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Original research article

Free form astaxanthin from yeast *Phaffia rhodozyma* fermentation reduces plasmatic triglycerides in a pre-obesity diet-induced dyslipidaemia mouse model

Myriam Mimoun-Benarroch[1], Justine Lallement[1], Larbi Rhazi[2], Camille Boroch[2], Cindy Hugot[1], Claude-Narcisse Niamba[1], Hassan Younes[1], Flore Depeint[1,2,*]

[1] *Département des sciences de la nutrition et santé, Institut Polytechnique UniLaSalle, 19 rue Pierre Waguet, 60000 Beauvais, France*
[2] *UR Transformation & Agro-ressources, Institut Polytechnique UniLaSalle, 19 rue Pierre Waguet, 60000 Beauvais, France*

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ABSTRACT

Previous work have indicated a lipid-lowering effect of esterified astaxanthin (AX) from algae *Haematococcus pluvialis*. The aim of this study was to set up a murine model of pre-obesity diet-induced dyslipidaemia to investigate the lipid-lowering effect of free-form AX from yeast *Phaffia rhodozyma* fermentation. After 6 weeks on high-fat diet, 32 male C57BL/6 mice were divided into 4 groups fed (1) a standard or (2–4) a high-fat diet (HFD) supplemented or not with AX at 0.03% or 0.06% for 8 weeks. Animals were anesthetized and sacrificed and biological samples (plasma and internal organs) collected and stored at −80 °C. The plasma concentrations of triglycerides and cholesterol were determined and gene expression measured for markers of energy metabolism in the liver and adipose tissue. Statistical analyses were performed using SPSS software and the significance set at $p < 0.05$. Primary results showed a lowering effect on the triglyceride levels (−35 to −45%, $p < 0.05$) in the groups fed high-fat-diet supplemented with AX, but no effect on cholesterol. Genetic expression showed a modulation of various mechanisms of energy regulation, from lipid transporters to beta-oxidation pathways and insulin resistance. The associated effects could also have implications for the prevention of metabolic syndrome.

1. Introduction

With the onset of metabolic disorders in the Western World, all their causative factors are potential targets for health promoting activity (O'Neill and O'Driscoll, 2015). Of these, dyslipidaemia can affect both fasting and post-prandial triglycerides and cholesterol levels. A recent national survey indicated that over 45% of the French population is overweight or obese (Obepi-Roche, 2012) and over 15% of the population is treated for dyslipidaemia. The same report suggested that overweight adults had twice increased risk of developing dyslipidaemia than normal weight comparators. While lipid lowering drugs and bioactive molecules have long been used, the carotenoid antioxidant astaxanthin has met more and more interest in the last decade (Fassett and Coombes, 2012). A few human clinical trials have been performed on this topic and Yoshida et al. have shown that esterified astaxanthin (AX) was able to reduce fasting triglyceride levels after 12 weeks supplementation (Yoshida et al., 2010). While most clinical and preclinical investigations have been focused on esterified AX from the algae *Haematococcus pluvialis*, our objective is to investigate whether free-form astaxanthin extract from fermentation of the yeast *Phaffia rhodozyma* could exert similar effects in dyslipidaemic mice.

2. Methods

2.1. Animals and diets

All standard and custom-made diets were produced by SAFE (Augy, France). Animal protocols were performed in LaSalle housing facility (C60-200-001) and have received prior approval from local ethics committee for animal care (CEEA-116). Male C57BL/6 mice (Janvier Laboratories, France) were housed, 4 per cage according to good laboratory practice.

2.2. Experimental protocol

Thirty-two 8-weeks old mice were fed either a standard diet (A04 offering 3.5 kcal/g and consisting of 60% carbohydrate, mostly from starch, and 3% fat; n = 8) or a high-fat diet (HFD offering 4.7 kcal/g

* Corresponding author.
E-mail address: flore.depeint@unilasalle.fr (F. Depeint).

