

Effects of β -carotene supplementation on adipose tissue thermogenic capacity in ferrets (*Mustela putorius furo*)

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We previously described that the intake of pharmacological doses of β -carotene (BC) resulted in higher body weight gain in the ferret (*Mustela putorius furo*), an animal model that resembles human subjects in terms of intestinal BC absorption and metabolism. These results were some way unexpected considering the condition of BC as a vitamin A precursor and the previous data in rodents showing these compounds as thermogenic activators. Here, we aimed to characterise in the ferret whether the mentioned changes in body weight could be explained by changes in adipose tissue thermogenic capacity. We studied the effects of 6-month supplementation with BC (0.8 and 3.2 mg/kg per d) on adipose tissue morphology and uncoupling protein-1 (UCP1) content. BC supplementation resulted in higher body weight (the high dose), induced depot- and dose-dependent hypertrophy of white adipocytes, decreased the amount of brown-like multilocular adipocytes in the retroperitoneal depot and decreased UCP1 content in different fat depots. To ascertain whether BC effects could be mediated by retinoic acid (RA), 1 week supplementation with RA (0.25 and 25 mg/kg per d) was also studied. RA treatment resulted in a slight decrease in adiposity, decreased cell lipid accumulation and increased UCP1 content, suggesting that the effects of BC on thermogenic capacity are not through RA. In conclusion, RA, but not BC, may have in the ferret comparable effects with those described in rodents, whereas differences concerning BC and RA treatments may be attributable to the different BC metabolism in the present animal model with a lower conversion of BC to RA compared with rodents.

Carotenoids: Uncoupling protein: Adiposity: Body weight: Retinoic acid

The worldwide increase in obesity prevalence and its associated medical complications has awoken great interest in the identification of the main factors involved in body weight control and in the identification of strategies for its prevention and treatment^(1,2). Knowledge of nutrients or food components able to influence energy balance, by altering energy expenditure, as well as through effects on the biology of adipose tissue, is potentially useful in designing functional foods or diets to help body weight control.

With respect to β -carotene (BC), confusing results in rodents and human subjects have been reported on its potential protective/promoting effects on lung cancer^(3–5) as well as on adiposity⁽⁶⁾, which can be related to species-specific differences in its metabolism. BC is one of the main provitamin A carotenoids in mammals, which has special interest by itself, as well as a vitamin A precursor. Vitamin A has many remarkable effects on adipose tissue biology and energy metabolism (reviewed in Bonet *et al.*⁽⁷⁾). Retinoic acid (RA), its carboxylic form, promotes⁽⁸⁾ or inhibits⁽⁹⁾ adipogenesis of preadipose cells in culture depending on the dose, and increases thermogenic capacity by inducing the expression of uncoupling protein 1 (UCP1) in cultured brown adipocytes^(10,11) and in brown adipose tissue of rodents⁽¹¹⁾.

Moreover, both RA treatment and vitamin A status influence body adiposity in rodents, with a low status favouring reduced expression of UCP and increased fat deposition^(12,13). Similar effects to that of RA on UCP1 induction have been described for BC and several other carotenoids with pro-vitamin A activity, such as α -carotene and lutein, in primary cultures of mice brown adipocytes, with an effectiveness that is related to their potency as vitamin A precursors⁽⁶⁾. BC also has features of a UCP1 activator, since its addition to cells increases the basal V_{O_2} of brown adipocytes (as does RA), which can be explained by a successive accumulation in the brown fat cells of RA obtained from BC cleavage⁽¹⁴⁾. However, whether dietary BC increases 'in vivo' thermogenic capacity is not known.

BC from the diet accumulates in adipose tissues and can be converted intracellularly to retinoids, including RA, which are also stored in adipose tissues⁽⁷⁾. Nevertheless, intestinal BC absorption as well as diet carotenoid conversion into retinoids is strictly species specific⁽¹⁵⁾. Rodents are extremely efficient converters and therefore do not absorb intact BC and do not accumulate appreciable tissue BC, whereas human subjects absorb significant amounts of uncleaved carotenoids and accumulate them in peripheral tissues, notably adipose tissues, where carotenoids may be metabolised to retinol

Abbreviations: BC, β -carotene; BC 0.8, 0.8 mg BC/kg body weight per d; BC 3.2, 3.2 mg BC/kg body weight per d; RA, retinoic acid; UCP, uncoupling protein.

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and RA^(15–17). In this sense, the ferret (*Mustela putorius furo*) offers an excellent animal model to use in mimicking the conditions of human subjects, because these animals, like human subjects, absorb dietary BC intact and accumulate it in tissues and serum in a dose-response manner^(18–21). In addition, tissue distribution of carotenoids in ferrets is similar to that of human subjects^(16,19,22). Therefore, use of the ferret as a model for studying human carotenoid effects seems well justified^(18,20,23).

