Odontella aurita-enriched diet prevents high fat diet-induced liver insulin resistance

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Abstract
The beneficial effect of polyunsaturated omega-3 fatty acid (w-3 FA) consumption regarding cardiovascular diseases, insulin resistance and inflammation has been widely reported. Fish oil is considered as the main source of commercialized w-3 FAs, and other alternative sources have been reported such as linseed or microalgae. However, despite numerous reports, the underlying mechanisms of action of w-3 FAs on insulin resistance are still not clearly established, especially those from microalgae. Here, we report that Odontella aurita, a microalga rich in w-3 FAs eicosapentaenoic acid, prevents high fat diet-induced insulin resistance and inflammation in the liver of Wistar rats. Indeed, a high fat diet (HFD) increased plasma insulin levels associated with the impairment of insulin receptor signaling and the up-regulation of toll-like receptor 4 (TLR4) expressions. Importantly, Odontella aurita-enriched HFD (HFOA) reduces body weight and plasma insulin levels and maintains normal insulin receptor expression and responsiveness. Furthermore, HFOA decreased TLR4 expression, JNK/p38 phosphorylation and pro-inflammatory factors. In conclusion, we demonstrate for the first time, to our knowledge, that diet supplementation with whole Odontella aurita overcomes HFD-induced insulin resistance through the inhibition of TLR4/JNK/p38 MAP kinase signaling pathways.

Key Words
- Odontella aurita
- insulin resistance
- inflammation
- high fat diet
- TLR-4

Introduction
Insulin resistance is a major risk factor for type 2 diabetes that is often associated with metabolic syndrome including obesity, cardiovascular diseases and dyslipidemia. The alarming worldwide augmentation of type 2 diabetes becomes a major public health concern. Thus, restoring insulin sensitivity or responsiveness becomes a real challenge to overcome type 2 diabetes and associated diseases. Several strategies have been used for this purpose. Indeed, among environmental factors, dietary fat plays an important role in the induction of insulin resistance (Storlien et al. 1997). Both the amount and the type of fatty acids ingested alter insulin sensitivity in target tissues (i.e., muscle, adipose tissue and liver) associated with glucose intolerance and obesity (Storlien et al. 1986, Storlien et al. 1991). Insulin resistance in the liver is also characterized by an increased flux through glucose-6-phosphatase (Oakes et al. 1997), whereas insulin resistance in muscle and adipose tissue results from an impairment...
of glucose uptake (Kraegen et al. 1986) and/or glucose phosphorylation followed by a reduction in the rate of both muscle glycogen synthesis and glucose oxidation (Roden et al. 1996). These effects mimic those reported in patients with type 2 diabetes (Cline et al. 1999, Clore et al. 2000). Furthermore, previous studies suggested that long chain saturated fatty acids promote insulin resistance through c-Jun N-terminal kinase (JNK) activation leading to the phosphorylation of insulin receptor substrate (IRS)1/2 on serine residues, then impairing insulin signaling (Nguyen et al. 2005, Solinas et al. 2006). However, not all types of fatty acids induce an alteration of insulin action during a high fat diet in rats (Storlien et al. 1991). Indeed, the substitution of omega-3 fatty acids (w-3 FAs) from fish oil for other types of lipids prevents insulin resistance (Kraegen et al. 1991, Jucker et al. 1999) and has been recognized as beneficial on human health (Kantha 1987). These beneficial effects cover several diseases and metabolic dysfunctions such as glucose homeostasis, insulin sensitivity, cardiovascular diseases and hepatic lipid metabolism (Sethan et al. 2008, Lee et al. 2009, Scorletti & Byrne 2013, Flachs et al. 2014). Indeed, previous studies reported that the effects of dietary w-3 FAs could be related to the subsequent changes in fatty acid content in membrane phospholipids of insulin target tissues (Kahn & Peersen 1993). In muscle, w-3 FAs might improve insulin sensitivity through a relative increase in the unsaturation of membrane phospholipids and/or a decrease in muscle content in triglycerides (Oakes et al. 1997, Wilkes et al. 1998). The alterations in membrane composition could affect insulin receptors (Liu et al. 1994) and/or IRS-1 and PI 3-kinase expression and protein abundance (Taouis et al. 2002). More recently, the beneficial effect of w-3 FAs has been attributed to their actions through G protein-coupled receptor 120 (GPR120) or free fatty acid receptor 4 (FFAR4) leading to anti-inflammatory and insulin sensitizing effects (Oh et al. 2010). Indeed, w-3 FAs bind to FFAR4 on macrophages recruiting then arrestin-2 followed by the internalization of FFAR4/arrestin-2 complex. This complex sequesters the pro-inflammatory TAK1 binding protein (TAB1) (Oh et al. 2010). Thus, the decrease in macrophage pro-inflammatory factor’s production and secretion promotes insulin responsiveness in insulin-dependent tissues such as adipose tissue (Oh et al. 2010). These data clearly indicate the beneficial anti-inflammatory and insulin-sensitizing effects of w-3 FAs when supplemented to human or rodent diets. The major sources of w-3 FAs are fish oils. However, the use of w-3 FAs from fish oil can have some disadvantages such as the presence of pollution residues (heavy metals), seasonal change in fatty acid composition and remnant smell, especially when used as a complement to the human diet. Several sources of w-3 FAs have been described such as those extracted from linseed and marine microalgae. Among marine microalgae, Odontella aurita (O. aurita) presents a high content in eicosapentaenoic acid (EPA) that is a bioactive w-3 FA. Furthermore, the supplementation of diet with O. aurita reduced high fat induced-metabolic syndrome risk including hyperlipidemia and oxidative stress (Haimeur et al. 2012). It has been also shown that feeding a rat a high fat diet supplemented with O. aurita increased the level of w-3 FAs content in different tissues such as the liver and platelets. Thus, these effects could be, at least partially, attributed to the high w-3 FAs content of O. aurita. Indeed, O. aurita contains anti-oxidants such as fucoxanthin (Moreau et al. 2006). However, the underlying mechanisms concerning the effects of O. aurita regarding the attenuation of metabolic syndrome risk and especially concerning insulin resistance are still not elucidated. Indeed, it is of great interest to compare the effect of w-3 FAs originated from fish oil and O. aurita regarding insulin sensitivity. Here, we aimed to investigate the effect of a high fat diet supplemented with O. aurita or fish oil on insulin signaling and pro-inflammatory factors.

