapted Folch method (16).

In vitro screening assays for SPF action on glucose metabolism and inflammation. L6 rat myoblasts (courtesy of Dr. Amira Klip, Hospital for Sick Children, Toronto, Canada) were grown and differentiated into myotubes, as previously described (17). Cells were plated in 24-well plates. Myotubes were serum deprived for 3 h and treated or not with SPF (1 ng/mL or 1 μg/mL) for 2 h without (basal) or with 100 nmol insulin during the last 45 min. The glucose uptake assay was performed as described previously (18). Cell-incorporated radioactivity was determined by scintillation counting.

FAO rat hepatocytes were grown and maintained in monolayer culture in Roswell Park Memorial Institute medium containing 10% FBS

[11] Abbreviations used: AA, amino acid; Akt, protein kinase B; CH, casein hydrolysate; CVD, cardiovascular disease; FO, fish oil, specifically ω-3 PUFAs; GPCR, G-protein-coupled receptor; HFS, high-fat and -sucrose diet; HFS+FO, high-fat and -sucrose diet supplemented with fish oil; HFS+SPF, high-fat and -sucrose diet supplemented with salmon peptide fraction; HFS+SPF+FO, high-fat and -sucrose diet supplemented with salmon peptide fraction and fish oil; HGP, hepatic glucose production; iNOS, inducible nitric oxide synthase; IRS1, insulin receptor substrate 1; LDLR$^{-/-}$/ApoB$^{100/100}$, ApoB^{100} only, LDL receptor knockout mice; MCP-1, monocyte chemoattractant protein 1; MetS, metabolic syndrome; mTORC1, mammalian target of rapamycin complex 1; NEFA, nonesterified fatty acid; p-Akt S473, phosphorylated protein kinase B serine 473; p-IRS1 S1101, phosphorylated insulin receptor substrate 1 serine 1101; p-S6 S240/244, phosphorylated S6 serine 240/244; p-S6 S235/236, phosphorylated S6 serine 235/236; RANTES, regulated upon activation, normal T-cell expressed and secreted; SC, standard feed pellet (chow) diet; SPF, salmon peptide fraction; S6, ribosomal protein s6; S6K1, S6 kinase 1; T2D, type 2 diabetes.

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in an atmosphere of 5% CO_2 at 37°C. FAO hepatocytes were plated in 24-well plates at 4×10^6 cells/plate. After a 16 h serum deprivation (with or without insulin, 0.1 nmol), cells were washed 3 times with PBS. Cells were then incubated for 5 h (37°C, 5% CO_2), in the presence or absence of insulin (0.1 nmol) in a glucose production medium [glucose-free DMEM containing 2 mmol sodium pyruvate, 20 mmol sodium L-lactate, and sodium bicarbonate (3.7 g/L)] in which SPF was present. Glucose production was measured in the medium by using the Amplex Red Glucose/Glucose Oxidase Assay kit (Invitrogen).

J774 mouse macrophages were grown and maintained in monolayer culture in DMEM high-glucose (25 mmol) medium supplemented with 10% FBS, in an atmosphere of 5% CO_2. Cells were plated at 4×10^6 cells/plate for 24 h before the experiment. Macrophages were stimulated with or without 2.5 ng/mL LPS in the presence of SPF for 16 h, and the accumulation of nitrite was used as an index of inducible NO synthase (iNOS) activity. Nitrite was measured by using the Griess method (19). Cells were lysed in 50 mmol NaOH, and protein content was determined. Total cellular protein for all experiments was determined by using the Pierce BCA Protein Assay kit (Thermo Scientific).

Statistical analysis. In vivo data were analyzed with Sigma Plot 12.0 by using a 2-factor ANOVA to determine the effect of SPF and ω-3 PUFAs and potential interactions between treatments. Homogeneity of variance was tested and found to be not significantly different for all measures. A 1-factor ANOVA with Tukey's post hoc test was performed between HFS diet groups when main treatment or interaction effects were significant. The SC group was included only as a reference group to show the presence of diet-induced obesity and was not included in the statistical analysis. In vitro data were analyzed by using SPSS version 22 with the use of a mixed-model ANOVA. Tukey's post hoc test was performed between control and SPF-treated cells when significant treatment effects were detected. Results are presented as means ± SEMs. P values ≤ 0.05 were considered significant.

