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Formation of Retinoyl-CoA in Rat Tissues[1]

Masashi Wada, Tetsuya Fukui, Yoshinori Kubo, and Noriko Takahashi[2]

Department of Health Chemistry, Hoshi University, Shinagawa-ku, Tokyo 142-8501

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Retinoylation (retinoic acid acylation) is a posttranslational modification of proteins occurring in a variety of cell types *in vitro* and in tissues *in vivo*. The widespread occurrence of retinoylation suggests that it may play a role in many effects of retinoic acid (RA) on cells. One metabolic pathway for retinoylation involves the intermediate formation of retinoyl-CoA and subsequent transfer and covalent binding of the retinoyl moiety to protein. However, such reactions are not well known. To gain further insight into retinoylation, we studied the synthesis of retinoyl-CoA, the first step in this multi-stage process. The formation of [^{3}H]-retinoyl-CoA was determined in incubation mixtures containing rat liver extract, [^{3}H]-RA, ATP, CoA, and $MgCl_2$. No retinoyl-CoA was formed in the presence of boiled extract, or in the absence of ATP, CoA, or $MgCl_2$ (a divalent cation). A greater amount of retinoyl-CoA was obtained from microsomal fractions of rat liver than from other subfractions. The presence of retinoyl-CoA was also detected in extracts prepared from rat testis, kidney, brain, spleen, and pancreas. The level of retinoylation in various tissue extracts was related directly to the amount of retinoyl-CoA formed. V_{max} and K_m values for RA in the formation of liver retinoyl-CoA were estimated to be 1.0×10^{-4} μmol/min/mg protein and 24 nM, respectively. Synthesis of retinoyl-CoA was suppressed by fatty acids and fatty acyl-CoAs. These results indicate that ATP-dependent generation of retinoyl-CoA occurs in rat tissues and may play a significant physiological role in RA actions mediated by retinoylation.

Key words: ligase, rat liver, retinoyl-CoA, retinoic acid, retinoylation.

Based on nutritional studies with vitamin A–deficient animals, all-*trans*-retinoic acid (RA) supports growth in animals and maintains epithelial tissues and bone, but does not function in vision and mammalian reproduction. More recently, studies with mice lacking specific RA nuclear receptors (RARs) have shown that RA plays a crucial role in spermatogenesis and reproduction (*1*). Underscoring the importance of RA is its potent induction of differentiation of some cell types and its utility in the treatment of patients with various malignancies, especially acute promylocytic leukaemia (*2*).

One mechanism for the activity of RA in a variety of cell types involves RA nuclear receptors (RARs and retinoid X receptors) (*1, 3–6*). The action of RA in development and cell differentiation is mediated by these receptors, which directly activate or repress transcription of their target genes by binding to specific DNA sequences. However, some effects of RA are non-genomic (*7–12*). Therefore it is possible that other mechanisms, in addition to RA binding to RA receptors, are involved in RA biologic effects.

Retinoylation (acylation by RA of protein) is another mechanism by which RA may act on cells (*13–17*). RA induces terminal differentiation of the human acute myeloid leukemia cell line HL60 to cells having many characteristics of mature granulocytes (*18*). In growing HL60 cultures, RA is linked *via* a thioester bond to protein, and the extent of retinoylation is dependent on the initial concentration of RA in a saturable manner (*14, 17*). The dose-response curves for RA-induced differentiation and for retinoylation are similar, with positive correlations (*14, 17*). Retinoylation occurs in other cell lines that respond to RA (*13–17*). In addition, the regulatory subunits of cyclic AMP-dependent protein kinase type I and type II (*15*) and vimentin (*19*) in HL60 cells and the cytokeratins in normal human keratinocytes (*16*) are retinoylated. Recently, we showed that retinoylation occurs *in vivo* primarily *via* the formation of an ester bond (*20*). It is becoming clearer that retinoylation may be a true physiological reaction of RA.

Retinoylation is one of a large number of diverse modifications affecting many proteins, including phosphorylation, palmitoylation, and myristoylation. Palmitoylation and myristoylation (*21, 22*) are known to involve the formation of CoA derivatives. Metabolic pathways for retinoylation also involve the formation of a retinoyl-CoA intermediate (*23*) with successive transfer of the retinoyl moiety to protein. However, the components responsible for retinoylation are not well understood.

