Morphology of ferret subcutaneous adipose tissue after 6-month daily supplementation with oral beta-carotene

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Abstract

Adipose tissue is an important retinoid depot and retinoids are known to influence white and brown adipocyte metabolism. Identifying nutrients that can affect the biological activity of the adipose organ would be of great medical interest in the light of the current obesity epidemic and related disorders in developed countries. The vast majority of mammal studies of chronic administration of oral beta-carotene have used murine models, while few have employed mammals exhibiting uptake and processing of intestinal beta-carotene similar to those of humans. While rodents transform practically all ingested beta-carotene into retinol, in ferrets, as in humans, part of the beta-carotene is absorbed and released into the circulation intact. We studied the effects of 6-month daily administration of two doses of oral beta-carotene (0.8 or 3.2 mg/kg/day) on ferret body weight, size of body fat depots, and, using morphological and morphometric methods, on subcutaneous (inguinal) white adipose tissue (WAT). Because of the oral mode of administration, liver, stomach, and small and large intestine were also studied. Control animals received the vehicle. Data show that at the end of treatment the higher dose induced significantly higher body weight compared with controls and significantly higher inguinal fat depot compared with animals treated with the lower dose. In addition, chronic treatment with beta-carotene induced a dose-dependent hypertrophy of white adipocytes and increased neoangiogenesis in subcutaneous WAT in all treated ferrets. Vasculogenesis was independent of adipocyte hypertrophy. We also found focally evident liver steatosis in the ferrets treated with the higher dose of beta-carotene. The other gastrointestinal tract organs studied were not significantly different from those of control animals.

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Keywords: beta-carotene chronic administration; WAT morphology; Ferret

1. Introduction

Obesity—a clinical condition that has been assuming epidemic proportions in the last years—is associated with several metabolic and cardiovascular disorders in the vast majority of patients [1,2]. The rising incidence of obesity in developed countries is not conceivable as a consequence of a sudden genomic change, underscoring the importance of the environmental component. The precise environmental factors involved in the rapid expansion of obesity are not known, but they are associated with food availability and composition and with the ever decreasing physical activity due to the new lifestyles and social requirements of affluent societies. The extraordinary progress made in the last few years in the molecular understanding of mechanisms regulating body weight is paving the way for new methods, especially pharmacological but also nutritional, of treating obesity [3].

Obesity is caused by a large increase in white fat due to energy unbalance. All factors able to influence the energy
and in vivo studies with murine models suggest that beta-carotenoids may be metabolized to retinol and retinoic acid (RA), which enter the circulation through the portal system bound to serum albumin. Intestinal beta-carotene absorption as well as diet carotenoid conversion to retinoids is strictly species-specific: humans and ferrets, but not rodents, absorb significant amounts of uncleaved carotenoids and accumulate them in peripheral tissues, notably adipose tissues, where carotenoids may be metabolized to retinol and RA [7,16,17]. The ferret thus provides an excellent model to mimic human metabolism to gain further insights.

We studied the effects of 6-month daily oral supplementation of ferrets with two doses of beta-carotene on body weight, size of body fat depots and WAT morphology. Because of the oral mode of administration, the morphology of the organs of the gastrointestinal tract was also studied.

2. Materials and methods

2.1. Animals and beta-carotene supplementation

Animals were 7-week-old female ferrets (Exopet AB, Glommen, Sweden). They were housed at 22 °C with a 12-h light/dark cycle (lights on at 08:00) and free access to food and water. The gross composition of the chow diet (Friskies, Spain) was 32% protein, 10% fat, 3% fibre, 9.5% moisture and 7.5% ash residue of total mass. Vitamin A content was 8500 IU/kg. After 1 week of adaptation, ferrets were randomized to three experimental groups of six animals to receive: (i) 200 µl of vehicle/day: control (C); (ii) 0.8 mg beta-carotene/kg body weight/day (BC 0.8); or (iii) 3.2 mg beta-carotene/kg body weight/day (BC 3.2) for 6 months. Beta-carotene was provided by DSM Nutritional Products Ltd. (Basel, Switzerland) as a water-soluble formulation (beadlets) containing beta-carotene crystalline, DL-α-tocopherol, ascorbyl palmitate, corn oil, fish gelatine, sucrose and corn starch. This formulation was given to the two BC groups dissolved in 200 µl of water according to the dose of beta-carotene. The same formulation was provided by the manufacturer also without beta-carotene and was administered to the control group. Body weight was recorded weekly. Food intake was measured several times a month. Animal care and maintenance (at the University of the Balearic Islands) were in line with the institution’s guidelines for the care and use of laboratory animals.