Results

Body weight, adiposity, and energy balance. We first assessed whether diet-induced obesity was present in the HFS-fed mice. As shown in **Table 1**, all HFS-fed groups showed a greater body weight and visceral adipose tissue accumulation when compared with the reference SC group. Mice in the HFS +SPF and HFS+SPF+FO groups had lower body weight, feeding efficiency (gross weight gain efficiency ratio), and liver weight compared with mice fed HFS and HFS+FO diets. However, this did not lead to significant differences between groups after post hoc analyses ($P \geq 0.05$). SPF and FO dietary components did not affect the weight of epididymal, retroperitoneal, inguinal, or brown adipose tissue depots ($P \geq 0.05$) (Table 1).

Glucose homeostasis and insulin concentrations. As expected with diet-induced obesity, all groups of HFS-fed mice had greater baseline blood glucose (**Figure 1**A), insulin (Figure 1B), and glucose intolerance (Figure 1C) than mice fed the reference SC (hatched lines). Statistical comparisons of the AUCs between HFS groups showed that glucose tolerance was markedly improved in HFS+SPF and HFS+SPF+FO groups compared with HFS and HFS+FO groups (Figure 1D).

Dyslipidemia. We next determined the impact of the dietary treatments on the plasma and liver lipid profile. As shown in **Figure 2**A, HFS+FO, HFS+SPF, and HFS+SPF+FO groups had significantly lower plasma TG concentrations, whereas cholesterol concentrations were only lowered in the HFS+FO mice (Figure 2B). Both HFS+FO and HFS+SPF+FO groups had lower plasma glycerol compared with the HFS group (Figure 2C). NEFA concentrations were reduced only in mice in the HFS+SPF +FO group (Figure 2D). We examined the effects of dietary treatments on hepatic TG concentrations (Figure 2E, F) but no significant differences were found.

Obesity-induced inflammation. We explored the possibility that SPF treatment would result in lower obesity-linked inflammation and thus contribute to the prevention of glucose intolerance. We found that HFS+SPF- and/or HFS+SPF+FO-fed mice had significantly lower concentrations of proinflammatory proteins, including the cytokines IL-1β, IL-6, IL-12, IFN-γ, and TNF-α and the chemokines monocyte chemoattractant protein 1 (MCP-1) and regulated upon activation, normal T-cell expressed and secreted (RANTES) in their visceral adipose tissue compared with HFS and HFS+FO groups (**Figure 3**).

Hepatic insulin signaling. We used S6 phosphorylation as a readout of mammalian target of rapamycin complex 1/S6 kinase 1/insulin receptor substrate 1 (mTORC1/S6K1/IRS1) activation in insulin-treated mice. We confirmed that activation of this nutrient-sensing pathway through S6 S240/244 and S6 S235/236

TABLE 1 Effects of dietary treatments on body weight, energy intake, GWGER, and organ weight after 12 wk of diet treatment in male LDLR$^{-/-}$/ApoB$^{100/100}$ mice[1]

	Diet groups					*P* (ANOVA)		
Variable	SC	HFS	HFS+FO	HFS+SPF	HFS+SPF+FO	P	L	P × L
Body weight, g	31.3 ± 0.76	36.6 ± 1.02	37.1 ± 1.35	34.1 ± 0.88	34.5 ± 1.13	0.024	NS	NS
EI, kcal/d	12.2 ± 0.30	12.1 ± 0.21	12.0 ± 0.29	12.3 ± 0.26	12.1 ± 0.24	NS	NS	NS
GWGER	9.82 ± 0.42	13.8 ± 0.61	14.4 ± 0.82	12.1 ± 0.56	12.2 ± 1,09	0.018	NS	NS
Liver, g	1.30 ± 0.04	1.59 ± 0.06	1.65 ± 0.16	1.36 ± 0.06	1.36 ± 0.06	0.003	NS	NS
EWAT, g	0.82 ± 0.07	1.68 ± 0.11	1.67 ± 0.13	1.44 ± 0.10	1.48 ± 0.14	NS	NS	NS
RWAT, g	0.39 ± 0.06	0.70 ± 0.06	0.66 ± 0.06	0.62 ± 0.07	0.61 ± 0.06	NS	NS	NS
IWAT, g	0.58 ± 0.05	1.12 ± 0.10	1.08 ± 0.14	0.89 ± 0.12	0.97 ± 0.10	NS	NS	NS
BAT, g	0.14 ± 0.02	0.13 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.15 ± 0.02	NS	NS	NS

