Fish oil prevents high saturated fat diet-induced impairments in adiponectin and insulin response in rodent soleus muscle.

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Running Head: Fish oil prevents loss of adiponectin and insulin response

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Abstract:

High saturated fatty acid (SFA) diets contribute to the development of insulin resistance, whereas fish oil-derived n-3 polyunsaturated fatty acids (PUFA) increase the secretion of adiponectin (Ad), an adipocyte-derived protein that stimulates fatty acid oxidation (FAO) and improves skeletal muscle insulin response. We sought to determine if fish oil could prevent and/or restore high SFA diet-induced impairments in Ad and insulin response in soleus muscle. Sprague-Dawley rats were fed: (1) low fat control diet (CON), (2) high SFA diet (SFA) or (3) high SFA with n-3 PUFA diet (SFA/n-3 PUFA). At 4 wk, CON and SFA/n-3 PUFA animals were terminated, and SFA animals were either terminated or fed SFA or SFA/n-3 PUFA for an additional 2 or 4 wk. The effect of diet on Ad-stimulated FAO, insulin-stimulated glucose transport, and expression of Ad, insulin and inflammatory signaling proteins was determined in soleus muscle. Ad stimulated FAO in CON and 4wk SFA/n-3 PUFA (+36%, +39%, respectively p ≤ 0.05) only. Insulin increased glucose transport in CON, 4wk SFA/n-3 PUFA, and 4wk SFA + 4wk SFA/n-3 PUFA (+82%, +33%, +25%, respectively p ≤ 0.05); this effect was lost in all other groups. TLR4 expression was increased with 4wk of SFA feeding (+24%, p ≤ 0.05) and this was prevented in 4wk SFA/n-3 PUFA. SOCS-3 expression was increased in SFA and SFA/n-3 PUFA (+33, +18%, respectively p ≤ 0.05). Our results demonstrate that fish oil can prevent high SFA diet-induced impairments in both Ad and insulin response in soleus muscle.

Key Words: n-3 PUFA, inflammation, lipid metabolism, glucose transport, fatty acid oxidation
Introduction:

Adiponectin (Ad), a 30 kDa adipokine secreted almost exclusively from adipose tissue (AT), is known to exert insulin-sensitizing effects that are largely attributed to its role in glucose and lipid metabolism in skeletal muscle and liver (7). The consumption of a diet high in saturated fatty acids (SFA), results in a marked decrease in circulating Ad concentrations, which may contribute to impairments in fatty acid metabolism and insulin responsiveness (7). In contrast, fish oil-derived n-3 polyunsaturated fatty acids (PUFA) have been shown to stimulate Ad secretion (20, 28), which may serve as an underlying mechanism by which dietary n-3 PUFA can improve insulin sensitivity (1, 10, 26).

High SFA diets have been shown to activate inflammatory pathways that interfere with insulin signaling (24). SFA act as ligands for Toll-like receptor (TLR)4, which initiates a downstream signaling cascade resulting in the activation of nuclear factor (NF)κB and upregulation of inflammatory gene expression (24). Importantly, the deletion of TLR4 in skeletal muscle has been demonstrated to protect against high SFA diet-induced insulin resistance, emphasizing the important connection between inflammation and insulin signaling (22). Fish oil-derived n-3 PUFA are thought to elicit their anti-inflammatory effects in part by downregulating NFκB (15); however, the effects of n-3 PUFA on the expression of inflammatory signalling mediators in soleus muscle has yet to be examined.

In addition to dysregulated adipokine synthesis and secretion, research suggests that tissue response to Ad may also be impaired in obesity (4). Both Ad and leptin are known to stimulate fatty acid oxidation (FAO) in rodent and human skeletal muscle (4). However, this effect is blunted in an obese state (3, 25), which may contribute to the accumulation of skeletal
muscle lipids that are known to interfere with insulin-stimulated glucose uptake (5).

Additionally, Mullen et al. recently demonstrated that Ad resistance not only develops rapidly in response to a high SFA diet, but also precedes both the accumulation of muscle lipid species and development of insulin resistance in soleus muscle (16). Interestingly, while fish oil-derived n-3 PUFA have been shown to stimulate Ad secretion (20, 28), their effects on the response of skeletal muscle to Ad, as well as any associated functional effects on insulin-stimulated glucose transport, are currently unknown.

