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Effects of antibiotics, short-chain fatty acids and amino acids on Apoliprotein A-I transcription and synthesis in normal and inflamed HepG2 and Caco-2 cells

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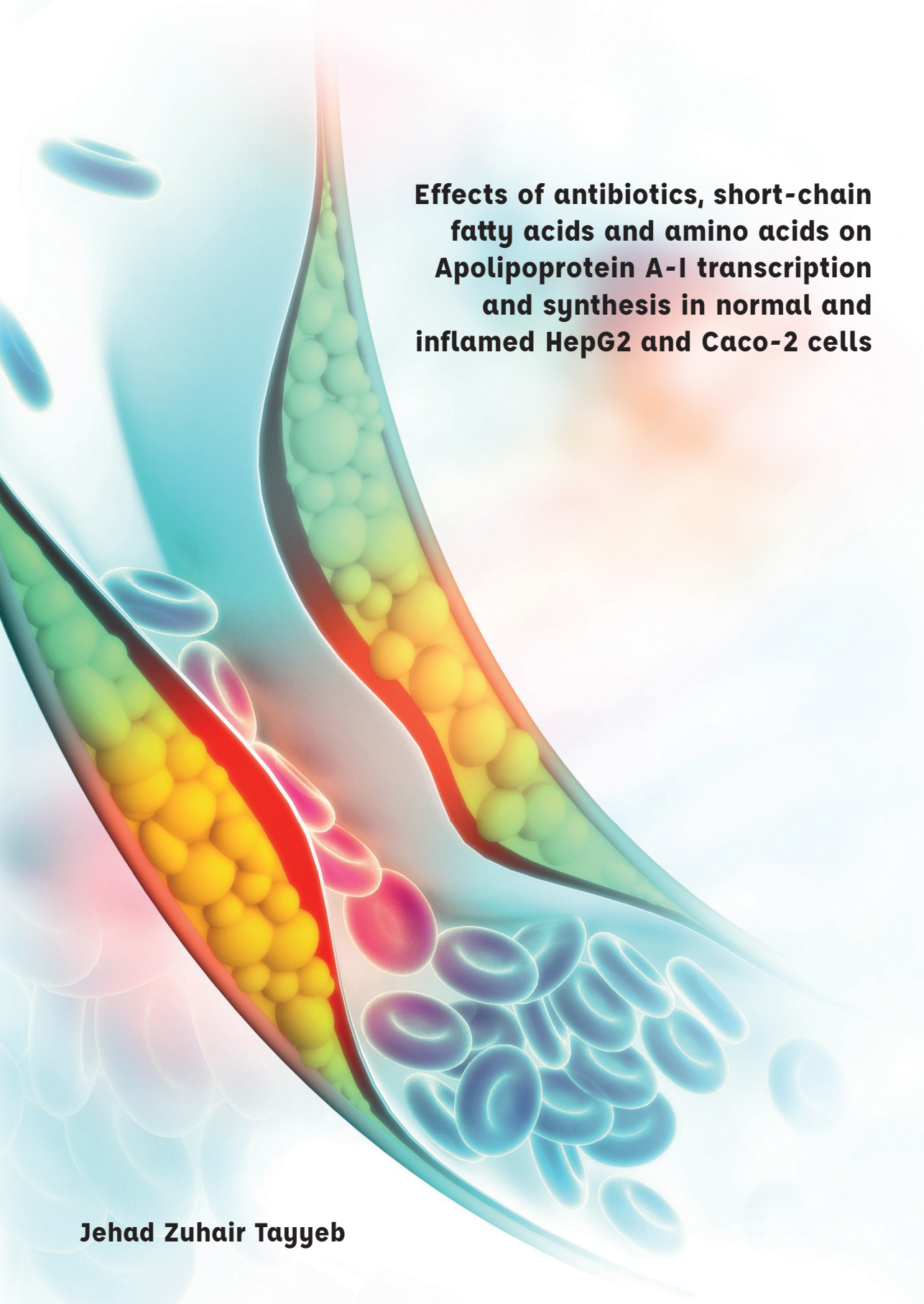

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Jehad Zuhair Tayyeb

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DISSERTATION

To obtain the degree of Doctor at the Maastricht University,
on the authority of the Rector Magnificus,
Prof. Dr. Rianne M. Letschert
in accordance with the decision of the Board of Deans,
to be defended in public on Tuesday 1st of June 2021, at 10.00 hours

by

Jehad Zuhair Tayyeb

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General introduction

Atherosclerosis and cardiovascular disease

Cardiovascular diseases (CVD) are still the leading cause of morbidity and mortality worldwide (1). The prevalence of CVD is estimated to even further increase by 18% in 2030 (2). Therefore, screening for existing CVD risk via established and novel biomarkers as well as developing new intervention strategies to prevent and treat CVD received a lot of the attention over the past decades. The most important strategy to reduce the occurrence of CVD is to prevent atherosclerotic lesion development. Known risk factors for atherosclerotic lesion development are dyslipidemia, hypertension, diabetes, elevated BMI, a lack of physical inactivity and genetic factors (3, 4). Dyslipidemia is a condition of a disturbed and unbalanced lipoprotein metabolism as shown by elevated low-density lipoprotein (LDL) cholesterol and triglyceride (TG) concentrations or reduced high-density lipoprotein (HDL) cholesterol concentrations (5). Moreover, these metabolic abnormalities contribute to the accumulation of (oxidized) cholesterol depositions within the artery wall, resulting in increased atherosclerotic lesion development (6). Moreover, atherosclerosis is not only characterized by the progressive accumulation of cholesterol but also of macrophages within the inner lining of blood vessels (7, 8), which relates to the fact that it is also a chronic inflammatory condition (9) in which many additional factors and immune cells play a role. Altogether these processes lead to a condition called endothelial dysfunction (Figure 1.1), which – when untreated – progresses further towards the formation of an atherosclerotic plaque.

The role of LDL and HDL in atherosclerosis

It is well-known that atherosclerotic plaque formation correlates with serum cholesterol concentrations. Individuals with high LDL cholesterol concentrations or low HDL cholesterol levels are considered at high risk of atherosclerosis (12, 13). There is an overwhelming amount of data showing that serum LDL cholesterol concentrations are strong and positively linked to CVD risk, and several reviews demonstrated the causal link between LDL-C and CVD (14, 15). Moreover, it was also shown that any decrease in LDL-C, independent of the size of the reduction, is associated with a decrease in CVD risk. An important message came from a meta-analysis including several drug and dietary intervention trials in which it was demonstrated that the benefit of lowering LDL-C is similar for all strategies, either via pharmacological or lifestyle approaches (16). Recent studies have shown that lowering LDL cholesterol concentrations alone is often not enough to fully prevent or stop atherosclerosis development. Therefore, an increased interest emerged to find additional therapies via targeting other lipids and lipoproteins that

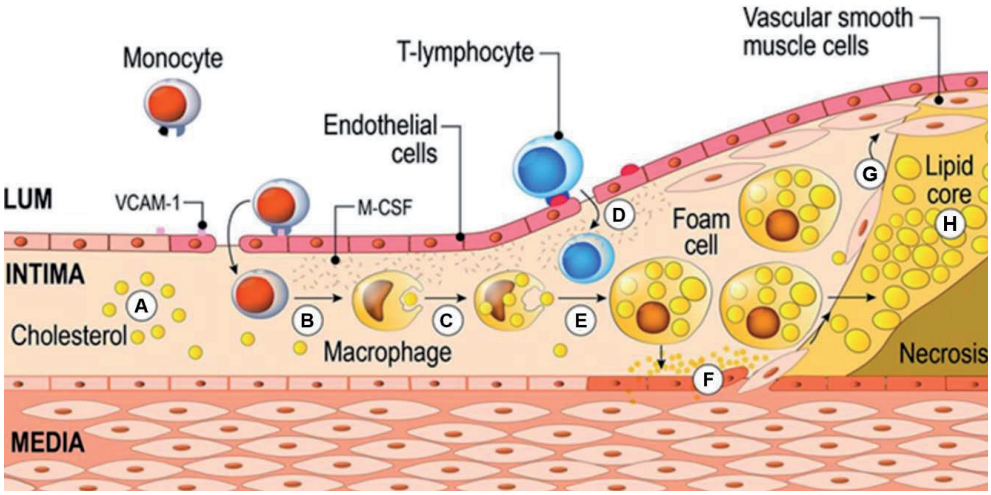

Figure 1.1. The process of atherosclerosis. When LDL becomes trapped in the sub-endothelial space (A), it may undergo oxidative modification, forming oxy-LDL. Consequently, the endothelial cells express various leukocyte adhesion molecules such as VCAM1 and stimulate mononuclear cells such as monocytes and T-cells to attach to the endothelium and enter subsequently the intima, where they differentiate (B, C) into macrophages (9, 10). T-lymphocytes join macrophages in the intima (D), and form lipid laden foam cells (E). The inflammatory condition stimulates the development of vascular smooth muscle cells, forming an atherosclerotic plaque (F, G). Eventually, the accumulation of cells debris, cytokines, chemokines, and apoptotic bodies results in necrotic core formation (H). Adapted from Minelli et al. (11).

also associated with CVD risk, such as HDL cholesterol (17) or triglycerides (13). HDL cholesterol concentrations are inversely correlated with CVD risk. Previous cross-sectional studies have reported that a 1 mg/dl (0.03 mM/L) higher HDL cholesterol concentration associates with a 3% lower CVD risk (18). Moreover, recent studies have also shown reductions in CVD risk by using HDL cholesterol elevating interventions, however this effect is not consistent (17). HDL particles are the smallest and densest lipoproteins, which differ in size, shape and lipid composition. Proteins form approximately 50% of an HDL particle, and 65–70% of this protein fraction is Apolipoprotein A-I (ApoA-I). In other words, ApoA-I is the main component of HDL (19). HDL has different functions: it can act as an antioxidant, anti-inflammatory, and antithrombotic agent. On the other hand, HDL particles can also become pro-inflammatory and pro-atherogenic, mainly when an acute phase or a chronic phase of the systemic inflammatory state is active (20). The main athero-protective function of HDL is to enhance the removal of cholesterol from macrophages within the artery wall and to transport this cholesterol to the liver for excretion (21). This process is called reverse cholesterol transport (RCT) (Figure 1.2). Therefore, a promising strategy to reduce the unfavorable atherosclerosis development is to increase the RCT and/or cholesterol efflux capacity.

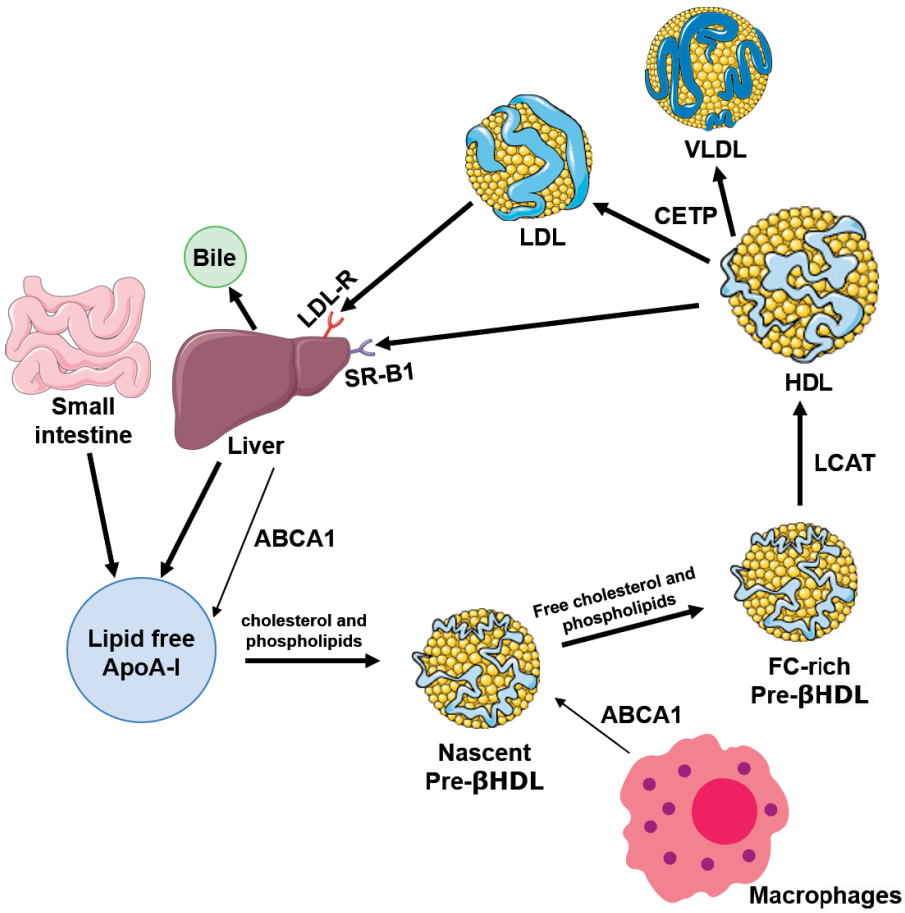

Figure 1.2. Schematic overview of HDL metabolism and the reverse cholesterol transport (RCT) pathway. First, lipid free ApoA-I is produced in the liver and small intestine, followed by obtaining cholesterol and phospholipids via ATP Binding Cassette Subfamily A Member 1 (ABCA1) mediated efflux from the liver to form nascent pre-β HDL particles (22). Next, pre-β HDL particles acquire additional free cholesterol and phospholipids from macrophages in the extrahepatic tissues via ABCA1-mediated efflux generating particles that are more cholesterol enriched (22). Then, lecithin cholesterol acyltransferase (LCAT) enzyme esterifies the free cholesterol molecules to form cholesteryl ester, which migrate to the core of the HDL particle to form mature HDL particles (23). Lastly, HDL particles bind to the SR-B1 receptor to bring the obtained cholesterol to the liver which is followed by the production of bile acids (23, 24). Additionally, HDL particles transfer its cholesterol content to LDL via CETP enzyme and then these lipoproteins can be bind to the liver receptors and deliver their cholesterol content (24). Obtained from Servier Medical Art (https://smart.servier.com).