Interestingly, and unlike the aforementioned effects described in rodents using RA, we previously described in ferrets that the intake of pharmacological doses of BC for 6 months resulted in a higher body weight gain compared with controls⁽²⁴⁾, although whether this effect could be explained by changes in the thermogenic capacity was not known. Thus, the aim of the present study was to characterise in ferrets the effects of chronic supplementation with two different doses of BC on thermogenic features (UCP1 content and morphology) of different adipose tissue depots. To ascertain whether the effects could be mediated by RA, the acute effects of supplementation with two different doses of RA were also studied in the present animal model.

Experimental methods

Animals and treatments

Expt 1: effects of chronic supplementation with two different doses of β -carotene. Eighteen, 2-month-old, female ferrets (Exopet AB, Glommen, Sweden) were housed at 22°C with a 12 h light–dark cycle (lights on at 08.00 hours) and free access to food and water. The gross composition of the chow diet (Friskies, Barcelona, Spain) used was the following: 32 % protein; 34.4 % carbohydrate; 10 % fat; 3 % fibre; 9.5 % moisture; 3.6 % minerals; 7.5 % as residue of total mass.

After 1 week adaptation, the animals were randomised to three experimental groups with six animals in each: control, 0.8 mg BC/kg body weight per d (BC 0.8) and 3.2 mg BC/kg body weight per d (BC 3.2). The animals in the BC 0.8 and BC 3.2 groups received a daily oral BC supplementation for 6 months with doses of BC 0.8 (a high physiological dose) and BC 3.2 (a pharmacological dose), respectively. These doses of BC were equivalent to supplemental doses of 10 and 40 mg BC/d in a 70 kg person⁽²⁵⁾. BC was provided by DMS Nutritional Products Ltd (Basel, Switzerland) as a water-soluble formulation (beadlets) containing BC crystalline, DL- α -tocopherol, ascorbyl palmitate as well as carriers such as maize oil, fish gelatine, sucrose and maize starch⁽²⁵⁾. This formulation of BC, instead of pure crystalline BC, was used to prevent BC oxidation and also because of previous results showing that this form is of better bioavailability than BC from other natural sources⁽²⁶⁾. This formulation was given orally dissolved in 200 μ l of water. The oral supplementation instead of the inclusion of BC in the diet also allowed us a better control of the doses given by the animals. The animals in the control group received the same formulation without BC, also provided by the manufacturer, exactly in the same way as the animals in the BC groups.

Expt 2: effects of acute supplementation with two different doses of retinoic acid. Eighteen, 10-month-old, female ferrets, from the same supplier and housed in the same

conditions as described in Expt 1, were randomised to three experimental groups: control, 0.25 mg RA/kg body weight per d and 25 mg RA/kg body weight per d (six animals per group). The animals in the 0.25 mg RA/kg body weight per d and 25 mg RA/kg body weight per d groups received oral RA supplementation for 7 d with doses of 0.25 mg RA/kg body weight per d and 25 mg RA/kg body weight per d, respectively. RA was provided by Sigma, Madrid, Spain, and was given to animals dissolved in a volume of 500 μ l of olive oil (Carbonell, Barcelona, Spain). The animals in the control group received the same amount of olive oil without RA.

National guidelines for the care and use of animals were followed, and experimental procedures involving animals were approved by the 'Bioethics committee of the University of the Balearic Islands'.

Sample collection

At the end of the treatments, ferrets from both experiments were anaesthetised using 10 mg/kg ketamine hydrochloride (Imalgène 1000, Merial Laboratorios SA, Lyon, France) and 80 μ g/kg medetomidine (Domtor, Orion Pharma, Espoo, Finland). Arterial blood was collected from the left ventricle in heparinised tubes and animals died by exsanguination. Afterwards, different adipose depots were rapidly removed and weighed. Samples for western-blot analysis were frozen in liquid N₂ and stored at –70°C until determinations were carried out. Samples for light microscopy analysis techniques were immediately fixed as described later.

Total lipid content extraction and quantification

Lipid extraction was performed as previously described^(27,28) with some modifications. Briefly, retroperitoneal, inguinal and interscapular adipose tissue samples were mixed with 1 ml of hexane–isopropanol (3:2, v/v). The tubes with the samples were gassed with N₂ before being closed to minimise lipid oxidation and then left overnight under orbital agitation at room temperature protected from light. The content of each tube was transferred into a new one and 0.3 ml of Na₂SO₄ (0.47 M) was added. Tubes were mixed for 5 min, left for 15 min in orbital agitation and centrifuged at 1000 g for 10 min at 4°C. The upper phase containing lipids was dissolved in hexane and transferred to a clean, previously weighed glass tube. Hexane extract was then dried with N₂ gas. Once the tube was dried, the percentage of lipids was determined as the weight difference between tubes with lipid extract and clean tubes, taking into account the initial amount of tissue present.

