Novel modulators of lipoprotein metabolism : implications for steatohepatitis and atherosclerosis

Wang, Y.

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SUMMARY

Atherosclerosis and non-alcoholic steatohepatitis (NASH) are the leading causes of cardiovascular disease (CVD) and chronic liver disease, respectively, both of which remain common reasons of morbidity and mortality in the Western world. Atherosclerosis and NASH share the same etiologies, of which disturbed lipid metabolism manifested by dyslipidemia is the most important one, hallmarked by increased plasma levels of (V)LDL-cholesterol (C) and triglycerides (TG), and decreased plasma levels of HDL-C. Pharmacological lipid-lowering agents improving dyslipidemia are effective tools to prevent and treat atherosclerosis. However, since mortality and morbidity due to cardiovascular disease are only partially improved by current lipid-lowering strategies, novel strategies are currently under development aimed at further reduction of atherosclerosis. Since HDL-C levels are inversely associated with cardiovascular risk, one of these strategies is to raise HDL levels. As no pharmacological agents have been identified thus far to treat NASH, and no biomarkers are available to detect NASH, the search for both treatment modalities and biomarkers for NASH is also warranted.

Among HDL-raising strategies, infusion of reconstituted HDL (rHDL) seems to be a promising one for the treatment of CVD, as rHDL has been shown to induce atherosclerosis regression in human studies. Studies in mice indicated that rHDL infusion adversely affects VLDL levels, but this effect is less apparent in humans. This discrepancy may be explained by the fact that humans, in contrast to mice, express CETP. CETP plays a vital role in lipid metabolism by mediating the transfer of TG and CE between (V)LDL and HDL. In **Chapter 2**, we investigated the role of CETP in the effects of rHDL on VLDL metabolism by using *APOE*3-Leiden (E3L)* mice, a well-established model for human-like lipoprotein metabolism, which had been crossbred with mice expressing human CETP under control of its natural flanking regions (*E3L.CETP* mice). At 1 hour after injection, rHDL increased plasma VLDL-C and TG in *E3L* mice, but not in *E3L.CETP* mice. This initial raise in VLDL was caused by competition between rHDL and VLDL for lipoprotein lipase (LPL)-mediated TG hydrolysis, and was thus prevented by the expression of CETP. At 24 hours after injection, rHDL caused a second increase in VLDL-C and TG in *E3L* mice, whereas rHDL even decreased VLDL in *E3L.CETP* mice. This secondary raise in VLDL was due to increased hepatic VLDL-TG production. Collectively, we concluded that CETP protects against the rHDL-induced increase in VLDL, and that treatment of atherogenic dyslipidemia by rHDL should thus not be combined with agents that aggressively reduce CETP activity.

Thiazolidinediones (PPARγ agonists) decrease plasma TG, increase HDL-C and reduce hepatic steatosis. Since previous studies in mice have shown that reduction of hepatic steatosis by lipid-lowering agents is accompanied by reduced hepatic CETP expression and plasma CETP levels, which may explain a secondary increase in HDL-C, we assessed in **Chapter 3** the effects of pioglitazone on plasma CETP levels in patients with type 2 diabetes mellitus (T2DM). Patients with T2DM were randomized to treatment with pioglitazone or metformin and matching placebos, in addition to glimepiride. At baseline and after 24 weeks of treatment, plasma HDL-C and CETP levels were measured, and hepatic TG content was assessed by proton magnetic resonance spectroscopy. Pioglitazone decreased the hepatic TG content, which was indeed associated with decreased plasma CETP levels and increased plasma HDL-C levels, whereas metformin did not significantly change any of these parameters. We concluded that the decrease in hepatic TG content by pioglitazone was accompanied by a decrease in plasma CETP concentration and, therefore, associated with an increase in HDL-C levels.

In contrast to pharmacological interventions, lifestyle interventions such as diet-induced weight loss and exercise are still the mainstays for the treatment of NASH. We recently reported that a 16-week very low calorie diet (VLCD) significantly decreased plasma total cholesterol (TC) and TG levels and markedly reduced the hepatic TG content in obese patients with T2DM and hepatic steatosis, but the potential beneficial effect of a VLCD on plasma CETP and HDL levels had not been studied. In **Chapter 4**, we investigated the effects of VLCD, resulting in a major reduction in hepatic TG content, on plasma CETP and HDL levels in obese patients with T2DM and hepatic steatosis. VLCD markedly decreased plasma CETP concentration and increased plasma apoAI levels, without significantly affecting plasma HDL-C and HDL-phospholipids levels. Although VLCD resulted in HDL that was less lipidated, the functionality of HDL with respect to inducing cholesterol efflux *in vitro* was unchanged. Therefore, we concluded that the marked decrease in hepatic TG content induced by VLCD was accompanied by a decrease in plasma CETP concentration and an increase in apoAI levels, without improving the cholesterol efflux properties of HDL *in vitro*.

