Detection of Retinyl Palmitate and Retinol in the Liver of Mice Injected with Excessive Amounts of Retinyl Acetate

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ABSTRACT. The transport of subcutaneously injected retinyl acetate (RA, 100,000 IU/mouse, 105.470 nM) was investigated in male ICR mice (10-week-old) at 0, 3, 6, 12, 18, 24 and 72 hr after a single injection. The retinol and retinyl palmitate levels of liver homogenates, bile in the gallbladder and serum from peripheral blood were measured by high performance liquid chromatography (HPLC) method. Retinyl palmitate in the lipid droplets of hepatocytes and Ito cells was localized by a modified gold chloride staining method. Accumulation of retinyl palmitate peaked at 12 hr post-injection and decreased thereafter until 24 hr post-injection. Fluorescence microscopy revealed many fluorescent vitamin A-containing lipid droplets in hepatocytes around central veins at 12 hr post-injection, but such droplets were not observed in the vehicle control mice or at in the RA-injected mice after 18 hr of injection. Electron microscopic observation also indicated that many retinyl esters-containing lipid droplets were observed in hepatocytes around the central veins at 12 hr post-injection, but no droplets were seen in the controls or 18 hr post-injection. The retinyl palmitate levels in liver homogenates assessed by HPLC decreased from 12 to 24 hr post-injection and increased significantly in bile, while retinol in liver homogenates and serum markedly decreased from 12 to 24 hr post-injection and increased significantly in bile, while retinol in liver homogenates and serum markedly increased. One of the morphological alterations was intense vacuolization in hepatocyte cell cords from the portal toward the central vein observed at 24 hr post-injection. Transitional lipid droplets between vacuoles and lipid droplets were identified in those hepatocytes. These results of HPLC analysis of retinol and retinyl palmitate in liver homogenates, serum, and bile, together with the results of gold chloride staining suggested that subcutaneously injected RA was first incorporated in hepatocytes at 12 hr and then partially metabolized through vacuoles, transferred into the blood and secreted into the bile over a 24 hr period. Many retinyl esters-containing lipid droplets were visualized in Ito cells at 72 hr post-injection. Most of vitamin A in the liver homogenates measured by HPLC was retinyl palmitate. Therefore, the contents in those lipid droplets might be retinyl palmitate. — KEY WORDS: gold chloride method, hepatocyte, HPLC, Ito cell, vitamin A.

Vitamin A (VA) and its metabolites are known to be essential for vision and to regulate diverse activities such as cell proliferation, differentiation, morphogenesis, and tumorigenesis [1, 3]. Pharmacological doses of synthetic VA (isoretinoin, acitretin) have been shown to have a beneficial effect in the treatment of a variety of skin conditions such as acne and psoriasis, as well as some neoplasms of epithelial origin [4]. However, excessive retinoic acid, retinol or retinyl acetate ingestion or injection has been linked to teratogenesis and to alterations in lipid metabolism in humans and animals [1, 7, 8, 18].

The liver is the major site of storage and metabolism of VA, containing more than 80% of the VA in the body, mainly in the form of retinyl palmitate [13, 20, 28, 29]. The uptake, storage, and mobilization of VA in the regulation of plasma levels of retinol and its delivery to extrahepatic organs are highly regulated, especially by a dynamic hydrolysis-esterification balance [20, 27, 28]. Hydrolysis and re-esterification occur in the liver, and the resulting retinyl esters are stored in both hepatocytes and Ito cells [11–13]. Hepatocytes and Ito cells contain VA, but Ito cells are particularly abundant in retinol-binding protein (RBP) and enzymes involved in VA metabolism [2].

Hypervitaminosis A resulting in toxicity occurs more frequently in developed countries as a result of excessive consumption of vitamin supplements [10, 15, 16]. Vitamin supplementation is frequently perceived as a method of preventing a number of diseases. The effects of hypervitaminosis A on liver metabolism have been extensively investigated by feeding or injecting multiple doses of synthetic derivatives of retinol or natural forms of VA over many days [5, 7, 18, 24]. The effect of subcutaneous injection of esterified forms of VA (retinyl acetate or palmitate) has been mainly reported with increase of VA lipid droplets in Ito cells after few days [29, 30].

