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Journal of Nutritional Biochemistry 12 (2001) 640–647

THE JOURNAL OF Nutritional Biochemistry

β-Carotene 15,15′-Dioxygenase activity in human tissues and cells: evidence of an iron dependency

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Received 14 March 2001; received in revised form 22 June 2001; accepted 20 July 2001

This work was presented in part at the Annual Meeting of Experimental Biology' 99 FASEB, Washington DC, MD, April 1999.

Abstract

The two objectives of this study were to investigate β-carotene 15,15′-dioxygenase activity in human tissues and to determine the effect of desferrioxamine on the dioxygenase activity. Two human *in vitro* models were used: the TC7 clone of the intestinal cell line Caco-2 and small intestinal mucosa preparations. β-Carotene 15,15′-dioxygenase activity in the small intestinal mucosa was (mean ± SD) 97.4 ± 39.8 pmol/h.mg protein for five adults (44–89 y) and 20 pmol/h.mg for an infant (17 months). No activity was detected in adult stomach tissue. We report for the first time the dioxygenase activity in human liver: 62 pmol/h.mg for a normal adult liver and 7 pmol/h.mg for a liver exhibiting gross pathology. The maximum capacity of β-carotene cleavage in an adult was estimated to be 12 mg/day (one fifth by small intestine and four fifths by liver), assuming an optimal β-carotene/retinal cleavage ratio of 1:2. The dioxygenase activity was decreased up to 80% with increasing desferrioxamine concentrations in the two *in vitro* models. Desferrioxamine was characterized as a noncompetitive inhibitor. In TC7 cells, the inhibitory effect of desferrioxamine was reversed by iron addition, suggesting that this effect was related to the ability of desferrioxamine to chelate iron, purported to be an obligate cofactor of the enzyme. In conclusion, these data report the presence of β-carotene 15,15′-dioxygenase activity in human small intestine and liver and demonstrate that desferrioxamine efficiently inhibits intestinal β-carotene cleavage in human tissues and cells.

Keywords: β-carotene; cleavage; intestine; liver; desferrioxamine; human.

1. Introduction

β-Carotene is the most abundant carotenoid found in plant-derived foods (mainly fruits and vegetables) consumed by humans and exhibits the highest biological activity of all provitamin A carotenoids. During absorption dietary β-carotene is transported from the intestinal lumen to the lymph as an intact molecule or it can be metabolized in the enterocytes into vitamin A esters or other compounds.

The first step of β-carotene metabolism is catalyzed by the cytosolic enzyme β-carotene 15,15′-dioxygenase (E.C. 1.13.11.21), which cleaves β-carotene at its central double bond to produce retinal in presence of oxygen [1,2]. The product of the reaction (retinal) is a direct precursor of both a) retinol (or vitamin A) by reduction through short-chain dehydrogenase and reductase activity and b) retinoic acid (active form of vitamin A) by irreversible oxidation through aldehyde dehydrogenase activity [3]. Thus, β-carotene 15,15′-dioxygenase is a key enzyme in the production of retinoids known to be involved in important biological functions such as vision, reproduction, and growth.

Although this enzyme is known for more than 40 years, its characterization on the molecular basis was done only recently. Indeed, during the last past year, three independent groups [4–7] were able to clone cDNA and to characterize the recombinant protein of the 15,15′-dioxygenase from *Drosophila melanogaster* [4], chicken [5], and mouse [6]. Furthermore, von Lintig et al. [7] clearly demonstrated that the encoded protein catalyzed vitamin A formation *in vivo* and the mutation in the resulting gene was responsible for the blindness of the *Drosophila* mutant *ninaB.*

β-Carotene 15,15′-dioxygenase has been extensively studied in various species, particularly in rat [1,2,8–13] and rabbit [13–15]. In rat the highest specific activity is in the

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0955-2863/01/$ – see front matter
PII: S0955-2863(01)00184-X

small intestine and next (in descending order) in liver, brain, lung, and kidney [1,12]. However, β-carotene cleavage data in nonprimates should be extrapolated to humans with caution; for example, the rat has greater β-carotene metabolism in enterocytes than do other species but does not transport intact β-carotene into lymph. The problem of establishing an appropriate animal model for carotenoid research relevant to humans was recently reviewed [16]; no single animal model completely mimics human absorption and metabolism of β-carotene.

Despite the importance of β-carotene 15,15′-dioxygenase in the formation of vitamin A, an essential nutrient for humans, studies of this enzyme in human tissues are limited. Lakshman et al. [17] first reported β-carotene cleavage to retinal by intestinal mucosa in autopsy samples of human neonates. We recently described that the TC7 clone of the human-derived intestinal cell line Caco-2 exhibited β-carotene 15,15′-dioxygenase activity and was able to convert β-carotene into vitamin A [18]. We also observed that vitamin A was esterified into retinyl esters in TC7 cells (unpublished data). Therefore, the TC7 clone can serve as an alternative model for the study of human β-carotene metabolism, particularly the mechanism of the enzymatic conversion and its regulation by other nutrients (e.g., trace minerals).