Niacin (nicotinic acid) is the most potent HDL-raising drug used in the clinic practice. In addition to raising the level of anti-atherogenic HDL-C, niacin also decreases plasma levels of pro-atherogenic lipoproteins and lipids including VLDL, LDL and TG. Therefore, niacin is regarded as a candidate for the treatment of atherosclerosis. Niacin has recently been shown in *E3L.CETP* mice to decrease the hepatic lipid content, accompanied with a reduction of the hepatic expression and plasma levels of CETP, thereby increasing

HDL-C. In **Chapter 5**, we investigated the mechanisms underlying the CETP-lowering effect of niacin by using human CETP transgenic mice. *In vitro* studies demonstrated that niacin did not directly attenuate CETP expression in macrophages. *In vivo* studies showed that niacin reduced the hepatic cholesterol content and attenuated Western type diet-induced hepatic inflammation. Furthermore, niacin reduced the hepatic gene expression and plasma level of CETP. Concomitantly, niacin decreased the hepatic expression of CD68 and ABCG1, both of which are specific markers for the hepatic macrophage content, as well as the actual hepatic macrophage content. In fact, the hepatic CETP expression was significantly correlated with the hepatic macrophage markers. We concluded that niacin decreases hepatic CETP expression and plasma CETP mass by attenuating liver inflammation and macrophage content in response to its primary lipid-lowering effect, rather than by attenuating the CETP expression level within macrophages.

Since CETP is a current target for treating dyslipidemia, it is crucial to understand the true origin of CETP in humans. Previous studies indicated that adipose tissue and liver are the two major sources of plasma CETP. However, the relative contribution of tissue-specific CETP expression to plasma CETP levels is unknown. Therefore, in **Chapter 6**, we aimed to elucidate the cellular origin of CETP using human cohorts and *E3L.CETP* mice. In a general population study, plasma CETP levels did not correlate with waist circumference, suggesting that central adipose tissue does not contribute to plasma CETP. Microarray analysis of liver and adipose tissue biopsies from bariatric surgery patients showed that CETP expression was highest in the liver, and correlated with inflammatory pathways. Immunohistochemistry revealed that CETP was primarily expressed by hepatic macrophages. CETP expression in liver, but not adipose tissue, positively correlated with plasma CETP levels, and inversely correlated with plasma HDL-C. Selective elimination of macrophages from liver versus adipose tissue in *E3L.CETP* mice virtually abolished hepatic CETP expression, but not adipose tissue CETP expression, accompanied by largely reduced plasma CETP concentration and increased plasma HDL-C. Treatment of *E3L.CETP* mice with lipid-lowering drugs that are known to reduce the plasma CETP concentration and to increase HDL-C in humans, reduced the hepatic macrophage content, thereby reducing plasma CETP and increasing HDL-C. We concluded that plasma CETP is primarily derived from liver macrophages and plasma CETP is a biomarker for the hepatic macrophage content, a hallmark of NASH for which no non-invasive diagnostic tool is currently available.

Accumulating evidence indicates that strategies targeting regulation of energy

homeostasis and food intake also beneficially affect lipid metabolism, and have the potential to treat atherosclerosis and NASH. The brain plays an important role in mediating energy homeostasis, with the hypothalamus being its key regulator. Two major neuronal populations within the hypothalamic arcuate nucleus are involved in the regulation of food intake, including pro-opiomelanocortin/cocaine- and amphetamine-regulated transcript-expressing neurons and neuropeptide Y (NPY)/agouti-related protein-expressing neurons. Previous studies suggested that central NPY administration increases hepatic production of VLDL-TG in rats. In **Chapter 7**, we set out to validate the effects of central NPY on hepatic VLDL production in mice, to ultimately investigate whether NPY, by inducing dyslipidemia, affects the development of atherosclerosis. Administration of NPY into both the lateral and third ventricle of the brain of mice increased food intake within one hour after injection, but had no effects on hepatic VLDL-TG or VLDL-apoB production. Likewise, antagonizing central NPY signaling did not affect hepatic VLDL production. We concluded that in mice, as opposed to rats, acute central administration of NPY increases food intake without affecting hepatic VLDL production. This apparent species difference in the effect of NPY on hepatic VLDL-TG production is of great significance for future animal studies on the central regulation of hepatic VLDL metabolism.

Human studies suggested that glucagon-like peptide-1 (GLP-1) receptor agonism not only modulates energy homeostasis and improves glucose metabolism, but also decreases the plasma TG level. However, the mechanism underlying the reduction in plasma TG remained unclear. In **Chapter 8**, we evaluated the effects of GLP-1 receptor agonism on TG metabolism in high fat diet (HFD)-fed *E3L* mice. Four weeks of treatment with GLP-1 receptor agonists (i.e. CNTO3649 and exendin-4) by using subcutaneous osmotic minipumps improved glycemic control by reducing fasting plasma glucose and insulin levels. In addition, both GLP-1 receptor agonists reduced hepatic VLDL-TG and VLDL-apoB production, indicating reduced production of VLDL particles rather than reduced lipidation of apoB. Moreover, GLP-1 receptor agonism markedly decreased the hepatic content of TG, cholesterol and phospholipids, accompanied by down-regulation of expression of genes involved in hepatic lipogenesis (*Srebp-1c, Fasn, Dgat1*) and apoB synthesis (*Apob*). We concluded that GLP-1 receptor agonism, in addition to improving glycemic control, ameliorates dyslipidemia and reduces hepatic steatosis.