Therefore, in the present study, the effects of an excessive amount of retinyl acetate (RA) were investigated at 0, 3, 6, 12, 18, 24 and 72 hr after a single injection by high performance liquid chromatography (HPLC) measurements and histochemistry using the modified gold chloride staining method reported in our previous publication [22].

MATERIALS AND METHODS

Animals and RA treatment: Male ICR mice, 10 weeks of age and weighing 45 to 50 g (Nippon Clea Co., Ltd., Osaka, Japan), were purchased, quarantined for 1 week, and housed in a room under controlled temperature (23 ± 1°C), humidity (60 ± 5%), and lighting (12 hr light, 12 hr dark). Access to food and tap water was ad libitum throughout the study. After a 1-week acclimation period on a basal diet (Oriental MF Diet; Oriental Yeast Co., Ltd., Chiba, Japan), the mice were divided into experimental groups. This experiment
was reviewed by the Ethics Committee on Animal Experiments of Yamaguchi University School of Medicine and performed in accordance with the Guidelines for Animal Experiments of Yamaguchi University School of Medicine and Law No. 105 and Government Notification No. 6.

The mice were given a single 100,000 IU (105470 nM)/mouse subcutaneous injection of RA (Merck Japan K.K., Tokyo, Japan) diluted in peanut oil. The control animals received a subcutaneous injection of the same volume (100 µl/mouse) of the peanut oil vehicle (Merck Japan K.K.). A total of 49 animals was euthanized under ether anesthesia 0, 3, 6, 12, 18, 24, or 72 hr after RA injection for analysis of retinol and retinyl palmitate levels. For the morphological study, three animals each were sacrificed at 12, 18, 24, and 72 hr post-injection and compared to the vehicle control animals. The animals were fasted for 24 hr, and blood specimens were collected from all animals by cardiac puncture, between 09:00 to 11:00 a.m., immediately prior to perfusion through the thoracic aorta of Gey’s balanced salt solution (Sigma-Aldrich Japan K.K., Tokyo, Japan) for 10 min.

**Electron microscopy:** The liver was fixed by perfusion through the thoracic aorta for 10 min with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at room temperature (20–26°C). The liver was then removed, and the middle portion of the liver was cut into pieces (1 mm3) or blocks (7 mm × 5 mm). The tissue pieces were fixed for an additional 2 hr in the same fixative at 4°C and washed in the same buffer containing 6% sucrose. Then, the tissues postfixed for 2 hr in 2% OsO4 were dehydrated in a graded series of acetone and embedded in Epon 812 resin (TAAB, Berkshire, UK). Ultrathin sections were cut on a ultramicrotome (Reichert-Jung, Vienna, Austria), double-stained with uranyl acetate and lead citrate (Katayama Chemical Co., Osaka, Japan), and examined with a transmission electron microscope (JEOL 200 CX, Jeol, Tokyo, Japan) at 80 kV accelerating voltage.

**Gold chloride method:** To examine for VA-containing lipid droplets at both the fluorescent and electron microscopic levels, fixed blocks were sectioned at 40 µm with a microslicer (DTK-200 Microslicer, Dosaka EM Co., Ltd., Kyoto, Japan) and postfixed for 10 min with a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The sections were then stained in 0.2% OsO4 buffered with sodium cacodylate (pH 7.4) for 90 sec or until they turned “caramel color” and incubated in 0.0001% gold chloride (Merck Japan K.K.) solution for 12 hr at room temperature in the dark. The sections were postfixed with 1% OsO4 for 1 hr. For light microscopic examination, the sections were dehydrated through a series of graded ethanol and mounted in Entellan (Merck Japan K.K.). For electron microscopic examination, the sections were dehydrated through a series of graded acetone and embedded in Epon 812 resin (TAAB) [22].