Earlier *in vitro* studies [1,19] reported that β-carotene 15,15′-dioxygenase activity was strongly inhibited by α, α′-dipyridyl and 1,10-phenanthroline, two effective chelators for ferrous iron (Fe^{2+}) and other metal ions. In addition, two different studies indicated that Fe^{2+} stimulated the dioxygenase activity when added to rabbit or guinea pig enzyme preparations [14,15]. These *in vitro* data suggested that the enzyme is metal dependent. Our recent *in vivo* data are also consistent with the hypothesis that iron is involved in the enzymatic β-carotene cleavage. Specifically, we reported that intestinal β-carotene 15,15′-dioxygenase activity was positively correlated with intestinal iron concentration in the rat [20,21]. Although these *in vitro* and *in vivo* data taken together suggest that the enzyme is metal dependent, no similar data were heretofore available for β-carotene 15,15′-dioxygenase in human tissues.

2. Materials and methods

2.1. Materials

Solvents were HPLC grade from Fisher Scientific (Pittsburgh, PA, USA). All-*trans* β-carotene (type IV), all-*trans* retinal, desferrioxamine mesylate (DFO), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *d*-α-Tocopherol was obtained as a generous gift from Dr. James Clark of Henkel (LaGrange, IL, USA). All-*trans* β-carotene was purified (>98.5%; *cis* isomers <1.5%) by passage through a neutral alumina column (Type WN-3, Sigma) in hexane and then stored with *d*-α-tocopherol (0.01 molar ratio) in hexane at –20°C. All-*trans* retinal was purified (>99%) by HPLC on a TSK gel SILICA 60 column, 4.6 × 250 mm (Tosoh Co.), with hexane/ethyl acetate (95:5, v/v) as the mobile phase.

2.2. Human tissue samples

Human tissue samples were collected from clinically living patients at the Department of Surgery of the Westchester Medical Center, Valhalla, New York. The study was approved by the Committee for the Protection of Human Subjects, New York Medical College, Valhalla. This committee is the institutional review board for the Westchester Medical Center. The approval stipulated that only tissue ordinarily discarded during the surgical procedure would be used. The tissues were immediately frozen at –20°C and then sent to our laboratory on ice. On arrival at the laboratory, the frozen samples were immediately stored at −80°C until used. On the day of the dioxygenase measurement, the sample was thawed and washed with cold NaCl solution (0.9 g/100 ml). Before the enzyme was prepared, the mucosa was scraped from each intestinal sample and kept on ice until analyzed.

2.3. Cell culture

The TC7 clone (passage 54) of the Caco-2 cell line was a generous gift from Dr. A. Zweibaum (Unite de Recherches sur la Differentiation Cellulaire Intestinale, INSERM U178, Villejuif, France). Cells were maintained as follows [22]: seeding density of 12×10^3 cells/cm^{2,} 7-day passage frequency, use of Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, 20% inactivated fetal bovine serum (FBS), and 1% nonessential amino acids (Gibco, Life Technologies Inc., USA). Cells were incubated at 37°C in a humidified atmosphere of air and carbon dioxide (95:5, v/v) and the medium was changed every 48 h using 10 or 20 ml for 25 or 75 cm^2 flasks, respectively (Corning Glassworks, Corning, NY, USA).

For each experiment, cells (in 75-cm^2 flasks) were grown on DMEM with 20% FBS and 1% nonessential amino acids for 1 week (up to confluency) and then maintained on serum-free medium for 2 weeks (15–17 days postconfluency), which yielded a differentiated cell monolayer showing the dioxygenase activity [18]. After a treatment period (≤72 h), medium was removed and cells were washed three times with 20 ml of the buffer A solution [50 mM *N*-(2-hydroxy-ethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES)-KOH buffer, pH 7.4, containing 154 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 mM dithiothreitol (DTT)]. Cells were then scraped in 10 ml of buffer A solution and centrifuged at 2000 rpm for 10 min (4°C). The resultant cell pellet was immediately used to make an enzyme preparation.

2.4. Enzyme preparation

The cell pellet (or tissue sample) in three (or five) volumes of buffer A solution was homogenized using a Potter-Elvehjem homogenizer at 600 rpm. The homogenate was then centrifuged (9000 *g* for 30 min). A fraction of the resultant supernatant S-9 was immediately subjected to a β-carotene dioxygenase activity assay as described below and analyzed for its protein concentration [23] using bovine serum albumin as standard. The rest of the supernatant S-9 was divided into portions and frozen at −80°C.

