Formation of all-*trans*-retinoic acid and 13-*cis*-retinoic acid from all-*trans*-retinyl palmitate in humans

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*Increments of levels of both 13-*cis- *and all-*trans-*retinoic acid in human plasma were observed after either a physiologic or a pharmacologic oral dose of all-*trans-*retinyl palmitate. Subjects receiving a physiologic dose showed mean ± SEM plasma rises over baseline as follows: all-*trans-*retinoic acid = 1.1 ± 0.3 nmol/L and 13-*cis-*retinoic acid = 4.7 ± 1.1 nmol/L, which represented increases in 1.3 fold and 1.9 fold over fasting plasma levels. Those receiving a pharmacologic dose showed mean ± SEM plasma rises over baseline as follows: all-*trans-*retinoic acid = 11.5 ± 2.6 nmol/L and 13-*cis-*retinoic acid = 37.5 ± 6.1 nmol/L, which represented increases of 3.9-fold and 8.4-fold over fasting plasma levels. Moreover, areas under the curve of the means of all-*trans- *and 13-*cis-*retinoic acid over 24 hours showed that larger amounts of 13-*cis-*retinoic acid appear in the circulation than all-*trans-*retinoic acid after feeding all-*trans-*retinyl palmitate. The increase in retinoic acid in the circulation may be an important source of retinoic acid for some organs.*

Keywords: vitamin A; retinoic acid; oral dose; humans; metabolism

Introduction

Retinoic acid (RA) is a naturally occurring retinoid in human blood and tissue.[1] However, unlike naturally occurring retinol, RA is a minor component of the diet. RA in blood, therefore, has been regarded as being derived from preformed vitamin A and/or provitamin A as a tissue metabolite.[2-11] In vitro studies have demonstrated that both all-*trans*-RA (all-tRA) and 13-*cis*-RA (13-cRA) are physiologic metabolites of vitamin A in rat blood and tissues.[2] Our laboratory has provided evidence that RA exists as a mixture of 13-cRA and all-tRA in human serum, and that the level of both 13-cRA and all-tRA in human serum is temporally related to diet.[12] Studies reported in the present paper provide evidence that both physiologic and pharmacologic oral doses of all-*trans*-retinyl palmitate affect the concentrations of 13-cRA and all-tRa in human plasma.

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Materials and methods

Chemicals

HPLC grade methanol, n-hexane, and water were purchased from J.T. Baker Chemical Co., Philipsburg, NJ. All-tRA and all-*trans*-retinyl palmitate were purchased from Sigma Chemcial Co., St. Louis, MO. Thirteen-cRA, all-*trans*-9-(4-methoxy-2,3,6-trimethyl phenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid (TMMP), 9-*cis*-, 11-*cis*-, and 13-*cis*-retinyl palmitate were generous gifts from Hoffmann-La Roche Inc., Nutley, NJ. All retinoids were stored at −70° C and handled under red light.

Supplement studies

The study included 7 subjects, who on different days were fed supplements of all-*trans*-retinol palmitate either in a physiologic dose of 3000 retinyl equivalents (RE) or a pharmacologic dose (2250 RE/kg body weight, not to exceed 105,000 RE). The doses were of an oil soluble preparation (Hoffmann-La Roche, Nutley, NJ) with 95% purity in all-*trans*-retinyl palmitate

(analyzed by HPLC, see HPLC method). The subjects were 31 to 54 years old (3 men and 4 women) in good health, and none of them were being treated with Accutane™ or Retin A™ or taking supplements containing β-carotene or vitamin A. For the physiologic dose test, subjects fasted (except water) for 14 hours, skipped breakfast, and consumed a lunch 5 hr after the oral vitamin A dose. The lunch consisted of a turkey breast sandwich on white bread, cranberry juice, almond butter, and a carbonated soda. This meal contained no detectable β-carotene or preformed vitamin A. For the pharmacologic dose test, subjects fasted for 14 hr, given a standardized test breakfast, and received the same lunch as in the physiologic dose test, 5 hr after the oral vitamin A dose. The test breakfast consisted of a scrambled egg, a slice of toast with butter, and a cup of tea or coffee with 15 g of heavy cream, and contained 226 RE of preformed vitamin A.

Following a blood draw for baseline analysis (t = 0), the oral supplement was administered. Blood was collected in 0.1% EDTA plasma tube from a forearm vein at intervals of 2, 4, 6, 8, and 24 hr after the physiologic dose and 4, 6, 9, 12, and 24 hr after the pharmacologic dose. The samples were protected from light, immediately placed on ice and centrifuged (800*g* and 10 min) within 2 hr to separate the plasma from red cells. Plasma was kept at −70° C in the dark before analysis.