Our objectives were to determine (1) if fish oil-derived n-3 PUFA could prevent and/or restore high SFA diet-induced impairments in Ad-stimulated FAO and maximal insulin-stimulated glucose transport in rodent soleus muscle, and (2) the potential mechanisms by which fish oil exerts these effects. We hypothesized that (1) fish oil-derived n-3 PUFA would prevent and restore high SFA diet-induced impairments in both Ad response and insulin response, and (2) fish oil-derived n-3 PUFA would mediate these effects via reduced activation of inflammatory pathways.
Methods:

Animals and Diets

Female Sprague-Dawley rats were purchased from Charles River (Charles River Laboratories, St-Constant, QC, Canada) at approximately 50 days of age and weighed 151 ± 0.67 g upon arrival. The animals were housed in groups of 6 with a 12:12 h reverse light dark cycle and allowed unlimited access to water. Rats were fed a standard Teklad (Harlan Laboratories, Mississauga, ON, Canada) rodent chow diet for 3 d, at which point they were randomly assigned to receive (1) low fat control diet (6% kcal from soybean oil, 4% kcal from lard, 4wk CON), (2) high SFA diet (55% kcal from lard, 5% kcal from soybean oil, 4wk SFA) known to induce both Ad resistance and insulin resistance (17), or (3) high SFA with n-3 PUFA diet (40% kcal from lard, 15% kcal from menhaden oil, 5% kcal from soybean oil, 4wk SFA/n-3 PUFA) for a period of 4 wk (Table 1). The SFA/n-3 diet contained a total of 7.9% long-chain n-3 PUFA in the form of eicosapentaenoic acid (4.1%), docosapentaenoic acid (0.8%), and docosahexaenoic acid (3.0%) (Table 2). At 4 wk, CON and SFA/n-3 PUFA animals were terminated, and SFA animals were either terminated or fed SFA or SFA/n-3 PUFA for an additional 2 (6wk SFA; 4wk SFA + 2wk SFA/n-3 PUFA) or 4 (8wk SFA; 4wk SFA + 4wk SFA/n-3 PUFA) wk. All diets were purchased from Research Diets (Research Diets Inc., New Brunswick, NJ, USA), stored at -20 °C, and administered fresh daily to the animals ad libitum. Body mass measurements were taken twice per wk, and the animals were fasted overnight prior to experiments. Approval for all experimental procedures was obtained from the University of Guelph Animal Care Committee (Guelph, ON, Canada).
Tissue and Blood Sampling

Rats were anaesthetized by an intraperitoneal injection with sodium pentobarbital at a dose of 6 mg per 100 g body mass. Intact soleus muscle was isolated and stripped longitudinally from tendon to tendon into 3 strips of approximate equal size. The outside 2 strips were used for either glucose transport or FAO assays and the remaining inside strip was used for insulin-stimulated signaling incubations or frozen immediately in liquid nitrogen for subsequent total protein analysis. Blood was obtained by cardiac puncture, transferred to heparinized tubes and centrifuged (10,000 x g, 5 min, 4 ºC) to isolate plasma for analysis of fasting insulin (Rat Insulin RIA kit, Millipore, Billerica, MA, USA), free fatty acids (Wako Chemicals Inc., Richmond, VA, USA), and Ad (Rat Adiponectin ELISA, Millipore, Billerica, MA, USA). Fasting plasma interleukin (IL)-6, leptin, monocyte chemoattractant protein (MCP)-1, and tumour necrosis factor (TNF)-α (Rat Cytokine/Chemokine Milliplex kit, Millipore, Billerica, MA, USA) were analyzed by Luminex xMAP technology (Bioplex-200, Bio-Rad Laboratories, Mississauga, ON, Canada). Fasting whole blood glucose values were obtained using a OneTouch Ultra2 glucometer (LifeScan Inc., Milpitas, CA, USA).

Fatty Acid Oxidation

Soleus strips were incubated in 2 mL of pregassed (95% O₂-5% CO₂) Krebs-Henseleit buffer (KHB), 4% BSA, at 30 ºC, with 1 mM palmitate and 5 mM glucose in a gentle shaking water bath for 30 min to equilibrate. Soleus strips were then incubated for an additional 60 min, in the presence or absence of recombinant globular adiponectin (gAd) (2.5 µg/mL, Peprotech, Rocky Hill, NJ, USA) with the addition of 0.5 µCi/ml [1-¹⁴C]palmitate (Amersham, Oakville, ON, Canada) to determine palmitate oxidation as previously outlined in detail (6). In brief, after the
incubations the soleus strips were blotted, tendons removed, weighed, and placed in a 14 mL tubes containing 5 mL of cold 2:1 chloroform methanol. Muscles were homogenized using a polytron (PT1200, Brinkman Institute, Mississauga, ON, Canada) and centrifuged (10,000 x g, 10 min, 4 °C). The supernatant was extracted and combined with 2 mL of water, shaken for 10 min, and centrifuged (10,000 x g, 10 min, 4 °C) to isolate the aqueous phase. The amount of $^{14}$C in 1 mL of the aqueous phase was quantified by liquid scintillation counting. Additionally, the $^{14}$CO$_2$ remaining in the buffer was released by transferring 1 mL of buffer into a sealed flask, acidifying the buffer with 1 mL of 1 M sulfuric acid and trapping it in 250 µL benzethonium hydroxide contained in a microcentrifuge tube suspended in the sealed flask. The tubes containing benzethonium hydroxide and the trapped $^{14}$CO$_2$ were quantified using liquid scintillation counting. Total palmitate oxidation was determined by summing the aqueous and gaseous $^{14}$C-labelled oxidation intermediates.