2.5. Enzyme assay

For human tissue samples the β-carotene dioxygenase assay procedure was identical to the method described previously for rat tissues [12]. The same procedure was used for cells with minor modifications as indicated below. Briefly, the reaction medium (final volume 0.36 ml instead of 0.5 ml) contained 3 nmol purified all-*trans* β-carotene, 40 μmol *N*-tris-(hydroxymethyl)-methylglycine (Tricine)-KOH buffer (pH 8.0), 0.2 μmol DTT, 0.3 mg Tween 40, 1.6 μmol sodium cholate, 20 nmol α-tocopherol, and the enzyme preparation (<1 mg protein). The reaction was initiated by adding 40 μl of 85 μM β-carotene suspension in Tween 40 and containing α-tocopherol. After incubation at 37°C for 60 min, the reaction was stopped by adding 50 μl of 37% (wt/wt) formaldehyde, followed by an incubation at 37°C for 10 min. Acetonitrile (500 μl) was added and insoluble substances were precipitated by centrifugation at 10,000 *g* for 5 min at 4°C. The resultant supernatant (200 μl) was directly subjected to HPLC analyses. The zero-time control containing both enzyme and substract followed the same procedure than assay, except that immediately after addition of β-carotene the reaction was stopped with formaldehyde.

For kinetic analyses (Km and Vmax determination), desired amounts (from 0.05 to 5 nmol) of β-carotene were incubated with enzyme preparation of either TC7 cells or human intestinal mucosa following the procedure described above. In these assays, only β-carotene concentration was varied, concentrations of other compounds (Tricine, DTT, sodium cholate, Tween 40 and α-tocopherol) present in the incubation mixture were kept constant.

2.6. HPLC conditions

Retinal formed in the enzyme assay was analyzed by HPLC using a system equipped with an 114M pump (Beckman Instruments, Inc., California, USA), a UV-970 ultraviolet and visible absorbance detector (Jasco, Tokyo, Japan), and a GOLD system for analyses (Beckman). Retinal was monitored at 380 nm and eluted with a retention time of 7.5 min on a TSK gel ODS-80Ts C18 reverse-phase column (5-μm particle size, 80-Å pore size, 4.6 × 150 mm) (Tosoh Co., Tokyo, Japan) attached to a precolumn (2 × 20 mm) of Pelliguard LC-18 (Supelco Inc., Bellefonte, PA, USA) with acetonitrile/water (90:10, v/v) containing 0.1% ammonium acetate as the mobile phase at a flow rate of 1.0 ml/min. Retinal formed was quantified from its peak area by using a standard curve prepared from purified all-*trans* retinal (0.2–50 pmol/200 μl injected). The limit of detection was 0.2 pmol/enzyme assay.

2.7. Effect of iron and desferrioxamine on β-carotene 15,15′-dioxygenase activity

Using cell culture system, differentiated TC7 cells at 15–17 days of postconfluency (passages 59–79) were incubated at 37°C with serum-free medium containing different concentrations of either iron (ferrous sulfate or ferric citrate: 0.1–4 mM) or desferrioxamine (DFO; 0.05–2 mM) up to 48 h. The control treatments were performed by incubation of TC7 cells with serum-free medium only. After each treatment, cells corresponding to two 75 cm^2 cell culture flasks were prepared into an enzyme preparation, assayed for β-carotene 15,15′-dioxygenase activity, and retinal formed was measured by HPLC. Both iron and DFO treatments did not affect cell viability, cell number as well as protein concentration in TC7 cells, compared to control treatment. Cell viability ranged between 75–85% and cell number between 4.10^5 and 6.10^5 cells/cm^2 for the three types of treatments (control, iron and DFO). In addition, independently of the treatment, the average of total cells and proteins recovered per 2 T75 flasks were 0.50 ± 0.08 g cells and 12 ± 1 mg proteins, respectively.

Using human intestinal mucosa preparations, various concentrations of either iron (0.1–1.5 mM) or DFO (0.25–3 mM) were directly added to the incubation mixture containing the enzyme preparation (<0.3 mg protein/assay) and incubated at 37°C for 1 h (enzyme reaction). To determine the type of inhibition by DFO, the enzyme preparation from small human intestinal mucosa was incubated with various amounts of β-carotene (from 0.25 to 15 μM) in presence or not of 1 mM DFO. Retinal formed in the incubation mixture was then analyzed by HPLC.

2.8. Statistical analysis

Results were analyzed by student's test.

