Regulation of synthesis and secretion of the lipoprotein by cultured eel hepatocytes

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SUMMARY: Eel hepatocytes actively synthesize and secrete a lipoprotein. It consists of about 60 % triacylglycerol and 10 % protein, and main apolipoproteins are apoB and apoA. Its average particle size is 750 nm. These characteristics show it is a chylomicron like lipoprotein. Thyroxine (T4) at 10^{-8} M activates the lipid synthesis in the hepatocytes and increases hepatocellular lipid. The synthesis of the chylomicron like lipoprotein is also stimulated by T4, but T4 does not affect the synthesis of other secreted proteins. T4 specifically stimulates the synthesis of the lipoprotein. Though docosahexaenoic acid (DHA) stimulates lipid synthesis in the hepatocytes, DHA inhibits the synthesis and secretion of the lipoprotein. However, DHA does not inhibit the synthesis of other secreted proteins. DHA specifically inhibits the synthesis and secretion of the lipoprotein, but eicosapentaenoic acid does not inhibit. HDL specifically binds HDL-binding protein (HBP) of the plasma membrane. The binding of HDL to the hepatocytes is saturated at concentrations over 100 μ g HDL/ml and Kd value is 20 μ g HDL/ml. HDL and apoAI are ligands of HBP. We suppose that HDL and free apoAI in eel serum take part in lipid transport through the mediation of the HBP.

KEY WORDS: hepatocytes, thyroxine, HDL, HDL-binding protein, DHA, EPA

INTRODUCTION

Cultured eel hepatocytes actively synthesize and secrete a chylomicron-like lipoprotein. In mammals chylomicron is synthesized and secreted by epithelial cells of small intestine. However, the characterization of the secreted lipoprotein by eel hepatocytes is chylomicron-like in its apolipoprotein component, particle size and chemical composition. This unique lipoprotein is synthesized and secreted under hormonal regulations, such as insulin, thyroxine, and estradiol, whilst serum very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) affect its synthesis and secretion. Furthermore, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) affect the synthesis and secretion.

PRIMARY CULTURE OF EEL HEPATOCYTES

Eel (*Anguilla japonica*) hepatocytes are prepared by collagenase digestion of a perfused liver as reported by Hayashi and Ooshiro¹⁾ and Hayashi and Komatsu.²⁾ Average yield of isolated hepatocytes is 10^8 cells per g liver. Isolated hepatocytes are cultured in William's medium E (pH 7.4) containing 5 % fetal bovine serum (FBS), insulin (0.16 μ M), NaHCO_3 (23 mM), penicillin (70 μ g/ml), and streptomycin (100 μ g/ml).^{2,3)} Hepatocytes of carp (*Cyprinus carpio*),⁴⁾ rainbow trout (*Oncorhynchus mykiss*),⁵⁾ salmon (*Oncorhynchus keta*),⁶⁾ channel catfish (*Ictalurus punctatus*),⁷⁾ tilapia (*Oreochromis niloticus*),⁸⁾ and Indian catfish⁹⁾ are also cultured.

Eel hepatocytes hardly attach on a dish if culture dishes are not

precoated with fibronectin (5 μ g/cm²) or poly-L-lysine (50 μ g/cm², 0.02 % solution).^{2,10)} Collagen coating is not useful for attachment of eel hepatocytes. However, carp hepatocytes can attach on a dish precoated with collagen.⁴⁾ Comparison of trout hepatocyte culture on different substrates was reported.¹¹⁾

Eel hepatocytes can be cultured in FBS-free William's medium.¹⁰⁾ The medium consists of William's medium E, insulin (10 μ g/ml), glucagon (1 μ g/ml), epidermal growth factor (50 ng/ml), prolactin (20 mU/ml), growth hormone (10 mU/ml), NaHCO_3 (24.5 mM), penicillin (70 μ g/ml), and streptomycin (100 μ g/ml). Insulin-deficient medium, particularly, affects the morphology and viability. The morphology was spherical during 11 days culture in insulin-deficient medium and the number of living cells was 53 % of that of control cells.¹⁰⁾

Fish liver has many important functions similar to those of mammalian livers. Perfused livers and isolated liver cells are available for investigation of gluconeogenesis.^{1,12)} Lipogenesis¹³⁾ and synthesis and secretion of serum proteins¹⁴⁾ were investigated in cultured eel hepatocytes. Induction, synthesis, and secretion of vitellogenin by cultured hepatocytes of rainbow trout and eel was reported by Meiter *et al.*¹⁵⁾ and Kwon and Mugiya,¹⁶⁾ respectively. Induction of detoxification enzyme of P450 was also investigated by using cultured hepatocytes of rainbow trout¹⁷⁾ and of Indian catfish.⁹⁾

