ORIGINAL

Hypercholesterolemia and effects of high cholesterol diet in type IIa sodium-dependent phosphate co-transporter (Npt2a) deficient mice

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Abstract : The type IIa sodium-dependent phosphate co-transporter (Npt2a) is important to maintain renal inorganic phosphate (Pi) homeostasis and the plasma Pi levels. It has reported that disorder of Pi metabolism in kidney can be risk factors for cardiovascular disease as well as hypercholesterolemia. However, the relationship between Pi and cholesterol metabolism has not been clarified. The current study investigated the effects of Npt2a gene ablation that is known as hypophosphatemia model on cholesterol metabolism in mice. Npt2a deficient ($Npt2a^{-/-}$) mice and wild type mice were fed diets with or without 2% cholesterol for 12 days. Plasma lipid and lipoprotein profile analysis revealed that plasma lipid levels (total, LDL and HDL cholesterol) were significantly higher in $Npt2a^{-/-}$ mice than wild type (WT) mice. Interestingly, high cholesterol diet markedly increased plasma levels of total, LDL and HDL cholesterol in WT mice, but not $Npt2a^{-/-}$ mice. On the other hand, there were no differences in body and liver weight, intake and hepatic lipid accumulation between WT and $Npt2a^{-/-}$ mice. These results suggest that ablation of Npt2a gene induces hypercholesterolemia and affects the ability to respond normally to dietary cholesterol. J. Med. Invest. 60 : 191-196, August, 2013

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1. INTRODUCTION

The type IIa renal sodium-dependent phosphate (Na/Pi) co-transporter Npt2a is the most important regulator of inorganic phosphate (Pi) homeostasis through reabsorption of Pi in renal brush border membrane (1). Beck *et al.* reported that Npt2a gene ablation leads not only to hypophosphatemia but also to hypercalciuria and high levels of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] and skeletal abnormalities. Also it is reported that renal Npt2a gene ablation induces renal calcification due to abnormalities of mineral metabolism (2, 3). Moreover, it is known that renal Npt2a expression is regulated by

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dietary Pi and hormones such as $1,25(OH)_2D_3$, parathyroid hormone (PTH), fibroblast growth factor-23 (FGF-23), dopamine, thyroid hormones, and glucocorticoids (2, 3). In addition, we have reported that 3,3',5-tri-iodothyronine (T_3), a known regulatory factor for hepatic cholesterol metabolism, induces hypocholesterolemia and hyperphosphatemia, and we revealed that T_3 transcriptionally up-regulates the Npt2a gene in renal proximal tubular cells (4). In contrast, hyperphosphatemia has emerged as a risk factor for vascular calcification and cardiovascular mortality (5). It has been also reported that hyperphosphatemia is associated with left ventricular hypertrophy and progression of chronic kidney disease (CKD) (6, 7).

Cholesterol is an important component of hormones and cell membranes in tissues and organs of the human body (8). On the other hand, it is also well known that an excessive cholesterol intake induces fatty liver, hypercholesterolemia and atherosclerosis (9, 10). In blood, cholesterol is carried by lipoproteins, chylomicrons (CM), very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). These lipoproteins vary not only in density but also in their functions. After dietary cholesterol was absorbed by the small intestine, it is then incorporated into the CM particle and delivered to the liver. VLDL cholesterol is formed in the liver and transported to the blood stream where it converted to LDL cholesterol and utilized in certain organs through LDL receptor. Excess cholesterol is then returned to the liver with HDL to be eliminated later in bile as bile acid. An elevated plasma LDL cholesterol level induces atherosclerosis, while high HDL cholesterol reduces risk of atherosclerosis (11-13). To get to understand these functions, it is necessary to measure plasma concentration of lipoproteins. In addition, it has been shown that abnormalities in lipid metabolism, especially cholesterol, are the major contributing factor in the induction of atherosclerosis (14, 15). It has been reported that higher serum Pi levels are associated with an increased risk of cardiovascular disease (CVD) in individuals free of CKD and CVD (16). Our recent studies also showed that dietary and serum Pi influence the progression of atherosclerosis *in vivo* and *in vitro* (17, 18). However, the effect of Pi on cholesterol metabolism remains to be elucidated.