**Animals and experimental protocol**

Twenty-four male Wistar rats weighing 120±10 g were purchased from the Centre d’Elevage Janvier (Le Genest Saint Isle, France). They were housed in pairs in cages in a room under standard conditions of temperature (22–24 °C), humidity (40–60%) and 12 h light:12 h darkness cycle. All of the animals were fed a standard diet (SAFE, Augy, France) for 1 week of acclimation. The animals were then randomly divided into four groups of six rats and were fed with the following diets for 8 weeks: the control group (C) continued to receive the standard diet; the HF group was fed a high-fat diet containing (on a per weight basis: 40% fat, 23% proteins, 23% carbohydrates, 6% cellulose, 7% mineral and 1% vitamins); the HFOA group received the high fat diet supplemented with 12% (w/w) of freeze-dried O. aurita (Innovation, Bouin, France); the HFFO group was fed with the high fat diet supplemented with 0.5% (w/w) of fish oil (Polaris, Pleuven, France). The composition of the experimental diets is shown in Table 1. All animals were allowed to feed ad libitum with free access to water. All study protocols complied with the institutional guidelines (Direction des services Vétérinaires de la Mayenne, France, N° B53500).
Table 1 Composition of the experimental diets (gram/kilogram diet). Analysis was provided by SAFE (Scientific Animal Food & Engineering, Augy, France). The mineral mixture provides the following amounts in milligram/kilogram of diet: CaHPO₄, 17.2; KCl, 4000; NaCl, 4000; MgO, 420; MgSO₄, 2000; Fe₂O₃, 120; FeSO₄ 7 H₂O, 200; trace elements, 400. Trace element mixture (mg/kg of diet): MnSO₄.H₂O, 98; CuSO₂.5H₂O, 20; ZnSO₄, 7 H₂O, 80; CoSO₄, 7 H₂O, 0.1; KI, 0.3. The vitamin mixture provides the following amounts in milligram/kilogram of diet: retinol, 39.600 IU; cholecalciferol, 5000 IU; thiamin, 40 mg; riboflavin, 30 mg; cyanocobalamin, 0.1 mg; ascorbic acid, 1600 mg; choline, 2.720 mg; folic acid, 10 mg; p-aminobenzoic acid, 100mg; biotin, 0.6 mg; inositol, 300 mg; pantothenic acid, 140 mg; pyridoxine, 20 mg; niacin, 10 mg; ergocalciferol, 0.1 mg; pyridoxine, 20 mg; retinol, 39.600 IU; cholecalciferol, 5000 IU; thiamin, 40 mg; riboflavin, 30 mg; cyanocobalamin, 0.1 mg; ascorbic acid, 1600 mg; choline, 2.720 mg; folic acid, 10 mg; p-aminobenzoic acid, 100 mg; biotin, 0.6 mg.