Previously, the formation of retinoyl-CoA from RA and

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[2] To whom correspondence should be addressed. Tel/Fax: +81-3-5498-5950, E-mail: t-noriko@hoshi.ac.jp

Abbreviations: RA, retinoic acid; PBS, phosphate-buffered saline (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136.9 mM NaCl, pH 7.2); EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; DTT, dithiothreitol; ATP, adenosine triphosphate; CoA, coenzyme A; RAR, retinoic acid nuclear receptor.

the incorporation of RA into proteins *via* retinoyl-CoA have been studied in rat kidney crude extracts (*23*). However, detection by HPLC of retinoyl-CoA formed in reaction mixtures was not clear, due to high background and low radioactivity. To date, the generation of retinoyl-CoA including its tissue distribution, localization in cells, kinetics and effect of inhibitors have not been well characterized. In this study, we investigate retinoyl-CoA formation in rat tissue fractions, as well as retinoylation *in vitro*.

MATERIALS AND METHODS

Animals—Eight-week-old male Sprague-Dawley (Slc. SD) rats (140–160 g) were obtained from Tokyo Laboratory Animals Science (Tokyo).

Preparation of Crude Extracts and Subcellular Fractions—The following procedures were performed at 0–4°C as described previously (*23, 24*). Rat liver, kidney, testis, brain, pancreas, and spleen were each homogenized in 5 volumes (w/v) of Buffer A [100 mM Tris-HCl buffer (pH 7.4), 0.25 M sucrose, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT) and proteinase inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.4 mg of aprotinin/ml, 0.2 mg of leupeptin/ml, and 15 μM 4-amidinophenylmethanesulfonyl fluoride (Sigma))]. Homogenates were fractionated by differential centrifugation. The membrane/nuclei (800 ×*g*, 10 min), mitochondrial (10,000 ×*g*, 10 min), and microsomal (100,000 ×*g*, 60 min) fractions were washed with and suspended in Buffer A. The supernatant obtained after centrifugation at 100,000 ×*g* for 60 min was used as the cytosol fraction. Homogenates (crude extracts) and subcellular fractions were used immediately or divided into tubes so as not to freeze-thaw and stored at –80°C for no longer than 2 months before use. Glutamate dehydrogenase and lactate dehydrogenase were also assayed as marker enzymes for mitochondrial and cytosolic fractions, respectively.

Protein Assay—Proteins were measured utilizing the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL) or the procedure of Bradford (*25*) with bovine serum albumin as a standard.

Preparation of Retinoyl-CoA—Labeled or radioinert retinoyl-CoA was synthesized and identified according to a modified method of Kutner *et al.* (*26*). Briefly, all-*trans*-[^{3}H]-RA ([11,12-^{3}H]-RA, 35.8–50 Ci/mmol (1.11 TBq/mmol); 1 Ci = 3.7 × 10^{10} Bq; Du Pont–New England Nuclear, Boston, MA) or radioinert RA (2 μmol) was dissolved in dioxane, diluted to a final concentration of 2.5 mM with an equimolar amount (2 μmol) of butylated hydroxytoluene (BHT) dissolved in dioxane, and taken to dryness three times from dioxane under argon gas. This preparation was treated with 400 μl (8 μmol) of 20 mM *N*-hydoroxysuccinimide and 400 μl (8 μmol) of 20 mM *N,N'*-dicyclohexylcarbodiimide dissolved in dioxane, and the reaction mixture was incubated at 37°C for 1 h under argon gas. After removing dioxane under an argon stream, 1% $NaHCO_3$ (0.5 ml) was added to the dry ester dissolved in tetrahydrofuran (THF) (1 ml), the resulting solution was added to a CoA solution [15 μmol (12.3 mg), 0.1 ml], and the pH was adjusted slowly to 8.0–8.5 by the addition of 1% $NaHCO_3$ (0.4 ml). The ratio of THF and water was two to one. The solution was mixed well, then incubated at 37°C with stirring under argon overnight. The reaction was stopped by adding 1 ml of $CHCl_3$/MeOH (2:1) containing BHT (50 μg/ml) and mixing well, and the mixture was centrifuged at 1,000 ×*g* for 10 min. The upper aqueous layer was lyophilized to remove solvent and the residue was dissolved in 200 μl of distilled water. The purity of the aqueous product was estimated and identified by high pressure liquid chromatography (HPLC). A peak corresponding to retinoyl-CoA was collected and the UV spectrum measured using a DU® 530 Life Science UV/Vis Spectrophotometer (Beckman Instrument, Arlington Height, IL).

Identification and Analysis of Retinoyl-CoA by HPLC—Chromatography was performed using a Beckman system GOLD radiochromatography system with Beckman 7725 injector, Beckman 125 solvent module, Beckman 168 detector module, and Beckman 171 radioisotope detector (Beckman Instrument). A Sepherisorb C_8 reverse-phase column (4.6 mm × 25 cm, Phenomenex, Rancho Palos Verdes, CA) was used to separate the aqueous and organic soluble materials. The column was eluted with 10 mM NH_4OAc in a methanol/water gradient (20–100%, 80 min) at a flow rate of 1.0 ml/min with UV monitoring at 340 nm.