[1] Values are means ± SEMs, n = 10–13 mice. The SC reference group is not included in statistical analysis. BAT, brown adipose tissue; CH, casein hydrolysate; EI, energy intake; EWAT, epididymal white adipose tissue; FO, fish oil; GWGER, gross weight gain efficiency ratio (body weight gain/energy intake × 1000); HFS, high-fat and -sucrose diet containing casein hydrolysate; HFS+FO, high-fat and -sucrose diet containing casein hydrolysate and fish oil; HFS+SPF, high-fat and -sucrose diet containing salmon peptide fraction; HFS+SPF+FO, high-fat and -sucrose diet containing salmon peptide fraction and fish oil; IWAT, inguinal white adipose tissue; L, lipid effect; LDLR$^{-/-}$/ApoB$^{100/100}$, ApoB^{100} only, LDL receptor knockout mice; P, protein effect; P × L, protein and lipid effects; RWAT, retroperitoneal white adipose tissue; SC, standard feed pellet (chow) diet; SPF, salmon peptide fraction.

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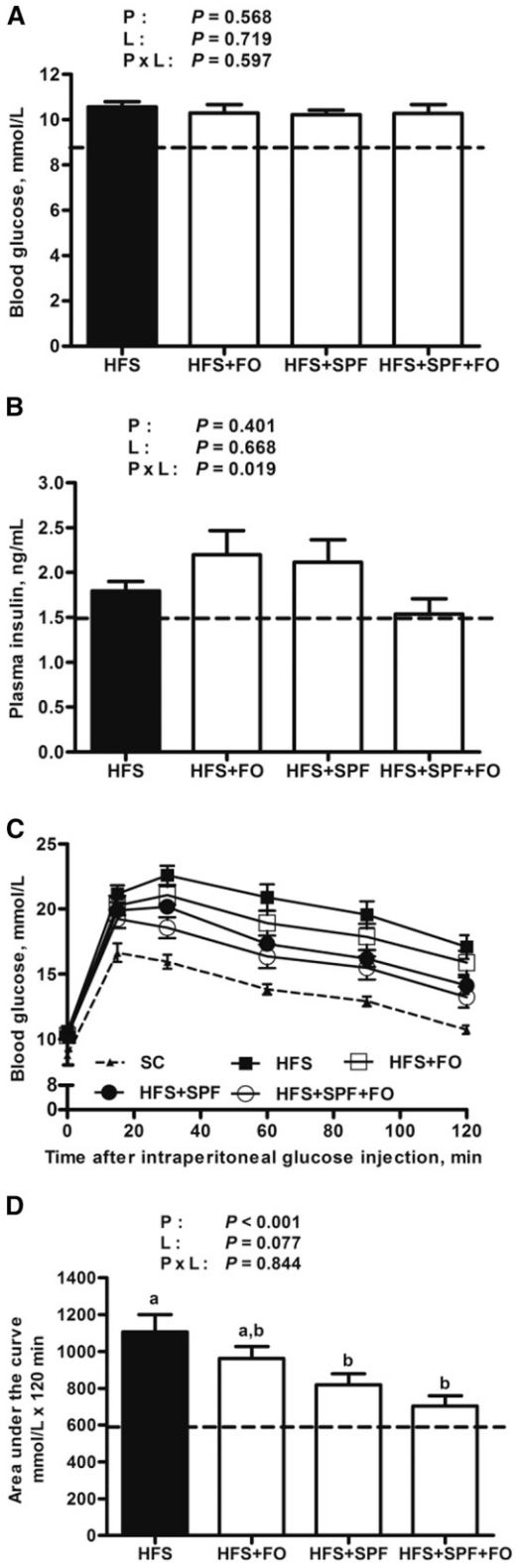