<table>
<thead>
<tr>
<th>Nutrient</th>
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<th>HFOA</th>
<th>HFFO</th>
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<td>Cellulose</td>
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</tr>
<tr>
<td>Lard</td>
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<tr>
<td>Coprah</td>
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<td>330</td>
</tr>
<tr>
<td>Freeze-dried O. aurita</td>
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<td>–</td>
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</tr>
<tr>
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<td>–</td>
<td>–</td>
<td>5</td>
</tr>
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</tbody>
</table>

C, control diet; HF, high-fat diet; HFOA, high-fat diet supplemented with 12% (w/w) of freeze-dried of Odontella aurita; HFFO, high-fat diet supplemented with 0.5% (w/w) of fish oil.

Supplementation rates of freeze-dried O. aurita (12%) and fish oil (0.5%) were selected to provide the same intake of EPA, consisting predominantly of PUFA n-3 for both sources, in the HFOA and HFFO diets.

The bodyweight (BW) gains of the rats were monitored at regular intervals, and their daily food intakes were estimated. At the end of the experiment, blood was collected from the abdominal aorta under diazepam/ketamine (4v/3v) anesthesia and the livers were removed, rinsed with ice-cold NaCl (0.9%), frozen in liquid nitrogen and stored at –80 °C before analysis.

Western blot analysis

Frozen liver samples were homogenized in 1 ml of solubilization buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% nonidet P40, 10% glycerol, protease inhibitors (0.35 mg/ml PMSF, 2 μg/ml leupeptin and 2 μg/ml aprotinin) and phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate, 20 mM sodium β-glycerophosphate and 10 mM benzamidine). Homogenates were incubated for 2 h at 4 °C then insoluble materials were removed by centrifugation and stored at −80 °C. Protein concentrations of the resulting lysates were determined using a protein assay kit (BCA Protein Assay Kit, Thermo Scientific, Illkirch, France).

Proteins were subjected to SDS-PAGE and transferred onto Immobilon-FL membrane (Life Technologies, Illkirch, France). Blots were then blocked with 5% BSA and incubated with primary antibodies raised against insulin receptor (IR) β-subunit, phospho (p)-IRS-1 (ser307), IRS-1, p-JNK, JNK, p-P38MAPK, P38MAPK, β-tubulin (Cell Signaling, Danvers, MA, USA), toll-like receptor 4 (TLR4), IL-6 and protein-tyrosine phosphatase (PTP)-1B (Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4 °C. The immunoblots were washed with PBS, incubated with the appropriated secondary antibody labeled with Alexa 488 (Life Technologies) then scanned and quantified using the Bruker Molecular Imaging System 4000MM Pro (Billerica, MA, USA).

In vitro insulin stimulation on liver membranes

The in vitro stimulation of the insulin receptor had been performed as previously reported (Benoit et al. 2013). Briefly, 500 mg of rat liver were homogenized in 1 ml buffer A (0.32 M sucrose, 2 mM HEPES (pH 7.4), protease and phosphatase inhibitors as described before) with Precellys 24/Cryolys apparatus (3000 g × 20 s). Homogenates were centrifuged 5 min at 1000 g at 4 °C. Supernatants were kept and pellets were suspended in 1 ml buffer A and centrifuged 5 min at 1000 g at 4 °C. The two supernatants were mixed and centrifuged 20 min at 10000 g at 4 °C. Pellets were suspended in 1 ml buffer B (50 μM CaCl₂, 2 mM HEPES (pH 7.4), protease and phosphatase inhibitors) and centrifuged 45 min at 17 000 g at 4 °C. Pellets, representing crude membranes, were then suspended in buffer C (50 mM Tris-HCl (pH 7.4), 1 mM MgCl₂, 2mM EGTA, protease and phosphatase inhibitors) and stored at −80 °C.

Crude liver membranes were incubated for 10 min at 37 °C with insulin (1 μM) and ATP (5 mM) or buffer C as control. Reaction was stopped by the addition of loading buffer. Western blots were performed as described before. Immunoblots were incubated with a primary antibody raised against IR β-subunit and phosphotyrosine (Millipore, Darmstadt, Germany).
Immunoprecipitation

Crude liver membranes (500 μg) were incubated for 10 min at 37 °C with insulin (1 μM) and ATP (5 mM) or buffer C as control. Reaction was stopped by the addition of 0.5 ml buffer D (Buffer C, 1% Triton X-100, 1% nonidet P40) followed by 2 h incubation at 4 °C. Lysate samples were then incubated with antibody raised against IR β-subunit overnight at 4 °C. The immune complexes were precipitated after incubation with a protein A-agarose (Sigma–Aldrich) for 2 h at 4 °C and then heated in loading buffer at 100 °C for 5 min. Western blots were performed as described before.