HDL functionality and ApoA-I

As already mentioned in the previous section, recent studies have shown that elevating HDL cholesterol concentrations is not always beneficial (25, 26). Moreover, when the composition of HDL is changed, it can even exert a negative effect on the vasculature (27). Therefore, over the past decade, it became evident that HDL functionality is more important in terms

of HDL-related athero-protective activities than the HDL cholesterol concentrations itself. The functionality of HDL is defined as its ability to show anti-atherogenic properties, such as regulating the RCT process, which is the main athero-protective function of HDL (28). Moreover, HDL cholesterol efflux capacity from macrophages shows an inverse correlation with the CVD (25). As mentioned above, ApoA-I is the main protein constituent of HDL particles and in contrast to HDL cholesterol concentrations, ApoA-I levels have so far remained a strong predictor of CVD risk, both cross-sectional (29) as well as in interventions aiming at an elevation of ApoA-I (30, 31). As ApoA-I is the ligand for ABCA1 mediated cholesterol efflux, ApoA-I is the main driver of RCT and therefore elevating ApoA-I concentrations is a new promising strategy to reduce atherosclerosis development. Newly synthesized ApoA-I is first produced as a Pre-pro-ApoA-I protein after the transcription of the ApoA-I gene within liver or small intestine cells. First, this Pre-pro-ApoA-I is cleaved in the endoplasmic reticulum in response to the signal peptidase process (32). Consequently, as a result of this cleavage, intracellular pro-ApoA-I is formed and secreted by the cells into the extracellular space. Next, proapoA-1 undergoes post-translational proteolytic cleavage after secretion resulting in removal of the pro-segment and conversion into mature ApoA-I, a process that is regulated by the action of bone morphogenetic protein-1 (BMP-1) and procollagen c-proteinase enhancer-2 protein (PCPE2) (33, 34). After that, the c-terminal domain of this lipid poor ApoA-I starts an interaction with the ABCA1 transporter within the macrophages, as such facilitating the uptake of the cholesterol which is the initial step of the RCT process (35, 36).

Elevating of ApoA-I therapeutic effects

The question is how strong the evidence is that elevating ApoA-I concentrations, and probably also HDL functionality, lowers CVD risk. Recent studies indeed suggest that elevating ApoA-I inhibits the progression of atherosclerosis and can protects against development of CVD (37, 38). Human clinical studies have shown that ApoA-I infusion associated with increased RCT, reduced cardiac inflammatory conditions and enhanced phospholipid oxidation (39-41). Therefore, new drugs targeting ApoA-I were developed and investigated in several clinical studies (42). Typical examples of such drugs are shown in Table 1.1, where they are summarized based on their therapeutic approaches.

As mentioned, numerous studies have shown that ApoA-I was associated with multiple beneficial effects on CVD. For example, Nissen et al. found that ApoA-I-Milano infusion significantly regressed atherosclerosis in patients with coronary heart disease (Table 1.1. A1) (43). Other studies showed a reduction in total atheroma volume by 4.2% in patients with

acute coronary syndrome after intravenous ApoA-I (ETC-216) treatment (44). Moreover, treatment with purified native ApoA-I (CSL-111) decreased atheroma volume by 3.4% in coronary disease patients (45). Furthermore, the use of RVX-208 increased ApoA-I and pre β-HDL concentrations in plasma by 10% and 42%, respectively, and additionally enhanced the cholesterol efflux capacity (Table 1.1. A2) (46). ApoA-I mimetics are short synthetic peptides with an amphipathic helix, that imitate the biological functions and the structure of ApoA-I (47). These peptides fragments which can be infused or taken orally (42), are very promising as a potential therapies for CVD (48, 49). In a recent study, Dunbar et al. have found that oral ApoA-I mimetic (D-4F) suppressed the HDL inflammatory index in high-risk CVD patients (Table 1.1. C1) (50). Thus, the evidence of these clinical trial clearly show that elevating ApoA-I concentrations lowers CVD risk and therefore it is evident that we need a better understanding of the process underlying ApoA-I transcription and production and identify novel compounds that can be used to elevate this process.

Table 1.1. ApoA-I different therapeutic strategies in the clinical studies (42)

A) Direct ApoA-I elevation	B) Indirect ApoA-I elevation	C) Mimic ApoA-I functionality
1 Intravenous ApoA-I therapy ➢ Recombinant ApoA-I Milano/phospholipids (ETC-216) ➢ Purified native ApoA-I/ phospholipids (CSL-111/112)	1 Cholesteryl ester transfer protein inhibitors ➢ Dalcetrapib ➢ Anacetrapib	1 ApoA-I mimetic peptides ➢ D-4F
2 Upregulation of endogenous ApoA-I production ➢ RVX-208	2 Niacin receptor (GPR109A) agonists	
	3 Endothelial lipase inhibitors	

ApoA-I transcription

ApoA-I contains 243 amino acids and is mainly produced in the liver and small intestine cells. The ApoA-I gene is located at chromosome 11 and its regulation mainly occurs at the transcriptional level (51). Several transcription factors and elements regulate ApoA-I transcription by activating or inhibiting ApoA-I gene expression in response to metabolic or hormonal changes (52) (Figure 1.3). This regulatory process also requires the presence of the ApoC-III enhancer, particularly for intestinal ApoA-I transcription (53). There is ample evidence that peroxisome proliferator-activated receptor alpha (PPARα) activators such as fibrates are able to induce ApoA-I gene expression (54, 55). Moreover, also Bromodomain and Extra-Terminal (BET) inhibitors significantly increased ApoA-I transcription and production. For example, the BET inhibitors RVX-208 and JQ1(+) increase ApoA-I

expression via inhibiting the binding of BET proteins to the acetylated histone residues (56, 57). In addition, several hormones were reported to modulate ApoA-I gene expression such as thyroid hormone, estrogen and glucocorticoid (58). Taken together, discovering natural compounds which can increase ApoA-I transcription and HDL functionality via these targets is needed.

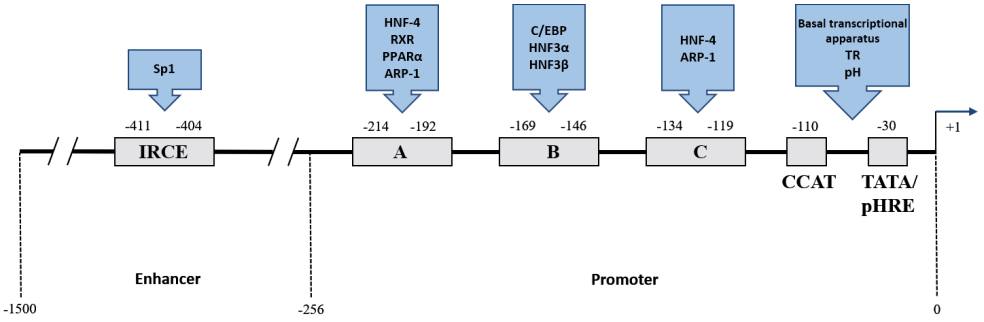

Figure 1.3. The ApoA-I promoter/enhancer model with selective activators or inhibitors. The sites A, B and C are essential for ApoA-I gene transcription. The PPAR response element (PPRE) is located on site A, which is a binding element for the transcription factor PPARα (59). Moreover, both sites A and C contain ARP-1 (ApoA-I repressor protein-1) and hormone response elements (HRE), which are binding elements of hepatic nuclear factor 4 (HNF-4) (60). Although both A and C sites contain HRE binding elements, the retinoid X receptor (RXR)/PPARα heterodimer binds only to site A (61). Furthermore, CCAAT-enhancer binding proteins (C/EBP), HNF3α and HNF3β bind to site B (62, 63). In addition, an insulin response core element (IRCE) is located on the enhancer site, which binds to the ubiquitous transcription factor Sp1, resulting on ApoA-I gene production by insulin (64).

Link between inflammation and ApoA-I concentrations

Inflammation leads to several structural and metabolic alterations in lipid and lipoproteins, which are associated with increased CVD risk (65). For example, inflammation results in a reduction of HDL cholesterol and HDL particle concentrations and most important even in the formation of dysfunctional HDL particles, which altogether eventually leads to inflammation induced RCT impairment (66). The innate immune system is known as the first line of defense against infections, and the main action of it is to fight against host of organisms by the activation of the oxidative state (67). This action is known as acute phase response, which occurs, amongst other tissues, in the liver (67). A number of cytokines such as Interleukin-8 (IL-8) stimulate the acute phase response in the liver (68). These inflammatory cytokines suppress the hepatic expression of ApoA-I and enhance the production of serum amyloid A, which becomes the major protein component of HDL (69-71). Therefore, the question is how to elevate ApoA-I concentrations during these inflammatory conditions.

SCFAs (physiology, production, absorption and function)

It is well-known that the composition of the intestinal microbiota plays a major role in the colonic health and contributes to the development of various conditions and diseases such as type 2 diabetes (72) and the metabolic syndrome (73). Besides a direct role on (intestinal) physiology for example via regulating the (local) host immune response (74), these microorganisms have enzyme activity which can break down carbohydrates and fibers and consequently catalyze the formation of different useful metabolites (74). For example, after intake of a meal that contains soluble fibers, the anaerobic intestinal microbiota produces short-chain fatty acids (SCFAs) by fermentation of these fibers (75). Notably, the fermentation of oat bran and resistant starch is assumed to result in the highest amounts of SCFAs (76). SCFAs are fatty acids that consist of carbon atoms with a length ranging from 2 to 6, i.e. acetic acid (C2), propionic acid (C3), butyric acid (C4), valeric acid (C5) and hexanoic acid (C6) (77, 78). The cecum is the major site of SCFAs production, in addition to the ascending, transverse, descending and sigmoid colon (76, 79). However, although SCFAs concentrations are considerably higher (80) (-131 mmol/kg of intestinal content) in the colon, there are certainly also SCFAs formed and present within the terminal ileum, though in lower concentrations (13 mmol/kg of intestinal content) (80). The absorption of SCFAs from the intestinal lumen is effective and fast with only 5% of SCFAs being excreted into the feces (79). The colonic absorption occurs via the apical membrane of the epithelial cells through several transporters, including the sodium-coupled monocarboxylate transporter 1 and hydrogen-coupled monocarboxylate transporters (MCT) (81, 82). Although most SCFAs are absorbed by the colonocytes and locally used as fuel, part of the SCFAs are released from the gut and transferred via the mesenteric veins to the portal vein and the liver (83). Eventually, the remaining SCFAs, which are not retained in the liver, are transported into the circulation (84). Human studies in which portal SCFAs concentrations were measured are scarce and only carried out in patients during surgery. For example, Bloemen et al. found that propionate and butyrate concentrations in the portal plasma were 30.3 and 30.1 μmol/l, respectively (83). Therefore, it is unknown whether these concentrations also reflect the healthy situation. In terms of underlying mechanisms, recent studies have revealed that SCFAs might affect enterocytes by binding to G protein coupled receptors (GPCRs) and activate intracellular signaling. For SCFAs, these GPCRs are FFAR2 (GPR43) and FFAR3 (GPCR41) (84). Interestingly, both receptors are also expressed in human liver, adipose tissue and skeletal muscles, which suggests that SCFAs could also influence (metabolic) processes in these peripheral tissues (85). The physiological functions of SCFAs include energy production for the colonocytes and the reduction of the gut luminal pH, which increases mineral absorption and inhibits

pathogens growth (86). In addition, SCFAs prompt expression of many genes and regulate the secretion of hormones and GI peptides such as glucagon-like peptide 1 (GLP-1) (87). Moreover, SCFAs might also lower cholesterol synthesis and modulate glucose metabolism (88) .

SCFAs role in metabolic and cardiovascular health

Many epidemiological studies have suggested that SCFAs could play a role in the prevention and treatment of metabolic diseases (76, 89-92). Systemic SCFAs are thought to contribute to a wide range of health benefits including improvements in glucose homeostasis, blood lipid profiles and reduction of body weight (77, 78). In addition, SCFAs exert anti-inflammatory effects, which might contribute to explaining the reduced development of atherosclerosis (93, 94). This is in line with earlier observations suggesting a protective role for SCFAs in CVD development and other inflammatory diseases (79). The anti-atherosclerotic effects of SCFAs are assumed to be mediated by modulating pro-inflammatory cytokine production, endothelial dysfunction and oxidative stress (95). In animal studies, Bartolomaeus and coworkers have recently shown that propionate supplementation reduced atherosclerosis (96). Moreover, the supplementation of propionate and butyrate in diabetic animals reduced hepatic lipid accumulation and improved glucose tolerance (97). A recent human study reported that dietary C4 supplementation was associated with improved blood pressure through a reduction in inflammation (98). Furthermore, colonic infusion of SCFAs increased energy expenditure and fat oxidation, while decreased adipose tissue lipolysis in overweight subjects (91), and propionate has been shown to modulate liver lipid content in NAFLD patients (99). Altogether, the findings so far are suggestive that SCFAs can influence metabolic processes that prevent the development of CVD, but intervention studies in humans are needed to come to more firm conclusions.

The possible relationship between SCFAs and ApoA-I

Clinical studies revealed that antibiotics disturbed gut microbiota and altered lipid and lipoprotein metabolism (100-102). In a recent human trial carried out in our group, we found that amoxicillin treatment reduced HDL cholesterol concentrations (101). Since ApoA-I is the major structural protein form HDL particles, the question is whether antibiotics could also modulate ApoA-I transcription and synthesis. Theoretically, the effects of antibiotics on ApoA-I expression and secretion can be the result from direct effects on ApoA-I gene transcription or indirect effects through affecting microbiota composition and subsequently

intestinal SCFAs production and SCFAs concentrations that reach the liver. For example, the intake of vancomycin resulted in a reduction of SCFAs concentrations in human's plasma (100). An additional indication supporting this assumption comes from a recent study in which it was shown that SCFA supplementation increased HDL cholesterol concentrations in hamsters (103). Another indication for a potential relationship between SCFAs and ApoA-I is based on the fact that fatty acids can bind strongly to PPARα, a transcription factor that plays an essential role in the regulation of ApoA-I expression (104, 105). Based on our previous data regarding the effects of long chain fatty acids on PPAR activation and ApoA-I transcription, it is likely to suggest that SCFAs also can bind to PPARα, which eventually led to PPARα transactivation and ApoA-I transcription (104).

