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Biosynthesis of retinoic acid from β-apo-14′-carotenal in ferret in vivo

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To determine whether β-apo-14′-carotenal (an excentric cleavage product of β-carotene) can serve as a source of intestinally derived retinoic acid, β-apo-14′-carotenal or retinal in micellar solution was perfused through 60-cm small bowel segments of ferrets in vivo. Both β-apo-14′-carotenoic acid and retinoic acid were identified in the ferret intestinal mucosa by comparing retention times in HPLC and by ultraviolet/visible spectra. The in vivo perfusion of ferret intestine with 10 μM β-14′-carotenal for 2 hr resulted in formation of β-apo-14′-carotenoic acid (218 ± 28 pmol/g) and retinoic acid (51 ± 4 pmol/g). Similarly, a 2-hr intestinal perfusion of 1 μM retinal resulted in formation of retinoic acid (30 ± 2 pmol/g). When coperfusing an inhibitor of retinal oxidation, 2 mM citral, retinoic acid was not detected in the intestinal mucosa after the perfusion of 1 μM retinal. However, retinoic acid (30 ± 3 pmol/g) was still formed from the intestinal perfusion of β-apo-14′-carotenal with 2 μM citral. Furthermore, retinol was formed after the intestinal perfusion of β-apo-14′-carotenal and increased markedly in the presence of citral. This study lends support for an excentric cleavage mechanism in the metabolism of β-carotene into retinoic acid in vivo. (J. Nutr. Biochem. 8:652–657, 1997) © *Elsevier Science Inc. 1997*

Keywords: retinoic acid; retinol; β-apo-carotenal; β-apo-carotenoic acid; β-carotene; ferret

Introduction

Among more than 500 carotinoids in nature, β-carotene has been well demonstrated, both in vitro and in vivo, to be the most active precursor of vitamin A.[1] Retinoic acid has been suggested to function as a key regulator of gene expression.[2] A number of studies have shown that β-carotene can generate retinoic acid, involving both central and excentric cleavage pathways for β-carotene metabolism.[3–6] Recently, 9-*cis*-β-carotene has been demonstrated both in vitro[7,8] and in vivo[9] to be a precursor of 9-*cis*-retinoic acid which is a ligand for both RAR and RXR nuclear receptors, and as such, play a role in regulating cell function. The ability of β-carotene to serve as a precursor to retinoic acid and determining whether a pathway exists for retinoic acid synthesis that does not require retinol as a substrate are prerequisites for understanding the metabolic relationship between carotenoids and retinoids.[3] Given that carotenoids provide about half of the daily vitamin A intake in the form of carotenoids in developed societies, and most of the total vitamin A intake in the form of carotenoids in developed societies, and most of the total vitamin A intake in the less developed societies of Africa and Asia, understanding of the mechanism(s) of carotenoid conversion into retinol and retinoic acid is an important issue.

There are at least two mechanisms for the biosynthesis of vitamin A (retinol) and retinoic acid from β-carotene.[10–12] The central cleavage pathway utilizes a 15,15′-dioxygenase to yield two molecules of retinal (retinaldehyde), which has been reconfirmed in a recent study.[13] Retinal in turn can be oxidized to retinoic acid.[14] This pathway also has been accepted for the formation of vitamin A from other provitamin A carotenoids. On the other hand, the excentric cleavage mechanism yields a series of β-apo-carotenals of different chain lengths,[15,16] which subsequently are oxidized to their corresponding β-apo-carotenoic acids. By a process analogous to fatty acid β-oxidation, β-apo-carotenoic acids are converted stepwise to retinoic acid.[5] After incubation of intestinal homogenates or intestinal perfusion

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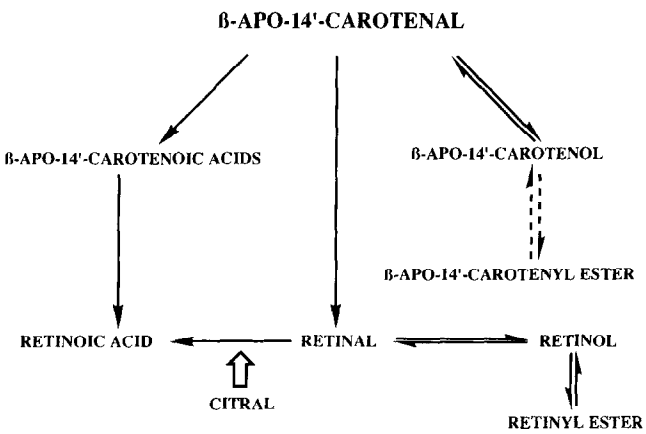