**Fluorescence microscopy:** In order to examine the VA-containing lipid droplets of hepatocytes, the 40 µm thick sections cut on a microslicer were mounted on a glass with glycerin:phosphate buffered saline (pH 7.4, 1:1, v/v) and immediately examined under a fluorescence microscope (Leica K.K., Tokyo, Japan) using a UV filter (BP 340–380 nm).

**Determination of hepatic VA levels:** VA standards (all-trans-retinol, retinyl acetate, and retinyl palmitate) and all organic solvents of HPLC quality were purchased from Nakalai Tesque Inc., Kyoto, Japan. Water was purified by adsorption, deionization, and filtration using a Milli-Q SP TOC (Nihon Millipore Ltd., Tokyo, Japan). Aqueous and organic solvents were filtered through a 0.45 µm millipore filter (Nihon Millipore Ltd.) and degassed prior to use.

After perfusion, the liver was cut into small pieces, about 1 mm3, with a razor blade and dehydrated by lyophilization (CentriVap Concentrator; Labconco Co., Missouri, U.S.A.) for 8 hr. In order to measure the concentration of retinyl palmitate secreted into the bile, the gallbladder was also removed, weighed, and stored at -80°C until HPLC analysis. The VA levels of serum, liver, and bile were determined within 2 days after sampling. The levels of retinol, retinyl acetate and retinyl palmitate in liver, serum, and bile were determined by the method of Furr [6]. Briefly, for 500 mg portions of liver, 2 g of anhydrous sodium sulfate were added, grounded, and extracted with 5 portions of dichloromethane. The extracts of each sample were filtered and diluted to 50 ml. A 5 ml aliquot of each was then gently evaporated under argon, and the residue dissolved in 1–2 ml dichloromethane. VA levels in liver homogenates, serum, and bile were determined in 10–µl aliquots using a gradient reversed-phase HPLC system (Hitachi L-7000; Hitachi Seisakusyo Co., Ltd., Tokyo, Japan) comprised of a rhodyne injector, a L-7100 pump, a L-7400 UV detector, and a L-7300 column oven. The chromatograms were plotted with a D-7500 plotter. Retinol, retinyl acetate, and retinyl palmitate were separated with a Nucleosil 100-5C18 [4.6 (i.d) x 150 mm] column (GL Sciences Inc., Hiroshima, Japan), and the respective retention times were 4.4, 5.5, and 11.7 min. Each sample was analyzed twice and the reproducibility was ±3.7% for retinol and ±2.9% for retinyl palmitate. Retinol and retinyl palmitate have equal molar absorptivities (52,275 M-1 cm-1 at 325 nm), and so standards were quantitated by absorbance.

**Statistical analysis:** Analysis of variance and student’s t-test (StatView 4.02; Abacus Concept Inc., Berkeley, U.S.A.) were used for statistical evaluation. The data of HPLC analysis from RA-injected and vehicle control animals were compared. The level of statistical significance was set at P<0.05.

**RESULTS**

After a single subcutaneous injection of RA, retinyl palmitate level in liver homogenates increased significantly from 6 to 12 hr post-injection (Fig. 1). When retinyl palmitate in the liver homogenates decreased between 12 to 24 hr post-injection (Fig. 1), the concentration of retinyl...
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Palmitate in bile (Fig. 2) and retinol in serum (Fig. 3) increased significantly. The retinol levels of the liver homogenates also increased significantly from 3 hr post-injection onward (Fig. 4).

Fluorescence microscopy revealed many fluorescent VA-containing lipid droplets in hepatocytes around the central vein at 12 hr post-injection (Fig. 5B). Very few fluorescent VA-containing lipid droplets were detected in the liver sections of vehicle controls (Fig. 5A) and those of RA-injected mice at 18 hr post-injection.

Using a modified gold chloride staining method, no retinyl esters-containing lipid droplets were observed at the electron microscopic level in the liver sections of controls (Fig. 5A) and those of RA-injected mice at 18 hr post-injection.