Plasma extraction

A Bond Elut™ aminopropyl column (500 mg/2.8 mL) and a Vac Elut™ vacuum elution apparatus obtained from Analytichem International (Harbor City, CA) were used to extract RA.[13] The internal standard TMMP in methanol (100 μL, 10^{-7}M) was added to plasma (1.5 mL). After the addition of 0.9% sodium chloride (2 mL) and chloroform/methanol (2:1, v/v, 5 mL), the mixture was vortexed and centrifuged at 800*g* for 10 min. The chloroform layer was removed and evaporated under N_2 to 0.2 mL. This extract was applied to an aminopropyl column which was placed in the Vac Elut™ apparatus and washed with 2 mL of hexane. The chloroform was pulled through the column by vacuum, and the plasma constituents that were retained on the column were then eluted with 4 mL of chloroform/2-propanol 2:1, (vol/vol). This eluate consisted of neutral lipid in plasma and was discarded. The column was then eluted with 4 mL of diethyl ether (with 3% acetic acid). This eluate contained RA and was evaporated to dryness under N_2. The residue was then redissolved in 150 μL of methanol, and a 40 μL aliquot was injected onto the HPLC column. All procedures for blood process and analysis were done under red light.

Peak indentification

For the identification of all-tRA and 13-cRA, a normal-phase HPLC and authenic all-tRA and 13-cRA were used.[12] Furthermore, the HPLC eluate of the plasma extract corresponding to the retention times of the authentic RAs was reacted with diazomethane. These products were also analyzed by HPLC.[12]

HPLC analysis

Plasma retinol and retinyl esters were measured by normal-phase HPLC as described by Bankson et al.[14] The HPLC system used for RA analysis consisted of a Series 410 LC pump, a LC-95 UV/visible spectrophotometer detector fixed at 340 nm at maximum sensitivity (0.001 AUFS), and a LCI-100 Laboratory Computing Integrator (Perkin-Elmer Co., Norwalk, CT) or HP-3390 integrator (Hewlett Packard, Avondale, PA). For sample injection, either a Rheodyne 7125 manual injector (Rheodyne Inc., Cotati, CA) or a Perkin-Elmer ISS-100 autosampler was used. For the determination of the purity of the all-*trans*-retinyl palmitate supplement, the method developed was able to separate all-*trans*-retinyl palmitate from 9-*cis*-, 11-*cis*-, and 13-*cis*-retinyl palmitate and used an HS-3 silica cartridge column (0.46 × 8.3 cm, Perkin-Elmer Co., Norwalk, CT) with n-hexane and toluene 99:1, (vol/vol) at a flow rate of 2.0 mL/min with the UV detector set at 325 nm. For quantitative analysis of plasma, a gradient HPLC procedure with a flow rate of 1.5 mL/min was used as follows: CH_3OH/H_2O (75:25) was eluted for 10 min, followed by a 3-min linear gradient to 100% CH_3OH, a 10-min hold at 100% CH_3OH, and then a 3-min linear gradient return to CH_3OH/H_2O (75:25). The concentration of 13-cRA and all-tRA in samples were calculated based on the recovery and the response factors which were quantified using authentic standards as previously described.[12]

Results

All-tRA and 13-cRA in plasma samples were identified by their retention times and the coelution with standards in both reverse-phase and normal-phase HPLC systems.[12] Their methylated derivatives were synthesized and analyzed using HPLC for comparison to the retention times of authentic standards. These results proved the identity of these compounds in human blood as all-tRA and 13-cRA.[12]

The means (± SEM) for plasma levels of all-tRA and 13-cRA during fasting were 4.0 (± 0.3) nmol/L and 5.1 (± 0.3) nmol/L, respectively, which were close to other reference values for serum.[12] Two hours after the physiologic dose, plasma samples showed mean ± SEM peak levels as follows: all-tRA = 5.1 ± 0.3 nmol/L and 13-cRA = 9.8 ± 1.0 nmol/L, which represented increases of 1.3-fold (P = 0.0005) and 1.9-fold (P = 0.007), respectively, over fasting levels (*Figure 1A*). Four hours after the pharmacologic dose, plasma samples showed mean ± SEM peak levels as follows: all-tRA = 15.5 ± 2.7 nmol/L and 13-cRA = 42.6 ± 6.0 nmol/L which represented increases of 3.9-fold (P = 0.007) and 8.4-fold (P = 0.001), respectively, over fasting levels (*Figure 1B*). After the physiologic or pharmacologic doses of retinyl palmitate, plasma concentrations of retinyl esters showed maximum increases of 40-fold (P = 0.00005, *Figure 2A*)