Assay for Retinoylation—Crude extracts (100 μg proteins) were incubated at 37°C for 5 min with 280 nM [^{3}H]-RA (1.11 TBq/mmol), 10 mM ATP, 0.15 mM CoA, 27 mM $MgCl_2$, 1 mM DTT, 50 mM sucrose, and 0.1 M Tris-HCl buffer (pH 7.4) in a final volume of 0.1 ml. Reaction mixtures were then extracted by the Bligh-Dyer procedure using $CHCl_3$:CH_3OH:H_2O (1:2:0.8) (*27*) and centrifuged at 10,000 ×*g* for 5 min in a microcentrifuge. This extraction was repeated approximately five times or until <300 cpm/ml was present in the supernatant fraction. The delipidated pellet was dried in a centrifugal vacuum device (Sakuma, Tokyo). Pellets were dissolved in Solvable [Packard Instrument (Meriden, CT)], and radioactivity was measured in a liquid scintillation spectrometer (Packard Instrument).

Assay of Retinoyl-CoA Formation—The amount of retinoyl-CoA produced was determined by measuring the radioactivity of [^{3}H]-retinoyl-CoA synthesized from [^{3}H]-RA. Mixtures containing various concentrations of [^{3}H]-RA (1.11 Tbq/mmol, 2–700 nM), 0.15 mM CoA, 10 mM ATP, 27 mM $MgCl_2$ (or 50 mM KCl), 1 mM DTT, 50 mM sucrose, 0.1 M Tris-HCl buffer, pH 7.4, and various amounts of crude extract or subcellular fraction were incubated in a final volume of 0.1 ml. Incubation was carried out at 37°C for 1–60 min, or 5 min, depending on the range giving a constant reaction rate. After terminating the reactions by the addition of 0.4 ml of Dole reagent (isopropanol:heptane = 4:1), the reaction mixtures were extracted with heptane (0.24 ml) and distilled water (0.14 ml), and centrifuged at 10,000 ×*g* for 5 min. This extraction was repeated until radioactivity in the supernatant fraction was <100 cpm. After removing supernatant, radioactivity in the lower aqueous layer was measured by liquid scintillation spectrophotometry.

Presentation of Results—Each experiment was performed at least 3 times, and most experiments were repeated at least 4 times with consistent results.

RESULTS

Production of Retinoyl-CoA from RA in Rat Liver—We first examined formation of retinoyl-CoA in rat liver using a high concentration of substrate, 280 nM [^{3}H]-RA (35.8–50.0 Ci/mmol), in order to detect retinoyl-CoA exhibiting high radioactivity. We analyzed the formation of [^{3}H]-retin-

oyl-CoA produced from [^{3}H]-RA using HPLC. As shown in Fig. 1A, a single peak of radioactivity eluted with a retention time of 48.5 min. This peak coeluted with a sample of authentic radioinert retinoyl-CoA (Fig. 1A, arrow). Boiling of microsomes (Fig. 1B) or omission of either ATP, CoA, or $MgCl_2$ (Fig. 1C) resulted in the absence of a peak corresponding to labeled retinoyl-CoA (48.5 min). In addition, retinoyl-CoA formation was not detected when KCl was used instead of $MgCl_2$ (Fig. 1C). Under the same conditions, authentic RA eluted with a retention time of 58 min. These results indicate that synthesis of retinoyl-CoA is enzymatic in nature and requires the presence of ATP, CoA, and $MgCl_2$.

Synthesis of Retinoyl-CoA in Subcellular Fractions of Liver and in Various Tissues—We examined the subcellular distribution of retinoyl-CoA formation in rat liver. The generation of retinoyl-CoA was found to be highest, approximately 18.2 pmol/min/mg protein, in microsomal fractions, and to be approximately 8.94 pmol/min/mg protein in membrane/nuclei fractions (Fig. 2A). In contrast, retinoyl-CoA formed by mitochondrial and cytosol fractions were somewhat lower than 3 pmol/min/mg protein (Fig. 2A). The total amounts of retinoyl-CoA observed in liver subfractions were 12.6 nmol/min/liver for membrane/nuclei fractions and 6.67 nmol/min/liver for microsomal fractions (Fig. 2B). Next, levels of retinoyl-CoA in microsomal fractions prepared from various tissues were measured. Results indicated that 18.2 pmol/min/mg protein of retinoyl-CoA were