3. Results and discussion

Our first objective was to investigate β-carotene 15,15′-dioxygenase activity in human tissues because few data are available in the literature. In the mid-1960s Goodman et al. [24] were the first to suggest the presence of β-carotene conversion by human enterocytes when they observed that 60–70% of total radioactive β-carotene ingested by patients was located in vitamin A esters of the lymph. However, only in 1993 did Lakshman et al. [17] report the enzymatic

Table 1
β-Carotene 15,15′-dioxgenase activity in different human tissues

Subject	Age	Sex	Diagnosis	Type of organ sample and size analyzed[a]	Storage[b] (wk)	Dioxygenase activity[c] (pmol/h.mg)
#1	57 y	M	Biliary structure	Small intestinal mucosa[d] (1.7 g)	19	152.9
#2	45 y	F	Gastric bypass	Small intestinal mucosa (5.4 g)	18	106.0
#3	89 y	M	Bladder cancer	Small intestinal mucosa (4.0 g)	8	91.9
#4	73 y	M	Colon cancer	Small intestinal mucosa (1.7 g)	9	41.2
#5	44 y	F	Obstructing pancreatic mass	Small intestinal mucosa (2.8 g)	19	95.0
#6	17 mo	F	Traumatic brain injury	Small intestinal mucosa (3.3 g)	27	20.3
#7	62 y	M	Dysplasia of esophagus	Stomachal mucosa (0.8 g)	14	n.d.[e]
#8	19 y	M	Trauma	Liver (9.2 g)	8	61.6
#9	63 y	M	Colorectal cancer	Liver[a] (9.9 g)	4	7.2

[a] Discarded surgical noncancerous tissues, except the liver of subject 9 was abnormal (cancer). All tissue samples were from clinically living patients and immediately frozen at −20°C.

[b] Duration of storage (at −20°C and −80°C) between the sample collection and the enzyme analysis.

[c] β-Carotene 15,15′-dioxygenase assay was determined using an enzyme preparation made from each sample as described in Materials and Methods. Values are means of triplicate enzyme analysis.

[d] The average ratio of scraped intestinal mucosa per small intestine sample was 0.40 ± 0.11 g/cm (n = 6 samples).

[e] n.d. = not detected.

conversion of β-carotene to retinal by intestinal enzyme preparations of autopsy samples from 14 premature infants (aged 3–60 days) and a fresh intestinal biopsy sample of one adult. In that study β-carotene 15,15′-dioxygenase activity varied widely: from 0 to 1210 pmol retinal formed/h.mg of protein in the samples from infants (mean ± SD, 123 ± 304 pmol/h.mg, n = 14) and 350 pmol/h.mg in the adult sample [17].

The present investigation provides additional data concerning the enzyme activity in the small intestine of five adults (aged 44–89 y) and one infant (aged 17 months). Also for the first time, the activity in liver tissue of two subjects (aged 19 and 63 y) is provided (Table 1). β-Carotene 15,15′-dioxygenase activity in the adults' small intestinal mucosa ranged from 41.2 to 153 pmol/h.mg (mean ± SD, 97.4 ± 39.8 pmol/h.mg); the lowest activity (20 pmol/h.mg) was found in the infant's mucosa (Table 1). This latter value was similar to values measured previously in intestinal samples of neonates, with the assumption that the one very high activity in the previously reported study (1210 pmol/h.mg) was an aberrant value [17]. Indeed, exclusion of that value decreased the mean ± SD to 40 ± 39 pmol/h.mg for 13 infants [17]. The data could suggest that the intestinal dioxygenase activity is age dependent, but additional analyses of samples would be necessary before such a definitive conclusion could be reached.

No dioxygenase activity was detected in human stomach in the single sample from a 62-y-old patient (Table 1). In previous studies [11,25] describing the enzyme distribution along the stomach-colon axis of the rat, the dioxygenase activity was mainly located in the upper half of small intestine (i.e., jejunum and duodenum); in contrast with the jejunum exhibiting the highest activity, no activity was detected in rat stomach or cecum. The fact that the enzyme is not uniformly distributed along the small intestine may contribute to variations observed among the values of intestinal dioxygenase activity determined for the six subjects (Table 1); only a section of small intestine (5–12 cm) was available and analyzed for the dioxygenase activity. In addition, as previously reported [17], the time between the subject's death and the enzyme assay may be an important factor, perhaps resulting in a decrease of the dioxygenase activity and thus an underestimation of the enzyme activity. To minimize that possible source of activity loss here, all tissue samples were from living patients and were immediately frozen at –20°C and then transferred to −80°C until they were analyzed. The intestinal dioxygenase activity was not correlated with the duration of storage at –20°C/−80°C (r = 0.012, p = 0.83, n = 6 intestinal samples) (Table 1).

To our knowledge, this is the first report of β-carotene 15,15′-dioxygenase activity in human liver: 7 and 62 pmol/h.mg for the two adult subjects (Table 1). The lower value was for a liver showing gross pathology and thus cannot be assumed to be a normal level.