In this study, we investigated the effects of Npt2a gene ablation on cholesterol metabolism in mice, and revealed that Npt2a gene ablation increased plasma total, LDL and HDL cholesterol levels and lose the responses to high cholesterol diet.

2. MATERIALS AND METHODS

2.1. Animals

Male and female $Npt2a^{+/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and crossing male and female $Npt2a^{+/-}$ yielded the $Npt2a^{-/-}$ mice, as described previously (2). Wild-type mice and $Npt2a^{-/-}$ mice were genotyped by PCR amplification of genomic DNA. PCR genotyping to confirm the Npt2a gene ablation was performed by using a thermal cycler (SHIMAZU, Kyoto, Japan) with a sense primer (5'-TGCCCAGGTTGGCACG-AAGC-3') located in exon 4 of Npt2a and either antisense primer 1 (5'-AGTCCTGTCCCTGCCTGCA-3') located in exon 6 of Npt2a or antisense primer 2 (5'-TGCTACTTCCATTTGTCACGTCC-3') located in the introduced neo^r gene cassette (2). Mice were maintained on 12 h light-12 h dark cycles (8 : 00-20 : 00) with free access to water and food. Mice were maintained under pathogen-free conditions and handled in accordance with the Guidelines for Animal Experimentation of the Tokushima University School of Medicine.

2.2. Diets

The experimental diets were based on the AIN-93G diet (Oriental, Osaka, Japan), with or without 2% cholesterol were prepared. The mice were randomly divided into two experimental groups of five mice each and were fed one of the two different diets for 12 days. The animals were allowed to eat *ad libitum* and given free access to distilled water. At the end of the experiment, all mice were sacrificed, and blood and liver samples were collected for analysis.

2.3. Quantification of hepatic total cholesterol and triglyceride (TG)

0.6-1.0 gram of frozen liver tissue from each animal was homogenized in a mixture of chloroform and methanol (2 : 1), and used for lipid extraction as described previously (19). Hepatic total cholesterol and TG levels were determined using the T-cholesterol E-test Wako and Triglyceride E-test Wako kits, respectively (Wako Pure Chemical Industries, Osaka, Japan).

2.4. Biochemical analysis

Plasma levels of Pi and calcium were determined using the Phospho C-test Wako, Calcium test Wako, respectively (Wako Pure Chemical Industries, Osaka, Japan). Plasma levels of TG and total cholesterol, CM, VLDL, LDL, and HDL cholesterol were determined using LipoSEARCH®, a high-sensitivity lipoprotein profiling system (Skylight Biotech, Akita, Japan).

2.5. Statistical analysis

Data are presented as means ± S.E.M. To assess the effect of genotype or diet, data were compared using student's t test. Statistical significance was determined by ANOVA followed by post-hoc testing using the Tukey-Kramer procedure for multiple comparisons and the Student's t test for effect of diet in hepatic cholesterol accumulation. Differences were considered significant for P value less than 0.05. Statistical tests were performed using Statcel2 (OMS Ltd., Saitama, Japan).

3. RESULTS

3.1. Effects of high cholesterol diet in Npt2a$^{-/-}$ mice

We elucidated whether Npt2a gene ablation and dietary cholesterol affect plasma lipid and lipoprotein profiles in mice. In Npt2a$^{-/-}$ mice, plasma total, LDL and HDL cholesterol significantly increased than in WT mice. In WT mice, high cholesterol diet markedly increased plasma levels of total cholesterol, CM, VLDL, LDL and HDL cholesterol. However, in Npt2a$^{-/-}$ mice, high cholesterol diet increased only plasma VLDL cholesterol levels. Moreover, high cholesterol diet-fed Npt2a$^{-/-}$ mice showed significant decrease of plasma CM and VLDL cholesterol levels when compared with high cholesterol diet-fed WT mice (Fig. 1). These data suggest that Npt2a gene ablation induces hypercholesterolemia and loses response to a high-cholesterol diet.