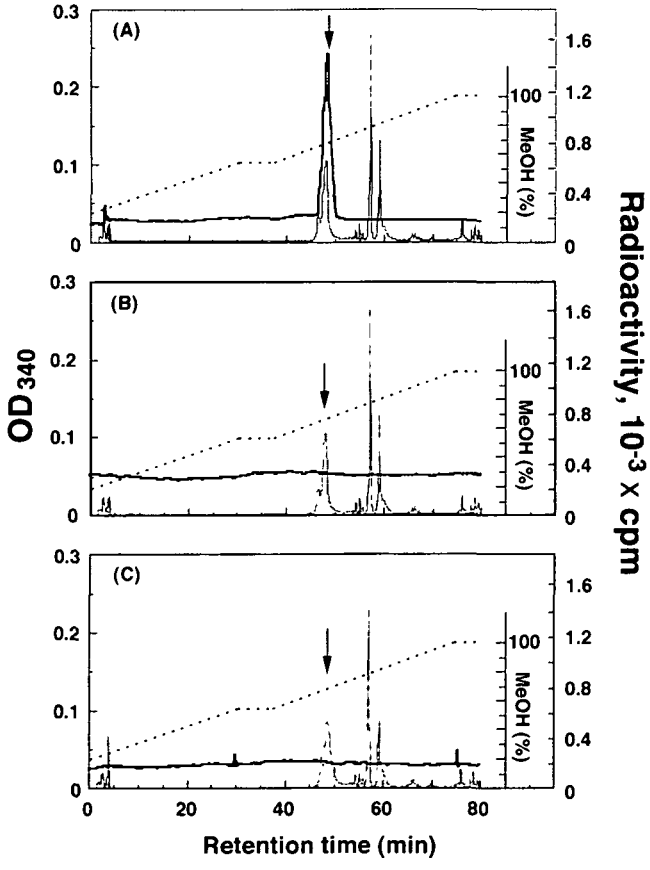

Fig. 1. **HPLC profile of retinoyl-CoA produced by rat liver microsomal protein.** Rat liver microsomes (6.3 μg) were incubated for 5 min at 37°C with reaction mixtures containing 280 nM [^{3}H]-RA, 0.15 mM CoA, 10 mM ATP, and 27 mM $MgCl_2$ as described in "MATERIALS AND METHODS" (complete assay system). After extraction with heptane, the aqueous phase was analyzed by HPLC. A Spherisorb C_8 column (4.6 mm × 250 mm, Phenomenex) was eluted using 10 mM NH_4OAc in a methanol/water gradient system at a flow rate of 1.0 ml/min with UV monitoring at 340 nm. The arrows indicate the elution position of retinoyl-CoA (retention time; 48.5 min). (A) complete; (B) boiled microsomes; (C) omission of CoA, ATP, or $MgCl_2$. —, OD_{340}; —, cpm; ---, concentration of MeOH.

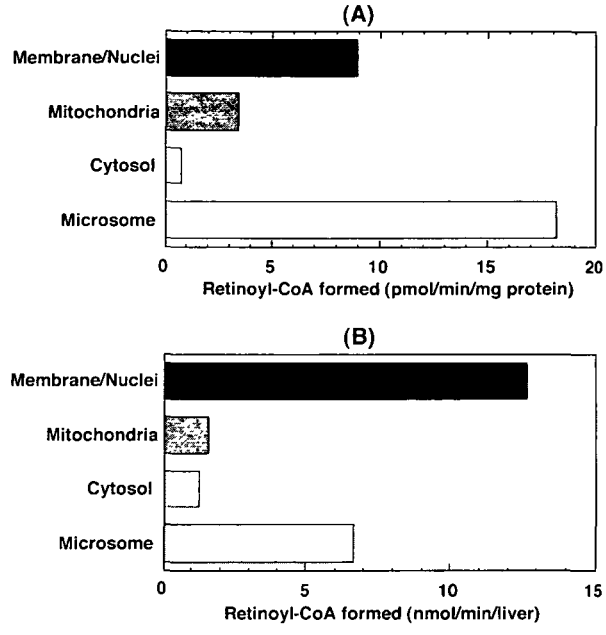

Fig. 2. **Retinoyl-CoA formed by subcellular fractions of rat liver.** Subcellular fractions (6.3 μg) of rat liver were incubated for 5 min at 37°C with reaction mixtures containing 280 nM [^{3}H]-RA and 0.15 mM CoA, 10 mM ATP, 27 mM $MgCl_2$. After extraction with heptane, radioactivity in the aqueous phase was measured using a liquid scintillation spectrometer as described in "MATERIALS AND METHODS." (A) specific activity; (B) total activities in liver subfractions.