Glucose Transport

Soleus strips were incubated in 2 mL of pregassed (95% O$_2$-5% CO$_2$) KHB (0.1% BSA, 30 °C) containing 8 mM glucose and 32 mM mannitol, for 30 min in the absence or presence of insulin (10 mU/mL) in a gently shaking water bath to equilibrate. The strips were washed twice in 2 mL glucose-free KHB (4 mM pyruvate, 36 mM mannitol) for 10 min each, and then incubated for 40 min (basal) or 20 min (insulin-stimulated) in KHB (4 mM pyruvate, 8 mM 3-O-[$^3$H]methyl-D-glucose, 28 mM [$^{14}$C]mannitol). Strips were blotted, tendons removed, weighed and then digested in 1 mL sodium hydroxide for 10 min at 95 °C. 200 µL of muscle digest was sampled and quantified by liquid scintillation counting, and glucose transport was determined from intracellular 3-O-[$^3$H]methyl-D-glucose as previously described (30).
**Insulin-Stimulated Signaling Proteins**

Soleus strips were incubated in 2 mL of pregassed (95% O₂-5% CO₂) KHB (0.1% BSA, 30 ℃) containing 8 mM glucose and 32 mM mannitol, for 30 min to equilibrate. Strips were then incubated in the same KHB for a subsequent 10 min in the presence of 10 mU/mL insulin and immediately frozen in liquid nitrogen for subsequent western blotting analysis.

**Western Blotting**

Soleus muscle strips were homogenized in ice cold buffer suitable for whole cell protein extraction, sonicated for 5 sec, and then centrifuged (1500 x g, 15 min, 4 ℃) to isolate the supernatant. Total protein was quantified using a bicinchoninic acid protein assay (Fisher Scientific, Ottawa, ON, Canada) according to the manufacturer’s instructions. Whole tissue lysate protein (50 µg) was solubilized in 4x Laemelli’s buffer, boiled (5 min, 95 ℃), resolved by SDS-PAGE, and wet transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 h at room temperature, washed, and then incubated overnight at 4 ℃ with specific primary antibodies for total Akt (Upstate, Billerica, MA, USA), Thr³⁰⁸ phosphorylated Akt (Santa Cruz Biotechnology, Santa Cruz, CA, USA), adaptor protein phosphotyrosine interaction pH domain and leucine zipper containing 1 (APPL1), 5' AMP-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC), TLR4, total and Ser³2 phosphorylated IκBα, total and Ser⁵³⁶ phosphorylated NFκB, and suppressor of cytokine signaling (SOCS)-3. All antibodies were purchased from Cell Signaling (Danvers, MA, USA) unless otherwise stated. The immune complexes were washed, incubated with the appropriate secondary antibody for 1 h at room temperature, detected using the enhanced chemiluminescence method (Syngene Chemigenius2; PerkinElmer, Waltham, MA, USA) and then quantified with densitometry (Gene Tools software,
PerkinElmer). Equal loading was confirmed by probing for α-tubulin (Abcam Cambridge, MA) and using nonspecific protein staining with Ponceau-S (Sigma Aldrich, Oakville, ON, Canada).

