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Original communications

Hepatic Subcellular Storage of Beta-Carotene in Rats Following Diet Supplementation

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Abstract: Beta-carotene (BC) storage was measured in liver and its subcellular fractions (plasma membranes, mitochondria, microsomes and nuclei) of rats fed BC added to diet. The BC supplementation dose was about 350 mg/week/rat. After 15 weeks of this supplementation, rats were killed and their livers were immediately excised and processed to obtain total liver tissue and its subcellular fractions. Their BC contents were measured by HPLC as pmols/mg protein. Intact BC was found to be stored in all the above subcellular fractions, thus showing that BC is probably taken up by liver cell lipid moiety. Interestingly, the mean BC concentrations in plasma membranes and mitochondria were significantly higher than that in total liver tissue. Our data confirmed that rodents are a good animal model for the study of BC metabolism and its effects on several pathologies, and cancer prevention and treatment in humans in spite of the fact that rodents are classified as white-fat animals because of their poor BC absorption and storage in fat and blood plasma, whereas humans are classified as yellow-fat organisms because of their opposite behavior in BC uptake and organ distribution.

Key words: Beta-carotene, rat liver, subcellular fractions, storage

Introduction

In a previous paper it was demonstrated that rats absorb beta-carotene (BC) and store the intact molecule in several organs (liver, small intestine, spleen and stomach), but not in the brain, fat or blood plasma, when BC supplementation was administered through pelletted diets. Conversely, when the same amount of BC was supplemented by gavage in crystalline form dispersed in peanut oil, the carotenoid was also taken up by the kidney, slightly by fat and

surprisingly in the lungs at high concentrations. Thus, the lung was indicated as a possible target organ for BC [1]. This striking drug kinetics has also been observed independently by Schweigert et al [2] in piglets supplemented with [14C] BC dispersed in olive oil.

The data obtained in these studies indicated that rodents would be a good model for the study of carotenoid metabolism, and it would hence be advantageous to ascertain not only carotenoid absorption and organ distribution, but also its subcellular localization. Accordingly, the present study was designed to measure BC storage in rat liver and its subcellular fractions from animals fed BC added to pelletted diet as reported below.

Materials and Methods

Animals and their handling: Twenty three adult female Wistar rats purchased from Nossan s.r.l. Correzzana, Milan, Italy, initially weighing between 150–180 g and after 1 month about 250 g, were used. Animals were handled following the statements on animal care in the Italian D.L. 116/92 and amendments. They were housed one per cage with free access to water and to standard or supplemented diet and were kept in a dimly lighted room. After 15 weeks, animals were anesthetized with ether, and the livers were excised and handled as described below.

Experimental design: Animals were randomly separated into groups as follows:

Control groups: a) 5 animals fed standard pelletted diet; b) 5 animals fed oil pelletted diet.

Experimental group: 14 animals fed BC pelletted diet.

After 15 weeks, the rats were sacrificed, and their livers were excised and processed for subcellular fractionation.

Diets (purchased from Nossan s.r.l, Correzzana, Milan, Italy) were as follows:

Control group a) **Standard pelletted diet:** Pellets consisting of raw proteins 21%, raw lipids 3.9%, raw cellulose 7.3% from corn, wheat bran, meat flour, soy , barley and oat flours, dried milk whey, sodium chloride, calcium carbonate, DL-metionine, completed by conventional addition of vitamins and minerals.

Control group b) **Peanut oil pelletted diet:** This diet contained 1 L of peanut oil per 100 kg of standard pelletted diet.

Experimental group BC pelletted diet: BC beadlets and crystalline BC were from Hoffmann-La Roche, Basel, Switzerland. One kg of BC beadlets and 50 g of crystalline BC dispersed in 750 ml of peanut oil, were added to 75 kg of standard diet at a temperature < 60°C in the dark. This preparation in pellets was kept in a freezer at –25°C until use; 25 g were given every day to each rat. Thus, the above BC oil diet preparation contained 2 g of BC/kg of diet, that is 50 mg of BC in 25 g of pellets, this being the average amount of food daily consumed by each rat weighing 250 g. Therefore, each rat ingested about 350 mg of BC/week.

Subcellular fraction preparation: The sacrificed control and experimental animals were immediately dissected, and their livers excised and processed to isolate plasma membranes, microsomes, nuclei and mitochondria.