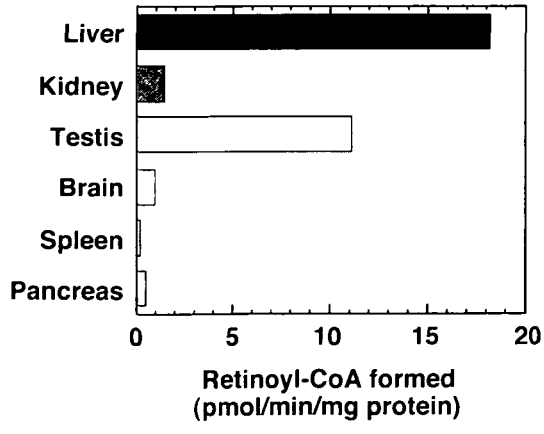

Fig. 3. **Retinoyl-CoA formed by microsomal fractions of rat tissues.** Microsomes (6.3 μg) from rat tissues were incubated for 5 min at 37°C with reaction mixtures containing 280 nM [^{3}H]-RA, 0.15 mM CoA, 10 mM ATP, and 27 mM $MgCl_2$. After extraction with heptane, radioactivity in the aqueous phase was measured using a liquid scintillation spectrometer as described in "MATERIALS AND METHODS."

formed for liver, 11.1 pmol/min/mg protein for testis, 1.4 pmol/min/mg protein for kidney, and 0.9 pmol/min/mg protein for brain (Fig. 3). Low amounts of retinoyl-CoA were seen in spleen and pancreas microsomes (Fig. 3). These results demonstrated retinoyl-CoA formation in various tissues.

Levels of Retinoyl-CoA Obtained from RA and Retinoylation—As shown in Fig. 3, microsomal fractions of liver and testis contained elevated amounts of retinoyl-CoA. Previous reports have shown that retinoylation involves two reactions, the formation of retinoyl-CoA and its transfer to protein, which occurs in rat kidney to a high extent (*20, 23*). Accordingly, synthesis of retinoyl-CoA was compared with retinoylation in crude extracts prepared from rat liver, testis and kidney. The amounts of retinoyl-CoA formed were approximately 200 fmol/min/mg of liver protein, 160 fmol/min/mg of testis protein, and 400 fmol/min/mg of kidney protein (Fig. 4). On the other hand, the levels of retinoylation were 50 fmol/min/mg of liver protein, 40 fmol/min/mg of testis protein, and 180 fmol/min/mg of kidney protein. The ratio of retinoyl-CoA formed to retinoylation was approximately 2.2:1 for kidney and 4:1 for liver and testis. These results indicated that the level of retinoylation was related directly to the amount of retinoyl-CoA formed.

Time Course and Dose-Response of Retinoyl-CoA Formation—Since quantities of retinoyl-CoA were highest in microsomal fractions of rat liver (Fig. 2), we next attempted to examine time- and dose-dependency in this fraction. In microsomal protein fractions (1 μg), the formation of retin-

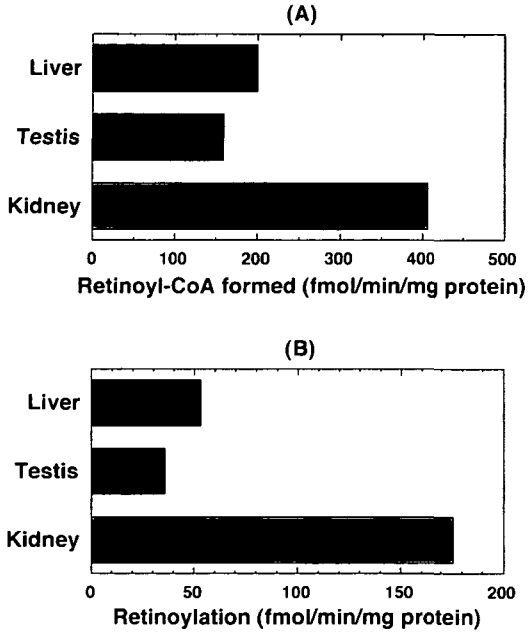

Fig. 4. **Levels of retinoyl-CoA formed and retinoylation in crude extracts of rat liver, testis, and kidney.** Crude extracts (100 μg) of various rat tissues were incubated for 5 min at 37°C with reaction mixtures containing 280 nM [^{3}H]-RA, 0.15 mM CoA, 10 mM ATP, and 27 mM $MgCl_2$. After extraction of the reaction mixture with heptane or by the Bligh-Dyer procedure, radioactivity was measured using a liquid scintillation spectrometer as described in "MATERIALS AND METHODS." (A) Retinoyl-CoA formed; (B) Retinoylation.