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consisting of 27.5% (53% kcal) fat, of which 25% saturated fat from lard, and 37.5% (32% kcal) carbohydrate, with 24% from sucrose; n = 24). After 6 weeks, HFD-fed mice were further divided to continue to receive HFD diet or HFD diet supplemented with 0.03 or 0.06% AX extracted from *Phaffia rhodozyma* (Ajinomoto Foods Europe, Mesnil-St-Nicaise, France) for 8 more weeks until sacrifice (n = 8 per group).

Fasted animals (12 h) were deeply anesthetized by intraperitoneal injection (Ketamine/xylazine at 87 and 13 mg/kg, respectively) and blood was collected by cardiac puncture before cervical dislocation. Plasma was separated by centrifugation (2000 rpm, 10 min) and stored (−80 °C) until further use. Internal organs (liver, adipose tissue, caecum, small intestine, colon, spleen and kidney) were collected and weighted before storage at −80 °C.

2.3. Lipid measurements

Fasting concentrations of total cholesterol and triglycerides in the plasma were quantified using colorimetric kits (K623-100 and K622-100, respectively, BioVision, France) and strictly following supplier instructions.

2.4. Genetic expression of target molecules

RNA was extracted from 20 mg of tissue (adipose tissue, liver, small intestine) using Rneasy plus mini kit (Qiagen, Courtabeuf, France) following suppliers instructions. Recombinant cDNA was obtained from 100 ng of RNA using the Quantitect reverse transcriptase kit (Qiagen, France) following supplier's instructions. Finally 5 µL of 10-fold diluted cDNA was amplified against designed primer sets (PrimerDesign, UK) against target genes listed in Table 1 using QuantiFast® SYBR® Green PCR kit (Qiagen, Courtabeuf, France). Following activation of the HotStart Taq DNA polymerase (95 °C, 5 min), 40 cycles of amplification (denaturation at 95 °C, 10 s followed by annealing/extension at 60 °C, 30 s) were performed with fluorescence detection at the end of each extension step. This was followed by a melting curve analysis (from 60 °C to 95 °C) for quality control of the amplicons. Relative expression was calculated against standard control diet and both GAPDH and ATP5 B as reference genes (qBase+, Biogazelle). The number and identity of reference genes were based on Genorm estimation of the best housekeeping genes (Vandesompele et al., 2002) using qBase+ software (Biogazelle) and using primers from geNorm 12 gene kit (PrimerDesign, UK).

2.5. Statistical analyses

All data sets were expressed either as mean and standard error of the means (SEM) or Median and confidence interval (CI95). Statistical comparisons between groups were performed using non parametric tests (SPSS v22, IBM) such as Kruskal-Wallis followed by pair comparisons between groups and significant value was set for $p < 0.05$.

Table 2
Phenotypic data.

	A04/A04	HFD/HFD	HFD/HFD-AX3	HFD/HFD-AX6
weight gain (g)	6.9(0.84)	9.7(4.52)	8.4(3.55)	10.2(3.33)
Food intake (g/animal/day)	4.34(0.621)	3.36(0.375)	3.10(0.232)	3.29(0.496)
Tissue weight (g)				
– Adipose tissue	0.27(0.078)	1.33(0.695)	1.22(0.875)	1.40 (0.564)
– Liver	1.17(0.073)	1.18(0.242)	1.16(0.104)	1.14 (0.128)
– Spleen	0.08(0.017)	0.11(0.033)	0.10(0.020)	0.11(0.030)
– Caecum	0.29(0.078)	0.19(0.038)	0.18(0.040)	0.18(0.045)
– Small intestine	0.71(0.089)	0.72 (0.084)	0.69 (0.089)	0.70(0.138)
– Colon	0.16(0.018)	0.12(0.008)	0.15(0.013)	0.13(0.013)

Animals (C57BL/6 mice, n = 8) were fed a range of treatments for 8 weeks. The phenotypic markers listed in the table are measured at the end of the study and expressed as mean and standard deviation. A04 (standard maintenance diet), HFD (high-fat diet), AX3 or AX6 (astaxanthin supplementation at 0.03 or 0.06% in high-fat diet).