CHARACTERIZATION OF LIPOPROTEIN SECRETED BY CULTURED EEL HEPATOCYTES

Main apolipoproteins of the secreted lipoprotein are apoB48, apoAI, and apoAII. It is known that chylomicron of mammals contains apoB48 and apoAI as the main apolipoproteins. Chemical composition of the lipoprotein secreted by eel hepatocytes is triacylglycerol (TG, 69.0 ± 5.1 %), phospholipid (PL, 14.7 ± 2.9 %), protein (12.0 ± 4.8 %), free cholesterol (FC, 3.8 ± 0.3 %), and cholesteryl ester (CE, 0.36 ± 0.50 %). TG is a main component, as that in mammalian chylomicron, but the protein ratio is rather higher than that of the chylomicron. The higher content of the protein reflects the density of the secreted lipoprotein, which is $0.95 < d < 1.063$. The mean particle size is 750 nm diameter. This particle size is almost the same size as that of the chylomicron.

The secreted lipoprotein by cultured eel hepatocytes is different from eel serum lipoproteins. Eel serum lipoproteins consist of two main lipoproteins. One is VLDL and the other is HDL. The VLDL contains apoAI and apoAII besides apoB48 as main protein components and TG as main lipid. This characterization is the same as that of the secreted lipoprotein. The HDL consists of apoAI and apoAII and contains PL and cholesterol (CH) as main lipid. As Babin and Vernier¹⁸ reported, HDL is predominant lipoprotein in plasma of many fishes.

EFFECT OF THYROXINE

Thyroid hormone is known to induce synthesis of lipogenic enzymes, malic enzyme, acetyl CoA carboxylase and fatty acid synthetase, in a liver. Promoting synthesis of lipogenic enzymes was confirmed by cultured hepatocytes with thyroid hormone¹⁹ and by in vivo experiments.²⁰ We then investigated whether stimulation of lipid synthesis by thyroid hormone induced promoting lipoprotein synthesis and secretion.¹³

Eel hepatocytes cultured with 10^{-8} M L-thyroxine (T4) for 6 days synthesized lipids from 14 C-acetate at a higher rate than control cells cultured without T4, and particularly CH and PL syntheses were stimulated about 2-fold. Quantities of CH, PL, and TG in the hepatocytes were also increased by T4 and the efflux of lipids into the culture medium was stimulated. Because cultured eel hepatocytes secrete only one kind of lipoprotein, a chylomicron-like lipoprotein, all lipids in the medium associate with the secreted lipoprotein. Therefore, the stimulatory effect of T4 on the efflux of lipids into the culture medium seems due to the stimulatory effect of T4 on synthesis and secretion of lipoprotein. When the hepatocytes were incubated with 3 H-leucine and 14 C-acetate, the incorporation of 3 H-leucine and 14 C-acetate into the secreted lipoprotein in the presence of T4 increased significantly compared to that of the control. However, the incorporation of 3 H-leucine into the proteins except lipoprotein was not different between T4-treated and control cells. These results suggest that eel hepatocytes cultured with T4 stimulate specifically synthesizing and secreting the lipoprotein.

Many reports on thyroid hormone effect are consistent with stimulatory effect on lipogenesis in the liver, but the effect on

lipoprotein synthesis and secretion is unclear. Daily injection of triiodothyronine (T3) to rats over a period of 6 days led to increases in serum TG and hepatic mRNA level for apoAI.²¹ Contrary to this, perfused livers from rats injected T3 reduced VLDL synthesis and secretion²² and TG synthesis and efflux.²³ We suggest that the primary function of T4 in eel hepatocytes is stimulation of lipogenesis and not stimulation of synthesis and secretion of lipoprotein. We consider that the stimulation of lipoprotein synthesis and secretion seems due to the increased lipids in the hepatocytes. Because the increased lipids caused by addition of eel serum VLDL or HDL to cultured eel hepatocytes induce the stimulation of lipoprotein synthesis and secretion.²⁴ In any experiments of the addition of serum VLDL, HDL or T4 to cultured eel hepatocytes, the increased lipid in the hepatocytes is PL and CH.

Thyroid hormone in teleosts is concerned with their migration and spawning, circulating concentrations of T4 in salmonids was reported during their migration²⁵ and spawning.²⁶ When juvenile salmonids in the river migrate to the sea, their concentration of T4 increased. Contrary to juvenile salmonids, when salmonids grown in the sea migrate to the river for spawning, their concentration of T4 decreases from 1.5×10^{-8} M to below 0.7×10^{-8} M.²⁶ Juvenile eels migrate from the sea to the river and eels grown in the river migrate to the sea for spawning, contrary to salmonids. If the increase of T4 concentration in eel serum induces the migration to the sea, stimulation of lipogenesis and the lipoprotein synthesis and secretion in the liver by T4 is supposed to reserve energy source for the migration.