**Statistical Analysis**

Body mass, blood measurements, and protein expression data were analyzed using a one-way ANOVA. FAO and glucose transport data was analyzed using a two-way repeated measures ANOVA for dietary treatment and time. A Tukey post-hoc test was used to identify significant differences between diet treatments within each time point. All data are expressed as mean ± SEM. Statistical significance was set at $p \leq 0.05$ and all statistical analysis was performed using Sigma Stat version 2.03.
Results

**Body Mass and Bloods**

Initial body mass did not differ between any of the groups (Table 3). Terminal body mass was higher (+18%, \( p \leq 0.05 \)) in 4wk SFA compared to CON (Table 3). Fasting whole blood glucose, plasma insulin, plasma free fatty acids, and plasma TNF-\( \alpha \) did not differ in any of the groups (Table 3). Fasting plasma Ad was elevated in 4wk SFA/n-3 PUFA compared to both CON (+38%, \( p \leq 0.05 \)) and 4wk SFA (+58%, \( p \leq 0.05 \)) (Table 3). Fasting plasma leptin and MCP-1 levels were increased in 4wk SFA (+74% and +23%, respectively \( p \leq 0.05 \)) and 4wk SFA/n-3 PUFA (+65%, +23%, respectively \( p \leq 0.05 \)) (Table 3) compared to CON. Fasting plasma IL-6 levels were elevated in 4wk SFA (+30%, \( p \leq 0.05 \)) compared to CON, but this increase was not seen in 4wk SFA/n-3 PUFA (Table 3).

**Fatty Acid Oxidation**

In CON, gAd increased FAO compared to the basal condition (+36%, \( p \leq 0.05 \)) (Fig. 1). The stimulatory effect of gAd was preserved in 4wk SFA/n-3 PUFA (+39%, \( p \leq 0.05 \)) but completely attenuated in all other groups (Fig. 1). There were no differences in either basal or gAd-stimulated FAO between diet groups for a given time point (Fig. 1).

**Glucose Transport**

Insulin increased glucose transport compared to basal in CON (+82%, \( p \leq 0.05 \)), 4wk SFA/n-3 PUFA (+33%, \( p \leq 0.05 \)), and 4wk SFA + 4wk SFA/n-3 PUFA (+25%, \( p \leq 0.05 \)) (Fig. 2). Insulin’s stimulatory effect was lost in all other diet groups (Fig. 2). There were no differences
in either basal or insulin-stimulated glucose transport between diet groups for a given time point (Fig. 2).

4 Ad Signaling Proteins

There was no significant effect of diet on the total protein content of APPL1 (Fig. 3a), AMPK (Fig 3b), or ACC (Fig. 3c).

5 Insulin Signaling Proteins

There was no significant effect of diet on the total protein content of Akt (Fig. 4a). Thr^{308} phosphorylation of Akt was impaired (-29%, p ≤ 0.05) in response to 10 min of insulin exposure in 4wk SFA compared to CON, and this impairment was not seen in 4wk SFA/n-3 PUFA (Fig. 4b).

6 Inflammatory Signaling Proteins

TLR4 protein expression in soleus muscle was elevated in 4wk SFA compared to CON (+24%, p ≤ 0.05) (Fig. 5a). There was no significant effect of diet on total protein content or phosphorylation of IκBα (Fig. 5b,c) or NFκB (Fig. 5d,e). SOCS-3 protein content was increased in both 4wk SFA and 4wk SFA/n-3 PUFA relative to CON (+33%, +18%, respectively p ≤ 0.05) (Fig. 5f).
Discussion

This study is the first to demonstrate that the partial substitution of long chain n-3 PUFA into a high SFA diet can modulate the response of rodent soleus muscle, an important tissue in the regulation of insulin sensitivity, to Ad. Specifically, we demonstrate for the first time that fish oil-derived n-3 PUFA can prevent, but not restore, impairments in Ad-stimulated FAO, and both prevent and restore impairments in insulin-stimulated glucose transport, in response to a high SFA diet. In addition, protein content of both TLR4 and SOCS-3 was increased in soleus muscle in response to the high SFA diet, and the increase in TLR4 expression was prevented by the partial substitution of long-chain n-3 PUFA in the diet. Taken together, these results suggest that dietary consumption of fish oil, rich in long-chain n-3 PUFA, confers beneficial effects on lipid metabolism and insulin response in soleus muscle.

Fish oil prevents but does not restore impairments in Ad-stimulated FAO.

Recently, Mullen et al. demonstrated that a high SFA diet impairs the acute increase in FAO in isolated soleus muscle in response to gAd in as little as 3 days (16). In the current study, we show that the high SFA diet-induced impairment in Ad response can be prevented, but not restored, by the partial dietary substitution of fish oil. While the inclusion of long-chain n-3 PUFA in the diet successfully prevented the development of Ad resistance in soleus muscle, it was unable to restore it. It is possible that if rats had been fed fish oil for longer than 4 wk, Ad responsiveness may have been recovered, but this requires further study. It is also interesting to note that feeding the high SFA diet containing fish oil for 4 wk significantly increased circulating Ad concentrations; however, when fish oil was supplemented into the diet subsequent
to an initial 4 wk of high SFA feeding, no change in circulating Ad concentrations was observed. Ultimately, the ability of fish oil to prevent, but not restore, impairments in Ad-stimulated FAO suggests that fish oil supplementation may be of greater therapeutic importance as a prevention, rather than treatment, for obesity-induced impairments in soleus muscle lipid metabolism.

