論文の内容の要旨

応用生命化学専攻
平成 29 年度博士課程進学
氏名：洪　子雯
指導教員：佐藤　隆一郎

論文題目

Study on novel metabolic regulation by bile acids

胆汁酸による新たな代謝調節に関する研究

Chapter 1: Introduction

Bile acids (BAs) are synthesized in the liver as cholesterol catabolites. They are released from the gallbladder to the intestine after food intake. In the ileum, bile acids are absorbed into enterocytes by the ileal bile acid transporter (IBAT) and then are exported into the portal vein. In the liver, they are re-taken up by Na+ taurocholate co-transporting polypeptide (NTCP), which fulfills the enterohepatic circulation. Currently, bile acids are recognized as ligands of a nuclear receptor FXR and a G protein-coupled receptor TGR5 and regulate not only bile acids metabolism but also energy metabolism. Therefore, BAs and their transporters are believed to have a potential role in improving metabolic syndromes. In the first part of this study, we aimed to screen the potential food factors that hamper NTCP activity and investigate their underlying mechanisms. We proposed a hypothesis that by inhibiting NTCP-mediated BA hepatic reuptake, the increase of BA activate the TGR5 signaling, and subsequently boost the energy expenditure and improve the glucose homeostasis.

Although the physiological benefits of TGR5 activation has been extensively studied, to date, the regulatory mechanism of TGR5 still remains largely unknown. We found an increasing tendency of TGR5 expression in liver and skeletal muscle during prolonged fasting. Fasting and subsequent refeeding are the events that cause the most dramatic fluctuations in energy metabolism. The lack of metabolic flexibility to adequately adapt to energy demands causes a fault of energy homeostasis, finally leading to many kinds of metabolic diseases. The second part is aimed to unveil the regulatory molecular mechanism of TGR5 expression during fasting and to investigate the novel physiological role of TGR5 in the liver after the BAs are secreted as postprandial signals.

Chapter 2: FST antagonizes HNF4 and inhibits hepatic NTCP-mediated bile acid transport

Several recent reports support the hypothesis that an increase in circulating BA levels improves energy and lipid metabolism in adipose tissues and skeletal muscles. Here, by the screening of 103 purified natural food compounds, we found that a flavonoid fisetin (FST) inhibits the transporter activity of human NTCP. *In vitro*

experiments revealed that FST accelerates human and mouse NTCP degradation and inhibits hepatic BA transport over a short period (< 2 h). FST treatment to mice increased serum BA levels, accompanied by changes in thermogenic gene expression in white/ brown adipose tissues and muscle, increased oxygen consumption, and decreased body weight gain. Unexpectedly, despite the reduced BA uptake, hepatic BA accumulation was markedly elevated, presumably because of an increase in expression of cytochrome P450 7 subfamily A member 1 (*CYP7A1)*, the gene encoding a rate-limiting enzyme for BA synthesis. In the liver, FST suppressed the expression of several hepatocyte nuclear factor 4 (HNF4) target genes, which are involved in modulating lipid metabolism indicating that FST functions as an HNF4 antagonist. Affinity binding experiments with FST-coupled agarose beads revealed an interaction between FST and HNF4α or FST and NTCP. These results indicate that FST functions as both an NTCP inhibitor and HNF4 antagonist, triggering metabolic changes in the liver, adipose tissues, and skeletal muscles.

Chapter 3: Regulatory mechanism of TGR5 expression during fasting

Previously, our group discovered that TGR5 expression is significantly up-regulated under different kinds of ER stress. Furthermore, the other study in our lab revealed that a food compound, luteolin treatment up-regulates TGR5 gene expression via JNK/ERK/p53 axis. p53 is known as a common mediator of the fasting responses; therefore, we proposed a hypothesis that p53 is involved in the regulation of TGR5 during fasting, and aimed to unveil the mechanism of BAs and TGR5 cross-talk during and after fasting. The fluctuation of TGR5 expression in fasted mouse liver was traced, and the elevation of TGR5 gene expression was observed at the 36 h fasted mouse livers. We further confirmed this up-regulation of TGR5 in both primary parenchymal and non-parenchymal hepatocytes. To investigate the detail molecular mechanism, we established an *in vitro* platform that mimics the fasting state *in vivo*, by treating the HepG2 cells with starvation medium contained 1 mM glucose and 1% FBS. We found that the TGR5 and p53 target genes expression were up-regulated in a time-dependent manner. To further examine whether the transcription factor p53 regulates TGR5 expression, we treated the HepG2 cells with a p53 activator (nutlin-3) or an inhibitor (pifithrinin-α) and found that TGR5 expression is regulated in a p53-dependent manner. It's reported that p53 binds to the p53 consensus-binding response elements (p53RE) in its target genes as a tetramer. To investigate whether the TGR5 gene contains p53RE and serves as a target gene of p53, the 5'-flanking region, 2000 bp genomic DNA sequence upstream of the translation start site (TSS) of hTGR5 gene was cloned, and the truncated analysis of reporter activity was conducted. The data revealed that the hTGR5 promoter (-2000 bp to -400 bp) activity was stimulated 5-10 folds when co-expressed with p53, and the deletion from -250 to -400 bp significantly abolished the promoter activity. Therefore, we examined the promoter sequence between -250 bp to TSS and found a putative p53 response like element with a 75% match. Furthermore, the results obtained from the ChIP assay provided evidence that the endogenous p53 in HepG2 cells directly interact with the putative p53 RE in the hTGR5 promoter (from -295 to -257 bp) region but not the distal region.