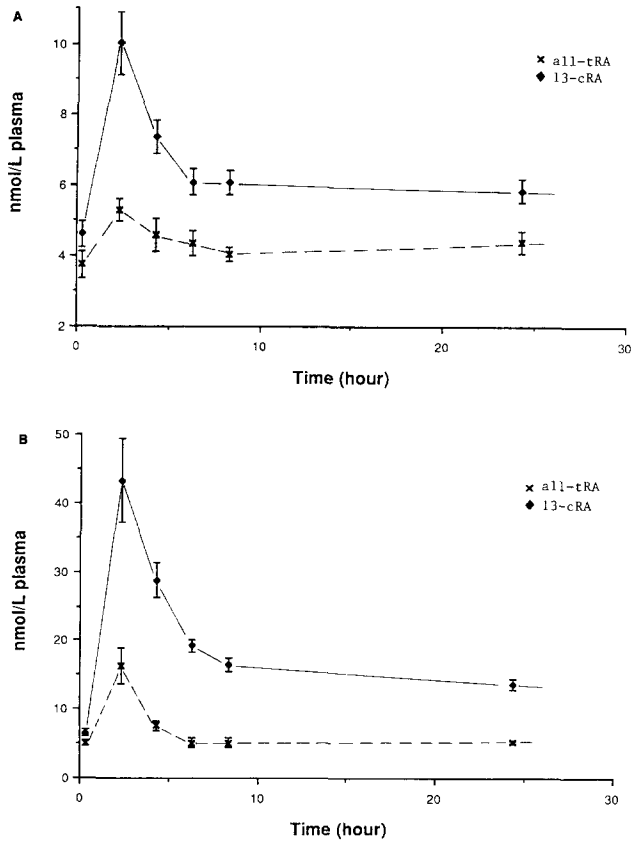

Figure 1 Changes in plasma levels of all-tRA (X) and 13-cRA (◆) in humans after a physiologic dose (A) or a pharmacologic dose (B) of all-*trans*-retinyl palmitate. Data and error bars represent mean ± SEM.

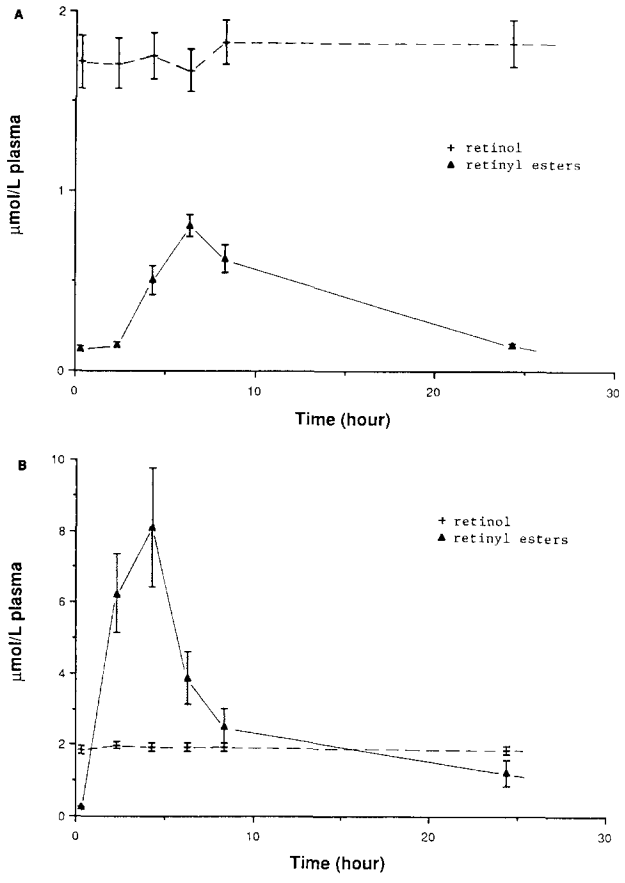

Figure 2 Changes in plasma levels of retinol (+) and retinyl esters (▲) in humans after a physiologic dose (A) or a pharmacologic dose (B) of all-*trans*-retinyl palmitate. Molecular weight of retinyl palmitate was used to calculate the concentration of retinyl esters in μmol/L. Data and error bars represent mean ± SEM.

and 76-fold ($P = 0.003$, *Figure 2B*), respectively, while the plasma concentrations of retinol remained unchanged (*Figures 2A & 2B*).