**Fish oil prevents and restores impairments in insulin-stimulated glucose transport.**

High SFA diets are known to impair insulin response in skeletal muscle (9, 16, 17, 32). In this study, we demonstrate that fish oil can prevent high SFA diet-induced impairments in insulin-stimulated glucose transport in isolated soleus muscle. This observation is consistent with Kim et al. who found that replacing 18% (of a 50% energy high fat diet) of corn oil, high in linoleic acid, with menhaden oil partially prevents high SFA diet-induced blunting in insulin-stimulated glucose transport in both epitrochlearis and soleus muscles (9). Additionally, our findings are supported by several groups (8, 10, 26) who report that fish oil supplementation can prevent high SFA diet-induced skeletal muscle insulin resistance in rodents as assessed by the hyperinsulinemic euglycemic, clamp technique.

Importantly, our study is the first to demonstrate that the SFA diet-induced impairment in insulin response in soleus muscle can be effectively restored with 4 wk of incorporation of long-chain n-3 PUFA into a high SFA diet. To the best of our knowledge, the ability of fish oil to reverse high SFA diet-induced impairments in insulin response has not been previously examined in skeletal muscle specifically. However, both Muurling et al. (19) and Podolin et al. (21) report that the inclusion of fish oil in a high fat diet is insufficient to reverse whole body insulin resistance as assessed by the hyperinsulinemic euglycemic, clamp technique. The inability of fish oil to reverse whole body insulin resistance in these studies suggests that
improvements in muscle insulin response may actually precede changes in systemic insulin sensitivity. In contrast to the aforementioned studies, Kalupahana et al. (8) demonstrated that 5 weeks of EPA supplementation into a high fat diet restored glucose tolerance in mice, suggesting that EPA may be of particular importance in n-3 PUFA-mediated improvements in whole body insulin sensitivity. Although we observed diet-induced changes in soleus muscle insulin response, fasting glucose and insulin concentrations were unaltered by the diets, suggesting that whole-body insulin sensitivity was unchanged. However, it must be recognized that a more sensitive measure of systemic insulin sensitivity (glucose and insulin tolerance tests, and/or a hyperinsulinemic euglycemic clamp) may have revealed diet-induced changes that existed; however, the focus of this study was on the response of soleus muscle to adiponectin and insulin. Furthermore, future work should examine the ability of n-3 PUFA to prevent and restore impairments in insulin response in glycolytic muscle types, such as the extensor digitorum longus.

It is noteworthy that the n-3 PUFA-induced restoration of soleus muscle insulin response occurred independently of a restoration in Ad response. This suggests that although high SFA diet-induced impairments in Ad-stimulated FAO precede the loss in insulin responsiveness (16), its restoration is not required for the restoration of insulin-stimulated glucose transport by n-3 PUFA. These findings are consistent with Ritchie et al. (23) who report an exercise-induced restoration of insulin-stimulated glucose transport prior to a restoration in leptin-stimulated FAO. Overall, our findings emphasize the importance of considering fish oil-derived long-chain n-3 PUFA as a therapeutic strategy in both the prevention and treatment of soleus muscle insulin resistance.
High SFA diet and fish oil have no effect on protein expression of Ad signaling proteins.

Given our findings that dietary fat composition altered Ad and insulin responses in soleus muscle, we analyzed the content of several proteins involved in the Ad, insulin and inflammatory signaling cascades to investigate potential underlying mechanisms. Globular Ad signals by binding to its receptor, AdipoR1 that then associates with APPL1, thereby increasing FAO (14, 33). Despite diet-induced functional changes in gAd-stimulated FAO, we found no effect of diet on the total protein expression of APPL1 or ACC. This suggests that high SFA-induced impairments in Ad-stimulated FAO are not attributable to changes in total protein content of these Ad signaling intermediates and is consistent with previous findings (16, 18). It is possible that the impairments observed with the high SFA diet are a result of changes in the phosphorylated state or cellular location of these proteins, as well as their co-localization with other proteins (31).

High SFA diet decreases protein expression of Thr$^{308}$ phosphorylated Akt.