2.2. Light microscopy

After 6 months, ferrets were sacrificed under anaesthesia using 10 mg/kg ketamine hydrochloride (Imalgène 1000, Merial Laboratorios SA, Lyon, France) and 80 µg/kg Medetomidine (Domtor, Orion Pharma, Espoo, Finland). Tissue specimens for morphological and morphometric analysis were fixed in 10% neutral buffered formalin and processed for routine histology. Sections of 5 µm were stained with hematoxylin/eosin and sections for immunohistochemistry were stained with retinoic acid receptor (RAR) and retinoid X receptor (RXR) antibodies as described previously [18].

Table 1

<table>
<thead>
<tr>
<th>Animal</th>
<th>Inguinal</th>
<th>Retropertioneal</th>
<th>Mesenteric</th>
<th>Gonadal</th>
<th>Parametral</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>42.0±4.3</td>
<td>19.2±2.5</td>
<td>46.1±5.3</td>
<td>2.41±0.40</td>
<td>3.40±0.39</td>
</tr>
<tr>
<td>BC 0.8</td>
<td>40.9±2.8</td>
<td>18.5±1.3</td>
<td>36.2±3.4</td>
<td>2.25±0.33</td>
<td>2.93±0.35</td>
</tr>
<tr>
<td>BC 3.2</td>
<td>48.8±1.6 #</td>
<td>23.5±2.3</td>
<td>45.3±4.6</td>
<td>2.80±0.12</td>
<td>3.14±0.37</td>
</tr>
</tbody>
</table>

See Table 1 for details on animal groups. Results are expressed in grams as means±SEM (n=6). #, p<0.05 BC 3.2 vs. BC 0.8, student’s t-test.
analysis were collected from subcutaneous (inguinal) WAT, liver, stomach, duodenum and colon.

2.2.1. Subcutaneous inguinal WAT
The tissue was divided into unilocular adipocyte areas: the right subcutaneous inguinal adipose tissue of each ferret was dissected whole and divided transversally into six to seven fragments. Representative fragments were fixed, reduced to smaller fragments and eight to ten of them were paraffin-embedded. One H&E section per fragment was studied for histology and one for area morphometry.

2.2.2. Liver
One random section was examined for each animal. All sections came from the same area of the left lobe.

2.2.3. Stomach, duodenum and colon
Each organ was examined in four different sections in order to perform a complete examination of the different areas of the organs.

Light microscopic observations and semi-quantitative assessment were performed independently by two experienced pathologists that agreed on the final form of the presentation of the results.

2.3. Morphometric analysis
For the morphometric analysis of inguinal subcutaneous adipose tissue, 100 unilocular cells/section, 800 cells/animal, and 4500 cells/experimental group were profiled at 20× and processed using an image analysis software (Lucia, v.4.6). Morphometric analysis was performed by digital acquisition of adipose tissue areas (Digital Still Camera DCM 1200 and Nikon 6000 Eclipse Microscope).

2.4. Vasculogenesis
To verify whether beta-carotene supplementation causes an increase in capillary number compared with control animals, capillary density (number of capillaries/area), endothelial cell nuclei density and total capillary area were measured at 100× on 2-μm-thick resin-embedded sections (10 areas from three different sections) of inguinal subcutaneous adipose tissue from each animal.

2.5. Statistical analysis
Data are expressed as mean±S.E. Student’s t-test was used to assess differences in body weight and size of WAT depots. Significance was set at P<0.05. One-way analysis of variance was used to analyze areas and the Kruskal–Wallis test to evaluate the results of vasculogenesis (GraphPad In Stat software, v.3.6, San Diego, CA, USA).

3. Results

3.1. Body weight and fat depots
At the end of the treatment, body weight was 14% higher (P<0.05) in animals receiving the higher dose of beta-carotene than in control ferrets (Fig. 1). The differences

![Fig. 2: Area of unilocular adipocytes in subcutaneous adipose tissue of ferrets treated during 6 months with beta-carotene (morphometric analysis). See Fig. 1 for details on animal groups. The results are expressed as means±S.E.](image)
induced by the lower dose of beta-carotene were not significant. Food intake was not affected in any experimental group.

At 6 months the ferrets on the higher dose also exhibited significantly (P<0.05) larger subcutaneous inguinal depots (+19%) than those receiving the lower BC supplementation and slightly, though not significantly, larger depots (+16%) than controls. Gonadal and retroperitoneal WAT were also slightly larger in these animals than in the control group, while the size of parametrial and mesenteric depots was ostensibly unaffected (Table 1).

3.2. Adipose tissue

Inguinal subcutaneous adipose tissue studied in eight different areas per animal (see Materials and methods for details) was composed of unilocular adipocytes. A morphometric study conducted on 800 adipocytes/animal showed significant hypertrophic adipocytes in the treated animals compared with controls. This hypertrophy was dose-dependent (Fig. 2).

The capillary vascular bed seemed to be increased in WAT of treated animals (Fig. 3). The morphometric study confirmed the vasculogenesis and showed it to be dose-dependent (Fig. 4).