Exploring effects of natural compounds (i.e. amino acids) on ApoA-I

As mentioned, increasing ApoA-I production is a promising strategy to reduce the CVD risk (37, 38) and therefore, studies exploring the potential effects of dietary compounds that can elevate ApoA-I transcription is needed. Thus, we also focused on natural compounds with the ability to stimulate PPARα, a transcription factor, that regulates ApoA-I expression (104, 105). Amino acids are interesting candidates since there are indications that amino acids can modulate PPARα expression (106). For example, leucine was found to significantly increase PPARα expression in skeletal muscle myotubes (106). Moreover, leucine also significantly reduced p-P65-NFκB expression leading to reduced IL6 mRNA expression (106). Furthermore, other studies showed that leucine, glutamine and proline all reduced IL-8 production, probably via NF-κB inhibition, in HepG2 cells (107), a process in which PPARα is also involved (108). Therefore, amino acids could be additional dietary compounds that play a role in regulating ApoA-I expression and there is a clear need to further explore this hypothesis.

Outline of this dissertation

The aim of this dissertation was to study the effects of a number of variables on ApoA-I production in vitro. For example, we observed in an earlier placebo controlled human intervention study carried out in our group that antibiotics changed serum HDL cholesterol concentrations. Since do novo ApoA-I production is a possible driver behind elevations in serum HDL cholesterol, we speculated that this observation could be a direct effect the antibiotic or otherwise an indirect effect related to changes in SCFA's due to antibiotic induced changes in microbiota composition. Therefore, in **chapter 2** we examined the dose-

response relationship between several SCFAs and ApoA-I transcription and secretion in HepG2 cells. In addition, we evaluated the molecular mechanisms underlying theses effects on ApoA-I such as PPARα transactivation and BET inhibition. In **chapter 3**, we investigated the direct effects of different classes of antibiotics on ApoA-I transcription and secretion in hepatocytes and enterocytes. Moreover, we addressed the potential involvement of BET inhibition, PPARα transactivation and ER stress on these antibiotic-induced changes in ApoA-I transcription. In **chapter 4** we focused on the potentially protective effects of SCFAs on inflammatory pathways (NF-κB transactivation and IL-8 secretion) in HepG2 cells exposed to a cytokine cocktail, which is known to lower ApoA-I expression. We also again evaluated the possible mechanisms underlying these anti-inflammatory effects of SCFAs on ApoA-I transcription. In **chapter 5**, we aimed to study the possible existence of a cross-talk between enterocytes and hepatocytes on ApoA-I transcription. Therefore, we compared the effects of C4 on ApoA-I transcription in Caco-2 and HepG2 cells when cultured alone to the effects when both cells were co-cultured together in a transwell system. Furthermore, till now we fully focused on the effects of dietary lipids on ApoA-I transcription. However, we broadened our horizon and explored in **chapter 6** the potential effects of different amino acids on ApoA-I transcription and secretion in HepG2 cells. We again also investigated the underlying pathways which might induce these effects of amino acids on ApoA-I production. Finally, in **chapter 7**, the main outcomes of all these studies are discussed and placed into broader perspective.

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The effects of short-chain fatty acids on the transcription and secretion of apolipoprotein A-I in human hepatocytes in vitro

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Abstract

Apolipoprotein-I (ApoA-I), the major component of high-density lipoprotein (HDL) particles, mediates cholesterol efflux by which it facilitates the removal of excess cholesterol from peripheral tissues. Therefore, elevating ApoA-I production leading to the production of new pre-β-HDL particles is thought to be beneficial in the prevention of cardiovascular diseases. Recently, we observed that amoxicillin treatment led to decreased HDL concentrations in healthy human volunteers. We questioned whether this antibiotic effect was directly or indirectly, via changed short-chain fatty acids (SCFA) concentrations through an altered gut microflora. Therefore, we here evaluated the effects of amoxicillin and various SCFA on hepatic ApoA-I expression, secretion, and the putative underlying pathways. Human hepatocytes (HepG2) were exposed to increasing dose of amoxicillin or SCFA for 48 hours. ApoA-I messenger RNA (mRNA) transcription and secreted protein were analyzed using quantitative polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. To study underlying mechanisms, changes in mRNA expression of KEAP1, CPT1, and PPARα, as well as a PPARα transactivation assay, were analyzed. Amoxicillin dose-dependently decreased ApoA-I mRNA transcription as well as ApoA-I protein secretion. SCFA treatment resulted in a dose-dependent stimulation of ApoA-I mRNA transcription, however, the ApoA-I protein secretion was decreased. Furthermore, SCFA treatment increased PPARα transactivation, PPARα and CPT1 mRNA transcription, whereas KEAP1 mRNA transcription was decreased. The results found indicate that direct treatment of HepG2 cells with amoxicillin has either direct effects on lowering ApoA-I transcription and secretion or indirect effects via modified SCFA concentrations because SCFA were found to stimulate hepatic ApoA-I expression. Furthermore, BET inhibition and PPARα activation were identified as possible mechanisms behind the observed effects on ApoA-I transcription.

Introduction

Several studies have suggested that antibiotics affect lipid and lipoprotein metabolism (1, 2). In fact, in a recent placebo-controlled trial we observed that amoxicillin treatment for 7 days lowered high-density lipoprotein (HDL) cholesterol concentrations in healthy volunteers (2). Whether this decrease is harmful as related to cardiovascular diseases (CVDs)-risk, is questionable because HDL functionality may be more important in this respect than HDL cholesterol concentrations (3). These functional, antiatherogenic effects have been mainly attributed to its most predominant structural protein particle, apolipoprotein-I (ApoA-I), which is produced by the small intestine and the liver (4). Amongst others, ApoA-I mediates cholesterol efflux facilitating the removal of excess cholesterol from peripheral tissues and the uptake by the liver for excretion into bile (5). Since ApoA-I is one of the most important HDL proteins in terms of functionality, a better understanding of ApoA-I transcription and production is warranted. Therefore, we explored in vitro the potential antibiotic-related effects on ApoA-I transcription and synthesis. Effects of antibiotics on de novo ApoA-I expression can be a direct effect of the antibiotic on gene transcription or an indirect effect by affecting microbiota composition. Effects of antibiotics on ApoA-I transcription have to the best of our knowledge never been studied. Regarding indirect effects, Reijnders et al. (1) have recently shown that the decreased bacterial diversity after the intake of vancomycin resulted in reduced circulating short-chain fatty acids (SCFA) concentrations. These SCFA are produced in the colon by the fermentation of dietary fibers such as resistant starches and non-starch polysaccharides (6). SCFA have been linked to multiple beneficial health effects including a reduced risk of inflammatory diseases, gastrointestinal disorders, cancer, and CVD (7). Furthermore, it was recently shown that dietary SCFA increase HDL cholesterol concentrations in hamsters (8). We therefore hypothesized that antibiotics directly, or indirectly via reduced SCFA concentrations, may relate to the changes in ApoA-I transcription, which eventually led to decreased HDL cholesterol concentrations.

Besides evaluating the effects of SCFA on hepatic ApoA-I expression, an additional question is how these potential effects are regulated at a molecular level. Previous studies reported that both bromodomain and extra-terminal domain (BET) inhibition and PPARα activation had a major effect on ApoA-I transcription (9, 10). For example, BET protein inhibitors such as JQ1(+) increased ApoA-I expression and protein secretion in human hepatocellular liver carcinoma (HepG2) cells (11, 12). BET inhibitors can bind to BET proteins such as BRD4, a general transcriptional regulator, which can regulate transcription of target genes such as KEAP1 (13, 14). PPARα is a nuclear receptor that forms a heterodimer with the retinoid X receptor, which then binds to specific response elements (PPREs) within promoter regions of target genes such as PPARα itself, CPT1 (15) and ApoA-I (16). Various

dietary components such as long-chain fatty acids have been recognized as natural ligands for PPARα (17) but there are indications that SCFA may have similar effects (18, 19). Therefore, except for effects on changes in ApoA-I messenger RNA (mRNA), we also evaluated changes in KEAP1, CPT1 and PPARα mRNA expressions during exposure of HepG2 cells to different SCFA to examine potential underlying pathways.

Material and methods

Materials

Human hepatocellular liver carcinoma (HepG2) cells were kindly provided by Sten Braesch-Andersen (Mabtech, Nacka Strand, Sweden). Cell culture flasks and plates were obtained from Corning (Cambridge, USA). Minimum Essential Medium (MEM), sodium pyruvate, non-essential amino acids (NEAA) and penicillin and streptomycin were all obtained from Thermo Fisher Scientific (Bleiswijk, Netherlands). Fetal bovine serum (FBS) was purchased from PAA (Toronto, Canada). Amoxicillin was obtained from Sigma (Uithoorn, Netherlands). Propionic acid (C3), butyric acid (C4), valeric acid (C5) and hexanoic acid (C6) were purchased from Sigma (Uithoorn, Netherlands). The BET inhibitor JQ1(+), was purchased from Bio-techne - R&D (Minneapolis, USA). Thapsigargin (Taps), an ER-stress inducer, was purchased from Sigma (Uithoorn, Netherlands). DMSO and Tri-reagent were obtained from Sigma (Uithoorn, Netherlands).

Cell culture and SCFA treatment

HepG2 cells were cultured at 37 °C in a humidified atmosphere of 5% carbon dioxide (CO_2) in MEM containing 10% heat inactivated FBS, 1% sodium pyruvate, 1% NEAA and 1% of penicillin-streptomycin mixture. In the amoxicillin experiments cells were cultured without penicillin-streptomycin mixture. For all experiments, cells were seeded in a 24-well plate at a density of 200,000 cells per well. Cell viability was daily inspected by microscope and when cells reached a density of 80–90%, they were incubated for 48 h in the medium (MEM without FBS) plus a concentration range of 0–7 mM SCFA (C3, C4, C5 or C6) or amoxicillin (0-200 μg/ml) or 3 μM JQ1(+). The positive control JQ1(+) was included – separate from the SCFA – in all experiments to ensure the cells were responsive and capable to produce sufficient amounts of ApoA-I mRNA. All SCFA as well as JQ1(+) were dissolved in dimethyl sulfoxide (*DMSO, cell culture tested*) and effects were expressed relative to those of the carrier control (DMSO only). Final DMSO concentration was always 0.2%. As amoxicillin was dissolved in water, effects of amoxicillin were compared to those of a water control. In previous study (20), it was shown that after oral administration of 500 mg

of amoxicillin (taken three times a day), serum peak levels were between 6.0 to 15.3 μg/ml and after intravenous administration of 500 mg of amoxicillin (taken three times a day), serum peak levels were between 30.1 to 52.1 μg/ml. It was therefore decided to test the effect of the antibiotics on HepG2 cells in concentrations of 3, 6, 12.5, 25, 50, 100 and 200 μg/ml. Medium was collected for analysis of ApoA-I concentrations and cells were harvested for analysis of mRNA expression after lysing with Tri-reagent. Both medium and lysed cells were snap frozen in liquid nitrogen and stored at −80 °C until further analysis.

Quantification of ApoA-I secretion levels in the culture medium

To investigate ApoA-I secretion by HepG2 cells, ApoA-I protein concentrations in culture medium were measured by an enzyme-linked immunoassay (ELISA) obtained from Mabtech (Nacka Strand, Sweden). Direct sandwich ELISA was performed according the manufacturer's instructions, with slight modifications, blocker BSA 10% (Thermo Fisher Scientific, Bleiswijk, Netherlands) was added to block buffer and to dilution buffer with final concentration of 1% and 0.1% respectively.

Quantification of gene mRNA transcription

To evaluate mRNA expression levels of ApoA-I, CPT1, KEAP1 and PPARα, total RNA was isolated using Tri-reagent, according the manufacturer's instructions. The RNeasy mini kit (Qiagen, Hilden, Germany) was used for RNA purification. For cDNA synthesis, 350 ng of total RNA was reverse-transcribed using RNAse inhibitor, dNTP's, random hexamers, MMLV reverse trans, DTT and 5xFS buffer (Thermo Fisher Scientific, Bleiswijk, Netherlands). The resulting cDNA was used for real time quantitative PCR using TaqMan Gene Expression Assays using Cyclophilin A (Hs99999904) as a housekeeping control. To quantify ApoA-I, KEAP1, PPARα and CPT1, the following TaqMan Genes Expression Assays Hs00163641, Hs00202227, Hs00231882 and Hs00912671 were used. Values are presented as relative gene expressions based on the Ct values, normalized for the internal control Cyclophilin A and compared to the control conditions.

Luciferase assay

To investigate PPARα transcriptional activity, HepG2 cells were transfected with X-treme gene 9 DNA transfection reagent (Sigma, Uithoorn, Netherlands) and the following plasmids: pcDNA3.1, pcDNA3.1_PPARα, pGL3 and pGL3_PPRE as previously described (17). Following transfection and 48 h SCFA treatment, cells were lysed in luciferase lysis buffer (Promega, Madison, USA), and measured for luciferase activity, reflecting

PPARα transactivation, using a GloMax® 96 Microplate luminometer, all according to the manufacturer's manual (Promega, Madison, USA).

Statistical analysis

All experiments were performed in Duplo and each experiment was repeated three times. To test the dose-response relationship with the gene of interest, a regression analysis was performed. When the analysis was not-linear, quadratic polynomial regression was performed. For all statistical analysis, the regression coefficient was considered statistically significant at $p < 0.05$. Statistical analysis was performed using SPSS v.25 (IBM Corp., Armonk, NY, USA).

Results

Effects of amoxicillin on ApoA-I mRNA expression and protein secretion

Amoxicillin significantly ($p < 0.05$) and dose-dependently decreased ApoA-I mRNA expression up to 30%. Interestingly, the lower ApoA-I mRNA expression translated into a lower ApoA-I protein secretion ($p < 0.01$) into the culture medium, i.e. ApoA-I protein concentrations followed mRNA expression. As expected, JQ1(+), a well-defined BET inhibitor, which was used as positive control, increased ApoA-I mRNA expression as well as ApoA-I protein secretion, while the negative control thapsigargin, an ER-stress inducer, inhibited both ApoA-I mRNA expression and protein secretion (Figure 2.1). Altogether, these findings suggest direct inhibitory effects of amoxicillin on ApoA-I transcription and secretion.