Quantification of DNA levels

For quantification of DNA levels, adipose tissue was homogenised in PBS (137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer, pH 7.4; dilution 1:3, w/v) using a polytron homogeniser, and was then centrifuged at 500 g for 10 min; the supernatant was collected and used for DNA quantification by a fluorometric method that uses 3,5-diaminobenzoic acid⁽²⁹⁾.

Western-blot analysis of uncoupling protein-1

UCP1 was determined by western blot in the retroperitoneal, inguinal and interscapular adipose depots of ferrets from each group as previously described⁽³⁰⁾ with slight modifications. The tissues were homogenised at 4°C in 1:5 (w/v) of PBS using a polytron homogeniser. The homogenate was centrifuged at 7000g and 4°C for 2 min and the supernatant used for UCP1 analysis. Total protein content in these samples was measured by Bradford's method⁽³¹⁾. Western blot was developed using the Laemmli method⁽³²⁾. Briefly, 80 µg of proteins were fractioned into a 10% SDS-PAGE and then transferred onto a 0.45 µm nitrocellulose membrane (BioRad, Madrid, Spain). Amido black staining was carried out to check equal loading/transfer of the proteins before blocking. The primary antibody solution used was a 1:1000 dilution in PBS + Tween 20 from an antiUCP1 antibody (alpha-Diagnostic, San Antonio, TX, USA). The secondary antibody was antirabbit IgG antibody conjugated to a streptavidin-biotinylated horseradish peroxidase complex (Amersham Biosciences, Barcelona, Spain), diluted 1:5000. The immunocomplexes were revealed using an enhanced chemiluminescence detection system (Amersham Biosciences) exposing the membrane to a Hyperfilm enhanced chemiluminescence (Amersham Biosciences). The films were scanned by Chemigenius BioImaging System (Syngene, Cambridge, UK), and the bands quantified using the GeneTools Software (Syngene, Cambridge, UK). Brown adipose tissue from rat was used as positive control.

The primary antibody used was designed for rodents, but has been previously checked to be effective for UCP1 analysis in ferrets⁽³³⁾.

Light microscopy techniques

Samples from several adipose depots from these animals were fixed overnight in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4), washed in phosphate buffer and then dehydrated in a series of alcohols (ethanol 75°, ethanol 95°, absolute ethanol and xylene) and paraffin embedded.

Immunohistochemistry analysis of uncoupling protein-1. After being paraffin embedded, the retroperitoneal adipose tissue from control animals and those treated with BC and RA were cut in 5 µm thick sections and immunostained by means of the avidin–biotin technique. These sections were incubated with primary antiUCP1 antibody (GeneTex, Inc., San Antonio, TX, USA) diluted 1:150 in PBS and with the corresponding biotinylated antirabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA), diluted 1:200. Finally, samples were incubated with ABC complex (Vectastain ABC kit, Vector) and peroxidase activity was revealed by 3,3'-diaminobenzidine hydrochloride as a chromogen (Sigma, St Louis, MO, USA) in water. Sections were counterstained with haematoxylin and mounted in Eukitt (Kindler, Germany).

The primary antibody used cross-reacts with mouse, rat and human UCP1 and has been previously checked to be also effective for ferret samples⁽³³⁾. Mouse interscapular brown adipose tissue sections were used as positive control, while negative control was performed by primary antibody omission.

Morphological analysis of adipose tissue. Five-micrometre sections of retroperitoneal, inguinal and interscapular adipose

tissues were stained with haematoxylin and eosin to assess morphology. Images from light microscopy were digitalised, and the area of 100 unilocular cells of each section was determined using AxioVision software (Carl Zeiss Imaging Solutions, Hallbergmoos, Germany). Percentage of multilocularity was also determined in retroperitoneal depot. Multilocular cells were considered with independence of their UCP1 expression.

Statistical analysis

All data are expressed as the means with their standard errors. The statistical significance of differences as effect of BC or RA treatment and the effect of the treatments and tissue were assessed by one-way and two-way ANOVA, respectively, followed by a least significant difference *post hoc* comparisons. The analyses were performed with SPSS for windows (SPSS, Chicago, IL, USA). Threshold of significance was defined at $P < 0.05$ and is indicated when different.

Results

Expt 1: effects of chronic supplementation with two different doses of β-carotene

Effects of β-carotene on body weight and fat depots. As previously described in the same cohort of animals⁽²⁴⁾, body weight of ferrets receiving the high dose of BC was, at the end of the treatment, significantly higher (14%) than controls, while the low dose did not result in significant changes (Table 1). Moreover, the size of the subcutaneous inguinal depot in animals treated with the high dose of BC was significantly higher ($P < 0.05$) than that of animals treated with the low dose and slightly higher than that of controls (19% and 16% higher, respectively)⁽²⁴⁾. In addition, other depots, particularly gonadal and retroperitoneal fat depots, were also slightly higher in animals treated with the high dose of BC compared with controls (Table 1). Food intake, measured on different days during the experimental period, was not different between the groups (data not shown).