3.3. Liver

Adult ferrets have a peculiar tendency to hepatic steatosis [18] and, indeed, the liver of control animals showed a diffuse vacuolization of hepatocytes. Liver steatosis was more evident in the central part of the lobules (near the centrolobular vein). Furthermore, the portal areas often showed a modest infiltrate of mononuclear cells. In some treated animals we observed a slightly increased portal infiltrate (see Table 2A for semi-quantitative analysis).

The portal infiltrate was not observed in 2/6 of the animals treated with the lower dosage of beta-carotene and in 1/6 of those treated with the higher dosage.

Hepatosteatosis was focally marked only in the livers of 3/6 ferrets treated with higher dose of beta-carotene (Table 2B and Fig. 5).

3.4. Stomach, duodenum and colon

Careful examination of serial sections of these organs did not evidence significant difference between treated and control animals.

In the pyloric part of the stomach, a mononuclear mucosal and submucosal infiltrate was evident both in

<table>
<thead>
<tr>
<th>Animal</th>
<th>Control</th>
<th>BC 0.8</th>
<th>BC 3.2</th>
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<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
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<tr>
<td>4</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>++++</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

See Fig. 1 for details on animal groups. (+) Diffuse lipid content; (+++) bigger abundant lipid droplets in hepatocytes in the acinar zone; (++++) very abundant lipid content.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Control</th>
<th>BC 0.8</th>
<th>BC 3.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

See Fig. 1 for details on animal groups. (+) Diffuse lipid content; (+++) bigger abundant lipid droplets in hepatocytes in the acinar zone; (++++) very abundant lipid content.
controls and in treated ferrets. Observation at higher
magnification to exclude possible infection with Helico-
bacter mustelae did not evidence any bacteria-like structure.

4. Discussion

Our study shows that a 6-month oral treatment with beta-
carotene increases body weight and size of the subcutaneous
fat depot in ferrets, and also suggests that beta-carotene
induces vasculogenesis in adipose tissues.

This is the first study where the effects of oral intake of
beta-carotene on adipose tissue morphology have been
assessed by using an animal model that mimics human beta-
carotene uptake. In general, rodents and other laboratory
animals break down beta-carotene in their intestine and thus
absorb almost none intact. Hence, rodents have low serum
carotenoid levels (about 1/1000 of human levels) that are
not related to dietary intake due to very active dioxygenase
cleavage to retinal. In man, roughly 20–75% of the beta-
carotene is absorbed intact [19].

Ferrets have been proposed as useful models to study
human beta-carotene absorption and cleavage as these
animals also absorb and release intact beta-carotene from
the enterocyte [19–22].

Although serum beta-carotene levels are normally very
low in these animals, dietary supplementation has been
shown to increase concentrations to levels similar to those
detected in human serum, and also to increase levels in the
liver, adipose and other tissues [20–25]. The results
concerning body weight and adiposity were in some way
unexpected considering the condition of beta-carotene as a
vitamin A precursor and previous in vitro results in our
laboratory showing that both beta-carotene and other
naturally occurring vitamin-A precursor carotenoids
increase the thermogenic capacity of brown adipocytes
[26]. RA is a natural vitamin A derivative known to have
profound effects on the growth and differentiation of many
mammalian cells [27]. Several studies performed in other
animal models (mice and rats) have evidenced an effect of
RA in decreasing body weight. In particular, treatment of
mice for 4 days with all-trans tRA given orally [12] or
subcutaneously injected [14] triggered a significant decrease
in body weight. Similarly, animals fed chronically with a
vitamin A-deficient diet tended to develop an excess body
weight [14]. Also in rats, chronic vitamin A supplementation
has been reported to cause a slight decrease of body
adiposity [28]. Of note, these mentioned studies have been
performed with RA and not with the precursor, beta-
carotene, and they have not been performed either in ferrets.
In a comparable study by Liu et al. [29] made also in ferrets,
treated during 6 months with lower doses of beta-carotene
(purchased from Sigma, St. Louis, MO, USA), no signifi-
cant effects on body weight were described.