RNA analysis

Frozen liver samples were homogenized with Precellys 24/Cryolys apparatus, and total RNA was extracted using TRIzol LS reagent (Invitrogen) according to the manufacturer’s recommendations. 1 μg of total RNA was reverse transcribed (F-572L M-Mulv) (Finnzymes, Illkirch, France), and the cDNAs were subjected to real-time PCR. Quantitative RT-PCR was performed using adequate primers: 18s forward, TCCCCGAGAAGTTTCAGC, and reverse, CTCCCATCTCAGTTCTCTC; CD68 forward, CTGGTGCTCATTGCCTTCTG, and reverse, GAGGGGCTGGTAGGTTGATT; IBA-1 forward, ATGAGCCAGAGGATTT, and reverse, ACCTCTCTTCGCTTTGATC; IL-1β forward, TAAGCCAACAAGTGGTATTC, and reverse, AGGTATAGATTCTTCCCCTTG; IL-6 forward, GTTGCACTTGAGCCCTCTC, and reverse, TCCCTCCGCTGCCCTCC; InsR forward, TGCCACCAATCCTGGAGG, and reverse, TGGAGGAGGAGGAGGAGGA; PTP1B forward, GCACAGCATGAGCAGTATGAGTC, and reverse, TCCACCCACCATCCGTTTCC.

Chemicals

Bovine serum albumin (fraction V) was purchased from Euromedex (Souffelweyersheim, France). All other chemicals were purchased from Sigma–Aldrich.

Statistical analysis

Statistical analyses were performed using a Mann–Whitney U test for physiological parameters, endocrine parameters data, signaling and gene expression experiments. Repeated-measures two-way ANOVA was used to test the changes of body weight and energy intake over time followed by Bonferroni post-hoc test. The results were expressed as mean ± S.E.M., and P value <0.05 was considered as statistically significant.

Results

High fat diet supplemented with O. aurita reduced body weight and adiposity

Adult Wistar rats were subjected during 8 weeks to control, HF, HFOA or HFFO diet. The HFOA group exhibited a lower body weight and lower body weight gain when compared to C, HF and HFFO groups as measured over time (Fig. 1A and 2B; Table 2). Food intake expressed as grams per day was significantly higher in the control group as compared to all groups, but when expressed as kilocalories per day, all groups consumed similar energy (Fig. 1D and Table 2). However, the weight of adipose tissue was significantly higher in the HF group as compared to all groups. In addition, adipose tissue weight was significantly lower in the HFOA group as compared to HFFO group.
the HFFO and C groups ($P<0.05$). When results were expressed as a ratio of adipose tissue weight to body weight, the HF group showed a significantly higher adiposity as compared to all groups (Table 2). In contrast, liver weight was not modified by diets (Table 2).

High fat diet supplemented with *O. aurita* or fish oil normalized plasma insulin and lipid levels

Wistar rats fed a high fat diet (HFD) during the 8 weeks exhibited a significant increase of plasma insulin levels as compared to the C, HFOA and HFFO groups without changes in plasma glucose ($P<0.05$, Table 3). Furthermore, both HFOA and HFFO improved the homeostatic model assessment (HOMA) index when compared to the HF group. Triglyceride liver content was lower in the HFOA group as compared to the HF and HFFO groups (Table 3). We also showed that the HFOA group exhibits significantly lower liver cholesterol content as compared to all groups including the C group (Table 3). In addition, plasma leptin was significantly increased in the HF group as compared to the C and HFOA groups. Plasma triglyceride and cholesterol levels were significantly higher in the C and HF groups as compared to the HFOA and HFFO groups (Table 3, $P<0.05$).

Supplementation of HFD with *O. aurita* or fish oil prevents the impairment of liver insulin receptor expression and tyrosine kinase activity

