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Original Article

Expressive Modulation of the Cyp7a1 and Ibat Protein by Cholesterol and its Contribution to the Maintenance of the Normal Cholesterolemia in Mouse

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Abstract

The aim of our study is to prove the existence of a very coordinated system of regulation between the route of excretion of taurocholic acid and cholesterol. We proceed to follow up physiologically animals, with no differences in water and food consumptions between the control group and the group of 5% diet of cholesterol. On the other hand dieresis has increased in mice with 5% diet of cholesterol. Cholesterol induced in the liver the transcribed rate of Cholesterol 7α-hydroxylaze (CYP7A1), limiting enzyme of bile acids synthesis. What's more, expression of CYP7A1 encoding gene is induced in mice with 5% diet of cholesterol. Also we noticed an increase of distribution and wealth expression related to the RNA and CYP7A1 in the liver reported by Northern Blotting. According to ileum role in intestinal re-absorption of bile acids, our interest was focused on the ileac transportation: the standard expression of Ileal Bile Acid Transporter (IBAT) has strongly reduced; in mice with rich diet of cholesterol the Western Blotting of proteins has quantitatively reduced. IBAT's oppression can be explained by the diminution of the ileac transportation of taurocholic acid (TCA).It results an increase of fecal flight of TCA in mice with a rich diet of cholesterol.

Key words: Bile acids, cholesterol, taurocholic acid, CYP7A1, IBAT, Mouse

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Abbreviations

Chol	Cholesterol
CYP7A1	Cholesterol-7α hydroxyl-aze
IBAT	Ileal Bile Acid Transporter;
TCA	Taurocholic Acid

Introduction

Cardio-vascular diseases (CVD) remain one of the main causes of mortality (Mathers CD et al., 2009) and lowering concentration of LDL-cholesterol remains a clinical and public health objective to reduce the impact of that pathology. A number of factor impact of risk has been now identified among which the hypercholesterolemia. It is generally accepted that elevation of LDL-cholesterol increases cardiovascular risk, although it's common today to speak about ¨bad cholesterol¨.

Facing this public health problem, the interest of cholesterol's absorption study continues to grow and remains in actuality with discovering of newly hypo-cholesterolemians acting not only by inhibition of the synthesis of cholesterol (Statins) but also by inhibition of its absorption.

Physiological, nutritional and genetic factors are involved in the metabolism of cholesterol but it is difficult to determine the relationship between causes and effect given that, the complexity of regulation mechanisms of cholesterol and simultaneously involvement of several factors. (Santosa S et al., 2007c)

Thus the metabolism of cholesterol is a key element of the development of cardio-vascular diseases (Russell DW 1992; Ros E 2000)

The homeostasis of cholesterol is implementing complex regulating mechanisms which involve both absorption and synthesis of cholesterol.

Mice are implementing a resistance system to hypercholesterolemia in case of alimentary overload in cholesterol. It is noticed the relation between the metabolism of cholesterol and the one of bile acids. Bile acids are synthesized from cholesterol in the liver. At the end of that synthesis, around 95% of bile acids are re-absorbed along intestine to be recycled in the liver trough portal vein. The remaining 5% are eliminated by fecal route. We have also noticed that bile acids represent the main way of elimination of cholesterol.

The transport of conjugated bile acids inside key organs for their metabolism requires several transporters.

The re-absorption of intestinal non conjugated bile acids held by passive way and easily at the duodenum and jejunum level, but the majority of bile acids is reabsorbed in the ileum by active transport. In the brush border of ileocytes, bile acids are supported by intestinal bile transporter (IBAT), a membrane protein cold also apical sodium-dependant bile acid transporter (ASBT). (Schneider BL et al., 1995)

In humans, some mutations of IBAT encoding gene cause serious bad-absorption of bile acids which is the origin of deep perturbation in lipid metabolism (Oelkers P et al., 1997).This protein is very important in ileac capture of bile acids. At that site, bile acids are supported by Ileal Bile Acid Binding Protein (IBABP).Bile acids are after secreted in portal vein according to three systems of transport among which truncated form of IBAT: the t-IBAT (Lazaridis KN et al., 2000).