3.2. Liver weight and hepatic cholesterol accumulation in Npt2a$^{-/-}$ mice

We found that plasma Pi levels in Npt2a$^{-/-}$ mice were significantly lower than in WT mice, but were not lower in Npt2a$^{-/-}$ mice with high cholesterol diet (Table. 1). As well as previous report (2), we also confirmed that plasma 1,25$(OH)_2$D levels were significantly increased in Npt2a$^{-/-}$ mice (data not shown). There were no differences in body weight, total chow consumption or plasma calcium and TG levels, between any of the groups (Table.1, 2). Although we could not find any differences in liver weight and hepatic lipid accumulation between WT and Npt2a$^{-/-}$ mice, high cholesterol diet increased significantly liver weight in WT mice, but not in Npt2a$^{-/-}$ mice. Furthermore, the quantification of hepatic total cholesterol and TG levels revealed that high cholesterol diet significantly induced hepatic cholesterol accumulation in Npt2a$^{-/-}$ mice, but its hepatic cholesterol levels tended to be lower than that of WT mice. In addition, high cholesterol diet-induced hepatic TG accumulations also tended to be lower in Npt2a$^{-/-}$ mice than WT mice (Table. 2).

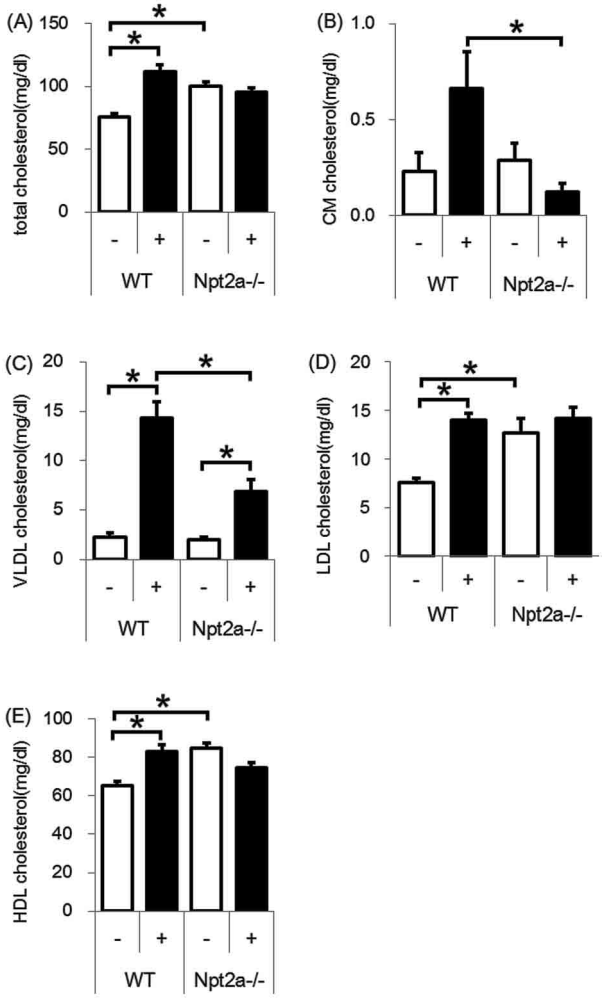

Figure 1 Effect of high cholesterol diet on plasma levels of cholesterol in Npt2a$^{-/-}$ mice
Diet with (black bars) or without (white bars) 2% cholesterol, were given to mice for 12 days. Levels of plasma cholesterol levels were measured as described in the Materials and Methods section. (A) Total cholesterol, (B) CM cholesterol, (C) VLDL cholesterol, (D) LDL cholesterol, (E) HDL cholesterol. Values are means ± SEM. n = 5 mice per group, *$p < 0.05$.

Table 1 Plasma levels of Pi, Ca and TG.

	WT		$Npt2a^{-/-}$	
mg/dl		+Chol		+Chol
Pi	8.01± 0.30	6.47± 0.25	5.72± 0.44[b]	6.19± 0.62
Calcium	8.05± 0.27	7.97± 0.15	7.25± 0.15	7.68± 0.18
TG	26.11± 7.00	17.32± 2.69	23.85± 4.68	14.56± 2.40

Values are means± SEM. n = 5 mice per group, $p< 0.05$. [a] effect of diet, [b] effect of genotype

Table 2 Body and hepatic weights, total chow consumption, and hepatic lipid accumulation.