Since AMPK activation is one of the major events that occur during prolonged fasting, and glucose starvation is known to increase p53 protein levels in an AMPK-dependent manner. Next, we examine whether the AMPK pathway is involved in the regulation of TGR5 expression. Indeed, the phosphorylation of AMPK was found in

the liver samples of 36 h fasting or the AICAR (200 mg/kg, 3 d)-injected mice, together with the increases of TGR5 and p53 target gene expression. The protein level of p-PKA substrates and PGC1α also increased in both 36 h fasted and AICAR-injected groups. PGC1α is a versatile transcriptional coactivator and plays a critical role in mediating cellular response to diverse stress. Previously, PGC1α is reported that it modulates the p53 transactivation activity by directly binds to p53 protein. Next, we examine the role of PGC1α in starvation-induced TGR5 regulation. Similar to the previous study (Sen et al., 2011); starvation treatment significantly induced the expression of PGC1α, p-AMPK, and both phosphorylated and total p53 protein in a time-dependent manner.

To further investigate the fluctuation of TGR5 expression during and after fasting, mouse livers harvested from *ad-lib*, 36 h fasting, and 36 h fasting plus 8 or 24 h normal chow-refed mice were analyzed. In consistence with our previous data, TGR5 gene expression was up-regulated by 36 h fasting compared to *ad lib* group. Interestingly, although the p53 mRNA level did not fluctuate upon fasting and refeeding, both total and phosphor-p53 showed an increasing tendency during and after fasting, suggesting this regulation is mainly at a post-translational level rather than at the transcriptional level. As a novel p53 target gene, the up-regulation of TGR5 gene expression during fasting may be explained by the regulation through the PGC1α-p53 axis.

Chapter 4: Feedback regulation of TGR5 by its ligands

To understand the regulatory mechanism of TGR5 expression upon the postprandial bile acid signaling, several reported TGR5 agonists were used in this study such as TLCA, INT777, Oleanolic acid, Maslinic acid, and Nomilin. The affinity of these compounds to TGR5 was examined using the CRE-Luc reporter assay. The previous observations in Chapter 3 imply that ligand of TGR5 might play a critical role in regulating the TGR5 protein level after the refeeding of prolonged fasting. To examine this effect, we attempted to reproduce the condition of re-feeding after prolonged fasting in the cell culture dishes, FLAG-hTGR5 was overexpressed in HepG2 cells then cultured with TLCA or INT777 for 24 h. Similar to the previous study (Masyuk et al., 2017), the immunoblotting results showed that the exogenous TGR5 protein level was increased by TLCA or INT777 treatment. To further investigate the regulatory effect of ligands on TGR5 protein abundance, a cycloheximide chasing assay was performed to track the degradation rate and found that TLCA or INT777 treatment prolongs the TGR5 protein half-life. The inhibitor assay further revealed that TGR5 ligands increased its protein stability in a proteasome-dependent manner.

Chapter 5: Physiological function of TGR5 during and after fasting

In the previous chapters, we elucidated the underlying mechanism that regulates the up-regulation of TGR5 during and after fasting. Since the endogenous TGR5 ligands, bile acids are released from gallbladder to the intestine and the peripheral circulation after food intake, as a refeeding signal parallel to insulin. We found that the concentration of total bile acids was around 20 μmol/L in portal vein serum upon fasting, and around 5 μmol/L in systemic serum with a peak seen 60-120 min after the food ingestion.

Next, we aimed to explicate the meaning of the up-regulation of TGR5 during fasting and the physiological functions of TGR5 activation by bile acids. It is known that lipogenesis is suppressed when the intracellular cAMP increased. When TGR5 is activated, the cAMP cascade will also be induced. To elucidate the role of TGR5 in regulating lipogenesis, we examined the expression level of lipogenic genes in the TGR5-KO and WT mice. Indeed, the SREBP1c, ACC, and CPT1 gene expressions in TGR5-KO mouse liver were significantly higher than the WT counterparts. Next, we examined whether the presence or absence of TGR5 alter the *de novo* synthesis of fatty acids and sterols from the [1-^{14}C] acetate. We found that both the *de novo* synthesis of fatty acid and the content of TG increased in the MPHs derived from TGR5-KO mouse. We also attempted to understand the underlying molecular mechanism by performing a microarray assay using TLCA treated Ad-hTGR5 HepG2 cells and found HDAC5 might be a potential mediator.

Previously, we found that TGR5 gene expression is only able to be increased by prolonged fasting (longer than 24 h). Therefore, we wondered whether the activation of TGR5 by postprandial bile acid signal after a prolonged (36 h) or intermittent (12 h) fasting would address different effect on the lipogenesis after refeeding we found that SREBP1c and ACC gene expression was suppressed and fully recovered after 24 h re-feeding (but not 8 h) in the 36 h-fasted mice, however, in the intermittent fasted group, 8 h was enough for both SREBP1c and ACC recovery. Furthermore, in the TGR5-KO mice, the recovery rate of lipogenic gene expression was significantly higher compared to the WT counterparts. Taken together, these data are implying a possible role of TGR5 in regulating lipogenesis after prolonged fasting as a survival mechanism.

Conclusion

Recently, the transporters and receptors of bile acids have got much attention as ideal therapeutic targets of metabolic syndromes. Our results demonstrate the regulatory mechanism of TGR5 expression and the physiological meaning of TGR5 activation by the BAs secreted from the gallbladder, which can be seen as an adaptive mechanism in response to fast and feast. Elucidating the potential role of BAs as feeding signals in terms of molecular nutrition suggests the possibility of achieving metabolic improvement effects by regulating feeding signal responses. It is expected to lead to innovation development by searching and creating functional food based on the above knowledge.

We also showed that moderate inhibition of BA transport through NTCP by FST increases plasma BA concentration and improves energy homeostasis, which may provide a potential therapeutic and nutraceutical target in the prevention of metabolic syndromes.