FIGURE 1 Glucose homeostasis in male LDLR$^{-/-}$/ApoB$^{100/100}$ mice fed CH or SPF with or without FO for 12 wk. Plasma concentrations, in the feed-deprived state, were measured after 12 wk of treatment. (A) Blood glucose. (B) Insulin. (C) Glycemic excursion curves during the IPGTT. (D) The area under the glucose curve (glucose-AUC) during IPGTT. The dotted line represents the SC reference group but is not included in statistical analysis. Values are means ± SEMs, n = 12–14. Means without a common letter differ, $P \leq 0.05$. CH, casein hydrolysate; FO, fish oil; HFS, high-fat and -sucrose diet containing casein hydrolysate; HFS+FO, high-fat and -sucrose diet containing casein hydrolysate and fish oil; HFS+SPF, high-fat and -sucrose diet containing salmon peptide fraction; HFS+SPF+FO, high-fat and -sucrose diet containing salmon peptide fraction and fish oil; IPGTT, intraperitoneal glucose tolerance test; L, lipid effect; LDLR$^{-/-}$/ApoB$^{100/100}$, ApoB^{100} only, LDL receptor knockout mice; P, protein effect; P × L, protein and lipid effects; SC, standard feed pellet (chow) diet; SPF, salmon peptide fraction.

was lower in HFS+SPF and HFS+SPF+FO groups (**Figure 4**A, B, E) than in HFS and HFS+FO groups. This was confirmed by the finding that phosphorylation of IRS1 S1101 was reduced but only in the HFS+SPF+FO group (Figure 4C, E). As expected, lower activation of mTORC1/S6K1/IRS1 was associated with significantly improved Akt phosphorylation (Figure 4D, E), suggesting improved hepatic insulin signaling in HFS+SPF-fed mice.

In vitro muscle glucose uptake, hepatic glucose production, and inflammation. We next sought to determine whether the metabolic and anti-inflammatory effects of SPF were explained by a direct effect of SPF on insulin target and inflammatory cells. We used L6 myocytes as a relevant cellular model of insulin action in skeletal muscle to test whether the SPF can directly modulate glucose uptake in vitro. We found that L6 myocytes treated with SPF at a concentration of 1 μg/mL had greater basal glucose uptake (+10%; **Figure 5**A, open bars). In addition, insulin-stimulated glucose uptake was also higher by ~25% (Figure 5A, solid bars). Hepatocytes treated with 1 μg/mL SPF showed an ~30% lowering of basal hepatic glucose production (HGP) as well as ~30% improvement in the ability of insulin to suppress HGP (Figure 5B).

We used nitrite production as an index of iNOS activation and inflammation in LPS-activated macrophages. SPF treatment inhibited iNOS activity in vitro, as shown by a 40% lowering of LPS-induced NO production (Figure 5C). Note that this anti-inflammatory effect was observed even at concentrations as low as 1 μg/mL.

Discussion

Although therapeutic drugs are currently available for the treatment of T2D, many have adverse side effects or become less effective with time. Currently, nutraceuticals or functional foods, which exhibit not only nutritional benefits but also biological activities, are attractive options for the prevention and management of T2D. Over the past decade, a variety of marine sources have been examined for bioactivity, which have therapeutic potential against several pathologies [reviewed in (20–23)].

Herein, we report on the isolation and biological activity of SPF, a novel fraction containing small peptides isolated from salmon striated muscle. Our goal was to assess whether small peptides derived from salmon could prevent the development of MetS, as measured by the determination of visceral obesity, glucose intolerance, dyslipidemia, and inflammation, all key features of MetS that lead to T2D and CVD (24, 25). Because ω-3 PUFAs have also been reported to improve similar MetS components, we also compared their individual effects as well as a potential synergy with SPF for alleviating MetS.

We found that SPF exerts remarkable glucoregulatory effects in HFS-fed LDLR$^{-/-}$/ApoB$^{100/100}$ mice, significantly improving glucose tolerance. The glucose-lowering effect of SPF was not explained by higher insulin secretion. The improvement in glucose

