Wax Esters from the Marine Copepod Calanus finmarchicus Reduce Diet-Induced Obesity and Obesity-Related Metabolic Disorders in Mice1–3

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Abstract
We showed previously that dietary supplementation with oil from the marine zooplankton Calanus finmarchicus (Calanus oil) attenuates obesity, inflammation, and glucose intolerance in mice. More than 80% of Calanus oil consists of wax esters, i.e., long-chain fatty alcohols linked to long-chain fatty acids. In the present study, we compared the metabolic effects of Calanus oil-derived wax esters (WE) with those of purified eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) ethyl esters (E/D) in a mouse model of diet-induced obesity. C57BL/6J mice received a high-fat diet (HFD; 45% energy from fat). After 7 wk, the diet was supplemented with either 1% (wt:wt) WE or 0.2% (wt:wt) E/D. The amount of EPA + DHA in the E/D diet was matched to the total amount of n–3 (ω-3) polyunsaturated fatty acids (PUFAs) in the WE diet. A third group was given an unsupplemented HFD throughout the entire 27-wk feeding period. WE reduced body weight gain, abdominal fat, and liver triacylglycerol by 21%, 34%, and 52%, respectively, and significantly improved insulin sensitivity, glucose tolerance, and hepatic steatosis. E/D primarily reduced inflammation (3). Calanus oil, which is extracted from the marine copepod Calanus finmarchicus, contains several components that might have contributed to these effects, among those the n–3 PUFAs EPA and DHA, long-chain MUFAs, antioxidants (astaxanthin), or phytosterols. EPA and DHA are reported to account for the biologic effects shown previously for the crude oil. However, these effects cannot exclusively be ascribed to the content of n–3 PUFAs in the wax ester fraction. J. Nutr. 144: 164–169, 2014.

Introduction
The prevalence of obesity has risen dramatically worldwide during the past 2–3 decades and has now reached epidemic proportions. Obesity, and in particular visceral/abdominal obesity, has been shown to correlate strongly with a number of pathologies, such as type 2 diabetes mellitus, cardiovascular disease, and stroke (1,2).

The increase in obesity is mostly due to sedentary lifestyle and increased energy intake, but changes in lifestyle designed to prevent obesity are difficult to implement at a scale necessary for bringing the epidemic under control. In this situation, nutritional supplements could be an alternative or additional intervention to adjust dietary imbalances and counteract harmful processes caused by obesity-facilitating diets. In a recent study on mice, we found that supplementation of a high-fat diet (HFD) with crude Calanus oil (1.5% wt:wt) resulted in improved glucose tolerance and at the same time reduced abdominal obesity, hepatic steatosis, and adipose tissue inflammation (3). Calanus oil, which is extracted from the marine copepod Calanus finmarchicus, contains several components that might have contributed to these effects, among those the n–3 PUFAs EPA and DHA, long-chain MUFAs, antioxidants (astaxanthin), or phytosterols. EPA and DHA are reported to
reduce obesity and inflammation in rodents (4–6). However, the daily supply of n–3 PUFAs in these studies was up to 35 times higher than the amount used in the study with Calanus oil (3). Of note, the n–3 PUFAs in Calanus oil are bound to aliphatic long-chain monounsaturated alcohols as wax esters, whereas most other studies apply n–3 PUFAs that are in the form of triacylglycerols (TAGs) (4,6) or ethyl esters (5).

The aim of the present study was to investigate whether Calanus oil-derived wax esters (WE) could provide the same beneficial health effects as crude Calanus oil and to compare the cardiometabolic effects of the wax esters with those of purified EPA + DHA ethyl esters.

Material and Methods

WE. The wax ester fraction of Calanus oil was obtained by sequential treatment of the crude oil. Polar substances, including pigments, monoglycerides and diglycerides, sterols, and tocopherols, were filtered off by solid-phase extraction, using silica particles. The portion of the remaining fraction was concentrated by low-temperature crystallization and solids were filtered off. Supplemental Table 1 shows analytical data of the wax esters. Analysis was done by gas chromatography by Unilab Analyse AS.