Our observation that the Thr$^{308}$ phosphorylation of Akt is impaired in response to a high SFA diet is consistent with previous findings (16). Diet had no effect on total Akt protein expression, which is also consistent with previous findings (16). Taouis et al. (27) demonstrated that skeletal muscle insulin-stimulated insulin receptor and insulin receptor substrate (IRS)-1 tyrosine phosphorylation was restored in rodents fed a high SFA diet supplemented with fish oil. To the best of our knowledge, the effects of dietary fish oil supplementation into a high SFA diet on the protein expression of insulin signaling mediators downstream of IRS-1 have not been previously examined. In the current study we found that the partial substitution of fish oil into a high SFA diet was able to prevent and restore impairments in phosphorylation of Thr$^{308}$ Akt.
induced by the high SFA diet. This finding is consistent with reports that EPA, one of the
bioactive n-3 PUFA in fish oil, stimulates phosphorylation of Akt in 3T3-L1 adipocytes (13), and
rodent adipose tissue (11).

High SFA diet increases TLR4 and SOCS-3 protein content.

Activation of TLR4 by SFA and subsequent stimulation of the NFκB inflammatory pathway
has been demonstrated in skeletal muscle and is thought to contribute to the development of
insulin resistance (22, 24). We observed an increase in soleus muscle TLR4 and SOCS-3 protein
content with the high SFA diet, which is consistent with several other reports (22, 23, 24, 29,
34). Importantly, we demonstrated for the first time that the high SFA diet-induced upregulation
of TLR4 expression in soleus muscle was completely prevented by the partial substitution of
SFA with fish oil. This observation is consistent with Lee et al. who reported that saturated, but
not unsaturated, fatty acids stimulated TLR4 in macrophages (12). Although there was a trend
towards a decrease in the expression of SOCS-3 with the partial substitution of n-3 PUFA into
the high SFA diet, SOCS-3 protein remained elevated compared to CON animals. Although
SOCS-3 is known to interfere with insulin signaling in muscle (34), it also acts to suppress
inflammatory cytokine signaling pathways (2). Therefore, it is possible that n-3 PUFA induced
SOCS-3 expression to feedback and downregulate cytokine signaling, which is consistent with
the observed decrease in plasma IL-6 concentrations in the SFA/n-3 PUFA fed animals, as well
as reports of DHA-induced increases in SOCS-3 expression in 3T3-L1 adipocytes (2).

Despite changes in TLR4 and SOCS-3 expression, we found no effect of any of our diets on
the total protein content or phosphorylation of IkBα or NFκB in soleus muscle. This is in
contrast to Yaspelkis et al. who recently demonstrated that feeding a high SFA diet for 12 weeks
significantly decreased total protein content and increased serine phosphorylation of IκBα (34).

It is possible that if we had extended our high SFA diet feeding for an additional 4 wk, we may have observed a similar result. Furthermore, although long-chain n-3 PUFA have been shown to inhibit NFκB activation (15), we found no effect of dietary fish oil on NFκB protein content or phosphorylation in soleus muscle. It is conceivable that any fish oil-induced inhibition of NFκB was masked by the high SFA diet, although this could not be resolved with the current study design. Finally, we must also acknowledge the possibility that, although there were no changes in total protein content or phosphorylation of IκBα and NFκB, the cellular localization of these proteins between the cytoplasm and nucleus may have been altered.

Perspectives and Significance:

In summary, we found that partial substitution of fish oil into a high SFA diet prevents impairments in Ad-stimulated FAO, and importantly, both prevents and restores impairments in maximal insulin-stimulated glucose transport in rodent soleus muscle. Additionally, high SFA diet stimulates TLR4 expression and this can be prevented, but not restored, by fish oil supplementation. Overall, our findings emphasize the importance of considering fish oil-derived n-3 PUFA as a therapeutic strategy in the prevention of high SFA diet-induced impairments in soleus muscle lipid metabolism and insulin responsiveness.

Grants:
This study was funded by the Natural Science and Engineering Research Council of Canada (LR and DD). Justine Tishinsky was supported by a Natural Science and Engineering Research Council of Canada Postgraduate Scholarship.

**Disclosures:**

The authors declare that there are no disclosures associated with this manuscript.
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Figure Legends:

**Fig. 1:** Basal and globular adiponectin-stimulated palmitate oxidation in soleus muscle. Values are means ± SEM, n=12. * = significantly different from basal, p ≤ 0.05. Open bars = basal, Closed bars = globular adiponectin-stimulated (2.5µg/ml). CON = control diet, SFA = high saturated fatty acid diet, SFA/n-3 PUFA = high saturated fatty acid with n-3 polyunsaturated fatty acid diet.