When expressed as total activity, β-carotene 15,15′-dioxygenase was 2.75 ± 0.85 nmol/h per gram of intestinal mucosa, or 1.27 ± 0.53 nmol/h per linear centimeter of small intestine (mean ± SD, n = 5 adult subjects) (data not shown). On the basis of its length, the upper half of small intestine (about 3 m) from a human adult is estimated to produce approximately 9 μmol retinal/24 h and has the capacity to cleave 4.5 μmol β-carotene/24 h if the molecular cleavage ratio is 1:2 (β-carotene:retinal) as demonstrated previously [10,26]. Thus, the maximum intestinal capacity for β-carotene cleavage approximates 5 μmol β-carotene (or 2.5 mg) per day for an adult. Similarly, in terms of total activity, the normal liver of an adult (about 1.4 kg) can produce a calculated 1.2 nmol/h per gram of liver, or 39 μmol retinal/24 h per total liver; this is four times the capacity of the small intestine. Finally, the two organs combined (liver plus small intestine) have an estimated β-carotene cleavage capacity of 24 μmol/day (or 12 mg/

Table 2
Comparison of β-carotene 15,15′-dioxygenase activities in human intestinal TC7 cells and in human small intestinal mucosa samples

Characteristics of the enzyme	Vmax[a] (pmol retinal/h · mg)	Km[a] (μM)
TC7 cells[b]	23.8	1.57
Small intestinal mucosa[c] (n = 5 adults)	112.1 ± 45.4	2.35 ± 0.73

[a] Apparent Vmax and Km values of β-carotene 15,15′-dioxygenase were determined by plotting inverse Lineweaver-Burk representations (1/specific activity = f[1/substrate concentration]).

[b] The TC7 clone was isolated from the human adenocarcinoma colon cell line Caco-2 in the laboratory of Dr. A. Zweibaum, INSERM U178, Villejuif, France [22].

[c] Values are means ± SD of the five adult patients (subjects 1–5 in Table 1).

day); this amount is markedly higher than the average daily dietary intake in the United States of 1.5 mg β-carotene/day [27]. Although it is well known that humans do not efficiently convert β-carotene to vitamin A in enterocytes (most of dietary β-carotene is absorbed intact), our data indicate that human liver has a large capacity for metabolizing β-carotene.

We recently reported the presence of β-carotene 15,15′-dioxygenase activity in the highly differentiated TC7 clone of the human intestinal cell line Caco-2 [18]. Thus, it was possible to compare the activities of human intestinal cells (TC7) and human small intestinal mucosa by applying the Michaelis constants (apparent Vmax and Km) to further assess whether TC7 cells might serve as a relevant model for human β-carotene metabolism. The data show that the enzyme in the transformed TC7 cells exhibited an apparent Vmax value nearly 5 times (4.7 times) less than the mean apparent Vmax value of the enzyme in adult small intestine (n = 5; Table 2). That difference could be due to the colonic origin of Caco-2 cells. Indeed, colonic tissue does not exhibit the dioxygenase activity in normal intact animals [11, 25]. Although the speed of retinal formation from β-carotene in TC7 cells was much lower than in small intestinal mucosa preparations, the apparent affinity of the enzyme for the substrate (β-carotene) was not significantly different for these two human intestinal models; apparent Km values were 1.6 and 2.4 μM, respectively (Table 2).

Our second objective was to examine the possible iron dependency of β-carotene 15,15′-dioxygenase activity in human intestine by using the two human models available in our laboratory. Dioxygenase activity was shown to be iron dependent in various species *in vitro*—by adding metal chelators [1,19] or iron [14,15] directly to the enzyme preparation (in test tubes)—and *in vivo*—by varying iron metabolism [20,21]. However, no data are available for the human β-carotene cleavage. Therefore, in this study the effect of iron on the dioxygenase activity was examined by using the two human *in vitro* models (TC7 cells and intestinal tissue preparations). The data indicate that in TC7 cells the dioxygenase activity (pmol/h. 2 T75 flasks) increased with increasing iron concentrations in cell culture medium up to 1 mM and plateaued for iron concentrations higher than 1 mM (Fig. 1A). More specifically, at 1 mM ferrous sulfate concentration, the enzyme activity was enhanced by approximately two times. Similar curves were obtained when the dioxygenase activity was expressed as either pmol/h. mg of proteins or pmol/h.g of cells (data not shown). Although the range of iron concentrations (0.5–2 mM) used in this study was estimated around 50-times the physiological level of iron found in the human gut (assuming a daily intake of 10 mg), these concentrations were in agreement with previous iron concentrations reported to have an effect on β-carotene 15,15′-dioxygenase activity [14,15]. Indeed, a maximum activity was observed with ferrous sulfate at the final concentration of 1 mM [14] or 0.5 mM [15], when using enzyme preparations of either guinea pig or rabbit.