3. Results and discussion

3.1. Lipid lowering effect

Animals fed the high-fat diet were significantly heavier than the standard diet controls ($p = 0.014$) and no differences were measured following AX treatment. Weight gain, food consumption and tissue weights are summarised in Table 2.The increase in body weight was mostly due to an increase in adipose tissue in HFD animals ($p = 0.001$) but again AX supplementation had no effect. When measuring fasting lipid concentrations in the plasma, however, both AX-supplemented diets significantly decreased triglyceride levels compared to HFD ($p = 0.004$) while cholesterol levels were not affected (Fig. 1).

C57BL/6 background is the most commonly used for genetic or diet-induced metabolic and obesity studies, however we did not aim for a development stage of the pathology and remained in non asymptomatic period, as validated by very small weight gain variation against standard diet. A number of transgenic models have been tested using AX from algae. Triglyceride levels in plasma are reduced by AX (0.03%) in ApoE-/- mice fed high-fat diet (HFD at 15% lipid content) for 4 weeks (Yang et al., 2011). While triglycerides are also reduced in ddY obese mice fed HFD at 40% lipid content and AX (6 mg/kg body weight (b.w)) for 9 weeks (Ikeuchi et al., 2007), no modulation is observed in db/db diabetic mice fed a standard diet with AX (up to 50 mg/kg b.w) for 8 weeks (Dong et al., 2013), suggesting that the AX effect may be dependent upon the co-treatment with high-fat diet.

Krill oil, which contains both n-3 fatty acids and AX shows a reducing effect on plasma cholesterol but not on triglycerides after 8 weeks on high-fat high-cholesterol diet (HCD) at 21% lipid and 1.25% cholesterol content, respectively in C57BL/6 mice (Tandy et al., 2009). While cholesterol lowering effect may be due to n-3 fatty acids, this suggests that a critical concentration of AX (0.03% in the diet or 6 mg/kg b.w if we recall the former authors) may be necessary to lower triglycerides, which is not reached in that study (0.01%).

The only study investigating AX from *Phaffia rhodozyma* is performed on CD-1 mice fed HFD at 10% lipid content supplemented with

Table 1
Listing of target genes.

	Metabolism	Transport	Storage	Toxicity
Liver	Gck, Acaca, Srebf1, Mlxipl, Crot, Pparγ, Cpt1a, Ucp2, Slc2a2, Acat1, Acat2, Pparα, RXRA	Fabp1, LDLR, Lrp8	IRS4	TNF, Gpt, Got1
Adipose tissue	LCAT, Pparα, RXRA, UCP1	Fabp4, Abca1, Glut4, LDLR	Adipoq, Adipor2	
Small intestine		Fabp2, Lpl, ApoB, ApoC3, SCARB1		

Following a 6-week pre-treatment, animals (C57BL/6 mice, n = 8) were fed a range of treatments for 8 weeks. Relative genetic expression of selected genes were measured by RT-qPCR after 8 weeks in selected tissues.