Effect of SCFA on ApoA-I mRNA expression and protein secretion

The positive control JQ1(+) strongly increased ApoA-I mRNA expression in all experiments with SCFA (Figure 2.2). This indicated that the cells were responsive and allows further analysis with SCFA. ApoA-I mRNA expression dose-dependently increased after C3 treatment ($p < 0.001$), with a maximum of a 3.2-fold increase at 7 mM. Treatment with C4 first dose-dependently increased ApoA-I mRNA expression which resulted in a 3-fold increase at 3 mM followed by a dose dependent decrease at higher concentrations down to ± 50% of basal ApoA-I mRNA expression at 7 mM ($p < 0.001$). C5 dose-dependently and significantly ($p < 0.001$) increased ApoA-I mRNA expression up to a 3-fold increase at 5.5 mM. C6 gradually increased ApoA-I mRNA expression with a maximum of 2-fold at 7 mM ($p < 0.01$) (Figure 2.2). Although ApoA-I mRNA expression was elevated with all types of

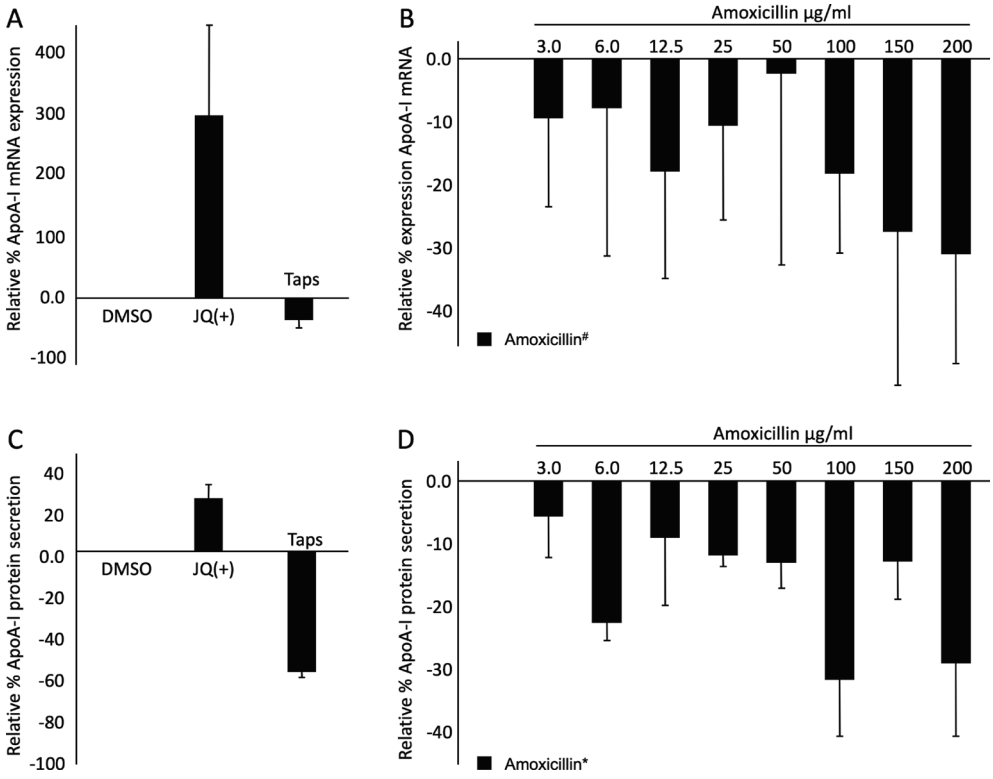

Figure 2.1. (A) Relative ApoA-I mRNA expression in HepG2 cells treated with JQ1(+) (3 µM) and Taps (0.01 µM). JQ1(+) was used as a positive control for ApoA-I transcription, whereas Taps was used as a control for reduced ApoA-I transcription. **(B)** Relative ApoA-I mRNA expression in HepG2 cells treated with different concentrations of amoxicillin. Increasing amoxicillin concentrations show significant regression coefficient deviated from zero ($p < 0.05$) in ApoA-I mRNA expression. **(C)** Relative ApoA-I protein secretion by HepG2 cells into the culture medium after treatment with JQ1(+) (3 µM) and Taps (0.01 µM). JQ1(+) was used as a positive control for ApoA-I protein secretion, whereas Taps was used as a control for reduced ApoA-I protein secretion. **(D)** Relative ApoA-I protein secretion by HepG2 cells into the culture medium after treatment with different concentrations of amoxicillin. Increasing amoxicillin concentrations show significant regression coefficient deviated from zero ($p < 0.01$) in ApoA-I protein secretion. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against expression of the control condition, which was arbitrarily set at 0%. Changes are indicated with * when regression coefficient significantly at ($p < 0.01$) or with # when regression coefficient significantly at ($p < 0.05$). ApoA-I, apolipoprotein-I; DMSO, dimethyl sulfoxide; mRNA, messenger RNA.

SCFA – except higher C4 doses –, we observed for all SCFA a dose-dependent decreases in ApoA-I protein concentrations in the culture medium of the HepG2 cells ($p < 0.05$) (Figure 2.3). In contrast, the positive control JQ1(+) not only elevated ApoA-I mRNA expression, but also increased ApoA-I protein secretion in the culture medium (Figure 2.3).

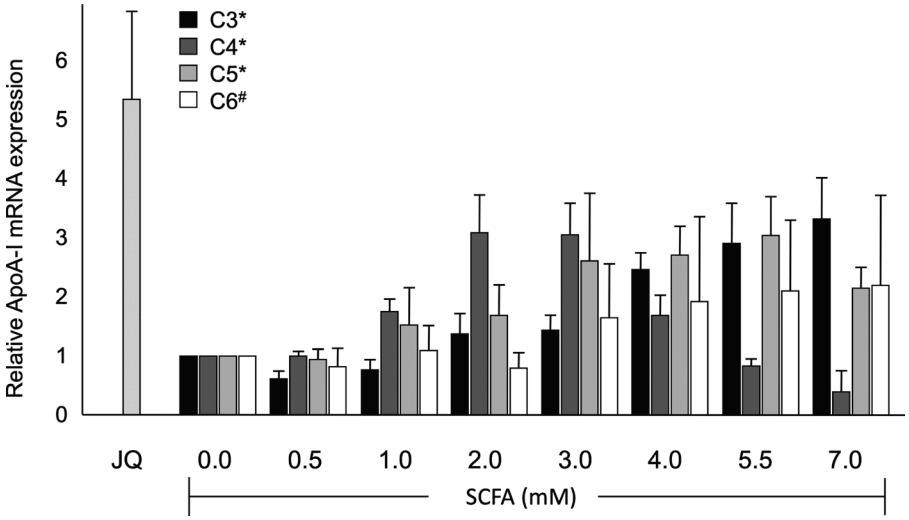

Figure 2.2. Relative ApoA-I mRNA expression in HepG2 cells treated with different concentrations of SCFA. Increasing C3, C4, and C5 concentrations show significant regression coefficient deviated from zero ($p < 0.001$) in ApoA-I mRNA expression. C6 also show significant regression coefficient deviated from zero ($p < 0.01$) in ApoA-I mRNA expression. Linear regression for SCFA dose-response effects was performed except C4, quadratic polynomial regression was performed for C4 dose-response effects. JQ1(+) (3 μM) was used as a positive control for ApoA-I transcription. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against expression of the control condition, which was arbitrarily set at 1. Changes are indicated with a * when regression coefficient significantly at ($p < 0.001$) or with a # when regression coefficient significantly at ($p < 0.01$). ApoA-I, apolipoprotein-I; mRNA, messenger RNA; SCFA, short-chain fatty acids.

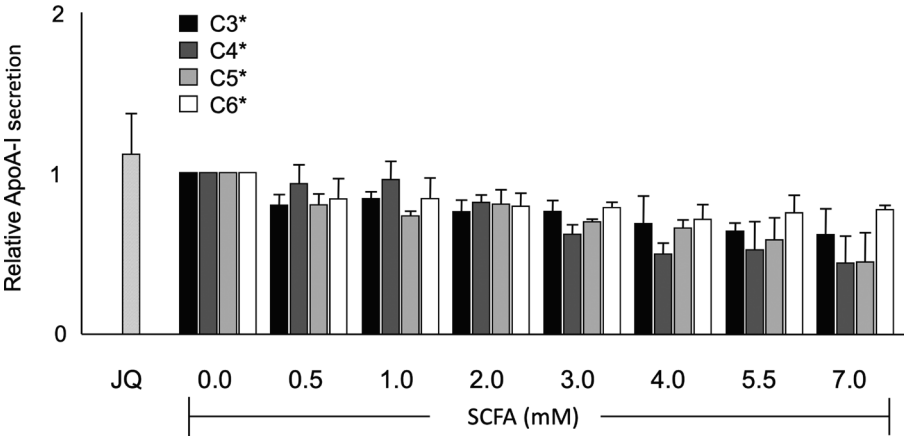

Figure 2.3. Relative ApoA-I protein secretion into the culture medium of HepG2 cells after treatment with different concentrations of SCFA. Increasing SCFA concentrations show significant regression coefficient deviated from zero ($p < 0.05$) in ApoA-I protein secretion. JQ1(+) (3 μM) was used as a positive control for ApoA-I protein secretion. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against expression of the control condition, which was arbitrarily set at 1. Changes are indicated with a * when regression coefficient significantly at ($p < 0.05$). ApoA-I, apolipoprotein-I; SCFA, short-chain fatty acids.

Effect of SCFA on KEAP1, CPT1 and PPARα mRNA expression

To evaluate potential underlying mechanisms, changes in activities on the BET and PPARα pathways were evaluated. In general, BET-inhibition is related to a decreased expression of the BET target gene KEAP1 and PPARα transactivation via an increased expression of the PPARα target genes CPT1 and PPARα itself.

In general, SCFA treatment significantly and dose-dependently decreased KEAP1 gene expression ($p < 0.001$). In more detail, C3 decreased KEAP1 gene expression maximally by 50% at 3 mM, whereas C4 lowered KEAP1 expression even up to 80% at 7 mM. Moreover, KEAP1 expression decreased by 60% and 50% after exposure to respectively 3 mM C5 and C6. Interestingly, KEAP1 was downregulated while ApoA-I mRNA expressions was increased for all SCFA, suggesting that BET inhibition could be related to the observed increases in ApoA-I mRNA levels (Figure 2.4).

Moreover, CPT1 mRNA expression dose-dependently and significantly increased after SCFA treatment. More specifically, C3 significantly ($p < 0.001$) increased CPT1 mRNA expression with a maximum of a 2.7-fold at 7 mM. C4 was the strongest activator, i.e. a 3.6-fold CPT1 expression at 3 mM. In line with ApoA-I, this C4-induced elevation in CPT1 expression was followed by gradual reductions at higher C4 doses ($p < 0.001$). C5 significantly ($p < 0.001$) increased CPT1 gene expression to 1.9-fold at 7 mM, whereas C6 was the weakest activator, i.e. only a slight but not significant 1.2-fold increase at 3 mM was observed (Figure 2.4).

In line with CPT1, all SCFA elevated PPARα gene expression, although effects of C4 fluctuated. Both C3 and C6 significantly increased PPARα expression to 4.0-fold and 4.4-fold respectively ($p < 0.001$). Like for ApoA-I and CPT1, C4 increased PPARα expression 1.9-fold with the most pronounced effect at 3 mM. At higher doses, a reduction of PPARα expression was observed. C5 significantly ($p < 0.01$) increased PPARα expressions to 1.8-fold at 7 mM (Figure 2.4).

To evaluate whether the effects of SCFA on CPT1 and PPARα mRNA expressions could be explained via PPARα activation, we evaluated changes in PPARα transcriptional activity with C4 as a model compound for all SCFA tested, in transfected HepG2 cells using a PPRE luciferase reporter system. In line with ApoA-I, CPT1 and PPARα mRNA expressions, C4 significantly increased PPARα transactivation, followed by a gradual dose-dependent decrease resulting in an inhibition of 90% of basal PPARα transactivation at 5.5 mM ($p < 0.001$). Interestingly, JQ1(+) markedly inhibited PPARα transactivation by 70% (Figure 2.5).

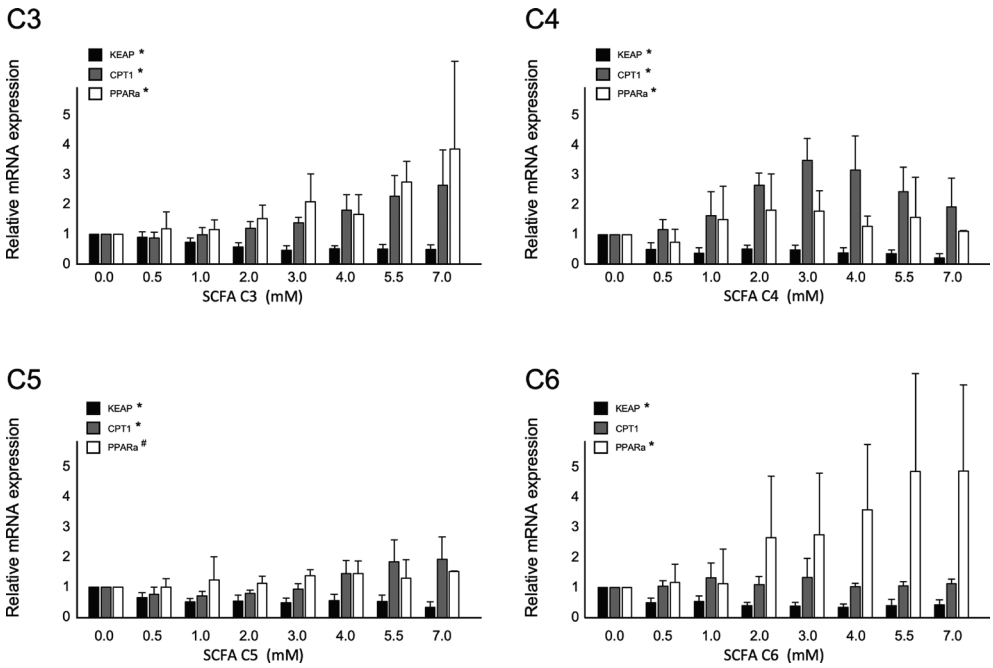

Figure 2.4. Relative KEAP1, CPT1 and PPARα mRNA expression in HepG2 cells treated with different concentrations of SCFA. C3 different concentrations show significant regression coefficient deviated from zero ($p < 0.001$) in KEAP1, CPT1 and PPARα mRNA expression. C4 different concentrations show significant regression coefficient deviated from zero ($p < 0.001$) in KEAP1 and CPT1 and PPARα mRNA expression. C5 different concentrations show significant regression coefficient deviated from zero ($p < 0.001$) in KEAP1 and CPT1 ($p < 0.01$) in PPARα mRNA expression. C6 different concentrations show significant regression coefficient deviated from zero ($p < 0.001$) in KEAP1 and PPARα mRNA expression. Linear regression for SCFA dose-response effects was performed except C4, quadratic polynomial regression was performed for C4 dose-response effects. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against expression of the control condition, which was arbitrarily set at 1. Changes are indicated with a * when regression coefficient significantly at ($p < 0.001$) or with a # when regression coefficient significantly at ($p < 0.01$). mRNA, messenger RNA; SCFA, short-chain fatty acids.