Effects of β-carotene on morphological and morphometric features of different fat depots. To approach morphological changes in adipose tissue as effect of BC, the retroperitoneal and inguinal adipose tissue depots were chosen as representative of internal and subcutaneous depots, respectively, and the interscapular depot was also selected because its location resembles the interscapular brown adipose tissue in rodents.

Adipose tissue morphology was different depending on the anatomical localisation. The retroperitoneal depot of control ferrets – unlike this anatomical depot in rodents (which is almost exclusively unilocular⁽³⁴⁾) – consisted of both unilocular- and multilocular-like adipocytes (Fig. 1(A)), the multilocular cells representing a relative area of 8.62% (Fig. 1(B)). The inguinal and interscapular depots consisted mostly of unilocular adipocytes (figures not shown).

In the retroperitoneal depot, the percentage of multilocularity decreased significantly with both doses of BC (Fig. 1(B)). In addition, although the total lipid concentration in this tissue was not significantly affected by BC treatment, the DNA content per g wet tissue decreased significantly as an effect of both doses of BC, and this resulted in a significant

Table 1. Effects of β-carotene (BC) supplementation for 6 months on body weight and size of adipose tissue depots†

(Means values with their standard errors of six animals per group)

	C		BC 0.8		BC 3.2		T
	Mean	SEM	Mean	SEM	Mean	SEM	
Body weight (g)							
Initial	571	20	561	28	587	14	
Final	922 ^a	44	898 ^a	25	1050 ^b	18	
Adipose weights (g)							
Retroperitoneal	19.2	2.5	18.5	1.3	23.5	2.3	
Inguinal	42.0	4.3	40.9	2.8	48.8*	1.6	
Mesenteric	46.1	5.3	36.2	3.4	45.3	4.6	
Gonadal	2.41	0.40	2.25	0.33	2.80	0.12	
Interscapular	4.41	0.50	4.26	0.49	5.45	0.62	

C, control group; BC 0.8, 0.8 mg BC/kg body weight per d; BC 3.2, 3.2 mg BC/kg body weight per d; T, effect of treatment ($P < 0.05$, one-way ANOVA).

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$; least significant difference *post hoc* test).

* $P < 0.05$ BC 3.2 v. BC 0.8, Student's *t* test.

† Animals were supplemented for 6 months with BC 0.8, BC 3.2 or with the vehicle (C). Initial body weight, before starting BC treatment, and final body weight, after 6 months treatment, are indicated.

increase in the ratio between both parameters (Table 2). In this way, the mean area of the white adipose unilocular cells in this depot, determined in representative sections, also tended to increase with both doses of BC (27% with BC 0.8 and 39%

with BC 3.2) compared with controls, although differences were not statistically significant.

Concerning the inguinal and interscapular depots, BC treatment did not significantly affect the total lipid concentrations