Figure 1 Proposed metabolic pathway of β-apo-14′-carotenal.

with β-carotene and citral (which inhibits the oxidation of retinal to retinoic acid), it was found that citral did not prevent the formation of β-apo-carotenals and retinoic acid.[4,6] These findings suggest that, in vitro, retinoic acid can be produced from excentric cleavage of β-carotene via a series of β-apo-carotenals. However, the biosynthesis of retinoic acid via β-apo-carotenals, which are products of β-carotene excentri cleavage, has not been demonstrated in the living animals.

The present study was undertaken to demonstrate that β-apo-14′-carotenal can be metabolized to retinoic acid in vivo during intestinal perfusion of the ferret despite the inhibition of retinal dehydrogenase by citral.

Methods and materials

Chemicals

All-*trans*-retinal, all-*trans*-retinol, citral, DMSO, α-tocopherol, retinyl acetate, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO USA). All-*trans*-β-apo-14′-carotenal, was gift from Drs. K. Schiedt and U. Hengartner of Hoffmann-La Roche, Inc. (Basel, Switzerland). Substrates were purified by passage through a 7% water-weakened alumina column or by HPLC, as described below.

Animals

Adult male ferrets (*Mustela putorius furo*, 900 to 1600 g, Marshall Farms, North Rose, NY USA) were housed in an AAALAC accredited animal facility at the USDA-Human Nutrition Research Center (HNRC) at Tufts University. Surgically manipulated ferrets were used for the perfusion study. The studies were acute in nature and the animals were killed 4 hr post surgery. Surgical procedures in the experiments were approved by the Committee on Animal Care at USDA-HNRC.

Preparation of micellar solutions

All-*trans*-β-apo-14′-carotenal and all-*trans*-retinal, dissolved in 0.5 mL DMSO, were prepared under red light in a mixed micellar solution containing 2.5 mM oleic acid and 10 mM sodium taurocholate in Krebs phosphate buffer at pH 7.0.[17] The micellar solution was formed by sonication for 15 min at 80 W of power before the perfusion.

Surgical and intestinal perfusion procedure

The surgical and perfusion procedures were the same as described previously with certain modifications.[17] The modifications were