We examined the effect of the supplementation of a high fat diet with *O. aurita* (OA) or fish oil on liver insulin receptor expression. We show that HFD induced the down-regulation (approximately −25%) of liver insulin receptor at both protein and mRNAs levels as compared to rats fed a chow diet (Fig. 2A, B and C). The supplementation of HFD with OA or fish oil maintained insulin receptor expression to the level of control rats (Fig. 2A, B and C). To determine whether OA or fish oil (FO) supplementation affected insulin receptor tyrosine kinase activity, we measured the insulin-dependent tyrosine phosphorylation in liver crude membranes prepared from HFD, HFOA and HFFO rats. We showed that HFD significantly reduced the insulin-dependent tyrosine phosphorylation of a band with around 180 kDa corresponding to IRS-1 and IRS-2 and a band around 95 kDa corresponding to IR as evidenced by the re-blotting using anti-IR antibodies as compared to control rats (Fig. 3A and B). Whereas, when HFD was supplemented with OA, the impairment of insulin-dependent tyrosine phosphorylation was prevented (Fig. 3C and D). To further investigate the impact of OA and FO on intrinsic insulin receptor tyrosine kinase activity, following crude liver membranes incubation in the presence or absence of insulin, samples were subjected to immunoprecipitation using anti-IR antibodies, and blots were revealed with anti-phosphotyrosine antibodies. We showed that the HFOA group exhibited a higher insulin receptor tyrosine phosphorylation in response to insulin with a higher amplitude when compared to C, HFD and HFFO groups (Fig. 3E and F). Because HFD induced the down-regulation of the insulin receptor,
we have also normalized insulin receptor phosphorylation to glycoprotein 130 (gp130) and confirmed the result obtained in Fig. 2F with lower insulin sensitivity in the HF group. Indeed, insulin receptor phosphorylation in response to insulin in the HF group was significantly lower as compared to the C, HFOA and HFFO groups (Fig. 3G).

Supplementation of HFD with OA prevents the augmentation of negative regulators of insulin signaling in the liver

We examined whether the insulin sensitizing effect of OA is attributed to the prevention of HFD-induced up-regulation of the insulin signaling negative regulator, PTP-1B and/or to the augmentation of IRS-1 phosphorylation on serine residues that impairs IRS-1 tyrosine phosphorylation. We showed that HFD increased PTP-1B expression as evidenced by western blot analysis using adequate antibodies and quantitative RT-PCR (Fig. 4A, B and C). The supplementation of HFD with OA maintained PTP-1B expression to the level measured in the liver of rat fed chow diet (Fig. 4A, B and C). The same effect was observed when HFD was supplemented with FO especially for PTP-1B mRNA expression (Fig. 4C). We have also investigated IRS-1 phosphorylation on Ser307. HFD significantly increased IRS-1 Ser307 phosphorylation as compared to rat fed chow diet (Fig. 4D and E). The supplementation of HFD with OA prevented HFD-induced IRS-1 Ser307 phosphorylation (Fig. 4D and E). When HFD was supplemented with FO, the IRS-1 Ser307 phosphorylation was similar to that obtained following OA supplementation (Fig. 4D).

OA supplementation prevented HFD-induced p38 and JNK1 phosphorylation

To investigate the effect of OA supplementation on insulin signaling, we investigated the impact on the phosphorylation of p38 MAPK and JNK1, known as hallmarks of insulin resistance. We showed that HFD induced both p38 and JNK1 phosphorylation (Fig. 4F, G, H and I). OA supplementation prevented the augmentation of p38 phosphorylation and maintained it to a level located between C and HF groups, but not significantly (Fig. 4F and G). A similar result was obtained with FO supplementation (Fig. 4F); however, the statistical analysis was not performed (n=2). OA supplementation to HFD significantly prevented HFD-induced JNK1 phosphorylation.

Table 2 Effects of the different diets given for 8 weeks on body and adipose tissue weights and daily food and energy intake. Mean values with different superscript letters (a, b and c) are significantly different (P<0.05, n=6)

<table>
<thead>
<tr>
<th>Metabolic parameters</th>
<th>C</th>
<th>HF</th>
<th>HFOA</th>
<th>HFFO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>466.8±9.4a</td>
<td>460.2±25a</td>
<td>400.2±13.3b</td>
<td>452.4±7a</td>
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<tr>
<td>Adipose tissue weight (g)</td>
<td>13.6±2.8b</td>
<td>17.1±3a</td>
<td>8.5±2.3c</td>
<td>11±1b</td>
</tr>
<tr>
<td>AT/BW (%)</td>
<td>2.7±0.7b</td>
<td>3.3±1.2a</td>
<td>2.3±0.5b</td>
<td>2.6±0.8b</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>12.4±0.7</td>
<td>11.8±1.7</td>
<td>10.6±1.6</td>
<td>11.4±1.4</td>
</tr>
<tr>
<td>Daily food intake (g/day)</td>
<td>26.6±0.5a</td>
<td>19.7±0.2b</td>
<td>18.4±0.4c</td>
<td>18.1±0.3a</td>
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<tr>
<td>Energy intake (Kcal/day)</td>
<td>107.2±1.9</td>
<td>111.6±1.1</td>
<td>110.8±2.3</td>
<td>110.7±2</td>
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</table>

C, control diet; HF, high-fat diet; HFOA, high-fat diet supplemented with 12% (w/w) of freeze-dried of O. aurita; HFFO, high-fat diet supplemented with 0.5% (w/w) of fish oil; AT, adipose tissue weight; BW, body weight.