Table I: Beta-carotene (BC) concentrations (pmols/mg protein) in totalliver tissue and in its subcellular fractions from 14 female Wistar rats fed BC in pelletted diet. Statistical analyses were performed according to Friedman test [9]

Rat	Total tissue	Plasma membranes	Microsomes	Mitochondria	Nuclei
1	64.3	0.0	0.0	182.6	0.6
2	117.0	1384.1	370.0	0.0	126.7
3	67.5	936.0	500.2	478.4	222.3
4	238.6	230.9	145.1	467.9	107.2
5	195.5	3430.7	9562.5	2791.4	259.8
6	30.8	441.3	930.4	253.8	117.3
7	233.1	853.8	0.0	0.0	426.3
8	113.0	194.8	10.4	5.5	2.2
9	117.8	93.6	17.2	2.4	0.0
10	0.0	303.2	0.0	699.7	74.2
11	61.2	799.3	0.0	334.1	61.2
13	0.0	296.9	509.9	904.5	8.0
14	41.6	68.8	0.0	0.0	26.6
Median	67.5	303.2	17.2	253.8	74.2
Minimum	0.0	0.0	0.0	0.0	0.0
Maximum	238.6	3430.7	9562.5	2791.4	426.3

Plasma-membrane preparation: The livers were processed as reported by Van Amelsvoort et al [3]. Briefly, the tissue was rinsed in buffer (0.25 M sucrose, 0.25 mM CaCl2, 10 mM Hepes at pH 7.6), minced with scissors and homogenized in 5 vol (v/w) of buffer in a tissue-homogenizer system (Glass-Col, Terre Haute, Indiana, USA). The homogenate was filtered through three layered gauze and diluted to 40 ml with buffer. After centrifugation for 10 min. at 540 g, the supernatant was collected. The pellet was resuspended in 40 ml of buffer-EDTA (0.25 M sucrose, 0.25 mM CaCl2, 10 mM Hepes, 0.5 mM-EGTA at pH 7.6) and centrifuged for 10 min. at 540 g. The supernatant and the fluffy layer on the top of the pellet put together with the previous supernatant, were collected and centrifuged for 30 min. at 20,000 g with a Sorvall ultracentrifuge, rotor T865 at 0–4°C. The resulting pellet was finally resuspended in buffer-EGTA layered (2 ml) on top of a discontinuous density gradient prepared with sucrose 39.5% (w/v) and sucrose 20% (w/v) and centrifuged for 2.5 h at 50,000 g at 0–4°C. The material at the gradient interface was collected, diluted in 2–3 ml of a suspending buffer (0.1 M KCl, 10 mM Hepes at pH 7.6) and centrifuged for 40 min. at 30,000 g. The pellet was finally resuspended in 1 ml of suspending buffer. All procedures were performed at a temperature between 0–4°C.

Microsome, nucleus and mitochondrion preparations: The experimental rat livers were immediately removed and processed as above. The tissue was rinsed in homogenation medium (0.25 M sucrose, 5 mM Hepes, 0.5 mM EGTA at pH 7.5), minced with scissors and homogenized in homogenation medium at 10% (w/v) in the same tissue homogenizer system as above, and filtered through gauze. After 40 min. centrifugation at 10,000 g in a Sorvall ultracentrifuge rotor T865, the pellet was resuspended in 2 ml of suspending medium (0.25 M sucrose, 10 mM TrisHCl at pH 7.4) and collected as nuclear fraction. The supernatant was then centrifuged at 25,000 g for 30 min; the collected pellet resuspended as above contained the mitochondria. This last supernatant was centrifuged at 13,500 g for 60 min.; the pellet washed in homogenation buffer (0.15 M KCl, 50 mM Hepes at pH 7.5) was centrifuged at 13,500 g for 30 min. and the resulting pellet resuspended in 1 ml of suspending medium, was the microsomal fraction. All procedures were performed at a temperature between 0–4°C [4].

Beta-carotene extraction and protein assay: BC was extracted from liver tissue using the Dimitrov method [5], as previously used [6], with minor variations [7]. Briefly, 0.5 g of wet tissue was homogenized in a tissue-homogenizer-system (Cole-Parmer International, Chicago, USA) diluted with 0.5 ml of bidistilled water, added to 2 ml of absolute ethanol and extracted twice through 2 ml of n-hexane; after mechanical shaking for 3 min., the sample was centrifuged at 300 g for 10 minutes. The extraction of BC from the subcellular fraction was conducted as above but without the water dilution step.