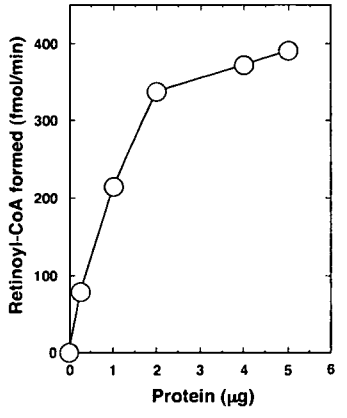

Fig. 6. **Dependency of retinoyl-CoA synthesis on protein concentration.** Retinoyl-CoA formation was measured as described in "MATERIALS AND METHODS." Rat liver microsomes at the indicated concentrations were incubated for 5 min at 37°C with 280 nM [^{3}H]-RA, 0.15 mM CoA, 10 mM ATP, and 27 mM $MgCl_2$.

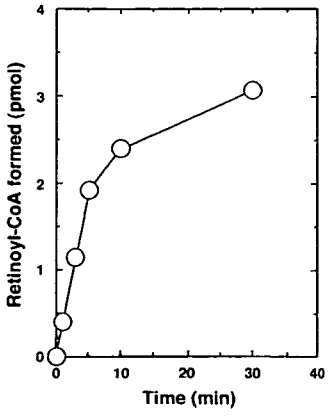

Fig. 5. **Time course of retinoyl-CoA synthesis.** Retinoyl-CoA formation was measured as described in "MATERIALS AND METHODS." Rat liver microsomes (1 μg) were incubated for the indicated time at 37°C with 280 nM [^{3}H]-RA, 0.15 mM CoA, 10 mM ATP, and 27 mM $MgCl_2$.

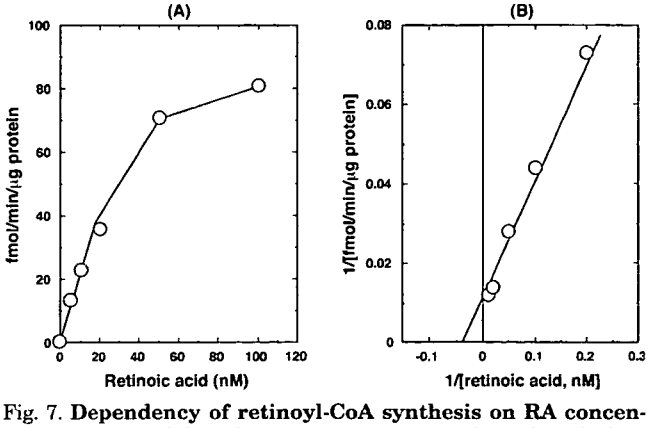

Fig. 7. **Dependency of retinoyl-CoA synthesis on RA concentration.** Retinoyl-CoA formation was measured as described in "MATERIALS AND METHODS." (A) Rat liver microsomes (1 μg) were incubated for 5 min at 37°C with the indicated concentrations of [^{3}H]-RA, 0.15 mM CoA, 10 mM ATP, 27 mM $MgCl_2$. (B) Double reciprocal analysis of the retinoyl-CoA formation reaction was performed by using the data in Fig. 7A.

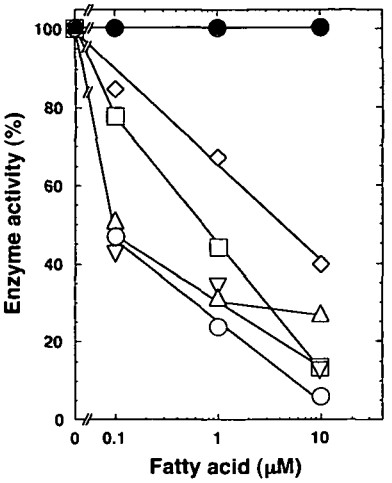

Fig. 8. **Inhibition of retinoyl-CoA synthesis by fatty acids.** Levels of retinoyl-CoA were measured as described in "MATERIALS AND METHODS." Rat liver microsomes (1 μg) were incubated for 5 min at 37°C with 280 nM [^{3}H]-RA and increasing concentrations of the following fatty acids: ●, control; ○, arachidonic acid; △, myristic acid; □, oleic acid; ▽, palmitic acid; ◇, stearic acid.