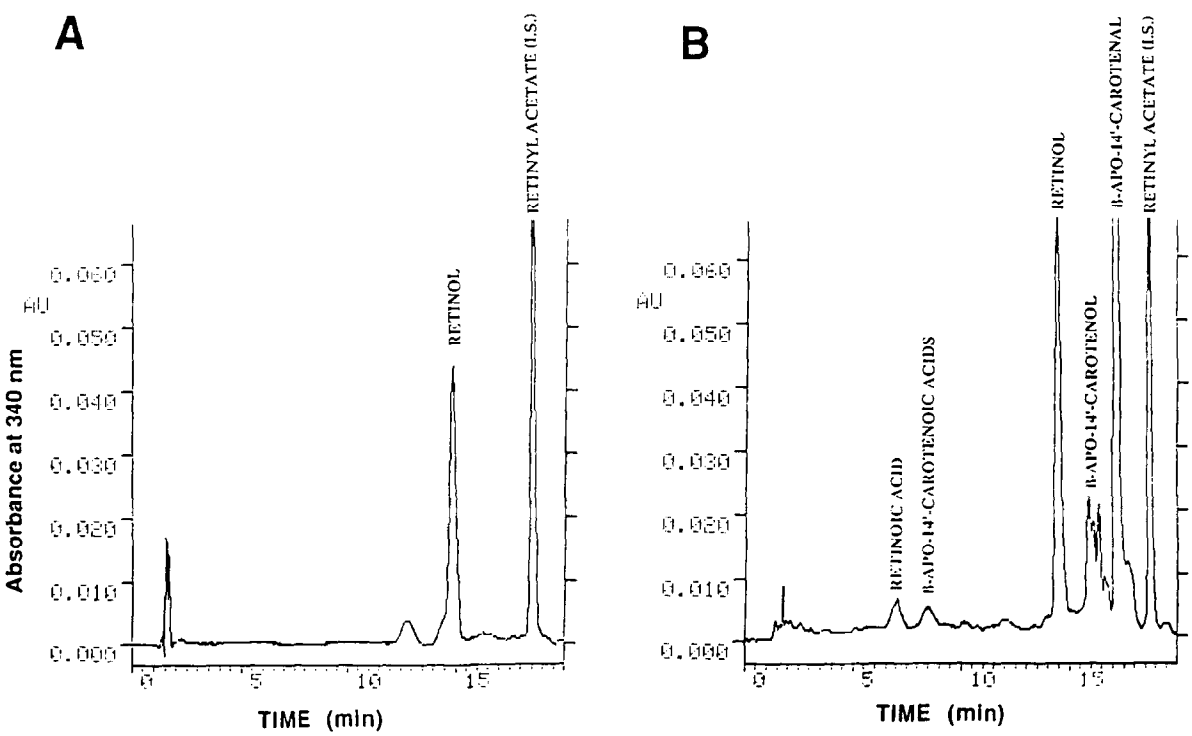

Figure 2 HPLC profile of the intestinal perfusion without β-apo-14′-carotenal (**A**) and with 10 μM β-apo-14′-carotenal (**B**) in the ferret.

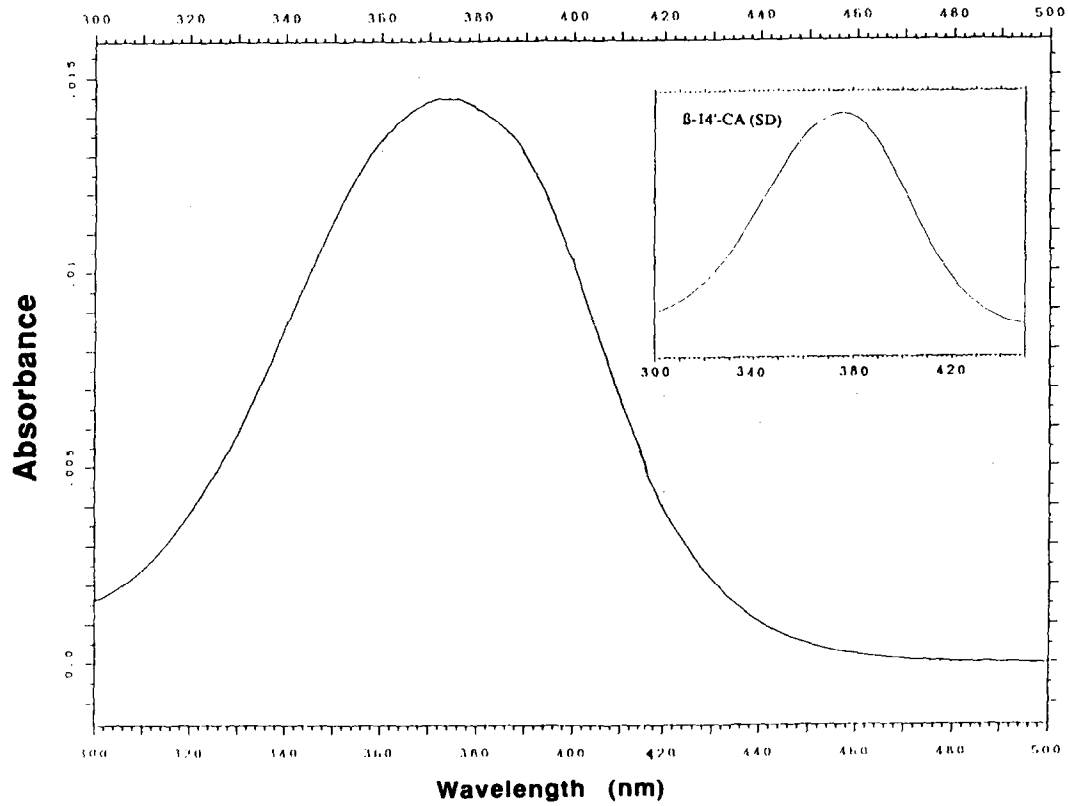

Figure 3 The spectrum of the compound derived from the intestinal perfusion of β-apo-14′-carotenal. The *inset* is the spectrum of authentic β-apo-14′-carotenoic acid.