Finally all the hexane fractions were mixed together in a vial and evaporated under reduced pressure in the presence of nitrogen before HPLC processing.

Protein assay was carried out by the Lowry method [8].

HPLC analysis: The HPLC line was equipped with the following modules: Pump: Mono piston, mod. T414, Kontron, Zurich, Switzerland. Mixer: mod. M800, Kontron. Injection valve with 20 ml loop. UV/VIS: programmable variable wavelength HPLC detector mod. 430, Kontron. Integrator: Data System mod. 430, Kontron. Plotter: mod. 800, Kontron. Column: stainless steel, length 15 cm, inner diameter 4.6 mm. Stationary phase: Ultrasphere ODS1 or Spherisorb C8 5 mm particle size. Mobile phase: acetonitrile/tetrahydrofurane/methanol/1% ammonium acetate (684:220:68:28). Flow rate: 1.5 ml/minute. HPLC reagents: Standard BC was kindly supplied by Hoffmann-La Roche, Basel, Switzerland. Acetonitrile, methanol, and tetrahydrofurane, all HPLC grade, were from Carlo Erba, Milan, Italy.

Statistical analysis: The statistical analysis to assess the significance of differences was performed with the Friedman test [9].

Results

In the rat control groups fed the standard diet and the standard diet plus peanut oil, no BC storage in the liver, apart from BC traces in 3 out of 10 animals, was detected by HPLC.

In the 14 female rats fed the peanut oil plus BC pelletted diet intact BC (pmols/mg protein) was found by HPLC analysis to have been absorbed and stored in total liver tissue and its subcellular fractions, namely plasma membranes, mitochondria, microsomes and nuclei, as reported in Table I, most probably following BC uptake by liver cell lipid moiety. The nonparametric statistical analysis of these data revealed non-homogenous distribution of BC storage among liver tissue and its subcellular fractions ($P = 0.036$) with major differences in BC concentration between nuclei and plasma membranes ($P = 0.0019$) and between total tissue and plasma membranes ($P = 0.0071$).

On the whole, these data demonstrated that intact BC is stored in all liver subcellular fractions, concentrations being at their highest in plasma membranes with respect to total liver tissue, and lower in nuclei. It should be noted, however, that the BC supplementation dose of

350 mg/week/experimental rat, which had no toxic side-effects, was not completely absorbed, since animal stools were constantly orange.

Discussion

Table I clearly shows that rats fed high dose BC through diet absorb and store intact BC in hepatic tissue, in accordance with previous results [1], and in its subcellular fractions.

The latter showed that rats, referred to as white-fat animals because of their poor absorption of BC, do in fact absorb and store intact BC in different organs, given high doses of BC. Thus, rodents can behave like yellow-fat organisms such as humans, who absorb and store low doses of BC in different organs, including fat and blood plasma.

The storage of both BC and canthaxanthin (CX) in rat liver and their concentrations in liver microsomes was also observed by others in a recent study on the effect of carotenoids on liver xenobiotic metabolizing enzymes. In this experiment, BC and CX were supplemented by diet or injected (5% emulsion) intraperitoneally [10]. Furthermore, intact BC has also been detected in the microsomes as well as in the mitochondria and nuclei of lymphocytes from the blood of female pigs after a single injection (i.m.) of a BC soluble preparation from BASF. In this experiment, BC was even detected in blood plasma, with a time course comparable to that for subcellular fractions [11].

Various data therefore substantiate the use of rats as animal models for the study of BC metabolism in humans. The findings also reinforce the significance of the earliest experiments on rats and mice which attracted attention to cancer chemoprevention by carotenoids in animals, and stimulated the many epidemiological studies and BC interventions in humans which have since been conducted [12, 13].

In the animal experiments mentioned, the mechanism of carotenoid anticarcinogenicity was even ascertained with respect to initiation, promotion and progression phases in different experimental carcinogenic models. This proved to be helpful to explain conflicting data in epidemiological trials [14].

Last but not least, the observation that "white-fat animals absorb dietary carotenoids only poorly, which frequently necessitates the use of pharmacological doses in order to see effects" [15], does not conflict with the significance of the data thus far obtained in rodents with reference to human interventions. Indeed, in humans, who are yellow-fat mammals, carotenoid supplementation should be applied at high dosage (≥ 40–100 mg/day) to meet positive expectations for prevention and treatment of either cancer or ARC/late AIDS stages [16].

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