Animals and dietary treatment. Five- to 6-wk-old (20 g) C57BL/J6 male mice (Charles River) were fed an HFD (catalog no. 58V8; TestDiet, IPS Ltd.), containing 18, 36, and 46% energy from carbohydrate, protein, and fat, respectively, for an initial period of 7 wk. Thereafter, mice were divided into three groups, receiving either HFD alone (HFD), HFD supplemented with 0.2% (wt/wt) purified EPA + DHA ethyl esters (OMACOR; Pronova Biopharma) (E/D) or 1% (wt/wt) WE. The content of EPA and DHA in the E/D-supplement diet was equivalent to the total content of n–3 PUFAs in the WE-supplemented diet. This dietary regimen was continued for another 20 wk (“treatment period”). The HFD group is identical to the one used in a parallel experiment in which we examined the biologic effects of crude Calanus oil (3). The HFD (catalog no. 58V8) was obtained from TestDiet, IPS Ltd., and addition of WE or E/D was compensated for by removing the same amounts of lard. This ensured the same percentages of macronutrients as in the HFD. For composition of the diet, see Supplemental Table 2.

The mice were treated in accordance to the guidelines on accommodation and care of animals formulated by the European Convention for the Protection of Vertebrate Animals for Experimental and Other Scientific purposes. All experiments were approved by the local authority of the National Animal Research Authority in Norway. All mice received food and drinking water ad libitum and were housed at 21°C on a reversed light/dark cycle with 3–4 mice per cage. The protocol lasted for 27 wk with weekly recording of body weight. At the end of the experiment, mice were killed by an overdose of pentobarbital, and organs were carefully dissected out, weighed, and snap frozen, fixed in Zinc-buffered fixative, or immersed in McDowell’s medium or RNA-protecting agents for later analysis.

Glucose tolerance tests and blood samples. An intraperitoneal glucose tolerance test (GTT) with 1.3 g/kg glucose was performed 5–6 wk before the mice were killed, as described previously (3). During the last 2 wk of the feeding period, blood samples were taken from both food deprived (4 h) and fed mice at 1300 h by puncture of the saphenous vein. Plasma insulin and non-esterified FAs were determined. Polar substances, including pigments, monoglycerides and diglycerides, sterols, and tocopherols, were filtered off by solid-phase extraction, using silica particles. The portion of the remaining fraction was concentrated by low-temperature crystallization and solids were filtered off. Supplemental Table 1 shows analytical data of the wax esters. Analysis was done by gas chromatography by Unilab Analyse AS.

Histology and immunohistochemistry. Histologic and immunohistologic sections were prepared as described previously (3). The histology sections were viewed at 10× magnification (Leica DM 2000 microscope; Leica Microsystems). Squares of 0.23 mm² were randomly selected, and adipocyte cross-sectional surface area was measured by manual tracing of each individual adipocyte in that square, using NIH Image J (7).

Results

Diet-induced obese mice. Mice fed the HFD developed a typical obese phenotype with excess body weight and intra-abdominal fat, as well as hepatic steatosis (Fig. 1A–D). They also became glucose intolerant, as evaluated by a standard intraperitoneal GTT (Fig. 2A). Adipocytes in perirenal white adipose tissue (pWAT), as a representative of intra-abdominal fat, were notably enlarged (Fig. 3), and the tissue was extensively infiltrated by macrophages, both with regard to total macrophage content (Fig. 4C) and number of CLSs (Fig. 4A, B). Finally, HFD mice also showed low aerobic fitness (Fig. 2B).

The effect of WE and EPA + DHA ethyl esters on obesity, glucose tolerance, and aerobic capacity. Body weight was similar in all groups before dietary supplementation with WE or E/D. Although there was no difference in energy intake among the groups (55.7 ± 1.8, 56.4 ± 2.74, and 53.0 ± 2.26 kJ/d for HFD, E/D, and WE, respectively), the body weight gain was significantly lower in the group receiving WE-supplemented HFD than in the HFD group (21.3 ± 1.1 vs. 27.0 ± 0.6 g, P < 0.001) (Fig. 1A). By comparison, body weight gain of mice fed E/D-supplemented diet was not different from mice given HFD (25.4 ± 1.0 g). Similarly, although pWAT was significantly reduced in the WE group, there was no difference among the E/D and HFD groups (Fig. 1B). Conversely, epididymal fat mass was not affected by either WE or E/D supplementation (Fig. 1C).
WE-supplemented mice showed significantly lower blood glucose, plasma insulin, and non-esterified FA concentrations (Table 1), as well as significantly improved glucose tolerance (Fig. 2A; Table 1) compared with HFD-fed mice. Dietary supplementation with E/D had no effect on these parameters, except on blood glucose, which was significantly lower than in the HFD group. Finally, compared with HFD-fed mice, only WE was found to reduce hepatic TAG content (Fig. 1D) and improve aerobic capacity, measured as VO₂max during treadmill running (Fig. 2B).

Mice fed wax esters from Calanus oil did not differ in behavior from the other groups. The mice, their organs, and feces appeared normal by visual inspection. The effect of WE and EPA + DHA ethyl esters on macrophage infiltration and cytokine expression in adipose tissue.