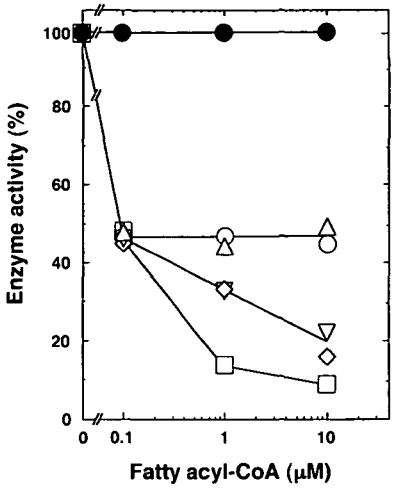

Fig. 9. **Inhibition of retinoyl-CoA synthesis by fatty acyl-CoA.** Synthesis of retinoyl-CoA was measured as described in "MATERIALS AND METHODS." Rat liver microsomes (1 μg) were incubated for 5 min at 37°C with 280 nM [^{3}H]-RA and increasing concentrations of the following fatty acyl-CoAs: ●, control; ○, acetyl-CoA; △, acetoacetyl-CoA; □, myristoyl-CoA; ▽, palmitoyl-CoA; ◇, stearoyl-CoA.

oyl-CoA was linear for at least 5 min and started to saturate at approximately 10 min (Fig. 5). After 30 min, reaction rates decreased and reached a plateau (constant value). At 5 min, approximately 2 pmol of retinoyl-CoA had formed (Fig. 5). On the other hand, the generation of retinoyl-CoA depended on the amount of liver microsome within the range of 0.2 to 5 μg (Fig. 6). Linear incorporation was seen within the range of 0.2 to 1 μg, and saturation occurred above a concentration of 2 μg (Fig. 6). Retinoyl-CoA formation rate was approximately 200 fmol/μg of liver microsomal protein/min (Fig. 6).

Effects of RA Concentration on the Synthesis of Retinoyl-CoA—Retinoyl-CoA was quantitated in reaction mixtures containing various concentrations of [^{3}H]-RA, 0.15 mM CoA, 10 mM ATP, 27 mM $MgCl_2$, and 1 μg of rat liver microsome. The rate of retinoyl-CoA formation was dose-dependent in a saturable manner (Fig. 7A). From double reciprocal plots of retinoyl-CoA formation (Fig. 7B), the K_m value for RA was calculated to be 2.4×10^{-8} M with the V_{max} value being 1.0×10^{-4} μmol/min/mg protein.

Inhibition of Retinoyl-CoA Production by Long-Chain Fatty Acids—RA consists of a cyclohexene ring connected to a conjugated long-chain tetraene carboxic acid. It therefore appeared significant to investigate whether long-chain fatty acids affect the rate of retinoyl-CoA formation. As shown in Fig. 8, generation of retinoyl-CoA in rat liver microsomes was inhibited in a dose-dependent manner (0.1–10 μM) by long-chain fatty acids of various chain lengths. At a low concentration (0.1 μM), palmitic acid ($C_{16:0}$), myristic acid ($C_{14:0}$), and arachidonic acid ($C_{20:4}$) inhibited the synthesis of retinoyl-CoA by approximately 50% as compared with approximately 20% inhibition elicited by oleic acid ($C_{18:1}$) and stearic acid ($C_{18:0}$). At a high concentration (10 μM), arachidonic acid, palmitic acid and oleic acid inhibited the production of retinoyl-CoA by more than 90%. Of these three fatty acids, arachidonic acid was the most potent inhibitor of rat liver retinoyl-CoA formation at a concentration of 10 μM (Fig. 8). In contrast, short-chain fatty acids had no effect on retinoyl-CoA formation in the range of 0.1 to 10 μM (data not shown). These results suggested that the specificity and potency of inhibition may be due to the three-dimensional structures of the fatty acids and be dependent on both carbon chain length and double bond position.

Effects of Fatty Acyl-CoA on the Formation of Retinoyl-CoA—On the assumption that fatty acyl-CoAs inhibit generation of retinoyl-CoA, the effects of acyl-CoAs were examined using rat liver microsomes. At a low concentration of 0.1 μM, all acyl-CoAs inhibited retinoyl-CoA formation by approximately 55% (Fig. 9). At higher concentrations of 1 or 10 μM, percent inhibition by both acetyl-CoA and acetoacetyl-CoA (short-chain fatty acyl-CoAs) did not increase further. However, myristoyl-CoA, palmitoyl-CoA, and stearoyl-CoA (long-chain fatty acyl-CoAs) suppressed production of retinoyl-CoA in proportion to their concentration (Fig. 9). Myristoyl-CoA in the range of 1 to 10 μM inhibited retinoyl-CoA formation by approximately 90%, being the most potent inhibitor of the three. Unsaturated long-chain fatty acyl-CoAs (oleoyl-CoA and arachidonoyl-CoA) inhibited the formation of retinoyl-CoA to the same extent as palmitoyl-CoA and stearoyl-CoA (data not shown). These results indicated that long-chain acyl-CoAs may affect the production of retinoyl-CoA more than short-chain acyl-CoAs.