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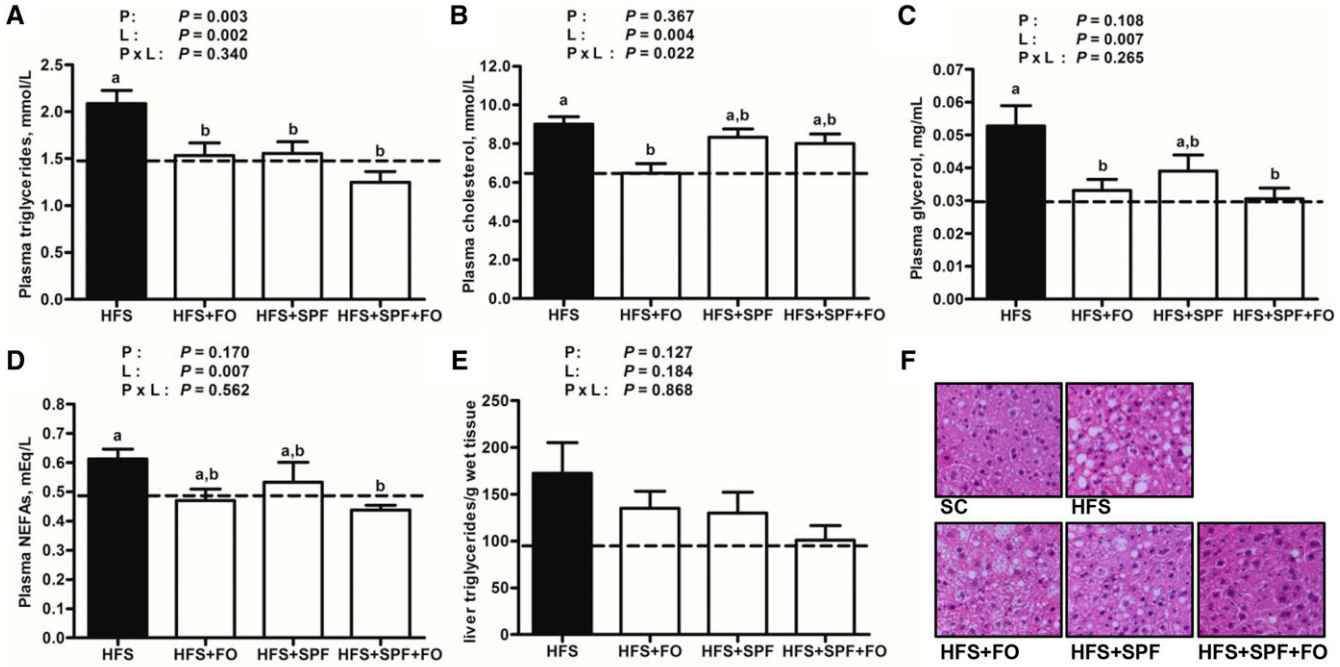

FIGURE 2 Lipid homeostasis in male LDLR$^{-/-}$/ApoB$^{100/100}$ mice fed CH or SPF with or without FO for 12 wk. Plasma concentrations, in the feed-deprived state, were measured after 12 wk of treatment. (A) TGs. (B) Cholesterol. (C) Glycerol. (D) NEFAs. (E) TGs in the liver. (F) Representative picture of liver fat droplets stained with hematoxylin and eosin. The dotted line represents the SC reference group but is not included in statistical analysis. Values are means ± SEMs, n = 12–14. Means without a common letter differ, $P \leq 0.05$. CH, casein hydrolysate; FO, fish oil; HFS, high-fat and -sucrose diet containing casein hydrolysate; HFS+FO, high-fat and -sucrose diet containing casein hydrolysate and fish oil; HFS+SPF, high-fat and -sucrose diet containing salmon peptide fraction; HFS+SPF+FO, high-fat and -sucrose diet containing salmon peptide fraction and fish oil; L, lipid effect; LDLR$^{-/-}$/ApoB$^{100/100}$, ApoB^{100} only, LDL receptor knockout mice; NEFA, nonesterified FA; P, protein effect; P × L, protein and lipid effects; SC, standard feed pellet (chow) diet; SPF, salmon peptide fraction.

tolerance in mice fed diets containing SPF may be partly related to the marginal reduction in body weight. However, these remarkable improvements in glucose tolerance were observed without a significant loss of adiposity. Furthermore, SPF was found to improve glucose metabolism in vitro, suggesting that at least part of its glucoregulatory effects cannot be explained by the marginal weight loss. Indeed, SPF treatment resulted in greater glucose uptake in vitro in L6 muscle cells, while lowering HGP in FAO hepatocytes, suggesting that the ability of SPF to improve glucose tolerance is linked to cell autonomous mechanisms.

We (5, 26–28) and others (22, 29–31) previously showed that fish proteins, FO, or both exert profound effects on plasma or hepatic lipid variables in rodents. The mechanisms of action behind the lipid-lowering effects of ω-3 PUFAs have largely been studied and reviewed (32, 33). Interactions with the transcription factors NF-κB and members of the PPAR family, as well as binding with G-protein-coupled receptors (GPCRs) have been implicated. However, the potential role of fish peptides in the regulation of lipid homeostasis remains unclear and is poorly studied.