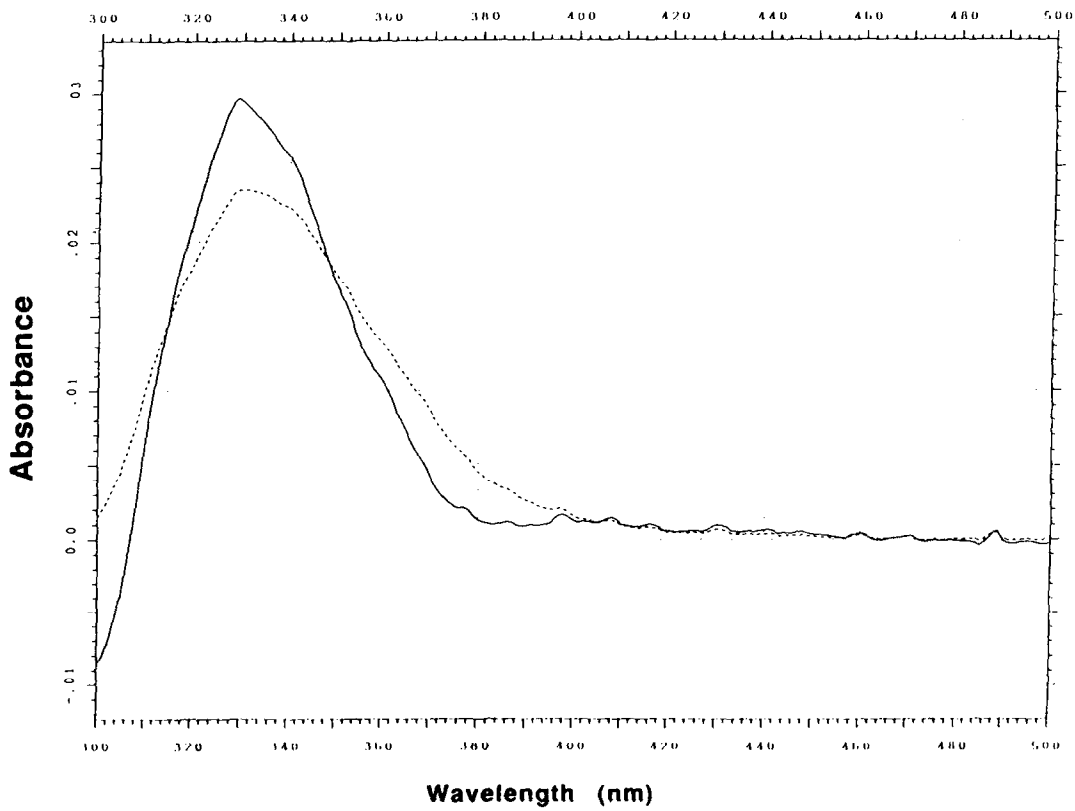

Figure 4 The spectrum of the compound (*solid line*) derived from the intestinal perfusion of β-apo-14′-carotenal and β-apo-14′-carotenol standard (*dashed line*).

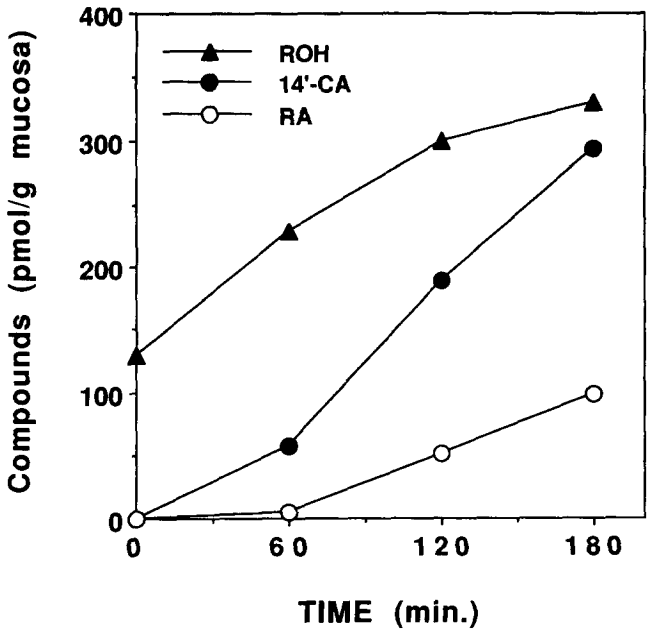

Figure 5 Effect of perfusion time on the formation of either retinol, β-apo-14′-carotenoic acid and retinoic acid after the intestinal perfusion of β-apo-14′-carotenal in ferrets. Data are average of two separate experiments.