**Fig. 2:** Basal and insulin-stimulated glucose transport in soleus muscle. Values are means ± SEM, n=12. * = significantly different from basal, p ≤ 0.05. Open bars = basal, Closed bars = insulin-stimulated (10 mU/mL). CON = control diet, SFA = high saturated fatty acid diet, SFA/n-3 PUFA = high saturated fatty acid with n-3 polyunsaturated fatty acid diet.

**Fig. 3:** The effect of high SFA diet and fish oil supplementation on total protein content of adiponectin signaling intermediates in soleus muscle. a) APPL1, b) AMPK, and c) ACC. Alpha tubulin was used as a loading control. Values are means ± SEM, n=12. * = significantly different from CON, p ≤ 0.05. CON = control diet, SFA = high saturated fatty acid diet, SFA/n-3 PUFA = high saturated fatty acid with n-3 polyunsaturated fatty acid diet.

**Fig. 4:** The effect of high SFA diet and fish oil supplementation on total protein content of insulin signaling intermediates in soleus muscle. a) Total Akt, and b) the acute effect of 10 min insulin exposure (10 mU/mL) on protein content of Thr^{308} phosphorylated Akt. Alpha tubulin was used as a loading control. Values are means ± SEM, n=12. * = significantly different from CON, p ≤ 0.05. CON = control diet, SFA = high saturated fatty acid diet, SFA/n-3 PUFA = high saturated fatty acid with n-3 polyunsaturated fatty acid diet.
**Fig. 5:** The effect of high SFA diets and fish oil supplementation on total protein content of inflammatory signaling intermediates in soleus muscle. a) TLR4, b) IκBα, c) Ser\(^{32}\) phosphorylated IκBα, d) NFκB, e) Ser\(^{536}\) phosphorylated NFκB f) SOCS-3. Alpha tubulin was used as a loading control. Values are means ± SEM, n=12. * = significantly different from CON, p ≤ 0.05. CON = control diet, SFA = high saturated fatty acid diet, SFA/n-3 PUFA = high saturated fatty acid with n-3 polyunsaturated fatty acid diet.
Table 1: Composition of Experimental Diets

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<td>Dicalcium Phosphate</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Calcium Carbonate</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Potassium Citrate, 1 H2O</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vitamin Mix, V10001</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>4057</td>
<td>4057</td>
<td>4057</td>
</tr>
</tbody>
</table>
Table 2: Fatty Acid Composition of Experimental Diets

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>CON</th>
<th>SFA</th>
<th>SFA/n-3 PUFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>0.4</td>
<td>0.9</td>
<td>2.8</td>
</tr>
<tr>
<td>C14:1</td>
<td>0.2</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>C16:0</td>
<td>16.8</td>
<td>23.2</td>
<td>21.2</td>
</tr>
<tr>
<td>C16:1</td>
<td>1.8</td>
<td>3.7</td>
<td>5.5</td>
</tr>
<tr>
<td>C18:0</td>
<td>8.4</td>
<td>13.2</td>
<td>10.4</td>
</tr>
<tr>
<td>C18:1</td>
<td>32.9</td>
<td>41.9</td>
<td>33.5</td>
</tr>
<tr>
<td>C18:2</td>
<td>33.9</td>
<td>13.3</td>
<td>12.0</td>
</tr>
<tr>
<td>C18:3</td>
<td>4.8</td>
<td>1.7</td>
<td>2.3</td>
</tr>
<tr>
<td>C18:4</td>
<td></td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>C20:0</td>
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<td></td>
<td>0.1</td>
</tr>
<tr>
<td>C20:1</td>
<td></td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>C20:4</td>
<td>0.8</td>
<td>1.6</td>
<td>1.8</td>
</tr>
<tr>
<td>C20:5</td>
<td></td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>C22:0</td>
<td></td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>C22:1</td>
<td></td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>C22:4</td>
<td></td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>C22:5</td>
<td></td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>C22:6</td>
<td></td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>C24:0</td>
<td></td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Saturated</td>
<td>25.6</td>
<td>37.3</td>
<td>34.8</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>34.9</td>
<td>46.1</td>
<td>39.8</td>
</tr>
<tr>
<td>n-6 Polyunsaturated</td>
<td>34.7</td>
<td>14.9</td>
<td>13.8</td>
</tr>
<tr>
<td>n-3 Polyunsaturated</td>
<td>4.8</td>
<td>1.7</td>
<td>10.2</td>
</tr>
</tbody>
</table>
Table 3: Body Mass and Blood Measurements