The MRP3 are also implicated (Kiuchi Y et al., 1998) and heterodimere formed by Organic Solute Transporters (OSTα and OSTβ) (Dawson PA et al., 2003). Then bile acids are recaptured at the apical pole of hepatocytes via Na^+ Taurocholate Co-transporting Polypeptide (NTCP) grace also to Organic Anion Transporter Polypeptide (OATP) (Mathers CD et al., 2009; Santosa S et al., 2007c ; Ros E 2000).At the baso-lateral pole, bile acids are spilled in the bile by the Bile Salt Export Pump (BSEP) and in few by Multidrug Resistance-Associated Protein 2 (MRP2).Ordinary a minority of non reabsorbed bile acids or excreted by the liver into the general circulation via MRP3 is filtered by kidneys. In epithelial cells of proximal tubules, at the apical pole the implicated proteins in bile acids transport are IBAT, OATP1 and MRP2; and at the baso-lateral pole MRP3 and OST. At ileal and renal apical pole, MRP2 can be responsible for the re-excretion of bile acids.

The excretion's modulation of the transporters can cause the excretion of bile acids within the context of cholesterol elimination. Cholesterol could be excreted ever since important transporters have been identified. In the liver, the formed heterodimer by ATP-Binding Cassette G5 & G8

(ABC G5/G8) excrete the cholesterol in the bile. At ileal level, Niemen Pick C1 like Protein 1 (NPC1L1) permitted the intestinal capture of alimentary and bile cholesterol.

The objective of this work is first: to study the impact of a rich diet of cholesterol on gene encoding cholesterol 7α-hydroxylase (CYP7A1) expression (which is the limiting enzyme of bile acid synthesis in the liver) on the involvement level of Ileal Bile Acid Transporter (IBAT) since ileum is the important site of intestinal bile acid re-absorption; secondly to evaluate the capture capacity of taurocholic acid and its consequence on fecal leakage of total bile acid.

It's to contribute to a better molecular and physiological knowledge which would be responsible of the metabolism of cholesterol, that we undertook in this work. Two different but complementary approaches have been used to better understand the metabolism of cholesterol as whole.

Materials and Methods

Animals

Eight (8) weeks male mice C57BL/6J have been used. Those mice have been fed during fifteen (15) days and divided into two (2) groups:

-The control group fed with standard diet: wheat, fish and vitamins

-The second group fed with a rich diet of 5%cholesterol called: group of 5%cholesterol

For physiological studies, fifteen (15) control mice and fifteen (15) 5%cholesterol mice were placed into two different metallic cages to monitor parameters such as weight, food consumption, water consumption, diuresis and faecal excretion. Then, mice are killed. Ileum and liver collected and frozen at -80°C.

Fifteen (15) other control mice and fifteen (15) other of the group of 5%cholesterol mice have been also placed in normal cages with intension to carry out test of ileal capture labeled taurocholic acids by isolated cove method.

The experimental protocol has been approved by the ethic commission in experimental research with animals according to the international conventions.

Total bile acids dosage

The dosage of bile acids is based on their oxidation by 3-α hydroxyl-steroid dehydrogenize. That reaction leads from the reaction of NADH and nitro-tetrazolium to a colored compound with absorbance 540nm.

RT-PCR quantification assay

Total RNA was prepared from treated T-cells using Trizol reagent (Invitrogen Life Technologies, Groningen, the Nederland) according to the manufacturer's instructions. The integrity of RNA was electrophoretically checked by ethidium bromide staining and by the OD absorption ratio OD_{260nm}/OD_{280nm}.A microgram of RNA was reversibly transcribed with Superscript II RNAze H-reverse transcriptase using oligo (DT) according to the manufacturer's instructions (Invitrogen Life Technology, France)

Real PCR time was performed on an icycler ,real detection time system(Bio-rad, Hercules, CA, USA),and amplification was done by using SYBR Green I detection(SYBR Green Jumpstart, Taq Ready Mix for quantitative PCR, Sigma-Aldrich, St Louis; MO USA). Oligonucleotide primers, used for mRNA analysis, were based on the sequences of mice gene in the Gene Bank database.

The amplification was carried out in a total volume of 25µl containing 12.5µl SYBR Green Taq Ready Mix, 0.3µM of each primer and diluted cDNA. Cycling condition consisted to an initial denaturation step of 95°C for 3minas a hot start followed by 40 cycles of 95°C for 30 sec or at 60°C for 30sec with a simple fluorescence detection point at the end of the relevant annealing or extension segment. At the end of the PCR, the temperature was increased from 60 to 90°C for 15sec and at 58$\pm$ 2°C for 60sec, and the fluorescence was measured every 15sec to draw the melting curve. The standard curves were generated for each protein or β-actin using serial dilution of positive control template in order to establish PCR efficiencies. All determinations were performed at least in duplicates using two dilutions of each assay to achieve reproducibility. Results were evaluated by icycler iQ software including standard curves, amplification efficiency(E) and threshold cycle(Ct).Relative quantization of mRNA expression was determine using the ΔΔCt in which