that the perfused length of the intestine was 60 cm, the perfusion rate was 0.5 mL/min, and the perfusion time was 2 to 3 hr. We perfused either β-apo-14′-carotenal (10 μM), and retinal (1 μM) with or without citral (2 mM). After perfusion, the animals were killed by puncturing the abdominal aorta under deep isoflurane anesthesia. The perfused intestinal segment was removed, freed of its mesentery and serosal fat, and weighed. The intestinal mucosa was scraped with a glass slide, and homogenized (Brinkmann (Westbury, NY USA) Polytron™) with ice-cold Hepes buffer and methanol (v:v = 2:1). After the intestinal scrapings were collected, the intestinal segments were suspended for 24 hr of drying with a 5 g weight tied to one end to insure a constant degree of stretching. At the end of the drying period, the length of each segment was recorded and there was no difference between the groups on the perfused length of intestine.

Extraction and HPLC assays

The samples (serum and intestinal mucosal scrapings) were extracted as follows: 100 μL of an ethanolic solution of 0.5 *N* KOH was added to either 0.8–2.0 ml of serum or 0.5 g of intestinal mucosal scrapings, followed by the addition of the internal standard, retinyl acetate in 100 mL of ethanol. The metabolites were extracted by adding 2 mL hexane, and the mixture was then centrifuged for 3 min at 320 g at 4°C. The hexane layer was removed and the residue was acidified by adding 50 μL 6 *N* HCl. A second extraction was performed with 2 mL hexane. The two extractions were pooled, dried under N_2, and resuspended in 50 μL ethanol for injection in the HPLC system described below. A gradient reverse-phase HPLC system described earlier[8] for the analysis of retinoids and carotenoids was used with minor modifications. The gradient procedure at a flow rate of 1 mL/min was as follows: 100% solvent A (acetonitrile (CH_3CN) : tetrahydrofuran (THF) : water = 50:20:30, v/v/v, with 0.35% acetic acid and 1% ammonium acetate in water) 3 min, followed by a 6 min linear gradient to 40% solvent A and 60% solvent B (CH_3CN : THF : water = 50:44:6, v/v/v, with 0.35% acetic acid and 1% ammonium acetate in water), a 12-min hold at 40% solvent A/60% solvent B, then a 7-min gradient back to 100% solvent A. Individual carotenoids and retinoids were identified by co-elution with standards, and quantified relative to the internal standard (retinyl acetate), by determining peak areas calibrated against known amounts of standards. In this HPLC system, retinoic acid, β-apo-14′-carotenoic acid, retinol, β-apo-14′-carotenol, β-apo-14′-carotenal and retinyl acetate eluted at 6.80 min, 8.6 min, 14.3 min, 15.6 min, 17.3 and 18.6 min, respectively. For analysis of retinyl esters, a gradient reverse-phase HPLC system described earlier[18] was used.

Statistical analysis

Results were expressed as means ± SEM and the significance of each of metabolites were compared between the groups using the Student's *t* test at $P < 0.05$.

Results and discussion

Retinoic acid produced from β-carotene represents one third of the total retinoids formed in vitro,[19] even when citral (an inhibitor of oxidation of retinal) was added to the incubation mixture.[4] Because earlier studies have only shown that β-apo-carotenals, formed from the cleavage of β-carotene, are rapidly oxidized to the corresponding β-apo-carotenoic acids in both animal[15,16] and humans,[20] in the present in vivo study, we demonstrated that both retinoic acid and retinol can be produced in the ferret after intestinal perfusion with β-apo-14′-carotenal (*Figure 1*).