We compared the plasma response curves for all-tRA and 13-cRA by measuring the areas under the curve (*Figure 1*) for the 24 hr period after the physiologic or pharmacologic doses (*Table 1*). The area under the curve was constructed by plotting the plasma RA concentration versus time after subtracting the fasting value (t = 0) from each concentration. The area under the curve of all-tRA after the physiologic dose of all-*trans*-retinyl palmitate was set as 1 unit. Relative to this, the area under the curve of 13-cRA increased 9.6 units after the physiologic dose of all-*trans*-retinyl palmitate. Although the pharmacologic dose was 35 times greater than the physiologic dose, the incremental areas in all-tRA and 13-cRA after the pharmacologic dose were 7.9-fold (7.9 units) and 6.5-fold (62.0 units) greater, respectively, than those after the physiologic dose.

Table 1 Areas under the curve of means of plasma all-tRA and 13-cRA concentrations versus time over 24 hr after feeding all-*trans*-retinyl palmitate to 7 normal whites. The fasting level was subtracted from the concentration at each time point. Results are expressed as a relative area to the all-tRA response after the physiologic dose, which was set at 1.0

	Physiologic dose (3000 RE)	Pharmacologic dose (2250 RE/kg BW[a])
all-tRA	1.0	7.9
13-cRA	9.6	62.0

[a] Body weight.

Discussion

The work described here shows that the total RA level in human plasma increased after either the physiologic or the pharmacologic dose supplement of all-*trans*-retinyl palmitate.

It is well known that shortly after a supplement of all-*trans*-retinyl palmitate, retinyl esters bound to various lipoproteins in chylomicrons, and chylomicron remnants are found in plasma. Although the number of time points is not sufficient to draw peak time precisely for retinyl esters and retinoic acid in plasma after the administration of oral retinyl palmitate, in our study, the concentration of retinyl esters in plasma reached a maximum 6 hr after dosing; however, all-tRA and 13-cRA levels in human plasma reached a maximum about 2 hr (after pharmacologic dose) to 4 hr (after physiologic dose) earlier than retinyl esters.

Since RA can be synthesized from retinol by homogenates of rat intestine,[15] it is possible that the earlier peak appearance of the RA was due to the direct absorption of RA formed from retinyl palmitate within the small intestinal epithelial cell. Alternatively, the RA could be the result of retinyl ester metabolism in other tissues.

The existence of all-tRA and 13-cRA in rats[2] and humans[12] during vitamin A steady state (i.e., fasting) conditions has been demonstrated. Moreover, 13-cRA has been shown to be a physiologic metabolite of all-tRA in rats.[2] In our previous work, we found the concentrations of 13-cRA and all-tRA in human serum to be diet-related.[12] The present study provides evidence that an all-*trans*-retinyl palmitate supplement increases the concentrations of both all-tRA and 13-cRA in human plasma. Futhermore, a larger amount of 13-cRA circulates than all-tRA after the feeding of all-*trans*-retinyl palmitate.

RA is more active than retinol in the maintenance of epithelial differentiation and its cellular transport protein, cellular retinoic acid binding protein (CRABP), is distributed widely in animal tissues. The source of RA in animal tissues could be from retinol or β-carotene since recent reports have demonstrated the conversion of retinol and β-carotene into RA by rat tissues in vitro.[4,10,15] However, it is known that there is no relationship between the ability of a tissue to convert retinol into RA and the presence or concentrations of cellular retinol binding protein (CRBP) and CRABP[15] and that the level of CRABP is higher than the level of CRBP in many tissues. For example, the levels of CRABP in pituitary, prostate, and uterus are 1.7-fold, 2.7-fold, and 2.4-fold higher, respectively, than the level of CRBP.[16] Moreover, homogenates of rat spleen cannot convert retinol to RA even though it contains CRABP.[15] Thus, it seems that in situ synthesis of RA from retinol is not a significant source of RA in some tissues and RA in the circulation (perhaps intestinally derived after a meal) may supply the RA demands of certain tissues.

The present results extend observations of vitamin A metabolism in humans. Both all-tRA and 13-cRA are quantitatively significant metabolites of dietary all-*trans*-retinyl palmitate. RA in the circulation could be an important RA source for some organs.

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