<table>
<thead>
<tr>
<th></th>
<th>4wk CON</th>
<th>4wk SFA</th>
<th>4wk SFA/n-3 PUFA</th>
<th>6wk SFA</th>
<th>4wk SFA + 2wk SFA/n-3 PUFA</th>
<th>8wk SFA</th>
<th>4wk SFA + 4wk SFA/n-3 PUFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arrival body mass (g)</td>
<td>153.1 ± 1.5</td>
<td>153.0 ± 2.1</td>
<td>153.2 ± 1.9</td>
<td>151.3 ± 1.9</td>
<td>149.2 ± 1.8</td>
<td>149.4 ± 1.8</td>
<td>150.3 ± 2.4</td>
</tr>
<tr>
<td>Terminal body mass (g)</td>
<td>254.8 ± 9.0</td>
<td>299.9 ± 10.5*</td>
<td>289.5 ± 7.7</td>
<td>330.3 ± 6.4</td>
<td>337.3 ± 10.2</td>
<td>345.8 ± 7.5</td>
<td>342.4 ± 9.6</td>
</tr>
<tr>
<td>Fasting blood glucose (mmol/L)</td>
<td>8.9 ± 0.3</td>
<td>9.8 ± 0.4</td>
<td>9.9 ± 0.4</td>
<td>8.8 ± 0.2</td>
<td>9.3 ± 0.3</td>
<td>8.5 ± 0.2</td>
<td>8.3 ± 0.3</td>
</tr>
<tr>
<td>Fasting plasma insulin (ng/ml)</td>
<td>5.2 ± 0.7</td>
<td>6.2 ± 0.5</td>
<td>4.8 ± 0.8</td>
<td>5.2 ± 0.6</td>
<td>4.3 ± 0.6</td>
<td>5.7 ± 0.5</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td>Fasting plasma free fatty acids (mmol/L)</td>
<td>0.64 ± 0.04</td>
<td>0.70 ± 0.04</td>
<td>0.72 ± 0.04</td>
<td>0.74 ± 0.05</td>
<td>0.74 ± 0.06</td>
<td>0.84 ± 0.07</td>
<td>0.88 ± 0.06</td>
</tr>
<tr>
<td>Fasting plasma adiponectin (µg/ml)</td>
<td>9.5 ± 0.4</td>
<td>8.3 ± 0.4</td>
<td>13.1 ± 0.6* ¥</td>
<td>8.4 ± 0.7</td>
<td>8.5 ± 0.5</td>
<td>8.2 ± 0.6</td>
<td>9.6 ± 0.4</td>
</tr>
<tr>
<td>Fasting plasma IL-6 (pg/mL)</td>
<td>13.4 ± 0.7</td>
<td>17.4 ± 0.7*</td>
<td>14.3 ± 0.7</td>
<td>19.6 ± 0.9</td>
<td>17.5 ± 0.7</td>
<td>17.8 ± 0.8</td>
<td>15.2 ± 0.6</td>
</tr>
<tr>
<td>Fasting plasma leptin (ng/mL)</td>
<td>3.1 ± 0.07</td>
<td>5.4 ± 0.4*</td>
<td>5.1 ± 0.1*</td>
<td>5.9 ± 0.2</td>
<td>5.9 ± 0.2</td>
<td>6.6 ± 0.4</td>
<td>6.1 ± 0.3</td>
</tr>
<tr>
<td>Fasting plasma MCP-1 (pg/mL)</td>
<td>22.8 ± 0.6</td>
<td>28.0 ± 1.3*</td>
<td>28.1 ± 1.3*</td>
<td>27.7 ± 1.0</td>
<td>26.7 ± 0.9</td>
<td>25.7 ± 1.0</td>
<td>27.1 ± 0.8</td>
</tr>
<tr>
<td>Fasting plasma TNF-α (pg/mL)</td>
<td>24.3 ± 0.7</td>
<td>24.0 ± 0.8</td>
<td>25.3 ± 0.7</td>
<td>23.8 ± 0.7</td>
<td>23.3 ± 0.5</td>
<td>21.7 ± 0.6</td>
<td>22.4 ± 0.7</td>
</tr>
</tbody>
</table>

Data are mean ± SEM, n = 10-12. * = significantly different from CON within the same time point, p ≤ 0.05; ¥ = significantly different from SFA within the same time point, p ≤ 0.05.
\[ \alpha\text{-tubulin} \]
\( \alpha \)-tubulin

![Image of Western Blots](image)

**Graph A:**
- CON
- 4wk SFA
- 6wk SFA
- 8wk SFA
- 4wk SFA + 2wk SFA
- 4wk SFA + 4wk SFA

**Graph B:**
- CON
- 4wk SFA
- 6wk SFA
- 8wk SFA
- 4wk SFA + 2wk SFA
- 4wk SFA + 4wk SFA

*Significance marker:*
α-tubulin