Overall, for all SCFA the patterns between CPT1 as well as PPARα and ApoA-I mRNA expressions and PPARα transactivation were comparable suggesting that PPAR activation might relate to the observed elevation in ApoA-I mRNA levels.

Discussion

We here report a series of in vitro experiments in HepG2 cells supporting our hypothesis that antibiotics could have direct and indirect effects on hepatic ApoA-I transcription and secretion. First, we showed that amoxicillin lowered ApoA-I transcription and secretion by HepG2 cells, whereas all SCFA tested, which are metabolites produced by microbiota,

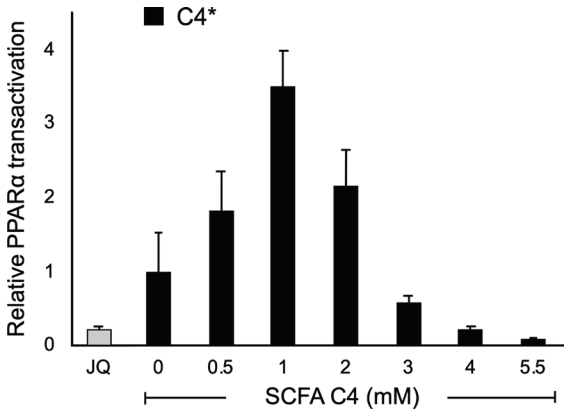

Figure 2.5. Relative PPARα transactivation in transfected HepG2 cells treated with different concentrations of C4. Increasing C4 concentrations show significant regression coefficient deviated from zero ($p < 0.001$) in PPARα transactivation. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against expression of the control condition, which was arbitrarily set at 1. Changes are indicated with a * when regression coefficient significantly at ($p < 0.001$).

clearly increased ApoA-I transcription, except for C4 at higher concentrations. This suggests that increasing the flux of SCFA from the portal vein towards the liver might translate into an increased hepatic ApoA-I transcription. Mechanistically, we showed that SCFA-induced BET inhibition as well as PPARα activation suggesting that these two pathways are potentially involved.

Recently, we observed in healthy human volunteers that amoxicillin, a broad-spectrum antibiotic, significantly lowered serum HDL-C concentrations (1). Amoxicillin is a member of β-lactam antibiotics, which negatively affects bacterial growth by binding to a primary receptor, called the penicillin-binding protein (PBP), on the bacterial membrane (21, 22). As a consequence, bacteria lose their control over autolytic enzymes leading to apoptosis (23). As such, amoxicillin has a major impact on microbiota composition and given the enormous number of observations regarding the role of microbiota on human metabolism, antibiotics could theoretically affect numerous processes (24). Besides a direct effect of amoxicillin, our findings also suggest that changing microbiota composition which theoretically also changes SCFA production, impact hepatic ApoA-I expression. In other words, besides the direct gene regulating effect, it is well possible that the observed effects of amoxicillin treatment in humans on HDL-cholesterol concentrations are indirect, i.e. mediated by altering the composition of the microbiota which are responsible for the production of SCFA (2).

ApoA-I mRNA expression was stimulated by C3, C4, C5 and C6 treatments. At the same time, BET was inhibited and PPARα transactivated, suggesting that both BET inhibition

as well as PPARα activation are involved in the effect of SCFA on ApoA-I expression. Interestingly, PPARα transactivation closely followed the same pattern, though not fully, which suggests that additional factors could be involved. These factors might include large families of co-activators and co-repressors molecules which together determine the net outcome of PPARα mediated effects (25). PPARα can be activated by recruitment of co-activators such as CBP and SRC1, but can be inhibited by binding of co-repressors such as SMRT and NCoR (26). On the other hand, increased ApoA-I expression with the BET inhibitor JQ1(+) was observed, even though JQ1(+) failed to induce PPARα transactivation. This indicates that ApoA-I expression could also increase due to other mechanisms besides PPARα transactivation, for instance via BET inhibition. This is also in agreement with our previous research in which we showed a clear role for BET inhibition, for example via exposure to JQ1(+), on ApoA-I expression (9). JQ1(+) binds to acetylated lysine binding sites within BET bromodomains, leading to BET proteins dissociation from chromatin, amongst others translating into elevated ApoA-I mRNA expression in HepG2 cells (27). Additionally, JQ1(+) also inhibited PPARα transactivation as PPARα and CTP1 expressions were both downregulated. This means that the net results of BET inhibitors on ApoA-I are actually very strong, since they also need to overcome the effect of a lower PPARα activation.

A puzzling question that remains is why after exposure to the SCFA ApoA-I concentrations in culture medium were in contrast to the elevated ApoA-I mRNA levels, significantly reduced. The lower secretion of ApoA-I into culture medium suggest that the higher level of ApoA-I mRNA is not translated, or ApoA-I protein is produced, but possibly remains trapped within the hepatocytes. Furthermore, it is also possible that ApoA-I was internalized and recycled back to the cell surface, which might be reason behind the reduced level of protein secreted (28). Moreover, the incubation period 48 h seems suitable for analyzing changes in ApoA-I mRNA expression, but for the changes in protein secretion this time frame could be relatively short. A study by Hahn and coworkers (29) has shown a significant effect on ApoA-I protein secretion only after three days of incubation. However, it should be mentioned that our positive control JQ1(+) did elevate ApoA-I protein secretion after 48 h.

We observed some variation in SCFA ability to increase ApoA-I expression. This variation might be due to differences in binding strength of the SCFA to PPARα or BET. As mentioned before, several of co-activators and co-repressors molecules mediate PPARα effects (25), SCFA (e.g. C4 at higher doses) may activate these co-repressor domains instead of activating the co-activator domains of PPARα, which can explain the inhibitory effects of C4 higher doses on PPARα transactivation and ApoA-I mRNA expression as well.

Of all SCFA evaluated, C4 was at lower doses the most promising SCFA regarding elevating ApoA-I expression. Therefore, it might be interesting to evaluate the effects of C4 on ApoA-I production in future human intervention studies. Previous study have demonstrated that specific fibers such as resistant starch increased C4 formation by the microbiota (30). Biscuits rich in resistant starch type 3 improved glucose and lipid profiles in diabetic mice (31). Furthermore, a positive correlation between starch-rich food consumption and increased number of HDL particles was observed, this correlation resulted in lipid profile improvement (32).

In summary, we have shown that amoxicillin treatment has either direct effects on lowering ApoA-I transcription and secretion, or indirect effects via modified SCFA concentrations, since SCFA were found to stimulate hepatic ApoA-I expression. Effects of all four SCFA tested on ApoA-I mRNA expression showed a dose-dependent increase – except for C4 higher doses –. Finally, based on findings regarding KEAP1, CPT1 and PPARα gene expression as well as PPAR transactivation, it is tempting to suggest that both BET inhibition and PPARα activation are potential mechanisms behind the observed SCFA induced effects on ApoA-I expression. A more detailed understanding of these mechanisms would add to new insights for approaches in elevating HDL levels to prevent and treat atherosclerosis and CVD.

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3

Amoxicillin modulates ApoA-I transcription and secretion, predominantly via PPARα transactivation inhibition

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Abstract

In a recent human study, we observed that amoxicillin treatment decreased HDL-C concentration. We hypothesize that antibiotics lower the transcription and secretion of ApoA-I, the responsible protein for HDL production. HepG2 and Caco-2 cells were exposed to increasing dose of amoxicillin, penicillin, and streptomycin. Secreted ApoA-I protein and mRNA transcripts were analyzed using ELISA and qPCR, respectively. To unravel underlying mechanisms, KEAP1, CPT1, and CHOP mRNA expressions were determined as well as PPARα transactivation. In HepG2 and Caco-2, amoxicillin decreased ApoA-I transcription and secretion. Effects on ApoA-I expression were clearly there for amoxicillin while no effects were observed for penicillin or streptomycin. KEAP1, CPT1, and CHOP mRNA expressions were reduced by amoxicillin treatments. Moreover, a significant correlation between ApoA-I and CPT1 mRNA expressions was found. Furthermore, amoxicillin lowered PPARα transactivation. All together, these data suggest that inhibited PPARα transactivation is involved in the effects of amoxicillin on ApoA-I. In conclusion, the direct effect of amoxicillin in treated HepG2 and Caco-2 cells was a lower ApoA-I secretion and transcription. Based on evaluating alterations in KEAP1, CPT1, and CHOP mRNA expressions plus PPARα transactivation, we suggest that a reduced PPARα activation is a potential mechanism behind the observed amoxicillin effects on ApoA-I expression.

Introduction

Frequently used antibiotics such as amoxicillin, penicillin, and streptomycin have been classified as essential drugs to treat many types of bacterial infections (1, 2). Besides treatment of bacterial infections, antibiotics also influence the quantity and composition of the natural microbiota, which may be involved in a wide variety of physiological processes (3). Over the last decades, evidence accumulated suggesting that the composition of the microbiota can be linked to cardiovascular disease (CVD) (4). Recent studies suggest that specific microbiota can influence atherosclerosis by altering inflammation processes and formation of microbial metabolites, and finally atherosclerotic plaques contain DNA of different bacterial species were found (5, 6). In addition, it has been found that gut microbiota is correlated with CVD biomarkers and lipid profiles including high-density lipoprotein–cholesterol (HDL-C) concentrations (7). The question is whether antibiotics might also affect CVD biomarkers, independent of effects on microbiota composition. In a recent placebo-controlled trial in healthy volunteers, we discovered that amoxicillin treatment significantly lowered HDL-C concentrations. Subjects were instructed to take 2 capsules of 250 mg amoxicillin 3 times daily after each meal. One week after treatment, the HDL-C concentration in the amoxicillin group was lower as compared to the concentration in the placebo group (8). However, the clinical relevance of this reduction as related to CVD is questionable since HDL functionality seems more important than HDL cholesterol concentrations (9). HDL functionality has been attributed to its main structural protein, ApolipoproteinA-I (ApoA-I), which amongst other effects mediates the process of cholesterol efflux. The cholesterol efflux capacity of the HDL fraction is representative of the removal of excess cholesterol from peripheral tissues by delivering it to the liver for excretion into bile (10). Since ApoA-I is the main functional protein in HDL, elevating the production of ApoA-I has become an interesting target for CVD prevention (11). In a recent overview, Smolders et al. have reviewed that ApoA-I mimetics as well as ApoA-I infusions (ApoA-I Milano, CSL-111, CSL-112, and CER001) and the BET inhibitor RVX-208, which elevates ApoA-I production, have promising effects with respect to CVD (11). The synthesis of HDL starts with ApoA-I particles, and therefore, it is considered essential to form new, fresh, functional HDL particles. We hypothesized that the reduction in serum HDL cholesterol concentrations as observed after amoxicillin treatment is related to a reduced ApoA-I transcription. Therefore, we examined here the effects of different antibiotics, including amoxicillin, on ApoA-I transcription and secretion in hepatocytes and enterocytes. Moreover, to understand the underlying mechanism, we addressed the potential involvement of BET inhibition (12), PPARα transactivation (13, 14), or ER stress (15) on the antibiotic-induced changes in ApoA-I transcription.

Results

Effects of JQ1(+) and Thap as controls on ApoA-I gene expression; ApoA-I protein secretion; and KEAP1, CPT1, and CHOP gene expressions in HepG2 and Caco-2 Cells

In agreement with previous results, JQ1(+) and Thap reacted as expected (16). To summarize, in HepG2 cells, the positive control JQ1(+) significantly increased ApoA-I gene expression whereas the negative control Thap decreased ApoA-I gene expression ($p < 0.05$). In line with ApoA-I gene expression, JQ1(+) significantly increased ApoA-I protein secretion whereas Thap significantly decreased ApoA-1 protein secretion ($p < 0.05$). Moreover, as expected JQ1(+), a BET inhibitor, significantly ($p < 0.05$) decreased KEAP1 gene expression and Thap increased KEAP1 gene expression. Furthermore, JQ1(+) significantly decreased CPT1 gene expression while Thap significantly increased CPT1 gene expression ($p < 0.05$). Finally, JQ1(+) decreased CHOP gene expression while Thap significantly ($p < 0.05$) increased CHOP gene expression (Figure 3.1).

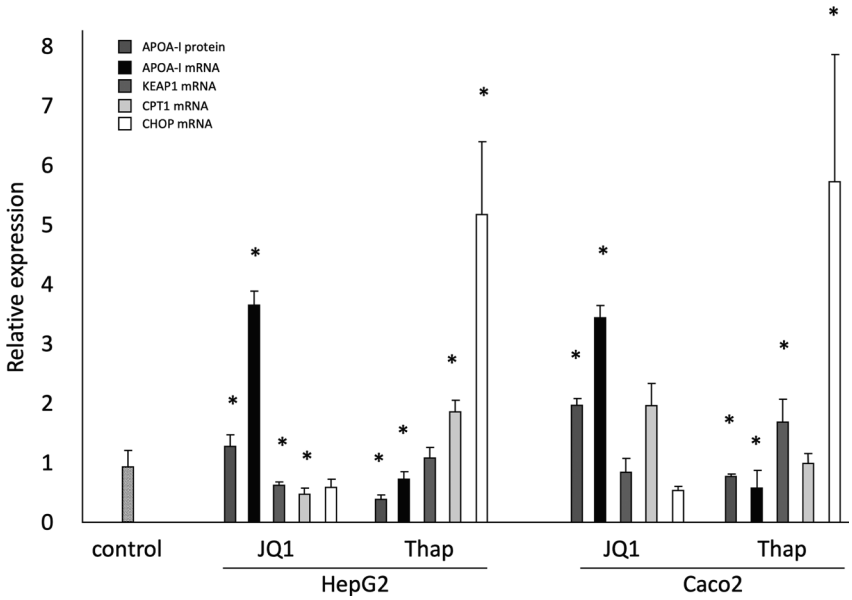


Figure 3.1. Relative ApolipoproteinA-I (ApoA-I) protein secretion and ApoA-I, KEAP1, CPT1, and CHOP mRNA expressions in HepG2 and Caco-2 cells treated with JQ1(+) (3 µM) or Thap (0.01 µM): Four biological (eight technical) replicates were performed for every condition. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against secretion or expression of the control condition (dimethyl sulfoxide (DMSO)), which was arbitrarily set at 1. Changes are indicated with * when JQ1(+) or Thap are significantly different from control ($p < 0.05$). DMSO, dimethyl sulfoxide; ApoA-I, apolipoprotein-I; mRNA, messenger RNA.