Table 3 Effects of the different diet for 8 weeks on glycemia, plasma insulin level, blood triglyceride and cholesterol levels. Mean values with different superscript letters (a, b and c) are significantly different (P<0.05, n=6)

<table>
<thead>
<tr>
<th>Metabolic parameters</th>
<th>C</th>
<th>HF</th>
<th>HFOA</th>
<th>HFFO</th>
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<tbody>
<tr>
<td>Glycemia (g/l)</td>
<td>0.58±0.01</td>
<td>0.56±0.02</td>
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<td>Cholesterolemia (g/l)</td>
<td>0.71±0.03a</td>
<td>0.76±0.03a</td>
<td>0.51±0.02b</td>
<td>0.47±0.05b</td>
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<td>Liver triglyceride (mg/g)</td>
<td>42.1±10.4a</td>
<td>94.8±22.4b</td>
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<td>Liver cholesterol (mg/g)</td>
<td>6.1±1.8a</td>
<td>22.2±3.1b</td>
<td>2.6±0.1c</td>
<td>4.05±0.7a</td>
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<td>Leptinemia (ng/ml)</td>
<td>2.14±0.55a</td>
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<td>Insulinemia (ng/ml)</td>
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<td>Glycerimia (g/l)</td>
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<td>HOMA-IR</td>
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<td>2.36±0.2a</td>
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C, control diet; HF, high-fat diet; HFOA, high-fat diet supplemented with 12% (w/w) of freeze-dried of O. aurita; HFFO, high-fat diet supplemented with 0.5% (w/w) of fish oil.
phosphorylation (Fig. 4H and I). Similar, tendency on JNK1 phosphorylation was also found when HFD was supplemented with FO (Fig. 4H and I). We also investigated the expression levels of NF-κB, known as one of key components involved in insulin resistance onset. We showed that HFD significantly increased NF-κB expression levels as compared to the C group (Fig. 4J). The HFOA and HFFO groups exhibited lower expression levels of NF-κB as compared to the HF group, but the difference was not significant (Fig. 4J).

OA supplementation prevents HFD-induced toll-like receptor 4 and IL-6 expressions in the liver

Recent studies have provided evidence for the implication of TLR4 and pro-inflammatory cytokines such as IL-6 in the pathogenesis of obesity and insulin resistance. Thus, we investigated the effect of OA supplementation to HFD on TLR4, IL-6 and IL-1 expression. HFD induced the overexpression of TLR4 but not the glycosylated form of the receptor (Fig. 5A and B). The supplementation of HFD with OA led to significantly lower levels of both forms of TLR4 as compared to the HF group (Fig. 5A and B). The supplementation of HFD with FO had a weaker effect on TLR4 expression as compared to OA (Fig. 5A). To further investigate the effect of OA supplementation on TLR4 receptor, (E) is a representative immunoprecipitation/immunoblot (IP/IB) analysis of insulin receptor phosphorylation normalized to insulin receptor and gp130. (F and G) are quantifications of band densities of phosphorylated insulin receptor normalized to IR (F) or to gp130 (G). All data are presented as means ± S.E.M. (n=4-6/group). *###denotes significant differences at P<0.05 by ANOVA test.

 signaling, we studied the co-immunoprecipitation of TLR4 with its signaling components. We showed that HFD increased both Myd88 and TIRAP co-immunoprecipitation with TLR4. This co-immunoprecipitation was diminished when HFD was supplemented with OA (Fig. 5C). However, the supplementation with FO seemed to have a weak impact on HFD-induced TLR4/Myd88/TIRAP co-immunoprecipitation (Fig. 5C).