ΔΔCt= ΔCt (gene of interest)−ΔCt(β-actin). ΔCt= Ct (interest group) –Ct (control group).Relative quantity (RQ) was calculated as follow: RQ= $(1+E)^{(-\Delta\Delta Ct)}$

Protein expression detection by Western Blotting

Levy of ileac mucosa were lysed in one hour time on ice with 40µLof buffer of cold lyse (Tris HCl, PH=8.2; 20mM final; NaCl 140mM final; NP-40 1%; Sodium orthovanadate PH=10,2mM final ; NaF 10mM; EDTA PH=8 5mM final; PMSF 1mM final, antiproteaze cocktail(sigma)2µL for 1ml of final volume)

After centrifugation (15000 rpm, 15 min at 4°C) the floated part were recovered for protein dosage (spectroscopic dosage BCA) before their separation by electrophoresis' migration. Denatured protein (25µg) were deposited on SDS-Polyacryl amide gel bis acryl amide (8%), and transferred on PVDF membrane.

The membrane were put on saturated 2hours in dilapidated milk solution diluted to 5% in TBS /(Tween- 20, 0.05%) and then after a brief rinse in a solution of TBS-Tween-20 has been 2hours reincubered with primary antibody. The membrane undergoes four (04) washings of five (5) minutes in Tween-20 0.05% buffer before one more to be incubate an hour time with secondary antibody(Dilution of 1/1000) conjugated to an enzyme HRP(Horse Radish Peroxydaze) diluted in the same previous solution.

The revelation of protein banding is finally performed thanks to the solution containing the substrate of HRP enzyme .To ensure that the quantity of the protein immunoassay detected were deposited uniformly, we have made the revelation of β-actin protein on the same membrane after dehybridization .

Every Western Blotting is representative of three experiments realized from three independent preparations.

Detection of CYP7A1's RNA distribution's expression in the liver and their relative plentiful study by Northern Blotting

20µg of total RNA have been denatured with formaldehyde in electrophoresis' gel like denaturant then storage on 1% agar's gel. We proceed then to the electrophoresis. After migration, the totals of RNA were fixed under UV light to the membrane. We proceed to a pre-hybridization which permits to saturate the non specific sites of the membrane. Finally the hybridization permits to pair the radioactive cDNA sand of the gene coding CYP7A1 to the corresponding mRNA. The sand 18S has been used to standardize the stored quantity. Once hybridized, membranes were washed in solutions to eliminate non specific absorbed radioactivity on the surface .The results are obtained by contacting the membrane with auto radiographic films.

Study of ileac capture of taurocholic acid

A group of 5 mice were feed with standard diet and another group subjected to a rich diet of 5% cholesterol during two weeks. An infused solution of taurocholic acid has been prepared at 3mM in the PBS. The operation proceeds under heat lamp in order to maintain the body temperature of animals. A distal segment of ileum has been cleared, ligatured and washed by PBS at 37°C.The solution of TCA has also been infused and the segment has rapidly been placed in the abdomen of the animal. The sufficient contact time is 1mn .At the end of that time, the cove was isolated, washed and digested in 0.2N of soda and the radioactivity in the ileocytes is measured using a scintilla-meter. The total quantity of taurocholic acid incorporate was standardized in relation to the total quantity of protein dosed by the kit of calorimeter.

Statistical Analysis

Results are shown as mean±SEM. The significance of the differences between mean values was determined by two ways: ANOVA (STATISTICA, Version 4.1, Stasoft, Paris France) followed by the least significant difference 'LSD' test. (Hsu JC; 1996) Differences were considered significant at $p < 0.05$; n=6

Results

Evolution of mice weight

We found after three (3) days of acclimatization to the cage that animal's weight

didn't change between the control group and the group subjected to 5%cholesterol diet. (Fig. 1)