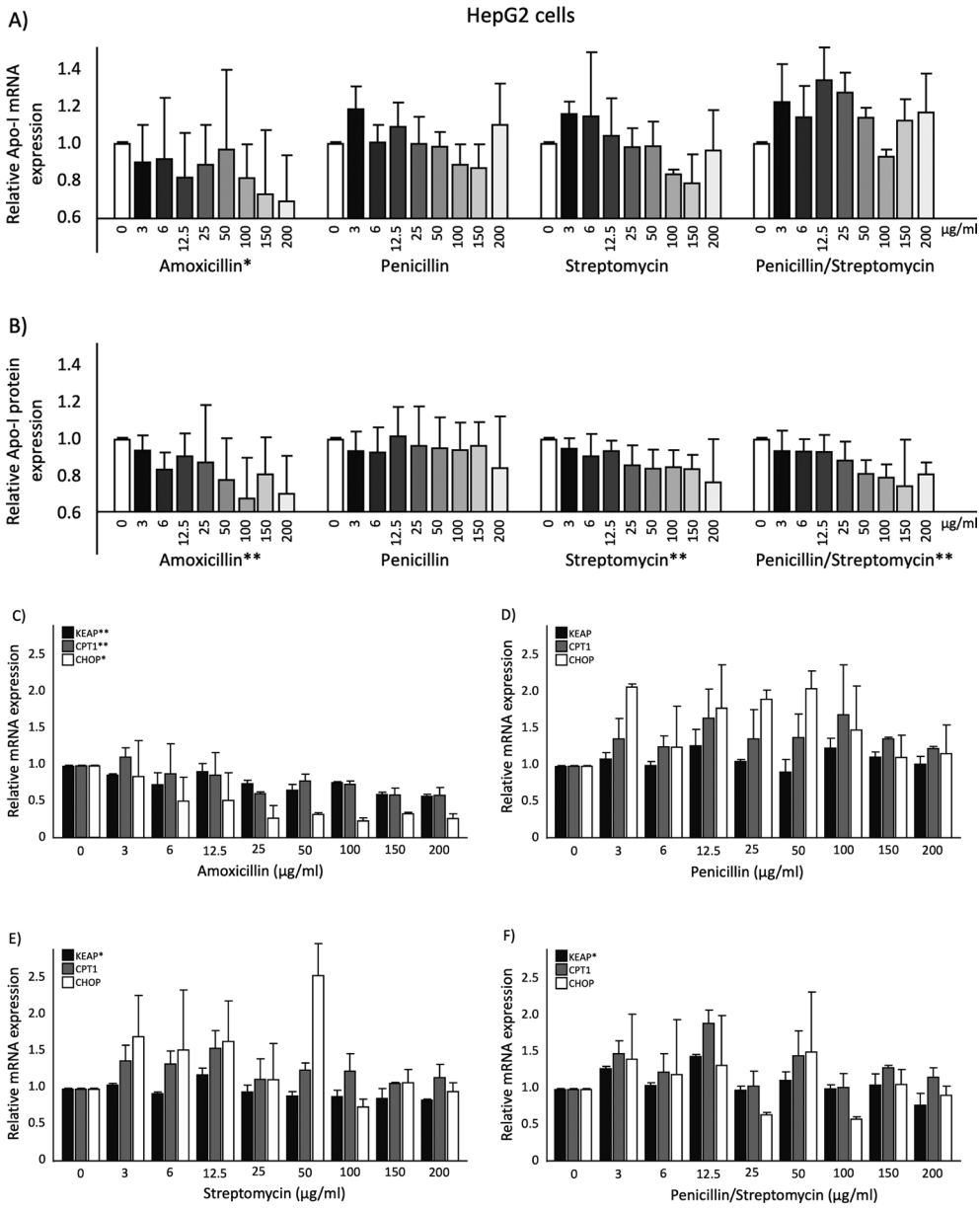
In line with the effects observed in HepG2 cells, in Caco-2 cells JQ1(+) showed a significant increase in ApoA-I mRNA expression and ApoA-I protein secretion while Thap decreased ApoA-I gene expression and ApoA-I protein secretion ($p < 0.05$). Moreover, JQ1(+) decreased KEAP1 gene expression, which did not reach statistical significance, whereas Thap significantly ($p < 0.05$) increased KEAP1 expression in Caco-2 cells. Furthermore, both JQ1(+) and Thap did not affect CPT1 gene expression in Caco-2 cells. Finally, JQ1(+) decreased CHOP gene expression while Thap significantly ($p < 0.05$) increased CHOP gene expression in Caco-2 cells (Figure 3.1).

Effects of antibiotics on ApoA-I gene expression and protein secretion in HepG2 Cells

Amoxicillin dose-dependently ($p < 0.05$) lowered ApoA-I mRNA expression (Figure 3.2). Both penicillin and streptomycin as well as the combination of penicillin and streptomycin did not change ApoA-I mRNA expression. Moreover, amoxicillin also significantly ($p < 0.01$), dose-dependently, decreased the amount of ApoA-I protein secreted into the culture medium (Figure 3.2). In contrast to ApoA-I mRNA, this dose-dependent reduction ($p < 0.01$) was also found for streptomycin as well as for the combination of penicillin and streptomycin. Penicillin alone, however, did not have an effect on ApoA-I protein concentrations in culture medium. For amoxicillin, a significant correlation ($r = 0.714$; $p < 0.05$) was found between ApoA-I mRNA expression and ApoA-I protein secretion.

Effects of antibiotics on KEAP1, CPT1, and CHOP gene expression and PPARα transactivation in HepG2cells

Amoxicillin decreased KEAP1 gene expression significantly ($p < 0.01$) and dose-dependently. Penicillin did not affect KEAP1 gene expression, whereas streptomycin and the combination of penicillin and streptomycin significantly ($p < 0.05$) decreased KEAP1 gene expression. Moreover, amoxicillin significantly ($p < 0.01$) decreased CPT1 gene expression, whereas penicillin, streptomycin, and the combination of penicillin and streptomycin did not change CPT1 mRNA expression. Furthermore, amoxicillin significantly ($p < 0.05$) decreased CHOP gene expression whereas penicillin, streptomycin, and the combination of penicillin and streptomycin did not change CHOP gene expression (Figure 3.2). In line with the lowering effect on CPT1 mRNA expression, amoxicillin also significantly ($p < 0.01$) lowered PPARα transactivation. Penicillin ($p < 0.05$) as well as the combination of penicillin and streptomycin ($p < 0.01$) also significantly ($p < 0.05$) decreased PPARα transactivation, whereas streptomycin alone did not have an effect on PPARα transactivation (Figure 3.3). Although amoxicillin lowered both ApoA-I and KEAP1 mRNA expressions,



changes in the expression of these two genes were not significantly correlated ($r = 0.510$; $p = 0.160$). ApoA-I mRNA expression was significantly correlated ($r = 0.753$; $p < 0.05$) with CPT1 mRNA expression, while both genes expressions were negatively correlated with the antibiotic treatment. Finally, a reduced CHOP mRNA expression was observed with amoxicillin treatment, but this effect was not significantly ($r = 0.552$; $p = 0.123$) correlated with the reduction of ApoA-I mRNA expression.

Figure 3.2. (A) Relative ApoA-I mRNA expression in HepG2 cells treated with different concentrations of antibiotics: Increasing amoxicillin concentrations showed a significant reduction in ApoA-I mRNA expression since the regression coefficient significantly different from zero ($p < 0.05$). **(B)** Relative ApoA-I protein secretion into culture medium of HepG2 cells after treatment with different concentrations of antibiotics: Increasing amoxicillin, streptomycin, and the combination of penicillin and streptomycin concentrations showed a significant reduction in ApoA-I protein secretion since the regression coefficient significantly different from zero ($p < 0.01$). **(C – F)** Relative KEAP1, CPT1, and CHOP mRNA expressions in HepG2 cells treated with different concentrations of antibiotics. **(C)** Increasing amoxicillin concentrations showed a significant reduction in KEAP1 and CPT1 mRNA expressions since the regression coefficient significantly different from zero ($p < 0.01$). Increasing amoxicillin concentrations showed a significant reduction in CHOP mRNA expression since the regression coefficient significantly different from zero ($p < 0.05$). **(D)** Increasing penicillin concentrations did not show any significant effects in KEAP1, CPT1, and CHOP mRNA expressions. **(E)** Increasing streptomycin concentrations showed a significant reduction in KEAP1 mRNA expression since the regression coefficient significantly different from zero ($p < 0.05$). **(F)** Increasing the combination of penicillin and streptomycin concentrations showed a significant reduction in KEAP1 mRNA expression since the regression coefficient significantly different from zero ($p < 0.05$). Four biological (eight technical) replicates were performed for every single dose treatment. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against expression of the control condition, which was arbitrarily set at 1. Changes are indicated with * when the regression coefficient was significant ($p < 0.05$) or with ** when the regression coefficient was significant ($p < 0.01$). ApoA-I, apolipoprotein-I; mRNA, messenger RNA.

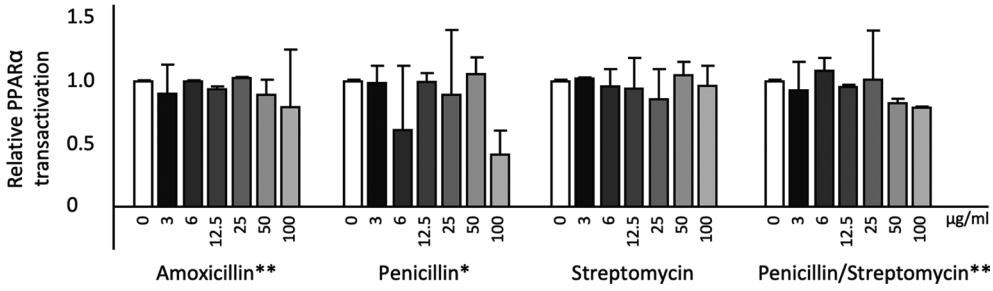


Figure 3.3. Relative PPARα transactivation in transfected HepG2 cells treated with different concentrations of antibiotics: Increasing amoxicillin and the combination of penicillin and streptomycin concentrations showed a significant reduction in PPARα transactivation since the regression coefficient significantly different from zero ($p < 0.01$). Increasing penicillin concentrations showed a significant reduction in PPARα transactivation since the regression coefficient significantly different from zero ($p < 0.05$). Four biological (eight technical) replicates were performed for every single dose treatment. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against expression of the control condition, which was arbitrarily set at 1. Changes are indicated with a * when the regression coefficient was significant ($p < 0.05$) or with a ** when the regression coefficient was significant ($p < 0.01$).

Effects of antibiotics on ApoA-I gene expression and protein secretion in Caco-2 Cells

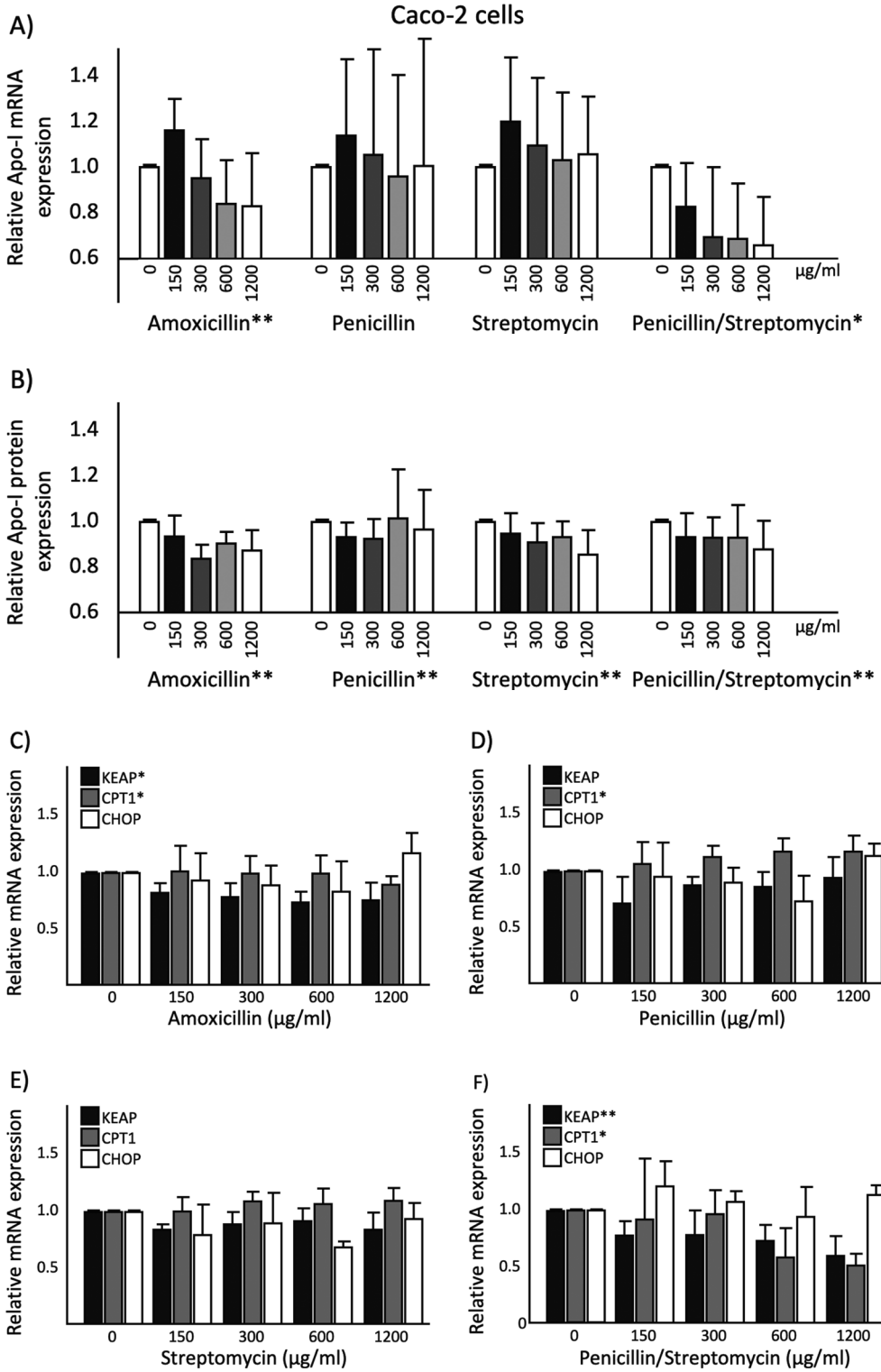
ApoA-1 mRNA expression was higher in differentiated Caco-2 cells as compared to HepG2 cells. As for HepG2 cells, amoxicillin significantly ($p < 0.01$) and dose-dependently lowered ApoA-I mRNA expression in Caco-2 cells. Although both penicillin and streptomycin did not change ApoA-I mRNA expression, the combination of penicillin and streptomycin

significantly ($p < 0.05$) lowered dose-dependently ApoA-I gene expression (Figure 3.4). ApoA-1 protein secretion was also higher in differentiated Caco-2 cells as compared to HepG2 cells. In line with the change in ApoA-I mRNA expression, amoxicillin significantly ($p < 0.01$) reduced dose-dependently the amount of ApoA-I protein secreted into the culture medium in Caco-2 cells. This dose-dependent reduction ($p < 0.01$) was also found for the other antibiotic treatments (Figure 3.4). Significant correlation was found between ApoA-I mRNA expression and ApoA-I protein secretion for the amoxicillin condition ($r = 0.900$; $p < 0.05$) as well as for the combination of penicillin and streptomycin ($r = 0.949$; $p < 0.05$).