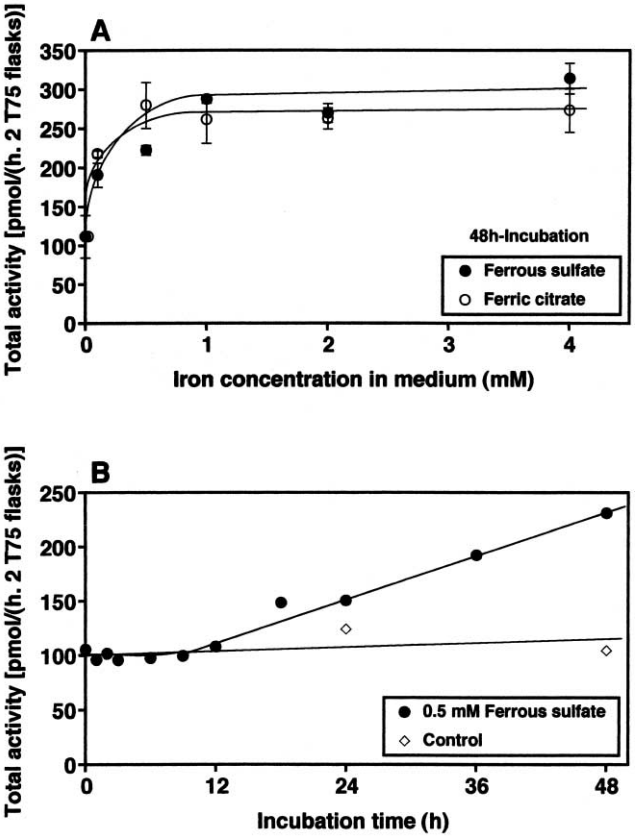

Fig. 1. Effects of iron on β-carotene 15,15′-dioxygenase activity of the TC7 cells (derived from the human intestinal cell line Caco-2) in relation to (A) increasing concentrations of ferrous sulfate (Fe^{2+}) or ferric citrate (Fe^{3+}) for 48 h incubation or (B) various incubation times with 0.5 mM of ferrous sulfate. Data in graph A are means (± range) of two independent experiments. Data in graph B are means of duplicate enzyme assays from two combined experiments.

These two studies [14,15] also reported that Fe^{2+} but not Fe^{3+} enhanced the dioxygenase activity. In contrast, under our experimental conditions using TC7 cells, ferric citrate (Fe^{3+}) resulted in an increase of the dioxygenase activity, probably because the iron state was interchangeable (Fe^{3+}

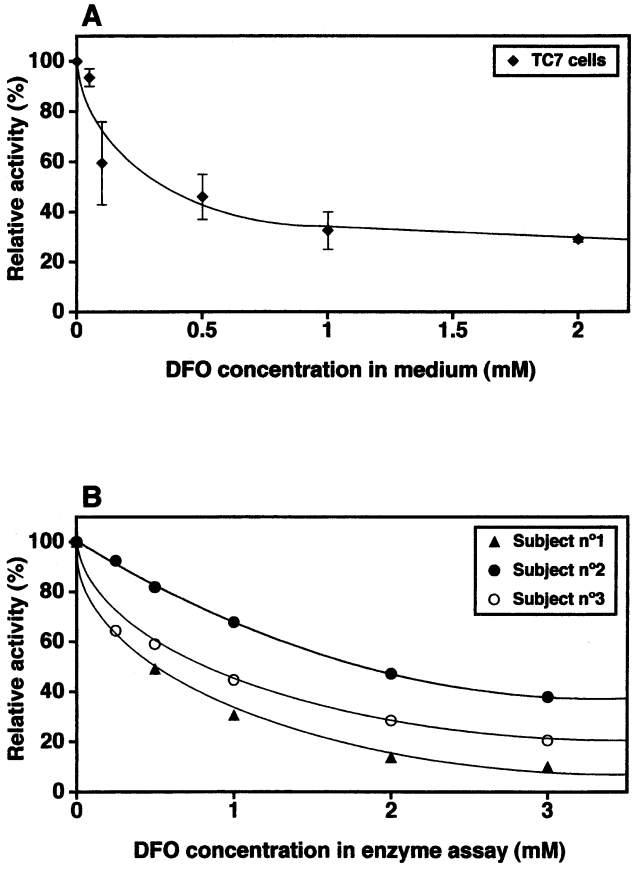

Fig. 2. Effect of increasing desferrioxamine mesylate (DFO) concentrations on β-carotene 15,15′-dioxygenase activity from either (A) the TC7 clone of the human intestinal cell line Caco-2 or (B) human small intestinal mucosa (subjects 1–3, see Table 1). Data in graph A are means (± range) of two independent experiments and values of each experiment were means of duplicate enzyme assays. Data in graph B are means of duplicate enzyme assays.