The HFOA group significantly exhibited lower IL-6 plasma levels as compared to the HF group (Fig. 5A). In addition, the expression level of IL-6 at both mRNA and protein levels in the liver was significantly reduced in HFOA and HFFO groups when compared to the HF group (Fig. 5B and 6C). Similar results were obtained when the expression of IL-1β was measured by quantitative RT-PCR. Indeed, HFD induced the up-regulation of hepatic IL-1β, and the supplementation of HFD with OA prevented this effect (Fig. 6D). However, FO supplementation had a weaker effect as compared to OA supplementation (Fig. 6D). We next investigated the impact of HFD supplementation with OA on other inflammation markers. We showed that HFD induced the up-regulation of IBA1, ICAM1 and CD68 (Fig. 6E, F and G). This effect was completely prevented by OA supplementation (Fig. 6E, F and G). However, FO supplementation was unable to down-regulate these markers (Fig. 6E, F and G).
Discussion

A high fat diet promotes overall insulin resistance through the increase of pro-inflammatory factors and the modification of adipokines secretion (e.g., leptin, adiponectin or resistin) mainly originated from adipose tissue. These factors impair insulin signaling mostly in insulin responsive tissues such as the liver, adipose tissue and muscle. The alteration of insulin signaling in the liver is a strong marker for overall insulin resistance as mirrored by increased hepatic glucose production that leads to increased insulin plasma levels in the early stages of insulin resistance. However, it is noteworthy that most studies that showed the deleterious effects of HFD on insulin responsiveness were performed using HFD enriched in saturated fat. In contrast, diet enrichment with polyunsaturated omega-3 partially prevents these effects. The aim of the present study was to investigate the effect of the supplementation of HFD with *O. aurita*, a marine diatom rich in EPA, on liver insulin responsiveness. The effect of OA supplementation has been also compared to the supplementation of HFD with fish oil. Here and as expected, HFD (mostly rich in saturated fatty acids) increased adipose tissue mass and plasma levels of insulin, triglycerides and cholesterol. Importantly, the HFOA and HFFO groups exhibited normal plasma insulin and lipid levels as compared to rats fed a control diet. Despite similar energy intake, HFOA showed a lower body weight and fat mass as compared to HFD and HFFO rats. This indicates that the OA supplementation of HFD has different effects on fat mass and body weight as those of

![Figure 4](http://joe.endocrinology-journals.org/C209)
fucoxanthin. To further analyze these differences between the supplementation of HFD with OA or FO, we focused on liver insulin responsiveness. We showed that HFD induced the down-regulation of liver insulin receptor and this effect is prevented when HFD was supplemented with OA and FO. Importantly, the effect on liver insulin receptor expression is concomitant with the restoration of endogenous insulin receptor tyrosine kinase activity. The prevention of insulin receptor and tyrosine kinase activity impairment in the HFOA group could be at least in part attributed to the effect of OA supplementation on plasma insulin and lipid levels. Indeed, the HFOA group exhibited lower plasma insulin levels as compared to all other groups including the HFFO group. We also showed that OA supplementation prevented HFD-induced up-regulation of tyrosine phosphatase PTP-1B, one of major negative regulators of insulin signaling (Koren & Fantus 2007).

HFO, and this is most likely attributed to the composition of OA. Indeed, OA contains EPA and other components such as anti-oxidants, especially fucoxanthin known for its beneficial effects (Maeda et al. 2009, Xia et al. 2013, Tan & Hou 2014). Indeed, fucoxanthin has been described as having anti-obesity, anti-diabetic and anti-inflammatory effects (Maeda et al. 2009, Tan & Hou 2014). These effects may contribute to the differential effects between the supplementation with OA and FO regarding liver insulin responsiveness. Importantly, we show that the HFOA group had a lower body weight even compared to the control group and clearly re-enforced the beneficial effect of OA supplementation that could attribute to both polyunsaturated omega-3 fatty acids and anti-oxidant fucoxanthin.

**Figure 5**

*Odontella aurita* supplementation prevents the up-regulation of TLR4 expression and TLR4/MyD88/TIRAP interaction in the liver of HFD rats. (A) is a representative western blot revealed with anti-TLR4 antibody that reveals both glycosylated and non-glycosylated TLR4. TLR4 expression was normalized to β-tubulin. Band densities are presented in (B). The results are expressed as ratio of TLR4/β-tubulin (white bars) and TLR4 glycosylated form/β-tubulin (black bars). (C) presents an immunoprecipitation/immunoblot (IP/IB) analysis of the interaction of TLR4 with MyD88 and TIRAP (*n* = 2). For western blot analysis, data are presented as means ± S.E.M. (*n* = 6/group, except HFFO with *n* = 2). **a,b,c,d** denotes significant differences for TLR4 gly at *P* < 0.05 by ANOVA test. **a,b,c,d** denotes significant differences for TLR4 at *P* < 0.05 by ANOVA test.