	WT		$Npt2a^{-/-}$	
		+Chol		+Chol
Final body weight (g)	23.03± 0.15	23.31± 0.32	22.91± 1.30	22.90± 1.22
Total chow consunption (g)	41.79± 0.09	46.16± 0.09	42.55± 0.11	44.51± 0.10
Liver weight (g)	0.95± 0.01	1.25± 0.07[a]	1.07± 0.06	1.08± 0.07
Hepatic total cholesterol (mg/g liver)	1.78± 0.40	3.93± 0.78	0.95± 0.16	2.98± 0.4 [a]
Hepatic TG (mg/g liver)	7.51± 1.90	12.40± 2.35	4.79± 1.43	8.01± 1.17

Values are means± SEM. n = 5 mice per group, $p< 0.05$. [a] effect of diet, [b] effect of genotype

4. DISCUSSION

In the present study, we examined the effects of Npt2a gene ablation on plasma lipid and lipoprotein profiles and response to a high cholesterol diet. We first observed that $Npt2a^{-/-}$ mice had hypophosphatemia and hypercholesterolemia. Indeed, it has been previously reported that plasma Pi levels and total cholesterol levels have negative correlation in the black sea bream *Sparus Macrocephalus* (20). This may indicate that plasma levels of Pi can affect plasma total cholesterol levels. In $Npt2a^{-/-}$ mice, high cholesterol diet did not increase plasma levels of total, LDL and HDL cholesterol. Reversely, high cholesterol diet-induced plasma levels of CM and VLDL cholesterol were lower in $Npt2a^{-/-}$ mice than WT mice. These lipoproteins that contain apolipoprotein, cholesterol, TG and phospholipid are synthesized and metabolized in the intestine and liver (8, 21). Pi deficiency in $Npt2a^{-/-}$ mice might affect the synthesis of lipoproteins and maintain their metabolism. Therefore, our findings could indicate that Npt2a gene ablation induces hypercholesterolemia and inhibits the ability of response to a high cholesterol diet.

Our results showed no significant differences in hepatic lipid accumulation, within all groups. However, in $Npt2a^{-/-}$ mice, the hepatic cholesterol and TG accumulation was decreased to lower levels than in WT mice with or without high cholesterol diet. We previously reported that renal Pi homeostasis is regulated by thyroid hormones (4). In fact, hypercholesterolemia is found in patients with hypothyroidism and resistance to thyroid hormone (22). However, it has not been clarified whether hypophosphatemia and high cholesterol diet affect on plasma levels of thyroid hormones. Liver X receptor (LXR) is known as a nuclear receptor to regulate cholesterol metabolism and lipid biosynthesis (23). In $Npt2a^{-/-}$ mice, LXR mRNA expressions were suppressed significantly by 70% than in WT mice (data not shown). Interestingly, recent study showed that LXR-activating ligands decreased plasma Pi levels and renal and intestinal NaPi transporters including Npt2a, Npt2c and Npt2b (24). These data might suggest that dietary cholesterol can regulate renal and intestinal Pi homeostasis through LXR, and hypophosphatemia by Npt2a gene ablation induce imbalance of cholesterol and lipid metabolism through suppression of hepatic LXR alpha gene expression. Moreover, Xie *et al.* reported Pi restriction diet could change glucose metabolism in liver (25, 26). It has been reported that the Npt2b known as intestinal Pi transporter is highly expressed in the intestine, lung and liver (27). Indeed, we demonstrated real-time quantitative PCR analysis and observed a higher hepatic Npt2b mRNA expression in $Npt2a^{-/-}$ mice (data not shown). These data suggest that hepatic Pi levels might be changed in $Npt2a^{-/-}$ mice to regulate the cholesterol metabolism. However,

further study is required to clarify the relationship of Pi and cholesterol metabolism in liver.

FGF-23 is known as a critical regulator of Pi homeostasis and renal Npt2a gene expression. Shimada *et al.* reported that FGF-23 deficient mice had abnormalities of mineral metabolism including hyperphosphatemia, increased plasma $1,25(OH)_2D$ levels, hypercholesterolemia and decreased plasma TG levels (28). Therefore, these results also suggest that the abnormality of Pi metabolism affects lipid homeostasis. As the relationship between the plasma levels of Pi and FGF-23 is well reported previously (1-3) ; the effects of high cholesterol diet on plasma FGF-23 levels should be considered in the further studies to understand the mechanism of hypercholesterolemia induced by Npt2a gene ablation.

In conclusion, we firstly reported hypercholesterolemia and the response to a high cholesterol diet in $Npt2a^{-/-}$ mice, and suggest that Npt2a gene ablation plays an important role in the control of cholesterol homeostasis.

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