Discussion

We reported recently that dietary supplementation with oil from the marine zooplankton C. finmarchicus reduces diet-induced obesity and attenuates obesity-related metabolic disorders in mice (3). The biochemical composition of this oil is notably different from that of other marine oils, containing ~80% wax esters, i.e., aliphatic long-chain fatty alcohols esterified to saturated or unsaturated FAs. In the present study, we show that dietary supplementation with the WE exerts similar effects as the crude oil. When compared with E/D, the former showed a superior effect.

Beneficial health effects of marine oils have traditionally been ascribed to their content of n–3 PUFAs, particularly EPA and DHA, and many studies have shown that n–3 PUFAs can counteract obesity-related metabolic disturbances (5,10–13). The present study on diet-induced obese mice demonstrated that supplementation of the diet with WE reduced body weight and abdominal (perirenal) fat stores. WE supplementation also reduced plasma free FAs and hepatic steatosis and improved glucose homeostasis and aerobic capacity in this model. It is important to emphasize that the amount of n–3 PUFAs in the WE-supplemented diet was markedly lower than that of diets used in previous studies with marine oils in rodents, in which anti-obesity and insulin-sensitizing effects were reported (5,12,13). Of particular note, when the diet was supplemented with an equally low amount of E/D, we did not observe a clear anti-obesity effect, in neither the form of body weight reduction nor reductions of abdominal fat mass or hepatic TAG content. WE was also superior to E/D with regard to improvements in glucose tolerance and stimulation of adiponectin expression in adipose tissue.

Conversely, both E/D and WE reduced the inflammatory state in adipose tissue, as demonstrated by less macrophage infiltration and lower expression of genes encoding inflammatory cytokines. Both supplements also reduced abdominal adipocyte size. Because increased adipocyte size is suggested to trigger the inflammatory response (14), it is reasonable to suggest that the anti-inflammatory effect of these supplements is related to the simultaneous reduction in adipocyte size.

The reduced abdominal fat deposition in WE-fed mice is difficult to explain, although we assume it is related to the FA composition of the WE. n–3 PUFAs are shown to increase FA oxidation and/or decrease lipogenesis in different tissues (12,15–19). Because anti-obesity effects were not observed in the E/D, we suggest that other components, alone or in synergy with n–3 PUFAs, could be responsible for the anti-obesity effect of the WE. We contain considerable amounts of MUFAs (mainly 20:1 n–9 and 22:1 n–11), and it is possible that this class of FAs may...
potentiate the effect of PUFAs because MUFAs are reported to have anti-obesity, anti-inflammatory, and anti-diabetic effects (20–23). In line with this notion, Saury oil, rich in long-chain MUFAs (primarily 22:1 n–11), has been shown to attenuate fat deposition in diabetic KK/Ay mice (24,25). Thus, we believe that MUFAs in combination with n–3 PUFAs contribute to the anti-obesity effects observed in response to dietary supplementation with WE.

MUFAs and PUFAs can also function as PPAR agonists (25, 26), and it has been shown that transgenic overexpression of PPARδ in mice resulted in a switch from type II toward the more aerobic type I skeletal muscle fibers (27). Therefore, one might speculate that the higher VO₂max observed in the WE-supplemented mice could possibly be explained in terms of FA-induced PPAR activation. However, the effect of PPAR activation on physical performance is complex (28), and additional studies are needed to settle this question.

In line with the well-established link between low-grade inflammation in adipose tissue and insulin resistance (29,30), the present study showed that the reduced inflammatory state after WE supplementation was accompanied by reduced circulating glucose and insulin concentrations, as well as improved glucose tolerance. The inflammatory state was also reduced in mice receiving E/D-supplemented diet, whereas plasma glucose, glucose tolerance, and insulin values were only modestly affected. The explanation for this finding is not clear, but the markedly lower expression of the insulin-sensitizing hormone adiponectin in adipose tissue of the E/D group might be one explanatory factor. However, the etiology of insulin resistance is multifactorial (31), and several studies have reported a positive correlation between liver fat and insulin resistance/glucose dysregulation (32–34). In contrast to WE treatment, hepatic steatosis was not reduced in response to E/D, which could be another factor explaining their less pronounced effect on glucose homeostasis.