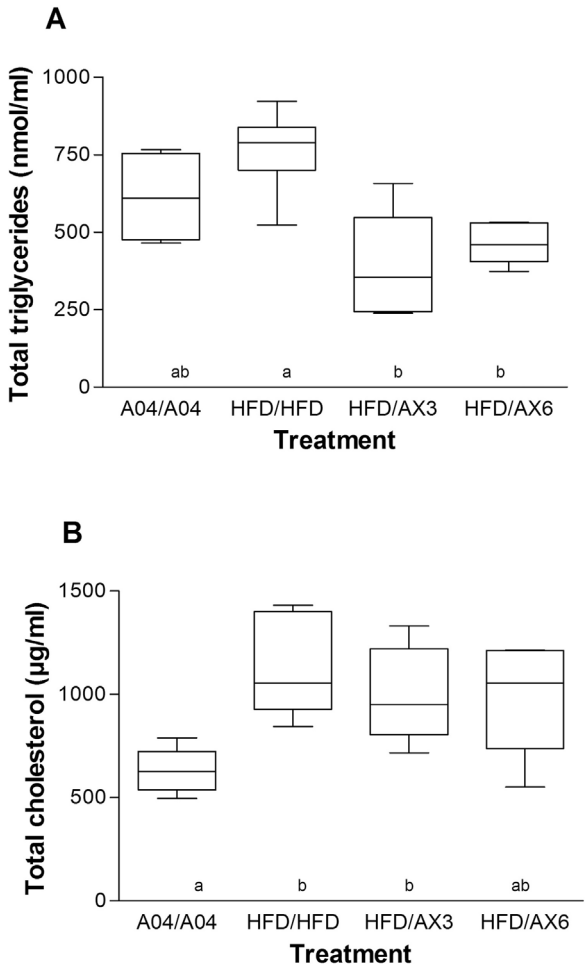

Fig. 1. Effects of astaxanthin on plasma lipids in mice fed high-fat diet for 8 weeks. Following a 6-week pre-treatment, animals (C57BL/6 mice, n = 8) were fed a range of treatments for 8 weeks. Fasting plasma concentrations of triglycerides (A) and cholesterol (B) were measured after 8 weeks. Data are expressed as Median and CI (95%). Different letters at the bottom of the boxes indicate statistically significant variations between groups ($p < 0.05$).

0.03% AX for 70 weeks (Kim et al., 2009). Fasting plasma triglyceride levels are once again significantly reduced by the AX treatment.

Only one other study used a pre-treatment (Ryu et al., 2012). The authors use high-cholesterol diet (HCD0.5%) and showed that AX (0.08%) reduce triglyceride levels after 4–9 weeks treatment in ApoE-/- mice. All those published data are consistent with our conclusion that sufficient AX supplementation in dyslipidaemic mice is able to reduce fasting plasma triglyceride levels within 8 weeks, irrespective of the source of astaxanthin.

3.2. Mechanisms of action

As listed in Table 1, 35 genes were investigated covering a range of pathways including lipid transport molecules, metabolic pathways, lipid storage molecules and hepatotoxicity. Although not necessarily significantly, AX at the dietary concentration of 0.03% increased expression of *Abca1* and *Adipor2* genes in the adipose tissue compared to HFD control mice. In addition, at the higher dose of 0.06% in the diet, AX reduced *Pparg* while increasing *Srebf1*, *Acat2* and *Lrp1* in the liver and at the same time increased *Glut4*, *Abca1* and *Rxra* in the adipose tissue. No significant modulation of the genetic expression was observed in small intestine samples (data not shown).

AX increased expression of ATP-Binding Cassette Transporter 1 (*Abca1*) gene in a dose-dependent manner ($p = 0.02$) in the adipose tissue of animals fed HFD diet, partially restoring control activity of the gene (Fig. 2A). *Abca1* is a gene coding for a cholesterol transport molecule, carrying free cholesterol from adipose tissue to high density lipoproteins (HDL). This is in agreement with the literature, where AX is shown to activate *Abca1* promoters in an LXR-independent manner (Iizuka et al., 2012). AX also increased Glucose Transporter type 4 (*Glut4*) expression in adipose tissue with final expression levels 2-fold from HFD control group and 4-fold from the standard diet control group (Fig. 2B). *Glut4* is coding for a glucose transporter, carrying glucose inside the cells in an insulin-dependent manner. In a similar way, AX is shown to enhance insulin-stimulated GLUT4 translocation to the plasma membrane (Ishiki et al., 2013). Still in the adipose tissue, Retinoid X Receptor Alpha (*Rxra*) RNA levels were increased in a dose-dependent manner in AX-treated animals compared to HDF controls, leading to levels close to standard diet controls (Fig. 2C). *Rxra* is coding for a nuclear receptor involved in the regulation of lipid metabolism. In this case, the nuclear receptor RXR-α forms an heterodimer with Peroxisome Proliferator-Activated Receptor α (PPAR-α). In a cell model, AX is a moderate agonist for PPAR-α (Jia et al., 2012). Although protein interaction was not measured in our study, the overall effect would be similar, increasing either the production of one of the nuclear receptors (RXR-α) or activating the other (PPAR-α) thus enhancing the activity of the heterodimer in regulating genetic expression of proteins involved in lipid metabolism.