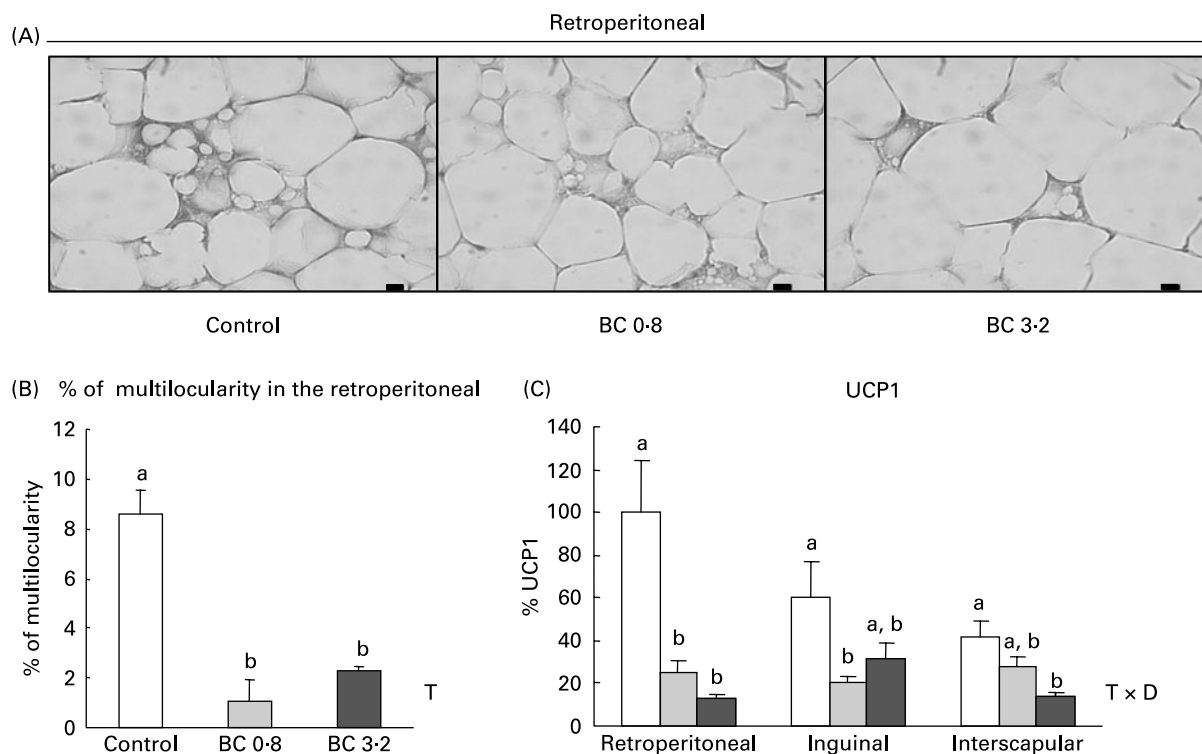


Fig. 1. (A) Immunostaining for uncoupling protein-1 (UCP1) in the retroperitoneal adipose tissue in ferrets supplemented for 6 months with 0.8 mg BC/kg body weight per d (BC 0.8), with 3.2 mg BC/kg body weight per d (BC 3.2) or with the vehicle (control group). This fat depot in control animals displayed substantial amounts of multilocular cells, positive for UCP1, surrounded by unilocular white cells. BC supplementation resulted in a reduction in the amount of multilocular cells. Light microscopy. Scale bar 12 μm. (B) Percentage of the area of multilocular cells in the retroperitoneal adipose tissue in control and BC-treated animals determined by using a morphological imaging system. Results represent the means with their standard errors ($n = 4-5$). A total area of 1 mm² was analysed for each animal. (C) Specific UCP1 in different adipose tissue depots (retroperitoneal, inguinal and interscapular) in control and BC-treated ferrets, measured by western blot. Data are expressed as percentage of the value of the retroperitoneal depot in control animals, which was set as 100%. Results represent the means with their standard errors ($n = 5-6$). T, effect of treatment ($P < 0.05$, one-way ANOVA); T × D, interaction of BC treatment and the adipose depot (two-way ANOVA, $P < 0.05$). ^{a,b} Mean values within each depot with unlike letters were significantly different (one-way ANOVA, $P < 0.05$). □, control; ▤, BC 0.8; ■, BC 3.2.

Table 2. Effects of β -carotene (BC) supplementation for 6 months on features of different adipose tissue depots* (Mean values with their standard errors (n 5–6))

	C		BC 0.8		BC 3.2		T
	Mean	SEM	Mean	SEM	Mean	SEM	
Retroperitoneal							
Lipid content (mg/g tissue)	721	105	938	133	1219	239	
DNA content (μ g/g tissue)	457 ^a	18	271 ^b	14	312 ^b	39	T
Lipid/DNA	1.56 ^a	0.19	3.48 ^b	0.49	4.24 ^b	1.26	T
Area of unilocular cells (μ m ²)	1960	483	2486	351	2717	384	
Inguinal							
Lipid content (mg/g tissue)	877	81	849	87	692	101	
DNA content (μ g/g tissue)	232 ^{ab}	14	189 ^a	25	289 ^b	19	T
Lipid/DNA	3.91 ^{ab}	0.44	4.88 ^a	0.86	2.51 ^b	0.47	T
Area of unilocular cells (μ m ²)	3152	519	3702	509	3780	349	
Interscapular							
Lipid content (mg/g tissue)	391	118	499	64	389	119	
DNA content (μ g/g tissue)	270	43	332	19	262	53	
Lipid/DNA	1.42	0.43	1.50	0.17	1.64	0.51	
Area of unilocular cells (μ m ²)	2962	477	3565	659	4041	629	

C, control group; BC 0.8, 0.8 mg BC/kg body weight per d; BC 3.2, 3.2 mg BC/kg body weight per d; T, effect of treatment ($P < 0.05$, one-way ANOVA).

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$; least significant difference *post hoc* test).

*Lipid and DNA contents were measured as described in the Experimental methods section. The mean area of unilocular cells was calculated by measuring the area of 100 unilocular cells for each tissue.

in these tissues, but DNA concentration and the lipid/DNA ratio in the inguinal depot of animals treated with the low dose of BC were significantly different (lower and higher, respectively) to that of animals treated with the high dose ($P < 0.05$, one-way ANOVA; Table 2). No significant changes were found in the mean area of the white adipose unilocular cells in these depots, although, similarly to what was observed in the retroperitoneal depot, there was a tendency to increase with BC treatment in a dose-dependent manner, particularly in the interscapular depot.

Effects of β -carotene on uncoupling protein-1 in different adipose tissue depots. UCP1 analysis by western blot revealed that this protein was present in the three adipose depots analysed: interscapular, inguinal and retroperitoneal (Fig. 1(C)). In control animals, retroperitoneal showed maximum levels of UCP1 compared with the inguinal and the interscapular adipose tissue depots. In this tissue, positive

UCP1 staining by immunohistochemistry was found, mainly in multilocular cells (Fig. 1(A)).