could be readily reduced to Fe^{2+}) in the cell culture medium. Indeed, the apical surface of Caco-2 cells exhibits a ferric reductase that reduces Fe^{3+} to Fe^{2+}, process required for Fe^{3+} uptake [28,29]. Moreover, the dioxygenase activity increased linearly with increasing incubation time (up to 48 h) in the presence of a fixed ferrous sulfate concentration (0.5 mM) in the cell culture medium (Fig. 1B). However, there was a period of latency (up to 12 h) for which Fe^{2+} did not change the dioxygenase activity. As discussed later (see below), this latency period may explain why we did not observe any significant effect on the dioxygenase activity when ferrous sulfate (from 0.1 to 1.5 mM) was added to the reaction mixture containing the intestinal enzyme preparations for subjects 5 and 6, followed by an incubation of 1 h at 37°C (data not shown).

To confirm the participation of iron in the enzymatic cleavage of β-carotene, the effect of desferrioxamine (DFO) on the dioxygenase activity was also investigated by using the two human *in vitro* models. DFO, known as a strong iron chelator and first introduced in the 1960s for treating iron overload, is the only agent presently licensed for clinical use in most countries [30]. The data demonstrate that β-carotene 15,15′-dioxygenase activity in TC7 cells was decreased with increasing DFO concentrations in the cell culture medium (Fig. 2A). A 50% inhibition of the enzyme activity was obtained in presence of 0.5 mM DFO; inhibition plateaued at 70% for DFO concentrations higher than 1 mM (Fig. 2A). Similar curves of inhibition were obtained for intestinal enzyme preparations of three human subjects (Fig. 2B). Under these experimental conditions, 50% inhibition of the dioxygenase activity required DFO concentrations of 0.5–2 mM depending on the subject. These inhibition curves obtained with DFO (Figs. 2A and 2B) were comparable to those reported with 1,10-phenanthroline and α, α′-dipyridyl and for a similar range of concentrations (0.2–1.5 mM) [19]. In contrast, Nagao et al. [31] reported that DFO as well as α, α′-dipyridyl did not affect the dioxygenase activity, but the final concentration tested was lower (0.1 mM). Thus, in both *in vitro* models derived from human intestine, DFO was an efficient inhibitor of the dioxygenase activity. Finally, the type of inhibition by DFO was determined by using the enzyme preparation from two subjects and varying β-carotene concentration with and without 1 mM DFO in the incubation reaction (Fig. 3). The Lineweaver-Burk representations revealed that DFO was a noncompetitive inhibitor (Vmax markedly reduced and Km unchanged).

Several independent groups [1,14,15,19] agreed with the fact that β-carotene 15,15′-dioxygenase activity was strongly inhibited by two effective chelators of ferrous sulfate (α, α′-dipyridyl and 1,10-phenanthroline); a 50% inhibition was found at the final concentrations of 0.5–1 mM for both chelators. Therefore, we postulated that β-carotene 15,15′-dioxygenase activity is inhibited by DFO as a result of its iron-chelating property. To investigate this hypothesis, we examined whether adding ferrous sulfate could reverse the inhibitory effect of DFO on the enzyme activity. The data clearly demonstrate that after a 24 h incubation with 0.5 mM DFO (activity reduced by 50%), adding 1 mM (or 2 mM) ferrous sulfate to the cell culture medium followed by another 24 h incubation restored the initial enzyme activity in TC7 cells (Fig. 4). Thus, the inhibitory effect of DFO was reversible by the addition of iron, indicating that DFO may act on the dioxygenase activity by reducing iron availability in the cell culture medium. However, adding 1 mM ferrous sulfate to an incubation mixture containing the enzyme preparation of subject 5 did not reverse the 50% inhibition of 1 mM DFO on the enzyme activity (data not shown), probably for one of the reasons indicated below.

In summary, using the TC7 cells, we found that β-carotene 15,15′-dioxygenase activity was activated by iron, inhibited by DFO, and that DFO inhibition was reversed by iron supplementation. We also reported an inhibition of the dioxygenase activity by DFO when using enzyme preparations from human intestinal mucosa. However, the addition of iron in the incubation mixture containing an enzyme preparation from human intestine did not result in an in-