Although mammals are considered to use wax esters to only a small extent (35), intestinal uptake of WE or of their hydrolytic products are reported in rats, mice, and dogs (36–40). Also, Gorreta et al. (39) reported similar bioavailability of n–3 PUFAs in rodents, regardless of whether the FAs were given in the form of TAG, ethyl ester, or wax ester. However, delayed digestion of wax esters has been proposed, suggesting that hydrolysis and liberation of biologically active components occurs primarily in the distal part of the intestine (41). Interestingly, Morishita et al. (42) showed that secretion of glucagon-like peptide-1 and the decline of plasma glucose after a glucose load were increased when DHA and EPA was delivered locally in the colon but not in the stomach or proximal jejunum. Glucagon-like peptide-1–producing L-cells have been localized in the distal intestine (ileum and colon), in which they are colocated with the n–3 PUFA G-protein–coupled receptor 120 (GPR120) (43). GPR 120 has been shown recently to mediate potent anti-inflammatory and insulin-sensitizing actions (11,44).

Therefore, it is tempting to speculate that the beneficial metabolic effects of WE observed in the present study could (at least in part) be explained in terms of intestinal GPR 120 activation attributable to delayed WE digestion.

Collectively, the data from the present study show that WE are superior to the ethyl esters of purified EPA + DHA. The biochemical differences of the 2 preparations, in terms of either esterification to an ethyl group (E/D) vs. a long-chain fatty alcohol (WE) or their different FA composition could possibly account for the observed differences between these 2 groups.

**TABLE 1** Circulating glucose, FA, and insulin concentrations and glucose tolerance in mice fed HFD, E/D, or WE for 27 wk

<table>
<thead>
<tr>
<th></th>
<th>HFD</th>
<th>E/D</th>
<th>WE</th>
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<tbody>
<tr>
<td>Blood glucose, mmol/L</td>
<td>8.5 ± 0.3a</td>
<td>7.1 ± 0.4b</td>
<td>6.5 ± 0.2b</td>
</tr>
<tr>
<td>Plasma Insulin, pmol/L</td>
<td>651 ± 77a</td>
<td>482 ± 64b</td>
<td>388 ± 77b</td>
</tr>
<tr>
<td>Plasma NEFA, μmol/L</td>
<td>535 ± 21a</td>
<td>459 ± 28b</td>
<td>408 ± 45b</td>
</tr>
<tr>
<td>AUCGTT, min·mmol/L</td>
<td>2420 ± 101a</td>
<td>2180 ± 82b</td>
<td>2070 ± 91b</td>
</tr>
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</table>

1 Values are means ± SEMs. HFD, n = 16; E/D, n = 10; WE, n = 10. Labeled means without a common letter differ, P < 0.05. E/D, EPA + DHA ethyl esters; HFD, high-fat diet; GTT, glucose tolerance test; NEFA, non-esterified FA; WE, Calanus oil–derived wax esters.

2 Glucose and insulin were measured in samples from feed-deprived mice and NEFAs in plasma from fed mice.
Finally, it is worth mentioning that hydrolysis of wax esters yields not only FAs but also fatty alcohols. Fatty alcohols can either be converted into FAs or might exert biologic effects per se (39,45). There seem to be no safety issues related to long-chain fatty alcohol intake in either animals (46) or humans (47). On the contrary, intake of the long-chain fatty alcohol octacosanol [CH₃(CH₂)₂₈CH₃OH] has not only been shown to improve lipid metabolism (48) but also to increase voluntary exercise in rats (49). Therefore, we might speculate that long-chain fatty alcohols could have contributed to the increased aerobic capacity in the WE group. However, in an ongoing study on mice receiving HFD supplemented with 2% (wt:wt) Calanus oil, considerable amounts of fatty alcohols appeared in the feces, whereas they were not detected in the tissues (Alice Pedersen, Norwegian College of Fishery Science, UiT The Arctic University of Norway, Tromsø, Norway, personal communication). Little is known about the absorption of fatty alcohols, but it is hypothesized that fatty alcohols are oxidized, possibly already in the intestinal mucosa, and the rate, it is an open question whether the fatty alcohols per se exert any biologic action in our mouse model.

Gastrointestinal side effects were not observed in mice receiving the WE diet; the feces appeared normal and not different from that of the other groups. It has been reported that wax ester concentrations of 30 g/kg are well tolerated in rodents (51). By comparison, the mice used in the present study received, at most, 1.4 g wax ester/kg. Furthermore, the equivalent human dose was calculated to be 0.11 g/kg (52), which is more than 10 times below doses that have been reported to cause gastrointestinal complications in humans (35,53).

In summary, this study demonstrates that dietary supplementation with WE has a marked anti-obesity effect, attenuates obesity-related inflammation, and improves glucose tolerance as well as aerobic capacity. Because these effects are similar to those after dietary supplementation with crude Calanus oil, it indicates that the active components of this oil are confined to its main lipid constituent (wax esters).

Acknowledgments

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Literature Cited