Specific UCP1 levels (per mg of total protein) were affected by BC treatment (Fig. 1(C)). BC treatment resulted in a significant decrease in UCP1 in the three depots studied; this decrease was already significant with the low dose of BC in the retroperitoneal and inguinal depots and with the high dose in the interscapular depot.

Expt 2: effects of acute supplementation with two different doses of retinoic acid

Effects of retinoic acid on body weight and fat depots. Body weight was not significantly affected by 7 d's RA treatment (Table 3). Neither were any significant changes found in the size of adipose tissue depots as an effect of RA treatment; however, a slight tendency to lower size was also found

Table 3. Effects of retinoic acid (RA) supplementation for 7 d on body weight and the size of adipose tissue depots*

(Mean values with their standard errors of six animals per group)

	C		RA 0.25		RA 25	
	Mean	SEM	Mean	SEM	Mean	SEM
Body weight (g)						
Initial	864	67	869	42	863	60
Final	855	97	862	47	884	63
Adipose weights (g)						
Retroperitoneal	22.1	3.7	19.0	2.6	19.4	3.6
Inguinal	41.0	6.5	35.4	4.9	39.5	4.3
Mesenteric	47.6	8.3	40.8	5.0	45.8	6.0
Gonadal	4.21	0.69	3.49	0.60	3.58	0.70
Interscapular	1.21	0.22	1.08	0.15	1.13	0.21

C, control group; RA 0.25, 0.25 mg RA/kg body weight per d; RA 25, 25 mg RA/kg body weight per d.

*Animals were supplemented for 7 d with RA 0.25, RA 25 or with the vehicle (C). Initial body weight, before starting RA treatment, and final body weight, after 6 months treatment, are indicated.

in the different depots with the low dose of RA compared with controls.

Effects of retinoic acid on morphological and morphometric features of different fat depots. RA treatment with the low dose resulted in changes in the retroperitoneal adipose tissue morphology, which showed a significant increase in multilocular cells (Fig. 2(A), 2(B)). Total lipid concentration and DNA content per g wet tissue were not significantly affected by the RA treatment in any of the depots studied, although the resulting lipid/DNA ratio decreased in the interscapular depots with both doses of RA (Table 4). The treatment with the low dose of RA also resulted in a significant decrease in the mean area of the white adipose unilocular cells in the inguinal depot.

Effects of retinoic acid on uncoupling protein-1 in different adipose tissue depots. UCP1 was also studied in the retroperitoneal, inguinal and interscapular adipose tissue depots of animals treated with RA. RA treatment with the low dose, but not with the high dose, resulted in a significant increase in UCP1 in the retroperitoneal depot, while no changes were found in the other depots studied (Fig. 2(C); see also UCP1 immunostaining in the retroperitoneal depot in Fig. 2(A)).

Discussion

The present study shows that BC supplementation of ferrets results in higher adiposity and body weight gain, depending on the dose, an effect that is associated to a decrease in the

thermogenic capacity of adipose tissues, particularly the retroperitoneal depot, which as previously described⁽³³⁾ is the one with the highest thermogenic capacity in the present animal model.

The recent description⁽³⁵⁾ that adult human subjects have well-defined brown adipose tissue depots that can be physiologically stimulated has revived the interest in this adipose tissue and its exclusive UCP1, as well as in factors, including nutrients or food components, which are able to induce or to activate UCP1. Vitamin A and its main dietary precursor, BC, have received particular attention as nutrients are able to stimulate thermogenesis⁽⁷⁾. The effects of vitamin A affecting the development and function of white adipose tissue and influencing the activity of brown adipose tissue have been described in several *in vitro* and *in vivo* animal models, particularly rodents^(11,12,36). Similar effects to those of RA have been described for BC and other carotenoids with pro-vitamin A activity on UCP1 induction⁽⁶⁾ and on V_{O_2} ⁽¹⁴⁾ in cultured brown adipocytes, and its effects have been associated to its cleavage and transformation to RA. While rodents do not readily absorb intact BC, ferrets mimic the intestinal absorption of BC in human subjects as they absorb intact BC to a significant extent^(18–20). Thus, by considering that carotenoids, particularly BC, are the main source of vitamin A in the body⁽³⁷⁾ to analyse whether dietary BC, by itself or as a vitamin A precursor, may increase *in vivo* thermogenic capacity becomes interesting in an animal model that resembles human subjects in terms of BC absorption and metabolism.