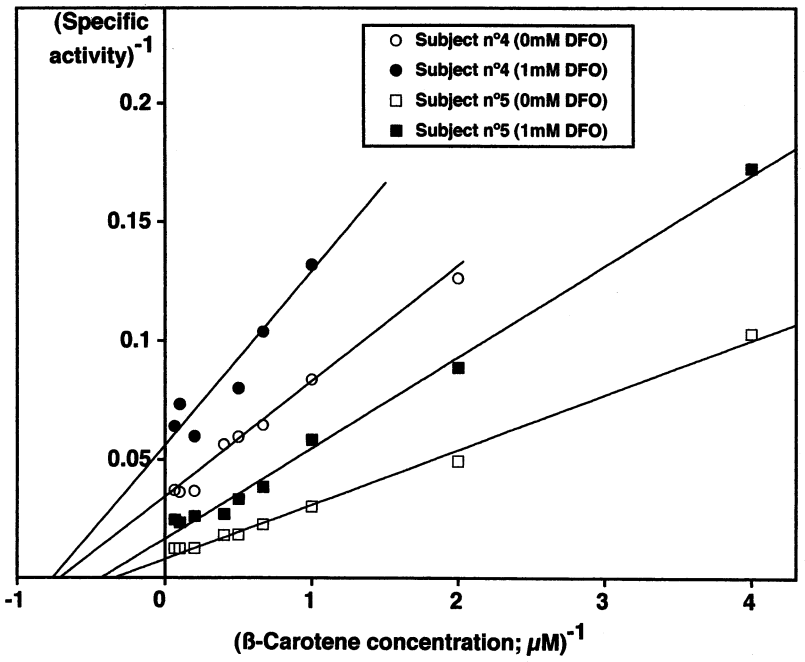

Fig. 3. Characterization of the type of inhibition by DFO on β-carotene 15,15′-dioxygenase activity from human small intestines (subjects 4 and 5, see Table 1) using inverse Lineweaver-Burk representation. Each value is the mean of duplicate enzyme assays.

crease of the dioxygenase as well as it did not reverse the effect of DFO. These different observations suggest that the process of activation of the enzyme by iron may either: a) require some time-dependent events occur (as suggested by the 12h lag time observed in TC7 cells), such an event might be the fixation of iron on the enzyme protein as recently speculated [21], b) require the participation of another compound, which may be absent in the enzyme preparations but present in TC7 cells, or c) be dependent on the enzyme assay used, since two earlier studies [14,15] were able to show (in test tube) an activation of the dioxygenase activity by ferrous sulfate when using a different enzyme assay. In contrast, the inhibition of the enzyme by DFO could involve a more simple mechanism by neutralizing iron already in place around the enzyme protein.

In conclusion, this study provides the first data concerning β-carotene 15,15′-dioxygenase activity in human small intestine and liver from clinically living subjects. These results clearly demonstrate that DFO is an efficient inhibitor of the dioxygenase activity in human intestine. The data suggest that DFO acts as iron chelator, supporting the hypothesis that β-carotene 15,15′-dioxygenase activity in human tissues is iron dependent. Finally, the TC7 clone of the human intestinal cell line Caco-2 is a relevant model to study intestinal β-carotene cleavage and its regulation.

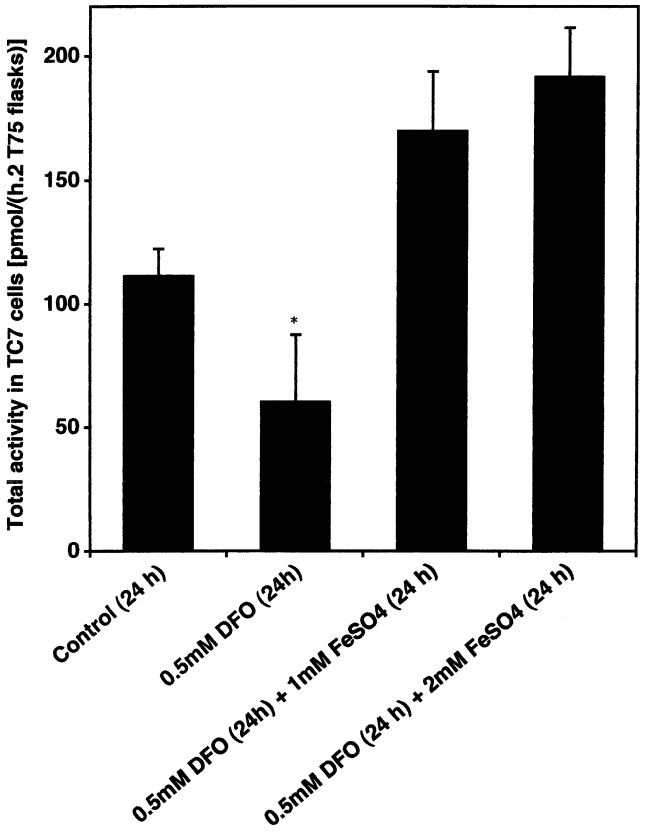

Fig. 4. Effect of DFO associated with effect of iron (ferrous sulfate) on β-carotene 15,15′-dioxygenase activity of TC7 cells. Differentiated TC7 cells were incubated at 37°C with serum-free medium containing 0 or 0.5 mM DFO for 24 h, followed by a second incubation at 37°C in presence of either 1 or 2 mM ferrous sulfate for 24 h. Values are means (± SD) of three independent experiments. * $P < 0.05$ compared to control.

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