DISCUSSION

RA is an active differentiation agent both in patients and in cell lines (*2, 18, 28*). Previous studies suggest that RA nuclear receptors (RARs) or the retinoylation of a nuclear protein, or both, may mediate its action on HL60 cells (*1, 3–6, 13, 15, 29*). To assess whether RA exerts its effects by retinoylation, we studied retinoyl-CoA formation, the first step in the process of retinoylation in rat tissues. In the current study, we have demonstrated the generation of retinoyl-CoA from RA in rat tissues, and shown that this reaction

has a relatively low K_m for RA which is close to the concentration at which RA is active in cells. We have also shown that synthesis of retinoyl-CoA is suppressed by long-chain fatty acids or acyl-CoAs that are similar in structure to RA.

RA has various effects on many cell types and tissues *in vivo* and *in vitro*. In frog embryos, RA affects the formation of an array of anterior structures, including brain, cement gland, and heart (*30, 31*) and the development of the central nervous system (*32*). In addition, previous studies have shown that one specific tissue change resulting from deprivation of vitamin A (vitamin A deficiency) is atrophy of the testis (*33*), and that the biosynthesis of RA from β-carotene occurs in kidney, testis, liver, lung, and intestine (*34*). In the current study, synthesis of retinoyl-CoA was shown to occur in rat liver, testis, brain, and kidney (Figs. 1–4), with liver showing the highest level of production (Fig. 3). The formation of retinoyl-CoA was approximately two to fourfold higher than that of retinoylation, and the extent of retinoylation correlated with levels of retinoyl-CoA produced (Fig. 4). These results suggest that retinoyl-CoA, an intermediate of retinoylation, may play important roles in these tissues. Though mRNA levels of RARs in these tissues (*35–38*) were induced by RA treatment (*39–41*), there was no evidence of a relationship between RA action and the extent of RARs (α, β, γ) expression or the amount of RA binding to RARs. It would be of interest to investigate in detail how the synthesis of retinoyl-CoA (retinoylation) and RARs are involved in RA action.

Recently, it was reported that retinoylation occurs *in vivo* (*20*). In RA-deficient rats, RA or retinol is incorporated covalently into proteins in the liver, kidney, and lung. The extent of retinoylation in kidney was highest, with the level of retinoylation in liver being approximately half that of kidney (*20*). These results agree with the present results, in which the extent of retinoylation was measured *in vitro* in crude extracts of kidney and liver (Fig. 4). On the other hand, levels of retinoyl-CoA were the highest in crude kidney extracts (Fig. 4) or liver microsomes (Fig. 2A). This indicates that the distribution of this enzyme in subfractions differs between kidney and liver. Taking these results into consideration, retinoyl-CoA formation in kidney is mainly in subfractions other than microsomes.

Time dependency studies on retinoyl-CoA synthesis in microsomes showed that reactions proceeded linearly within the first 5 min, but not afterward (Fig. 5). It is possible that the enzyme is very unstable and loses activity during the course of the reaction. Enzymatic activity increased linearly in a dose-dependent manner up to 2 μg of microsomal enzyme protein, but with increasing enzyme protein, the rate of retinoyl-CoA synthesis decreased (Fig. 6) and reduced (data not shown). These results suggest that microsomal fractions may contain components which inhibit retinoyl-CoA formation. Furthermore, levels of retinoyl-CoA increased linearly up to 20 nM and were saturable upon increasing the concentration of RA as a substrate (Fig. 7). The K_m and V_{max} values for RA were 2.4×10^{-8} M and 1×10^{-4} μmol/min/mg, respectively. In contrast, acetoacetate exhibits a K_m value of 8 μM in rat liver for acetoacetyl-CoA synthetase (*24*); arachidonic acid, 30 or 12 μM for arachidonoyl-CoA synthetase in human platelets (*42, 43*); and palmitic acid, 42 μM for palmitoyl-CoA synthetase in guinea-pig liver (*44*). Generation of retinoyl-CoA exhibited a much lower K_m than these fatty acyl-CoA synthetases. Apopropionyl-CoA carboxylase ligase showed a lower K_m (13 nM) for its substrate than was observed in the formation of retinoyl-CoA (*45*), and retinal had a K_m value of 600 nM for cytosolic aldehyde dehydrogenase 1 in rat liver (*46*). Production of retinoyl-CoA may play an important role in metabolite regulation with trace levels of substrate in the body.

Our results suggest that retinoyl-CoA may be formed in various tissues and be involved in biochemical reactions mediating the response of some cell-types to RA. It is now becoming clearer that RA may exert its effects by different mechanisms. The major retinoylated protein of HL60 cells binds to DNA and is palmitoylated and myristoylated (*47*). Palmitoylation and myristoylation have major effects on the function or intracellular localization of known proteins (*48–51*). Further study of retinoyl-CoA formation should contribute to a better understanding of the functional role(s) of retinoylation.

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