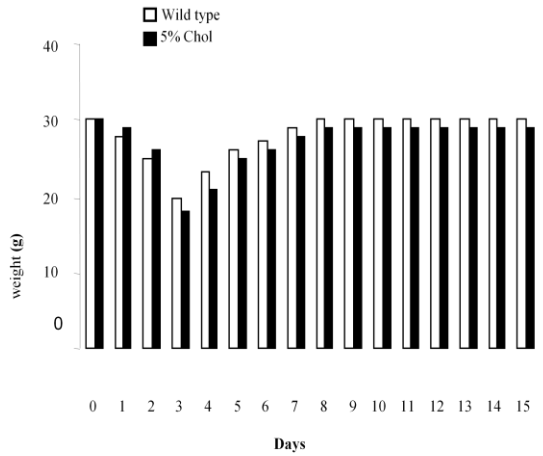

Figure .1: Evolution of mice weight during the fifteen days of diet

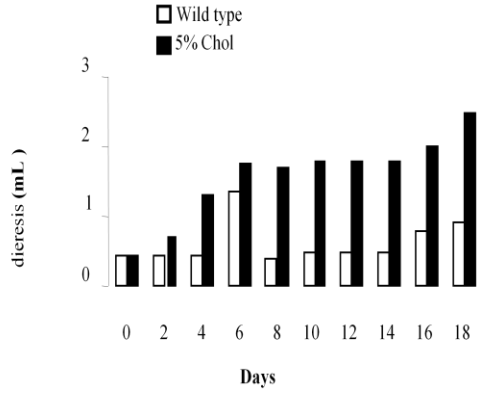

Figure. 2 Evolution of dieresis in the two groups of mice

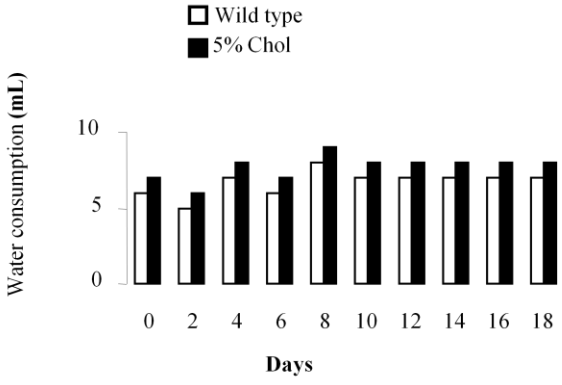

Figure. 3 Water consumption

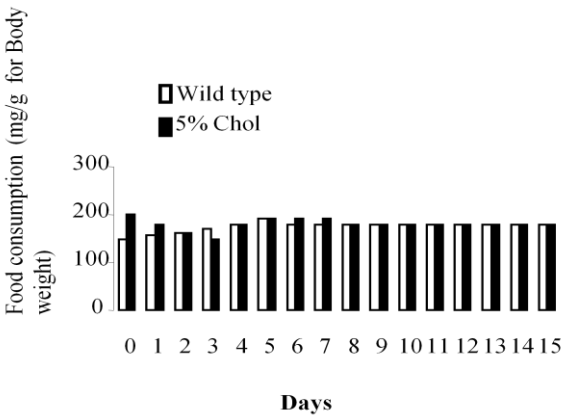

Figure. 4 Food consumption

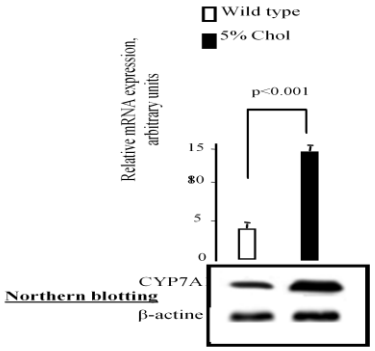

Figure .5 CYP7A1 expression in liver

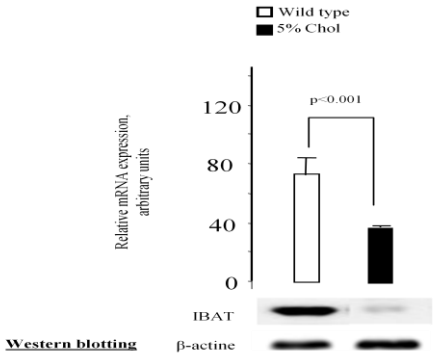

Figure. 6 Intestinal IBAT's RNA expression and its protein

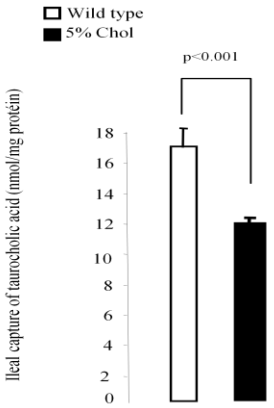

Figure .7 Ileal capture of taurocholic acid

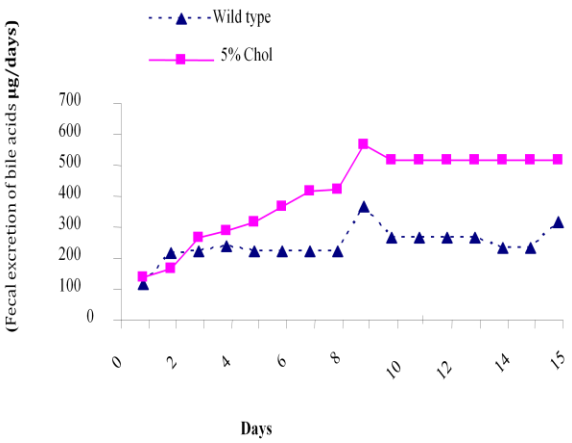

Figure. 8 Fecal excretion of bile acids

Evolution of dieresis in mice

Diuresis in mice has increased in mice subjected to 5% diet of cholesterol. (Fig. 2)

Water consumption in mice

Fig.3 shows that water consumption didn't change in the two groups

Food consumption

Food consumption didn't change in the two groups of animal (Fig. 4)

Expression of the CYP7A1 gene encoding in the liver

The expression of the gene which encodes CYP7A1 is induced in mice subjected to 5%diet of cholesterol. Also the expression of the distribution and abundance relative to RNA in the liver is induced. (Fig. 5)

The cholesterol decreases the transcripted rate of the intestinal IBAT and the expression of its protein

The expression level of the transcripted rate of IBAT has sharply felled and the Western Blotting of its protein has quantitatively reduced in mice subjected to 5% diet of cholesterol (Fig. 6)

The cholesterol induced the diminution of ileac uptake of taurocholic acid

Cholesterol induced in lower capacity of the captivity of taurocholic acid (Fig. 7)

The cholesterol induces an increase of fecal excretion of bile acids

Cholesterol induced an increase of the quantity of bile acids excreted in feces of mice (Fig. 8)

Discussion

In this study, we have shown in experimental conditions that:

Mice subjected to 5%cholesterol diet didn't change their weight in comparison to the control group. On the other hand the dieresis has been increased in those mice.