The present results suggest that SPF improves the lipid profile of HFS-fed LDLR$^{-/-}$/ApoB$^{100/100}$ mice, which implicates that small peptides may in part contribute to the TG-lowering effects of fish proteins. However, SPF feeding did not improve lipid homeostasis beyond ω-3 PUFAs alone in the present study. It is possible that the TG-lowering effect of SPF is linked to its AA composition. Indeed, it has been suggested that high amounts of taurine and glycine in fish proteins could contribute to an increase in fecal cholesterol and/or bile acid excretion (22, 31, 34), thus contributing to improvement in plasma lipid variables. Although we have not determined taurine concentrations in SPF, we found that it contains relatively high concentrations of glycine. Other potential mechanisms may include an increase in lipoprotein lipase activity in the adipose tissue, as previously suggested in rabbits (28).

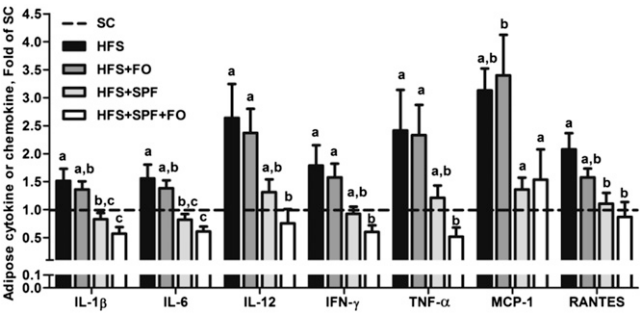

FIGURE 3 Visceral adipose tissue cytokines and chemokines in male LDLR$^{-/-}$/ApoB$^{100/100}$ mice fed CH or SPF with or without FO for 12 wk. The dotted line represents the SC reference group but is not included in statistical analysis. Values are means ± SEMs, n = 12–14. Means without a common letter differ, $P \leq 0.05$. The effect of protein was significant for all variables ($P \leq 0.003$), but the effect of lipid and the interaction were not. CH, casein hydrolysate; FO, fish oil; HFS, high-fat and -sucrose diet containing casein hydrolysate; HFS+FO, high-fat and -sucrose diet containing casein hydrolysate and fish oil; HFS+SPF, high-fat and -sucrose diet containing salmon peptide fraction; HFS+SPF+FO, high-fat and -sucrose diet containing salmon peptide fraction and fish oil; L, lipid effect; LDLR$^{-/-}$/ApoB$^{100/100}$, ApoB^{100} only, LDL receptor knockout mice; MCP-1, monocyte chemoattractant protein 1; P, protein effect; P × L, protein and lipid effects; RANTES, regulated upon activation, normal T-cell expressed and secreted; SC, standard feed pellet (chow) diet; SPF, salmon peptide fraction.

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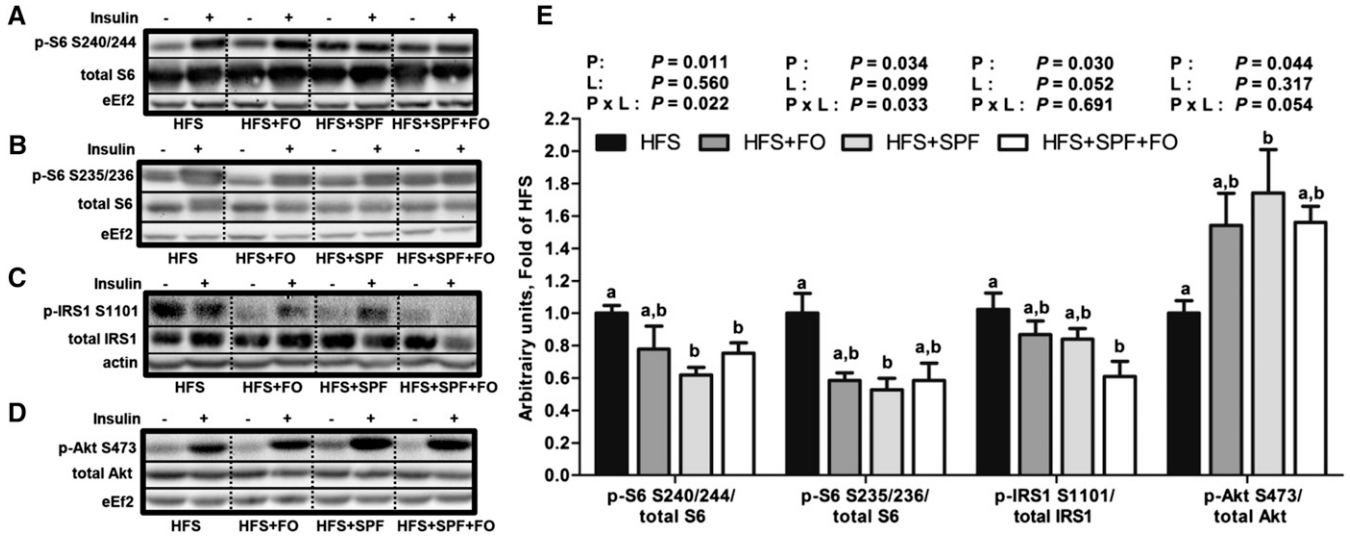