Hepatic gene expression is also affected by AX. *Pparg* is inhibited in a dose-dependent manner up to 4-fold compared to both the HFD and standard diet controls ($p = 0.002$) (Fig. 2D). *Pparg* is coding for a nuclear receptor PPAR-γ acting as a transcription factor involved in a number of physiological functions including lipid metabolism or inflammation. *In vitro* cellular data show that AX is a moderate antagonist for PPAR-ɣ (Jia et al., 2012). The same authors have also shown an inhibition of PPAR-γ hepatic expression in steatic C57BL/6 mice fed AX at 6 to 30 mg/kg b.w (Jia et al., 2016). Although our data are not so clear, they agree with the analyses presented in that paper. The lower modulation in our experiment may be due to differences in the delivery of AX, gavage may be more suitable to induce a clear reproducible response or in the nature of the AX being esterified algae extract in that paper. Sterol Regulatory Element Binding Transcription Factor 1 or Sterol regulatory element-binding protein 1 (*Srebf1*) gene was increased in a dose-dependent manner by AX treatment ($p = 0.002$) leading to recovery of expression close to that of standard diet controls (Fig. 2E). *Srebf1* is coding for a transcription factor controlled by sterol concentrations in the cell and its environment. Its target genes are involved in biosynthesis and absorption of lipids. Acetyl-CoA AcylTransférase 2 (*Acat2*) genetic expression was similarly partially recovered following AX treatment ($p < 0.001$). *Acat2* gene is coding for an enzyme catalysing the esterification of cholesterol in the liver. Esterified cholesterol is then transported via the Apolipoprotein B (ApoB) to very low density lipoprotein cholesterol carriers (VLDL). Low density lipoprotein receptor-related protein 1 (*Lrp1*), finally was partially recovered but non significantly (Fig. 2F) in the liver. *Lrp1* is coding for and apolipoprotein (ApoE) located on the surface of chylomicrons and intermediate density lipoproteins (IDL).

In addition, AX at both concentrations reduced the concentrations of C18:1 trans fatty acids in the adipose tissue compared to the HFD control group ($p = 0.001$). Trans-fatty acids may be associated with increased risks of cardiovascular disorders through increased LDL and decreased HDL cholesterol. Although we did not see any modulation of cholesterol levels, this may still be a positive impact for the animals.

4. Conclusion

Although the sum of the information and pathways that seem to be impacted by AX treatment remains partially hypothetical, it gives us preliminary information on the possible pathways that are affected leading to lipid lowering properties of the carotenoid. Further