HDL BINDING PROTEIN

Although the function of HDL binding protein is still unclear,²⁷ its presence of plasma membranes of cells such as hepatocytes,²⁸ epithelial cells of small intestine²⁹ and platelets³⁰ has been confirmed. The cloning of the HDL binding protein was reported.³¹ We demonstrated the specific binding of serum HDL to eel hepatocytes by using a fluorescent lipophilic dye labeled HDL.³² The dye is N,N-dipentadecylaminostyrylpyridinium iodide (di-15-ASP). Freshly isolated hepatocytes bind to di-15-ASP-HDL under the incubation at 0 °C for 2 h under the dark, the fluorescence of the cells bound to it can be detected by a flow cytometer. Unlabeled HDL inhibits the binding. The binding of di-15-ASP-HDL to the hepatocytes is saturated at concentrations over 100 μ g HDL protein/ml and Kd value is 20 μ g HDL protein/ml. The Kd value is almost the same as those of rat liver³¹ and epithelial cells of rat intestine,²⁷ which are 10 to 50 μ g/ml.

HDL binding protein (HBP) is isolated from the microsomal fraction of eel liver homogenate by affinity chromatography with a HDL-column.³³ The HBP isolated by a HDL-column consists of at least three proteins with low molecular weights of 18.5, 14.5 and 13.5 kDa; the main component is 14.5 kDa. These products are not products of protease digestion, as the procedure was

carried out in the presence of protease inhibitors including (p-aminophenyl) methanesulfonyl fluoride, 4-(2-aminoethyl)-benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, aprotinin and EDTA. The HBP specifically binds to FITC-apoAI and faintly binds or does not bind to FITC-apoAII. Furthermore, binding of di-15-ASP-HDL to isolated eel hepatocytes is inhibited by the antibody to apoAI, but not inhibited by the antibody to apoAII. These results strongly suggest that the HBP isolated from the microsomal fraction is present on the plasma membrane of eel liver. On the other hand, we demonstrated the presence of free apoAI in the eel serum and that free apoAI is secreted in the culture medium by cultured eel hepatocytes.³⁴⁾ Therefore, we can suppose that HDL and free apoAI in eel serum take part in lipid transport through the mediation of the HBP.

EFFECT OF DHA AND EPA

The first characteristic on the effect of DHA or EPA is that hepatocellular lipid synthesis is stimulated by DHA. DHA at 1 mM increased lipogenesis from ¹⁴C-acetate by 3.5 fold and the hepatocellular TG, FC, and CE syntheses increased 9.7, 1.6, and 8.5 fold, respectively.³⁵⁾ Furthermore, addition of 1 mM DHA to the cultured hepatocytes increased the amount of hepatocellular lipid such as TG, PL, and CH significantly compared to that of control cells. Particularly, the TG increased 4.8 fold in DHA treated cells. Similarly, EPA at 1 mM increased hepatocellular lipid by 1.64 fold.

The second characteristic is that DHA inhibits the secretion of the lipoprotein. When DHA treated cells were incubated with ¹⁴C-acetate and ³H-leucine, the incorporation of ¹⁴C and ³H into the lipoprotein reduced to 55 and 60 %, respectively, compared to those by control cells. However, DHA did not affect the incorporation of ³H-leucine into other secreted proteins and the hepatocellular proteins.³⁶⁾ Though the incorporation of ¹⁴C-acetate into the secreted lipoprotein was reduced by DHA, the distribution of radioactivity between TG, PL, FC, and CE of the secreted lipoprotein was the same between DHA treated and control cells. The amount of the lipoprotein secreted by 1 mM DHA treated cells was 16.6 % of that by control cells, but the chemical composition of the lipoprotein was also the same between the DHA treated and controls. Similarly, the profile of apolipoproteins determined from protein staining or autoradiogram after SDS-PAGE of the lipoprotein was the same between the DHA and controls. It was reported that DHA and EPA stimulated degradation of apoB protein and this degradation was the reason to reduce lipoprotein secretion since apoB is essential for lipoprotein assembly.³⁶⁾ The lipoprotein secreted by eel hepatocytes contains apoB corresponding to apoB48 as a main apolipoprotein, but the high specific activity of the apolipoproteins including apoB48 secreted by DHA treated cells would be difficult to explain the increase of apoB48 degradation. Recently, we confirmed that apoB48 detected by

immunoblotting was much more present in the hepatocytes treated with DHA than in the control cells. Inhibition of the lipoprotein secretion by DHA seems due to inhibition of secretion itself and does not due to apoB48 degradation.

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