FIGURE 4 The mTORC1/S6K1/IRS1 pathway and insulin signaling to Akt in liver of male LDLR$^{-/-}$/ApoB$^{100/100}$ mice fed CH or SPF with or without FO for 12 wk. Representative gels and quantification of densitometric analyses of p-S6 S240/244 (A), p-S6 S235/236 (B), p-IRS1 S1101 (C), and p-Akt S473 (D) are shown. (E) Graphic compilation of p-S6 S240/244, p-S6 S235/236, p-IRS1 S1101, and p-Akt S473. Values are means ± SEMs, n = 4–7 mice injected with insulin 5 min before being killed. Dashed lines indicate that samples were conducted on the same gel but were noncontiguous. Means without a common letter differ, $P \leq 0.05$. Akt, protein kinase B; CH, casein hydrolysate; eEf2, eukaryotic elongation factor 2; FO, fish oil; HFS, high-fat and -sucrose diet containing casein hydrolysate; HFS+FO, high-fat and -sucrose diet containing casein hydrolysate and fish oil; HFS+SPF, high-fat and -sucrose diet containing salmon peptide fraction; HFS+SPF+FO, high-fat and -sucrose diet containing salmon peptide fraction and fish oil; L, lipid effect; LDLR$^{-/-}$/ApoB$^{100/100}$, ApoB^{100} only, LDL receptor knockout mice; mTORC1/S6K1/IRS1, mammalian target of rapamycin complex 1/S6 kinase 1/insulin receptor substrate 1; P, protein effect; P × L, protein and lipid effects; p-Akt S473, phosphorylating state of protein kinase B serine 473; p-IRS1 S1101, phosphorylating state of insulin receptor substrate 1 serine 1101; p-S6 S235/236, phosphorylating state of S6 serine 235/236; p-S6 S240/244, phosphorylating state of S6 serine 240/244; S6, ribosomal protein s6; SPF, salmon peptide fraction.

SPF treatment reduced adipose tissue inflammation in HFS-fed LDLR$^{-/-}$/ApoB$^{100/100}$ mice as revealed by decreased concentrations of chemokines, such as MCP-1 and RANTES, and of several proinflammatory cytokines. Both MCP-1 and RANTES were shown to be involved in the macrophage infiltration in the adipose tissue of animal models of obesity (35–37). Furthermore, SPF decreased LPS-induced iNOS activation in macrophages, suggesting that at least part of the anti-inflammatory effects of SPF may be linked to inhibition of activated macrophages recruited in the visceral fat of the obese animals. Importantly, the inhibition of NO production occurred at SPF amounts that were much lower than those required to demonstrate changes in metabolic variables of MetS.

Our results are consistent with a previous study in which we found that a nonfractionated salmon protein hydrolysate dampens adipose tissue inflammation (7). However, in the previous work these effects were linked to reduced body weight gain and visceral adiposity in HFS-fed rats fed the nonfractionated salmon hydrolysate. The antiobesity effect of this diet was likely explained by its calcitonin content. The low-molecular-weight SPF also blunted adipose tissue inflammation, but without significant effects on visceral fat mass in the present study. The absence of a significant effect of SPF on visceral obesity is likely explained by the fact that SPF, which contains peptides <1 kDa, is free of calcitonin (a 32-kDa polypeptide), which was found in the nonfractionated salmon protein hydrolysate.