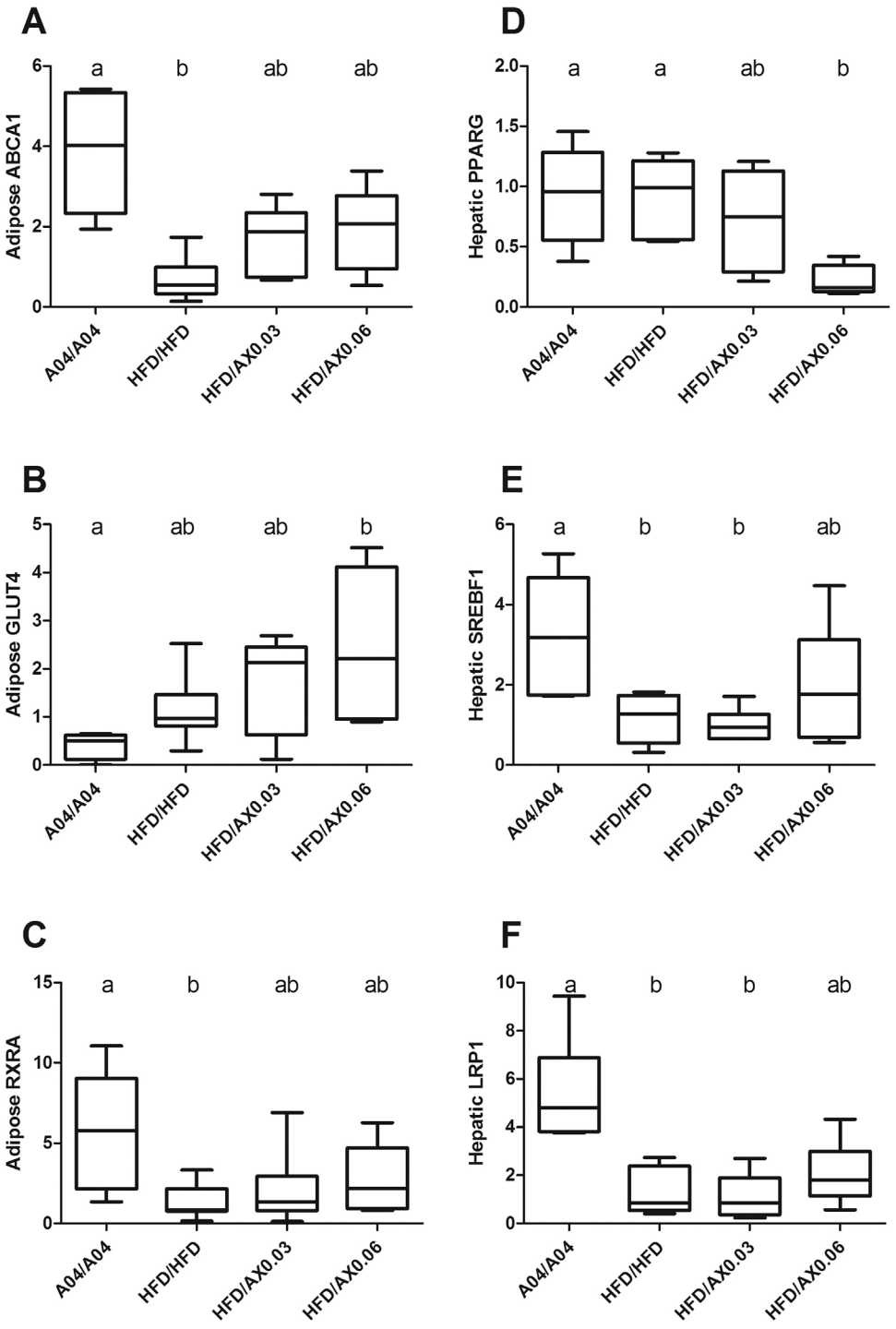

Fig. 2. Effects of astaxanthin on genetic expression in mice fed high-fat diet for 8 weeks.
Following a 6-week pre-treatment, animals (C57BL/6 mice, n = 8) were fed a range of treatments for 8 weeks. Relative genetic expression of selected genes were measured by RT-qPCR after 8 weeks in adipose tissue (A–C) and liver (D–F). Data are expressed as Median and CI (95%). Different letters above the boxes indicate statistically significant variations between groups ($p < 0.05$).

investigations are necessary in order to validate some of the mechanisms proposed here.

Conflict of interest

The authors declare that they have no conflict of interest.

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