Of interest, our present results are different from those
obtained in a previous pilot study (unpublished results)
where we treated ferrets with the same doses of beta-
carotene as in this study during 3 months. However, in this
pilot study, beta-carotene was purchased from another
company (all-trans-beta-carotene, type II; Sigma cat. no.
C4582), and dissolved, according to the doses decided for
each experimental group, into 1 ml of olive oil. This study
was performed with a restricted number of ferrets (five
animals: two, two and one for control, BC 0.8 and BC 3.2,
respectively). Considering the limited number of animals, in
this pilot study we did not find apparent changes in body
weight or size of fat depots, and we did not find adipocyte
hypertrophy either. In addition, microscopy analysis of
adipose tissue showed the appearance of a significant

<table>
<thead>
<tr>
<th>Section number</th>
<th>Unilocular cell number</th>
<th>Mean area (µm²)</th>
<th>Multilocular cell number</th>
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</thead>
<tbody>
<tr>
<td>C1</td>
<td>10</td>
<td>1855</td>
<td>3886.45</td>
</tr>
<tr>
<td>C2</td>
<td>10</td>
<td>2155</td>
<td>3295.06</td>
</tr>
<tr>
<td>BC 0.8–1</td>
<td>10</td>
<td>2131</td>
<td>3279.17</td>
</tr>
<tr>
<td>BC 0.8–2</td>
<td>10</td>
<td>2313</td>
<td>3239.98</td>
</tr>
<tr>
<td>BC 3.2</td>
<td>10</td>
<td>1789</td>
<td>3981.25</td>
</tr>
</tbody>
</table>

The number of multilocular adipocytes is increased in treated animals compared to controls.
number of multilocular adipocytes (dose-dependent) among unilocular adipocytes with a size comparable to that of controls (Table 3). Furthermore, electron microscopy showed the typical features of brown adipocytes in the multilocular cells (mainly numerous big mitochondria rich in lamellar cristae). Mitochondrial population was not homogeneous (as usually seen in brown adipocytes from BAT) [30,31] and several mitochondria with transitional aspects between those found in white adipocytes and those in brown were observed. Of note, often in close association to these brown-appearing adipocytes, numerous unilocular adipocytes with thick (instead that the normal thin) cytoplasmic rim rich in mitochondria with transitional aspects between white and brown as above described were found (Fig. 6). These aspects were absent in controls and reminded the white into brown adipocyte transdifferentiation aspects described previously by our and other groups with different animal models [30,32]. Here, by using the formulation of beta-carotene from DSM, we have not seen any signal of transdifferentiation.

Vasculogenesis was also found in the previous pilot study (Fig. 7), although without adipocyte hypertrophy, suggesting that vasculogenesis is induced by beta-carotene regardless of its effects on the size of adipose tissue and on lipid accumulation. The adipocyte hypertrophy could also contribute to the observed vasculogenesis due to the fact that bigger adipocytes secrete more known vasculogenic adipokines such as leptin itself [33,34]; however, circulating leptin levels have not been measured in this study.

The chronic oral administration of drugs could induce adverse effects on gastrointestinal tract. We were not able to observe any significant difference between control and treated animals. Only high doses of beta-carotene induced evident, but focal, hepatosteatosis in 3/6 ferrets; on the other hand, the portal infiltrate present in all controls seemed to improve in 2/6 and in 1/6 of the treated animals treated with low or high dosages, respectively.

Fig. 6. Electron microscopy of a unilocular adipocyte in subcutaneous adipose tissue of the animal of the BC 3.2 group, treated during 3 months, showing transdifferentiation phenomena (see discussion section and Table 3 for details). (A) The thick peripheral rim of cytoplasm contains numerous mitochondria. (B) Enlargement of the framed area in A showing mitochondria similar to those present in the classic brown adipocytes. A: bar=1.4 μm; B: bar=0.7 μm.

Fig. 7. Morphometric analysis of capillaries in subcutaneous adipose tissue in control and beta-carotene-treated animal during 3 months. See Table 3 for details on animal groups. The values are expressed in percentage.
In conclusion, this study suggests effects on subcutaneous WAT visible by morphological techniques after prolonged oral administration of beta-carotene in ferrets. A high-dose administration of beta-carotene also increases body weight and size of the inguinal subcutaneous fat depot. These results differ from preliminary studies performed by our groups also in ferrets, giving the same doses of beta-carotene during 3 months; but in that case beta-carotene was from another pharmaceutical supplier. We could speculate that the formulation of beta-carotene (e.g., the presence of alphatocopherol or other antioxidant compounds) might be important in determining some of the effects of this compound, although other factors, such as differences in the duration of the treatment (3 months in the preliminary vs. 6 months in this present study) or the age of the animals when killed (5 vs. 8 months), could also be significant. Unfortunately the knowledge on beta-carotene metabolism in both humans and ferrets is scarce and the vast majority of studies on mammalian beta-carotene have used rodents or other laboratory small mammals, species not absorbing carotenoids such as beta-carotene. Thus, care has to be taken to avoid extrapolating the results to humans. In this paper, the most striking effect induced by oral beta-carotene (obtained with both the molecules used: Sigma and DSM) on the ferret’s adipose tissue has been the increase of vasculogenesis.

Acknowledgements

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References


I. Murano et al. / Biochimica et Biophysica Acta 1740 (2005) 305–312
otene supplementation on cell proliferation and histopathological changes in the lungs of cigarette smoke-exposed ferrets, Carcinogenesis 21 (2000) 2245–2253.