Physiologically it's noticed that an increase of the quantity of bile acids in urines has consequences to the excretion of cholesterol. Cholesterol can be directly excreted or converted into bile acids which elimination can be urinary or fecal. We have noticed in our experimental conditions that, the elimination of bile acids by those two ways was significantly increased in mice subjected to 5% cholesterol diet. At the same time, we observed that the overload of cholesterol induced gene encoding CYP7A1 which really induces the synthesis of bile acids (Torchia EC, 1996). The biosynthesis of bile acids is subjected to different type of modulation. One of the most important is the effect of bile acids on their own biosynthesis. The recycling of bile acids via entero- hepatic cycle is well described in the control of that synthesis. Then, oral administration of resins as cholestyramine which is trapped in intestinal tracts bile acids with consequences to limit the flux back of bile acids to the liver and increase their fecal excretion. It results

the stimulation of the activity of CYP7A1 (Chyang JYL et al., 1990**)**

A rich diet in cholesterol provokes in mice an increase of the activity of CYP7A1 without change the activity of CYP27 (Pandak WM et al**.,** 1998).The synthesis of cholesterol-7α hydro laze has increased in mice with 2% cholesterol diet. The increase of the enzyme CYP7A1 synthesis will induce the transformation of alimentary cholesterol to bile acids.

Bile acids poured in the intestine are reabsorbed in ileum grace of IBAT transporter located in apical membrane of enterocytes. This transporter is an interesting target because its inhibition can increase bile acids elimination from the body and activate cholesterol transformation into bile acids. (Bhatt BG et al., 2003)

Different IBAT inhibitors have been studded in animals and have been proved to be efficient hypo-cholesterolemians. (Root C et al., 2002)

However it is noticed that the increase of the concentration of free bile acids in intestine can cause minor side effects as diarrhea and also develop some intestinal diseases. (Izzat NN et al., 2000)

Evidences of the extreme decrease of IBAT expression can explain the observed decrease of ileac capture capacity of taurocholic acid. It may justify the increase of fecal flight of bile acids. The fecal flight has been increased in mice with invalid IBAT- encoding gene and decreased total pool of bile acids (Dawson PA et al., 2003). Thus the expression's decrease of that protein (IBAT) participates to fecal elimination of bile acids and consequently cholesterol elimination. Urinary flight of bile acids has increased what can be explained by the decrease of IBAT expression at epithelial cells level of proximal tubules.

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