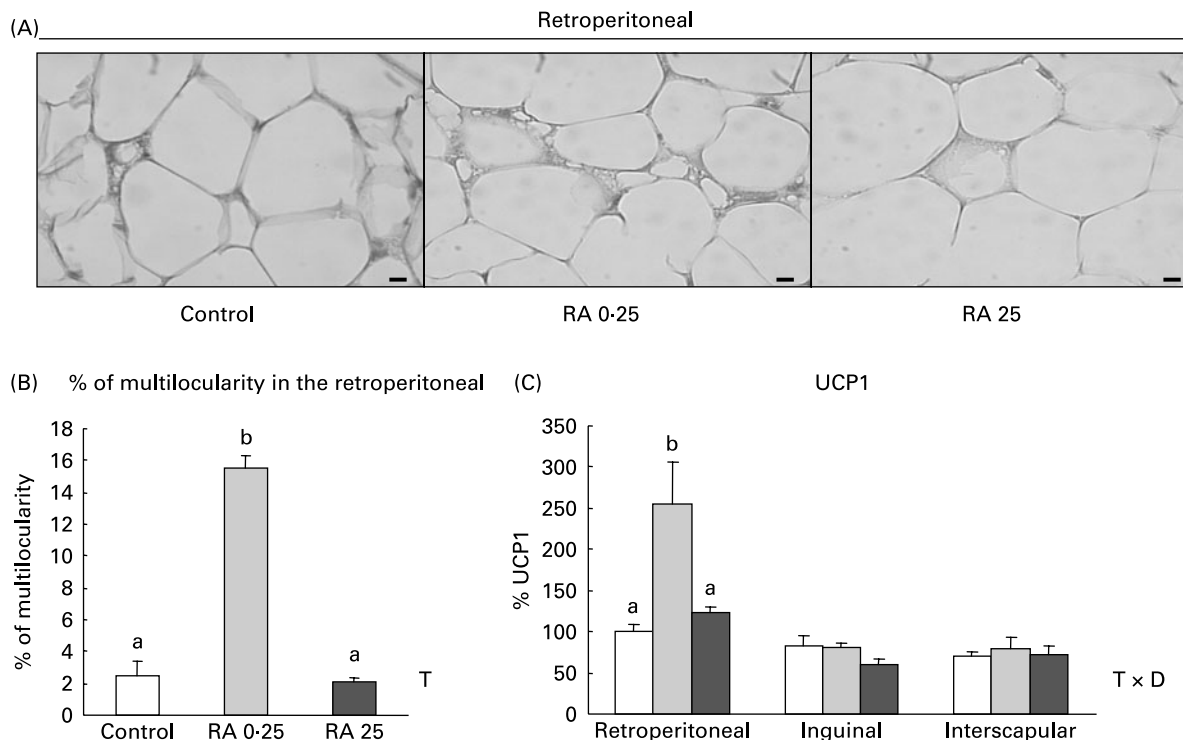


Fig. 2. (A) Immunostaining for uncoupling protein-1 (UCP1) in the retroperitoneal adipose tissue in ferrets supplemented for 7 d with 0.25 mg retinoic acid/kg body weight per d (RA 0.25), with 25 mg RA/kg body weight per d (RA 25) or with the vehicle (control group). RA resulted in an increase in the amount of multilocular cells, positive for UCP1. Light microscopy. Scale bar 12 μm. (B) Percentage of the area of multilocular cells in the retroperitoneal adipose tissue in control and RA-treated animals determined by using a morphological imaging system. Results represent the means with their standard errors (n 4–5). A total area of 1 mm² was analysed for each animal. (C) Specific UCP1 in different adipose tissue depots (retroperitoneal, inguinal and interscapular) in control and RA-treated ferrets, measured by western blot. Data are expressed as percentage of the value of the retroperitoneal depot in control animals that was set as 100%. Results represent the means with their standard errors (n 5–6). T, effect of treatment (P<0.05, one-way ANOVA); T × D, interaction of RA treatment and the adipose depot (two-way ANOVA, P<0.05). ^{a,b} Mean values within each depot with unlike letters were significantly different (one-way ANOVA, P<0.05). □, control; ▤, RA 0.25; ■, RA 25.

Table 4. Effects of retinoic acid (RA) supplementation for 7 d on features of different adipose tissue depots* (Mean values with their standard errors (*n* 5–6))

	Control		RA 0.25		RA 25		
	Mean	SEM	Mean	SEM	Mean	SEM	
Retroperitoneal							
Lipid content (mg/g tissue)	623	44	587	32	545	47	
DNA content ($\mu\text{g/g}$ tissue)	415	69	505	110	657	115	
Lipid/DNA	1.93	0.57	1.61	0.46	1.02	0.25	
Area of unilocular cells (μm^2)	3024	361	2259	549	2777	534	
Inguinal							
Lipid content (mg/g tissue)	490	37	547	51	555	32	
DNA content ($\mu\text{g/g}$ tissue)	376	36	474	112	351	81	
Lipid/DNA	1.35	0.14	1.78	0.64	2.21	0.62	
Area of unilocular cells (μm^2)	3808 ^a	344	2536 ^b	349	3927 ^a	330	T
Interscapular							
Lipid content (mg/g tissue)	501	114	517	51	439	52	
DNA content ($\mu\text{g/g}$ tissue)	230	49	369	21	260	30	
Lipid/DNA	2.41 ^a	0.35	1.43 ^b	0.17	1.73 ^b	0.15	T
Area of unilocular cells (μm^2)	4189	548	3824	921	3783	804	

C, control group; RA 0.25, 0.25 mg RA/kg body weight per d; RA 25, 25 mg RA/kg body weight per d; T, effect of treatment ($P < 0.05$, one-way ANOVA).