Since our studies showed that exendin-4 improves glycemic control and lipid metabolism, and reverses HFD-induced hepatic steatosis (chapter 8), we anticipated that GLP-1 receptor agonism has the potential to treat atherosclerosis and fatty liver disease.

However, the impact of GLP-1 receptor agonism on NASH, especially with respect to hepatic inflammation, was still uncertain. Since the development of atherosclerosis and NASH share common etiologies, in **Chapter 9** we evaluated the effects of exendin-4 on the development of atherosclerosis and NASH simultaneously by using *E3L.CETP* mice fed a Western-type diet. Although four weeks of treatment with exendin-4 only slightly reduced plasma lipid and lipoprotein levels, it markedly decreased atherosclerotic lesion severity and area, accompanied with a reduction in monocyte adhesion to the vessel wall and macrophage content in the plaque. Furthermore, exendin-4 reduced the hepatic cholesterol content as well as the hepatic $CD68^+$ and $F4/80^+$ macrophage content indicating that exendin-4 attenuated diet-induced NASH. This was accompanied by less monocyte recruitment from the circulation as the hepatic $Mac\text{-}1^+$ macrophage content was decreased. Finally, exendin-4 reduced chemokine expression *in vivo* and suppressed oxLDL accumulation in peritoneal macrophages *in vitro*, dependent on the GLP-1 receptor, suggesting that exendin-4 reduces both atherosclerosis and NASH by reducing macrophage recruitment and activation. We concluded that exendin-4 could be a valuable strategy to treat atherosclerosis and NASH in addition to T2DM, especially in patients who display a combination of these diseases.

In addition to lipid-lowering strategies, anti-inflammatory agents that are aimed at reducing the risk of CVD are now under development. Glucocorticoids are one of the strongest anti-inflammatory drugs widely used as immunosuppressive agents in the clinical practice. However, glucocorticoids excess can also induce adverse metabolic effects in adipose tissue, such as central obesity and insulin resistance, which may attenuate the potentially protective effects of glucocorticoids on CVD. In **Chapter 10,** we investigated the effects of both transient and continuous glucocorticoid treatment on atherosclerosis development by using *E3L.CETP* mice, which display human-like lipoprotein metabolism upon feeding a Western-type diet. Although both 5 weeks (transient) and 17 weeks (continuous) of corticosterone (CORT) treatment increased body weight and food intake for the duration of the treatment, only continuous CORT treatment induced changes in body composition with lower adrenal weight and higher gonadal and subcutaneous fat pad weights at 17 weeks. Moreover, the group that continuously received CORT displayed increased plasma insulin levels and HOMA-IR index, indicating that long-term administration of glucocorticoids induces insulin resistance. Strikingly, both transient and continuous CORT treatment decreased total atherosclerotic lesion area after 17 weeks to a similar extent, without affecting either plasma lipid levels or lipoprotein profiles, accompanied by decreased macrophage content in the atherosclerotic plaque. We concluded that CORT treatment *per se* has

long-lasting beneficial effects on atherosclerosis development. Therefore, in clinical practice, glucocorticoids schemes as used for anti-inflammatory indications might benefit from adjustments towards higher doses for a shorter period of time instead of lower dosages for a prolonged period of time.

Taken together, the studies described in this thesis have contributed to the discovery of CETP as a biomarker for the hepatic macrophage content, a hallmark of NASH for which no non-invasive diagnostic method is currently available, and discovery of novel therapeutic modalities for atherosclerosis and NASH. First of all, we gained more insight into the true cellular origin of CETP (i.e. the liver macrophage), and the mechanisms underlying the CETP-lowering effects of HDL-raising agents (i.e. by reducing the hepatic macrophage content). We extrapolated the association between the reduction of hepatic lipid content and plasma CETP concentration upon lipid-lowering interventions from mice to humans. Furthermore, we demonstrated the role of CETP in discrepant effects of rHDL on VLDL metabolism between mice and humans, and reported a species difference in the central regulation of hepatic VLDL metabolism by NPY between mice and rats, which underscores a general concern in animal research in view of extrapolating findings from specific animal studies to explain observations done in humans. Additionally, we demonstrated that CORT has long-lasting beneficial effects on atherosclerosis development suggesting a possibility for therapeutic application of anti-inflammatory agents in CVD. Finally, we described GLP-1 receptor agonism as a novel strategy to improve lipid metabolism and hepatic inflammation, which may result in novel strategies to treat both atherosclerosis and NASH.