**Figure 6**

*Odontella aurita* supplementation prevents the up-regulation of pro-inflammatory markers in the liver of HFD rats. (A) presents plasma IL-6 levels. (B) is a representative western blot revealed with anti-IL-6 antibody normalized to β-tubulin and a quantification of band density expressed as ratio of IL6/β-tubulin. (C, D, E, F and G) present the mRNA expression levels of IL-6, IL-1β, IBA1, ICAM1 and CD68 measured by qRT-PCR and normalized to 18S mRNA. All data are presented as means ± S.E.M. (*n* = 4–6/group). **a,b,c,d** denotes significant differences at *P* < 0.05 by ANOVA test.
In addition, it is well established that Ser307 phosphorylation of IRS-1 impairs insulin signaling (Aguire et al. 2000). Indeed, the serine phosphorylation of IRS-1 impairs the tyrosine phosphorylation of IRS-1 and thus the alteration of the IR/IRS-1/P13-kinase/Akt pathway. Here, we show that HFD increased liver IRS-1 Ser307 phosphorylation, and this effect is completely abolished in the HFOA and HFFO groups. The phosphorylation of IRS-1 on serine residues is also promoted by the increase of JNK1 (a serine kinase) phosphorylation. JNK1 phosphorylation has been reported in the insulin resistance state in various cellular models and in vivo (Aguire et al. 2000, Solinas et al. 2006). Here, we show that HFD-induced augmentation of JNK1 phosphorylation is prevented in the HFOA group. In addition, p38MAPK phosphorylation induced by HFD is prevented by OA supplementation as well as the expression of NF-κB but not significantly. However, this tendency could also promote the insulin sensitizing effect of OA. Indeed, p38MAPK phosphorylation contributes to the onset of insulin resistance (Shen et al. 2006). Taken together, these data indicate clearly that the supplementation of HFD with OA promotes liver insulin responsiveness through the amelioration of insulin signaling through the inhibition of several negative regulators of insulin signaling such as PTP-1B and the phosphorylation of IRS-1 on serine residues. This leads to increased insulin sensitivity that could explain the diminution of plasma insulin levels. In addition, we also demonstrate that the supplementation of HFD with OA prevents the augmentation of IL-6 pro-inflammatory cytokine and the up-regulation of TLR4. Indeed, TLR4 is known to bind lipopolysaccharide (LPS) and resistin, which both strongly contribute to insulin resistance as previously reported (Benomar et al. 2013). We have recently shown that resistin-induced inflammation is associated with the up-regulation of TLR4 and the impairment of insulin signaling leading to overall insulin resistance. Thus, the role of OA in preventing the HFD-induced up-regulation of liver TLR4 is of great importance because this will reduce HFD-induced inflammation restoring then insulin responsiveness. Indeed, we show here that HFD induces the up-regulation of TLR4 in the liver, and this up-regulation was completely prevented when diet was supplemented with OA or FO. In addition, we show that HFD induced the co-immunoprecipitation of TLR4 and Myd88/TIRAP. Interestingly, this interaction is reduced in the HFOA group but not in the HFFO group. The interaction of TLR4 with TIRAP and Myd88 initiates overall inflammation (Benomar et al. 2013). These findings are reinforced by the effect of OA supplementation on HFD-induced up-regulation of other liver inflammation markers such as IL-1β, IBA1, ICAM1 and CD68. We show that HFD supplementation with OA prevented the expression of IL-1β that is known as a marker of inflammation and insulin resistance (Stanton et al. 2011). Furthermore, we also show that OA supplementation prevented the expression of markers of hepatic macrophages or liver macrophage infiltration such as IBA1, ICAM1 and CD68 (Deininger et al. 2002, Xu et al. 2003, Cai et al. 2005, Koller 2007). All of these markers are up-regulated in HFD rats. Importantly, the supplementation of HFD with FO has a weaker effect as compared to OA regarding inflammation markers. This difference could be attributed to the composition of OA that contains EPA and other components such as anti-oxidants (Moreau et al. 2006, Haimeur et al. 2012) that are absent in FO. In conclusion, the originality of our findings is that O. aurita added as whole microalgae without any processing or purification to HFD is sufficient to overcome HFD-induced liver insulin resistance and inflammation.

Taken together, we show for the first time that the supplementation of HFD with OA prevents HFD-induced insulin resistance by acting through several mechanisms: normalizing plasma insulin and lipids, preventing the down-regulation of the insulin receptor, maintaining insulin receptor tyrosine kinase activity, preventing HFD-induced up-regulation of negative insulin receptor regulators, preventing TLR4 signaling increase and preventing the augmentation of pro-inflammatory factors.

Declaration of interest
We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
H A has performed experiments and participated in writing; Y B participated in performing experiments and discussions; A H measured metabolic parameters; H M participated in discussions; N M participated in performing experiments and discussions; and M T supervised experiments, wrote the manuscript, acted as guarantor.

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