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$; least significant difference *post hoc* test).

* Lipid and DNA contents were measured as described in the Experimental section. The mean area of unilocular cells was calculated by measuring the area of 100 unilocular cells for each tissue.

Our previous results concerning the effects of BC on body weight and adiposity⁽²⁴⁾ were in some way unexpected, considering the condition of BC as a vitamin A precursor and the previous data in rodents showing these compounds as thermogenic activators. In ferrets, BC treatment resulted in an increase in body weight and adiposity, depending on the dose, and here we show that this was associated to depot-specific changes in adipose tissue morphology. In particular, these changes involved adipocyte hypertrophy (particularly in the retroperitoneal depot) evidenced by an increase in the lipid to DNA ratio, as well as hyperplasia (particularly in the inguinal depot) suggested by an increase in the tissue content of DNA and in the size of this fat depot, particularly with the high dose of BC. Moreover, there was a tendency to an increase in the size of unilocular cells in different fat depots. In addition, and particularly in the retroperitoneal tissue – which has a considerable amount of brown adipocyte-like multilocular cells – BC treatment resulted in a significant decrease in the amount of these multilocular cells. These changes in adipose tissue morphology were also associated to changes in UCP1 content. It must be pointed out that UCP1 is distributed in the ferret differently than in rodents, since it is widely present, although at low levels, in different depots, and the retroperitoneal depot exhibits relative higher levels than the interscapular depot, as previously described⁽³³⁾. BC treatment resulted in a decrease in the UCP1 content, which was more marked in the retroperitoneal depot but was also present in the other depots studied, the inguinal and the interscapular ones. All these changes reflect decreased thermogenic features, contrary to what is seen in rodents, and could be explained by a lower conversion of BC to RA, at least not converted in the amounts needed to induce UCP1.

To further document that the observed effects are due to BC by itself, we analysed in another group of ferrets the effects of 7 d supplementation with RA. Results showed that RA treatment, particularly with the low dose used, resulted in a

slight decrease in the size of the different fat depots. RA also resulted in a decrease in cell lipid accumulation, evidenced by a decrease in the lipid to DNA ratio (in the interscapular depot and with both doses) and in the size of adipocyte cells (in the inguinal depot and with the low dose). Interestingly, the low dose of RA resulted in a significant increase in UCP1 content in the retroperitoneal depot, while no effects were found in the other depots studied. All in all, these results evidence that RA may have in the ferret comparable effects with those described in rodents^(11,12,36). Therefore, although the two studies are not directly comparable mainly because of differences in the period of treatment, differences concerning BC and RA treatments seem to be attributable to the different BC metabolism in the present animal model compared with rodents, particularly to the low conversion of BC to RA.

In this sense, in a previous study in the same cohort of animals, we showed that BC supplementation resulted in a dose-dependent increase in plasma BC levels (control: 5.0 (SEM 3.0) nM; BC 0.8: 36 (SEM 12) nM; BC 3.2: 102 (SEM 18) nM)⁽²⁵⁾; but levels of RA in plasma were very low and it was not possible to consistently measure them (A Fuster, C Picó, J Sánchez, *et al.*, unpublished results). Similarly, in human subjects, the intake of higher amount of fruits and vegetables has also been found to be associated with higher BC plasma levels, while no significant changes in plasma retinoid (retinol, retinyl esters and RA) concentrations have been found⁽³⁸⁾; this means that there may be little tissue metabolism of BC to retinoids. Other authors have also described that dietary BC supplementation of ferrets may also increase circulating BC concentrations to levels similar to those detected in human serum, and also resulted in increased levels in the liver, adipose tissue and other tissues^(19–22,39). This is different to what has been reported in rodents that have low serum carotenoid levels (about 1/1000 of human levels), which are not related to dietary intake⁽¹⁸⁾.

However, even considering that BC cleavage to RA occurs in small amounts, why chronic BC treatment increases adiposity and decreases UCP1 content in the adipose tissue is not known and deserves further investigation.

All in all, these findings in the ferret, which are more likely to be extrapolated to human subjects than the results from rodents, do not support the potential usefulness of BC-rich foods in helping to prevent body fat accumulation as potential thermogenic regulators. Actually, the present study shows that chronic supplementation with BC of ferrets results in increased adiposity and lowers adipose tissue thermogenic capacity.

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