Reverse-phase HPLC analyses of extracts of ferret intestinal mucosa after the perfusion of β-apo-14′-carotenal are

Table 1 Retinoids and β-apo-14′-carotenoic acid in ferret intestinal mucosa after perfusion of 10 μM of β-apo-14′-carotenal and 1 μM of retinal in the absence or presence of 2 mM citral for 2 hr

	β-apo-14′-carotenal		Retinal	
pmol/g mucosa	−citral	+citral	−citral	+citral
Retinoic acid	51 ± 4	30 ± 3	30 ± 2	ND
β-Apo-14′-carotenoic acid	218 ± 28	245 ± 37	ND	ND
Retinol	329 ± 11	884 ± 10*	896 ± 35	2120 ± 57*
Retinyl esters	1523 ± 17	1655 ± 43	4035 ± 186	6938 ± 192

Values (mean ± SEM) are expressed in pmol/g mucosa ($N = 5$).
*Significantly different at $P < 0.05$ (−citral vs. +citral).

shown in *Figure 2*. As seen in *Figure 2A*, only retinol was detected in the ferret intestinal mucosa after perfusion of micellar solution alone. However, the retinol concentration increased in the ferret intestinal mucosa (*Figure 2B*) after the perfusion of 10 μM β-apo-14′-carotenal for 2 hr. This indicated that the conversion of β-apo-carotenal to vitamin A is by oxidative cleavage of the central 15, 15′ double bond. An additional three peaks were detected in the ferret intestinal mucosa (*Figure 2B*) after the perfusion of 10 μM β-apo-14′-carotenal for 3 hr. Peak 1 comigrated with authentic retinoic acid, and showed maximal absorbance at 340 nm, which has been identified as retinoic acid as described in our previous paper.[6,9] Peak 2 comigrated with authentic samples of β-apo-14′-carotenoic acid, and showed maximal absorbance at 376 nm (*Figure 3*), which is identical with authentic β-apo-14′-carotenoic acid (*Figure 3*, inset). Peak 3 co-eluteed with β-apo-14′-carotenol, with maximal absorbance at 330 nm, which is similar to the standard β-apo-14′-carotenol (*Figure 4*). These data confirm earlier studies that β-apo-carotenals, formed from cleavage of β-carotene, are rapidly metabolized to the corresponding β-apo-carotenoic acids, and for the first time, show that retinoic acid can be produced from β-apo-carotenal in vivo.

In two separate experiments, the intestinal mucosa was sampled at every hour after the perfusion of β-apo-14′-carotenal (*Figure 5*). It can be seen that formation of retinol increased linearly during the first 2 hr of perfusion, and then reached a plateau. In contrast, β-apo-14′-carotenoic acid increased linearly throughout whole perfusion period. The formation of retinoic acid increased also linearly; however, there was a lag period in the formation of retinoic acid (*Figure 5*), which suggests that β-apo-14′-carotenoic acid is a precursor of retinoic acid. This in vivo observation is in agreement with our in vitro study that β-apo-carotenoic acids can undergo a type of β-oxidation to form retinoic acid.[5]

To provide further evidence that β-carotene may be a significant precursor of retinoic acid in vitro in a process in which retinal is probably not involved as an intermediate, as indicated by Napoli and Race,[3] we coperfused retinal or β-apo-14′-carotenal with citral, which is an inhibitor of retinal oxidation. The in vivo perfusion of ferret intestine with 10 μM β-14′-carotenal and 1 μM retinal for 2 hr resulted in formation of retinoic acid (51 ± 4 pmol/g, 30 ± 2 pmol/g, respectively). When co-perfusing citral and retinal together, retinoic acid was not detected in the intestinal mucosa (*Table 1*). However, retinoic acid was still formed (30 ± 3 pmol/g) from the intestinal perfusion of β-apo-14′-carotenal with 2 μM citral (*Table 1*). This indicates that the retinoic acid produced by the excentric cleavage pathway of β-apo-14′-carotenal represents most of the retinoic acid formed in vivo.

Retinol increased significantly in the presence of citral (*Table 1*) after intestinal perfusion of β-apo-14′-carotenal, apparently because of blocking retinal oxidation. The intestinal accumulation of retinol caused by perfusion of β-apo-14′-carotenal with citral was almost 6-fold higher than baseline (*Figure 5*) which probably reflects the activity of the 15, 15′-dioxygenase enzyme. We did not see any difference in systemic plasma concentration of retinol and retinyl esters in the ferrets before and after intestinal perfusion of β-apo-14′-carotenal (data not shown), perhaps because of vitamin A homeostastic mechanisms and/or the fact that the intestinal perfusion time was only 2 to 3 hr.

Our data lend support to the idea that an excentric cleavage mechanism is involved in the metabolism of β-carotene and β-apo-carotenals into retinoic acid in vivo.

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