Effects of antibiotics on KEAP1, CPT1, and CHOP gene expression in Caco-2 Cells

As for HepG2 cells, amoxicillin decreased KEAP1 gene expression dose-dependently in Caco-2 cells ($p < 0.05$). Although penicillin and streptomycin did not affect KEAP1 gene expression, the combination of penicillin and streptomycin also significantly lowered KEAP1 gene expression in a dose-dependent manner ($p < 0.01$). Amoxicillin as well as the combination of penicillin and streptomycin significantly decreased CPT1 gene expression dose dependently ($p < 0.05$). Moreover, penicillin significantly ($p < 0.05$) increased CPT1 gene expression whereas streptomycin did not. Furthermore, in Caco-2 cells, CHOP mRNA expression was not changed after treatment with amoxicillin, penicillin, streptomycin, and the combination of penicillin and streptomycin (Figure 3.4). Although both amoxicillin and the combination of penicillin and streptomycin lowered ApoA-I and KEAP1 mRNA

Figure 3.4. (A) Relative ApoA-I mRNA expression in Caco-2 cells treated with different concentrations of antibiotics: Increasing amoxicillin concentrations showed a significant reduction in ApoA-I mRNA expression since the regression coefficient significantly different from zero ($p < 0.01$). Increasing the combination of penicillin and streptomycin concentrations showed a significant reduction in ApoA-I mRNA expression since the regression coefficient significantly different from zero ($p < 0.05$). **(B)** Relative ApoA-I protein secretion into culture medium of Caco-2 cells after treatment with different concentrations of antibiotics. Increasing amoxicillin, penicillin, streptomycin, and the combination of penicillin and streptomycin concentrations showed a significant reduction in ApoA-I protein secretion since the regression coefficient significantly different from zero ($p < 0.01$). **(C – F)** Relative KEAP1, CPT1, and CHOP mRNA expressions in Caco-2 cells treated with different concentrations of antibiotics: **(C)** Increasing amoxicillin concentrations showed a significant reduction in KEAP1 and CPT1 mRNA expressions since the regression coefficient significantly different from zero ($p < 0.05$). **(D)** Increasing penicillin concentrations showed a significant increase in CPT1 mRNA expression since the regression coefficient significantly different from zero ($p < 0.05$). **(E)** Increasing streptomycin concentrations did not show any significant effects in KEAP1, CPT1, and CHOP mRNA expressions. **(F)** Increasing the combination of penicillin and streptomycin concentrations showed a significant reduction in KEAP1 mRNA expression since the regression coefficient significantly different from zero ($p < 0.01$). Increasing the combination of penicillin and streptomycin concentrations showed a significant reduction in CPT1 mRNA expression since the regression coefficient significantly different from zero ($p < 0.05$). Four biological (eight technical) replicates were performed for every single dose treatment. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against expression of the control condition, which was arbitrarily set at 1. Changes are indicated with * when the regression coefficient was significant ($p < 0.05$) or with ** when the regression coefficient was significant ($p < 0.01$). ApoA-I, apolipoprotein-I; mRNA, messenger RNA.


A)
Caco-2 cells
Relative Apo-I mRNA expression
1.4
1.2
1.0
0.8
0.6
0
150
300
600
1200
µg/ml
Amoxicillin**
Penicillin
Streptomycin
Penicillin/Streptomycin*
B)
Relative Apo-I protein expression
Amoxicillin**
Penicillin**
Streptomycin**
Penicillin/Streptomycin**
C)
KEAP*
CPT1*
CHOP
Relative mRNA expression
1.5
1.0
0.5
Amoxicillin (µg/ml)
D)
KEAP
CPT1*
CHOP
Penicillin (µg/ml)
E)
KEAP
CPT1
CHOP
Streptomycin (µg/ml)
F)
KEAP**
CPT1*
CHOP
Penicillin/Streptomycin (µg/ml)

expressions, there was no significant correlation ($r = 0.800$; $p = 0.104$). However, significant correlations ($r = 0.975$; $p < 0.01$ and $r = 0.900$; $p < 0.05$) were found between ApoA-I mRNA expression and CPT1 mRNA expression after exposure with amoxicillin and the combination of penicillin and streptomycin, respectively.

Discussion

Antibiotics are classified based on their chemical structures and modes of action. The generally used classes of antibiotics contain the aminoglycosides, beta-lactams, glycopeptides, macrolides, oxazolidinones, quinolones, sulphonamides, and tetracyclines (17). Here, we focused on amoxicillin and penicillin, which both belong to the beta-lactams, and on streptomycin, which belongs to the aminoglycosides class. Although these three antibiotics are considered as safe (18), there are some known "acceptable" side effects such as nausea, diarrhea, vomiting, allergic reactions, and hepatotoxicity (19–21). In addition, the effect of antibiotics on intestinal microbiota composition recently received major attention. These effects have been linked to diabetes development and CVD. Regarding CVD, we show here that one of these frequently used antibiotics, amoxicillin, had in vitro a direct negative effect on ApoA-I transcription and secretion by enterocytes and hepatocytes. Recently, we observed that amoxicillin treatment for 7 days significantly lowered serum HDL cholesterol in healthy subjects (8). Potential direct effects of antibiotics on human lipid and lipoprotein metabolism have, to the best of our knowledge, not been studied in humans before. Therefore, this is the first study to investigate the potential association between antibiotics treatment and ApoA-I gene expression and protein secretion. However, there are observations concerning a potential link between antibiotics and lipid metabolism in animal studies. Significant correlations were found between amoxicillin intake and changes in serum total cholesterol, triglycerides, and HDL and LDL concentrations in male rabbits (22). Moreover, Sato and coworkers (23) recently showed a significant reduction of intestinal ApoA-I secretion after four days of streptomycin and penicillin intake by Sprague–Dawley Rats.

Besides analyzing changes in ApoA-I transcription and secretion, we also explored the possible mechanisms behind this relation such as BET inhibition, PPARα transactivation, or ER stress. BET protein inhibitors such as JQ1(+) increased ApoA-I expression (24, 25). BET inhibitors can regulate transcription of target genes such as KEAP1 (12, 26). PPARα is a nuclear receptor which binds to specific response elements (PPREs) within promoter regions of target genes such as CPT1 (14). PPARα activation is identified as targets to elevate ApoA-I transcription (16). ER stress markers like C/EBP homologous protein (CHOP) is activated by components that negatively influence the function of the endoplasmic

reticulum (ER) (27). A previous study by Neam et al. reported that, in HepG2 cells, Thap resulted in an increase of ER-stress and a decrease of ApoA-I protein secretion (28).

Interestingly, by evaluating the mutual effects of the used agonists on the three pathways (PPARα activation, BET inhibition, and ER stress), some interesting issues concerning the relevance of the different pathways in ApoA-I transcription emerged. In line with our previous data, the BET inhibitor JQ1(+) significantly decreased KEAP1 gene expression; JQ1(+) also inhibited PPARα transactivation in HepG2 cells as CPT1 expressions were downregulated (16). This shows that the sum of effect by BET inhibitors on ApoA-I are quite strong, especially as they also need to counter its negative effect of a lower PPARα activation. JQ1(+) also decreased CHOP, suggesting that BET inhibition reduces ER stress. Interestingly, Thap not only elevated ER stress (higher CHOP expression) but also increased KEAP1 gene expression. This finding was expected since ER stress has opposite effects to BET inhibition in ApoA-I expression (16), meaning that ER stress is more likely to limit BET inhibition. Thap also increased PPARα transactivation in HepG2 cells as CPT1 expressions were increased, similar to JQ1(+); this means that the net results of ER stress on ApoA-I are potent, as they also need to counter the positive effect of a higher PPARα activation (Figure 3.5).

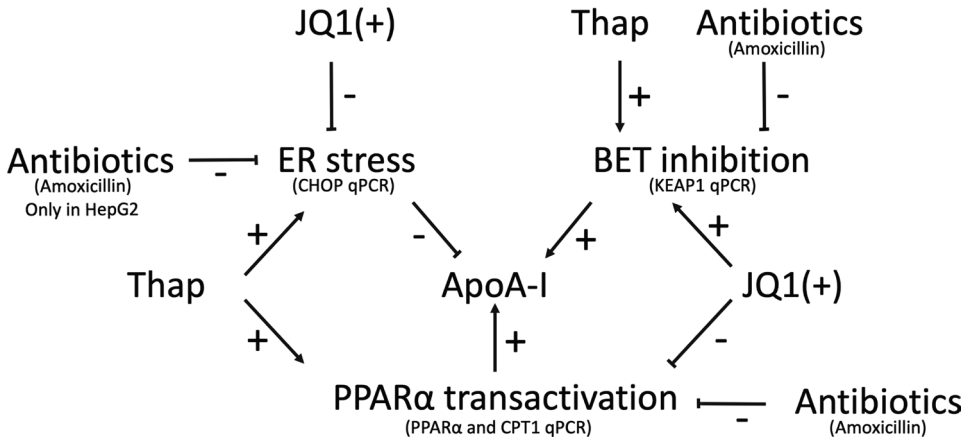


Figure 3.5. Schematic summary of the effects of antibiotics, JQ1(+) and Thap on pathways involved in ApoA-I mRNA expression: The lines represent effects responses (+ positive or – negative) in HepG2 and Caco-2 cells. ApoA-I, apolipoprotein-I; mRNA, messenger RNA.

We observed differences in the ability of individual antibiotics to stimulate ApoA-I expression. As mentioned, the effects on ApoA-I expression were particularly evident for amoxicillin while no effects were observed for penicillin or streptomycin. The question now arises what differentiates amoxicillin from the other evaluated antibiotics. Amoxicillin, a β-lactam antibiotic, prevents bacterial growth through binding to the penicillin-binding

protein (PBP) that is present in the membrane of susceptible bacteria (29, 30). Moreover, amoxicillin induces protein damage; inhibits microsomal Ca2+ ATPase and G-6-Pase; and elevates membrane lipid peroxidation as result of increased production of reactive nitrogen species, reactive oxygen species, and free radicals. Finally, there is evidence that effects of amoxicillin might be the result of the attack of its β-lactam ring on the protein membrane and enzyme thiol groups (31). Although penicillin belongs to the same class as amoxicillin (beta-lactams), penicillin had no effects on the ApoA-I pathways studied. The possible reason is the presence of the NH2 functions in amoxicillin chemical structure. Amoxicillin has an additional amino acid chain (–NH2) in the amide side chain and (OH) in the para position. This makes amoxicillin acid resistant and more hydrophilic than penicillin (32). The hydrophilic force which regulates the molecular interactions between lipoproteins and enzymes (33) might be the reason behind the negative effects of amoxicillin on ApoA-I.

The experiments in HepG2 and Caco-2 cells presented here support our hypothesis that antibiotics elicit direct effects on ApoA-I secretion as well as transcription. We determined that amoxicillin lowered ApoA-I secretion and expression in both cell lines, and the question is how this effect can be explained mechanistically. As for ApoA-I expression, KEAP1, CPT1, and CHOP expressions were all reduced by amoxicillin treatments. Furthermore, we found a significant correlation between ApoA-I and CPT1 mRNA expressions after amoxicillin treatment. Moreover, amoxicillin had lowered PPARα transactivation in HepG2 cells. All together, these data support our previous finding that PPARα transactivation is involved in the effects of ApoA-I expression and secretion (16). Additionally, both BET inhibition and ER stress are most likely not involved in the relation between amoxicillin and ApoA-I expression.

There were some limitations of the in vitro system in this study, which could have some effects on the examined biomarkers outcome. For instance, cell lines are different from primary cells (in vivo) in some of their features and physiological responses (34). Additionally, the cell line responses to the antibiotics could be different compared to the patients' responses to same dose treatment; also, cells might have variations in their responses based on their passage numbers. Moreover, the cells were exposed to culture medium and serum, which can affect cells behavior, functions, and genetic information (35).

In summary, we have shown that amoxicillin treatment has direct effects by lowering ApoA-I secretion and transcription. Based on evaluating alterations in KEAP1, CPT1, and CHOP mRNA expression plus PPARα transactivation, it is tempting to suggest that a reduced PPARα transactivation is a potential mechanism behind the observed amoxicillin-induced effects on hepatic and intestinal ApoA-I expression.