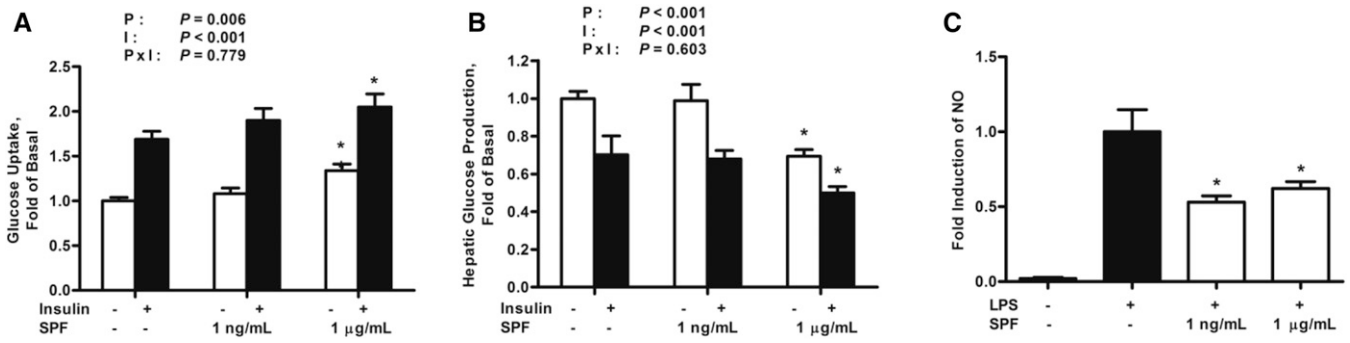

FIGURE 5 Screening assays for SPF action on glucose metabolism and inflammation with the use of L6 myocytes, FAO hepatocytes, and J774 macrophages. (A) L6 myocytes treated or not treated with SPF at the indicated concentration without (basal) or with 100 nmol insulin. (B) FAO hepatocytes treated or not treated with the indicated concentrations of SPF with or without 0.1 nmol/L insulin. (C) J774 macrophages were treated with 2.5 ng/mL LPS with or without SPF at the indicated concentrations. Values are means ± SEMs, n = 4–7 independent experiments performed in triplicate. *$P \leq 0.05$ vs. control in panels A and B or vs. LPS alone in panel C. I, insulin effect; P, protein effect; P × I, protein and insulin effects; SPF, salmon peptide fraction.

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To explore potential mechanisms that could explain the glucoregulatory effects of SPF, we first tested whether SPF-treated mice had reduced activation of the mTORC1/S6K1/IRS1 nutrient-sensing pathway. We previously showed that dietary proteins and AAs modulate this pathway and that overactivation of mTORC1 and S6K1 both promote insulin resistance in obesity (15, 38). We found that SPF treatment blunted the activation of the mTORC1/S6K1/IRS1 pathway in liver, as shown by reduced phosphorylation of S6 and IRS1 S1101, a well-known target of S6K1 (15). As expected, this was associated with improved hepatic insulin action as shown by enhanced Akt signaling in the HFS+SPF group. We previously showed that the mTORC1/S6K1/IRS1 pathway is activated by high AA concentrations, and it is well known that BCAAs are particularly potent in activating this negative feedback loop and contribute to obesity-linked insulin resistance (39). The bioactivity of our new SPF isolate, given its composition, is most likely explained by the action of small peptides <1 kDa, although the role of free AAs cannot be completely discounted. It is also possible that the lower amount of BCAAs in the SPF contributes to its beneficial action through a limited activation of the mTORC1/S6K1/IRS1 negative feedback loop, leading to enhance insulin action in target tissues.

Another interesting finding of the present study is that at least some of the beneficial effects of SPF on MetS features can be explained by cell-autonomous action on key insulin target and inflammatory cells. Indeed, we found that SPF could exert rapid modulatory effects on glucose metabolism and iNOS activation in vitro, suggesting that peptides in this fraction can directly exert glucoregulatory and anti-inflammatory effects.

In conclusion, we have isolated a new low-molecular-weight SPF with unique glucoregulatory and anti-inflammatory properties. SPF prevented glucose intolerance, dyslipidemia, and adipose tissue inflammation in HFS-fed obese mice, and the anti-inflammatory effects were potentiated by the addition of ω-3 PUFAs to the diet. The glucoregulatory effects of SPF were linked to blunted activation of the mTORC1/S6K1/IRS1 nutrient-sensing pathway in liver. Dietary SPF supplementation and ω-3 PUFAs may thus represent an alternative treatment for the prevention of MetS and reduce T2D and CVD risk in obesity.

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