The vacuolization observed around the sinusoid area at 18 hr post-injection was more pronounced at 24 hr post-injection (Figs. 7B and 7C). Numerous vacuoles with various size were seen in the vacuolated area of hepatocytes of RA-injected mice, but no lipid droplet was seen (Fig. 7D).

Many Ito cells were observed around the central vein 72 hr post-injection (Fig. 8A). At the electron microscopic level, the cytoplasm of those Ito cells contained many gold chloride reacted lipid droplets (Fig. 8B).

DISCUSSION

In the present study, alterations of the retinol and retinyl palmitate levels of liver homogenates were monitored by a HPLC technique at 0, 3, 6, 12, 18, 24 and 72 hr after a single subcutaneous injection of RA. All animals sacrificed between 09:00 and 11:00 a.m. had a very constant concentration of retinyl palmitate levels in bile of gallbladder with a low standard deviation. After RA injection, many fluorescent lipid droplets and gold chloride reacted lipid droplets were detected in hepatocytes at 12 hr post-injection. These findings indicate that most of RA-injected subcutaneously is present in the lipid droplets of the hepatocytes in the form of retinyl palmitate up to 12 hr.
Between 12 and 24 hr post-injection of RA, the retinyl palmitate levels of the liver homogenates decreased, but its secretion into bile increased. Secretion of retinol and retinoic acid metabolites in the bile, feces, and urine of rats has been reported in studies using labeled compounds [20, 21, 26]. The results of these studies indicated that the retinol or retinoic acid administered was metabolized in extrahepatic organs prior to being secreted in the bile and then in the feces and urine. Increased activity of alkaline phosphatase, an enzyme involved in transport processes through the bile canaliculus membranes of hepatocytes has also been reported in high dose VA-treated rats [17]. These findings indicated increased transport of retinyl palmitate from hepatocytes into the bile.

A significant increase in liver and serum retinol was also observed from 6 hr post-injection of RA onward. It has been reported that retinyl esters taken up by hepatocytes as chylomicron remnant retinyl esters are hydrolyzed to retinol [27]. Some of the retinyl palmitate present in hepatocytes may be hydrolyzed to retinol, stored in the Ito cells, and transferred into the blood [27, 28]. On the other hand, a portion of the excessive retinyl palmitate present in hepatocytes would be secreted into the bile in unaltered form, and partially explain the decrease in retinyl palmitate in the liver homogenates between 12 to 24 hr post-injection.
Intense vacuolization was observed at 24 hr post-injection of RA, and transitional lipid droplets between vacuoles and lipid droplets were identified. Vacuoles varying in number and size and containing acid phosphatase activity were observed within hepatocytes after intraportal injection of retinol [14]. Autophagic vacuoles increased markedly in number under a variety of conditions including starvation, administration of toxicants, hypoxia, and injuries, such as irradiation [9, 19]. Autophagy protects the remainder of the cells from the possible destructive effects of sequestration and digestion. A portion of the excessive retinyl palmitate present in hepatocytes at 12 hr post-injection of RA may be metabolized through vacuoles to protect them from the toxic effects of VA. Increased numbers of peroxisomes [23] and catalase activity [25] have also been associated with increased antioxidant capacity against radical scavengers [17]. At 72 hr post-injection of RA, retinyl palmitate in the liver homogenates and the number of Ito cells around the central veins increased, indicating that most of the retinyl palmitate determined by HPLC in the liver homogenates might originate from retinyl palmitate stored in Ito cells.

In conclusion, injected RA first appeared as retinyl palmitate in lipid droplets in hepatocytes at 12 hr. Most of the retinyl palmitate present in hepatocytes at 12 hr post-injection was partially metabolized in vacuoles, transferred into the blood and secreted into the bile over a 24 hr period. It was then stored mainly as retinyl palmitate in lipid droplets in Ito cells at 72 hr post-injection. Determination of retinol-binding protein (RBP) and retinyl ester hydrolase
(REH) levels in isolated hepatocytes and Ito cells will confirm the process postulated based on the results of the present HPLC and cytochemical studies.

REFERENCES