Material and methods

Materials

Human hepatocellular liver carcinoma (HepG2) cells were obtained from Sten Braesch-Andersen (Mabtech, Nacka Strand, Sweden). Human epithelial colorectal adenocarcinoma (Caco-2) cells were obtained from ATCC (Molsheim, France). Flasks and plates for cell culture were derived from Corning (Corning, NY, USA). Minimum Essential Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), sodium pyruvate, and nonessential amino acids (NEAA) used were purchased from Thermo Fisher Scientific (Bleiswijk, The Netherlands). Fetal bovine serum (FBS) was derived from PAA (Toronto, Canada). Amoxicillin, penicillin, streptomycin, DMSO, Tri-reagent, and Thapsigargin (Thap; an endoplasmic reticulum (ER) stress inducer) were obtained from Sigma (Uithoorn, The Netherlands). The BET inhibitor JQ1(+) was obtained from Bio-techne—R&D (Minneapolis, MN, USA).

Cell culture and antibiotics treatment

HepG2 and Caco-2 cells were cultured under a humidified atmosphere with 5% carbon dioxide (CO_2) in antibiotic free medium (MEM for HepG2 and DMEM for Caco-2) containing 1% sodium pyruvate, 10% heat inactivated FBS, and 1% NEAA at 37 °C. For experiments, cells were seeded at a density of 200,000 cells per well in a 24-well plate setup. HepG2 cells were grown to confluence for 48 h; when cells reached a density of 80–90%, they were exposed to the different antibiotics or JQ1(+) or Thap. Caco-2 cells were grown for 21 days to allow them to differentiate towards a small intestinal phenotype (36) before exposure to the same compounds as mentioned for the HepG2 cells. We used amoxicillin and penicillin G (benzyl penicillin), both β-lactam antibiotics, and streptomycin, an aminoglycoside antibiotic (37-39). In addition, the cells were exposed to the combination of streptomycin and penicillin, which is often used prophylactically in cell cultures. In our previous human study where the reduction in serum HDL cholesterol concentrations was observed (8), capsules of 500 mg amoxicillin were taken three times daily. If 500 mg is dissolved in an assumed maximal volume of 3 L of stomach content (40), the cells of the small intestine will be exposed to a minimal concentration of about 167 μg/mL amoxicillin. Moreover, antibiotic local concentrations in the gastrointestinal tract might differ due to the presence of food or variable fluid consumption. Therefore, it was decided in this study to test the effects of the antibiotics on differentiated Caco-2 cells in concentrations of 150, 300, 600, and 1200 μg/mL. On the other hand, following oral administration of 500 mg of amoxicillin, serum peak levels were between 6.0 to 15.3 μg/mL, and after intravenous administration of 500 mg of amoxicillin, serum peak levels were found to be between 52.1

to 30.1 μg/mL (41). After oral administration of 500 mg penicillin, blood serum levels were 3.8 μg/mL and intravenous administration resulted in serum levels between 13.9 μg/mL and 17 μg/mL (42). Intramuscular injection of 1 g of streptomycin showed a peak serum level of 25 to 50 μg/mL (43). Therefore, the effects of the selected antibiotics on HepG2 cells were initially tested at concentrations of 3, 6, 12.5, 25, 50, 100, 150, and 200 μg/mL. Both HepG2 as well as Caco-2 cells were exposed for 48 h in culture medium without added FBS enriched to the abovementioned concentration range antibiotics. In all experiments, JQ1(+) (3 μM) and Thap (0.01 μM) were used in separate wells as positive and negative controls for ApoA-I production, respectively (16). All antibiotics were dissolved in water, and their effects were expressed relative to a water control. JQ1(+) and Thap were both dissolved in dimethyl sulfoxide (DMSO) and their effects were therefore expressed relative to a DMSO control. Cell culture medium was collected after 48 h for analysis of ApoA-I protein concentrations. The cells were harvested to determine mRNA expression levels of ApoA-I, KEAP1, CPT1, CHOP and cyclophilin A. All samples, culture medium and lysed cells, were snap frozen in liquid nitrogen and stored at −80 °C prior to further analysis.

ApoA-I protein concentration in cell culture medium

ApoA-I protein concentrations in culture medium of both HepG2 and Caco-2 cells were measured by a direct enzyme-linked sandwich immunoassay (ELISA) obtained from Mabtech (Nacka Strand, Sweden) following the manufacturer's instructions, with small adaptations, e.g., blocker BSA 10% (Thermo Fisher Scientific, Bleiswijk, Netherlands) was added to the block buffer (final concentration 1%) and the dilution buffer (final concentration 0.1%).

mRNA expression quantification

Total RNA was isolated using Tri-reagent to evaluate mRNA expression levels of ApoA-I, KEAP1, CPT1, and CHOP according to the manufacturer's instructions. The isolated RNA was further purified using the RNeasy mini kit (Qiagen, Hilden, Germany). Next, 350 ng of total RNA was reverse transcribed for cDNA synthesis, using RNAse inhibitor, dNTPs, random hexamers, MMLV reverse trans, DTT, and 5×FS buffer (Thermo Fisher Scientific, Bleiswijk, Netherlands). This cDNA served as a template for real time quantitative PCR using TaqMan Gene Expression Assays, ApoA-I (Hs 00163641), KEAP1 (Hs 00202227), CPT1 (Hs 00912671), and CHOP (Hs 00358796). Expression of the housekeeping cyclophilin A (Hs 99999904) was used as a control. Gene expression levels were presented as relative values based on the Ct values, normalized for the cyclophilin A, and compared to their respective control conditions.

Luciferase assay

PPARα transcriptional activity was analyzed by transfection of HepG2 cells using X-treme gene 9 DNA transfection reagent (Sigma, Uithoorn, Netherlands) with the following plasmids: pcDNA3.1, pcDNA3.1_PPARα, pGL3, and pGL3_PPRE as previously described (44). Following transfection and 48-h antibiotics treatment, cells were harvested by lysis in 1× luciferase lysis buffer (Promega, Madison, USA) and luciferase activity, reflecting PPARα transactivation, and was determined by a GloMax® 96 Microplate luminometer, following the manufacturer's instructions (Promega, Madison, WI, USA). This transfection assay was only performed in HepG2 cells, since Caco-2 cells need 21 days to be fully differentiated, as cells only remain transfected for 7 days maximally and the differentiated cells are typically resistant to transient transfection.

Statistical analysis

All independent experiments contained duplicate samples; furthermore, each independent experiment was repeated at least twice. Four biological (eight technical) replicates were performed for every single dose treatment. With regression analysis, the dose-response relationship between the antibiotic and the gene of interest was examined using a regression coefficient. Spearman correlations between ApoA-I protein secretion and ApoA-I mRNA expression or the mRNA expression of KEAP1, CPT1, and CHOP were calculated. The regression coefficient and spearman correlation coefficient were considered to be statistically significant at $p < 0.05$. All statistical analyses were performed using SPSS v.25 (IBM Corp., Armonk, NY, USA).

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4

Short-chain fatty acids (except Hexanoic acid) lower NF-kB transactivation, which rescues inflammation-induced decreased Apolipoprotein A-I transcription in HepG2 cells

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Abstract

Concentrations of apolipoprotein A-I (ApoA-I) decrease during inflammation, which may lead to dysfunctional ApoA-I-poor high-density lipoprotein (HDL) particles, and as such, elevate cardiovascular risk. Therefore, rescuing ApoA-I concentrations, especially during inflammation, seems beneficial. Recently, short-chain fatty acids (SCFAs) have received more attention as a strategy in reversing atherosclerosis. We here evaluated the effects of SCFAs on inflammatory pathways in relation to ApoA-I transcription. SCFAs dose–response studies were performed in the presence and absence of inflammatory cytokines. ApoA-I and interleukin 8 (IL -8) mRNA expression were analyzed using qPCR and ELISA, respectively. To study underlying mechanisms, nuclear factor kappa B (NF-κB) transactivation and changes in mRNA expressions of the genes targets of bromodomain and extra-terminal (BET) inhibition, peroxisome proliferator-activated receptor-alpha (PPARα) transactivation and activator protein 1 (AP -1) pathway were analyzed. SCFAs (except hexanoic acid) increased ApoA-I mRNA transcription in both normal and inflammatory conditions and lowered IL-8 mRNA expression. This anti-inflammatory effect of SCFAs was confirmed by inhibition of NF-κB transactivation. Moreover, butyric acid increased carnitine palmitoyltransferase 1 (CPT1), PPARα target gene, mRNA transcription in both conditions, and there was a negative correlation between CPT1 and NF-κB. Therefore, PPARα transactivation is probably involved in the anti-inflammatory effects of SCFAs, which rescues ApoA-I transcription. In conclusion, propionate, butyrate and valerate elicit anti-inflammatory effects which might rescue ApoA-I transcription in inflammatory conditions via PPARα transactivation mediated NF-κB inhibition.

Introduction

Inflammation has clear effects on lipid and lipoprotein metabolism, which contributes to the association between inflammation and increased cardiovascular risk as seen for example in metabolic syndrome (1). It is known that inflammation strongly reduces high-density lipoprotein (HDL) cholesterol and particle concentrations and, more importantly, introduces the formation of dysfunctional HDL particles which consequently leads to impaired reverse cholesterol transport (2). Therefore, elevation of HDL particle concentration and more particularly its functionality, especially in inflamed conditions, might help to prevent the development of atherosclerosis and cardiovascular diseases (CVD) (3). The potential anti-atherogenic effects of HDL have been linked to its main functional and structural protein, apolipoprotein A-I (ApoA-I) (4). Amongst others, ApoA-I is the acceptor for ATP-binding cassette transporter (ABCA1)-mediated cholesterol efflux and as such, regulates cholesterol efflux capacity (5). In addition, ApoA-I has many other functional and cardioprotective effects such as blunting inflammation (6) and lowering coagulant activity (7, 8). This explains the cross-sectional associations between ApoA-I and lower CVD risk. Lately, short chain fatty acids (SCFAs) have received more attention as they may be an attractive strategy in reversing the pathophysiology of metabolic disorders such as chronic inflammation and atherosclerosis (9). SCFAs are either absorbed and utilized by gut epithelial cells or transported directly to the liver via the portal vein (10). We have shown earlier that exposure to SCFAs elevates in vitro ApoA-I production in HepG2 cells (11). Moreover, Bartolomaeus and coworkers have recently shown that propionate (C3) supplementation reduced atherosclerosis in experimental animal models (12). This is in line with earlier observations suggesting a protective role for SCFAs in CVD development and inflammatory diseases (13). Furthermore, SCFAs can inhibit the proliferation and activation of T-cells and block the adhesion of antigen presenting cells in obesity-associated systemic inflammation (14). Given these effects of SCFAs on inflammation, here we evaluated the effects of SCFAs on the inflammatory pathways in relation to ApoA-I transcription in inflamed HepG2 cells and attempted to unravel the mechanism underlying these effects.

Results

Effects of SCFAs on ApoA-I mRNA expression in normal and inflammatory conditions

ApoA-I mRNA expression in HepG2 cells was lower under inflammatory conditions as compared to normal conditions (Figure 4.1). In agreement with our previous studies (15), the

positive control JQ 1(+) and the negative control thapsigargin (Thap) respectively increased and decreased ApoA-I mRNA expression (both $p < 0.001$). Also, under inflammatory conditions, JQ1(+) significantly ($p < 0.001$) increased ApoA-I gene expression, whereas Thap even further decreased ApoA-1 gene expression ($p < 0.001$) compared with the normal condition (Figure 4.1). For the SCFAs, we observed that both under normal and inflammatory conditions, ApoA-I mRNA expression dose-dependently increased after C3, butyric acid (C4) and valeric acid (C5) treatment ($p < 0.05$), whereas hexanoic acid (C6) did not change ApoA-I mRNA expression. Interestingly, all SCFAs (except C6) were able to rescue the reduced levels of ApoA-I mRNA under the inflammatory condition. Both C3 and C5 fully rescued ApoA-I mRNA expression at 5.5 mM, and even increased ApoA-I mRNA expression at 7 mM, while C4 partly rescued ApoA-I mRNA expression up to 80% at 4 mM (Figure 4.1).

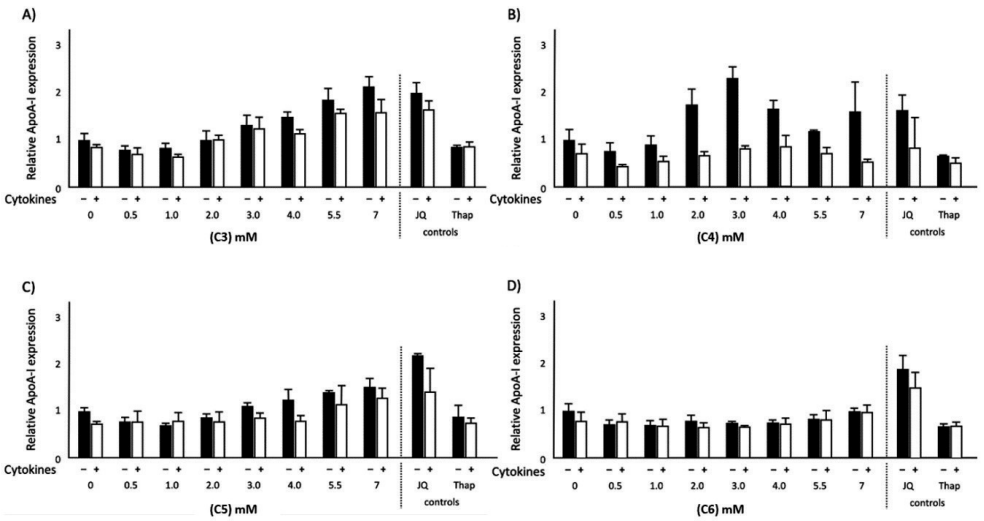

Figure 4.1. Relative apolipoprotein A-I (ApoA-I) mRNA expression in HepG2 cells treated with different concentrations of short chain fatty acids (SCFAs), JQ 1(+) (3 μM) or thapsigargin (Thap) (0.01 μM). Six biological (12 technical) replicates were performed for every condition. **(A)** Increasing C3 concentrations showed a significant increase in ApoA-I mRNA expression in both normal and inflammatory conditions ($p < 0.05$). **(B)** Increasing C4 concentrations showed a significant increase in ApoA-I mRNA expression in both normal and inflammatory conditions ($p < 0.05$). **(C)** Increasing C5 concentrations showed a significant increase in ApoA-I mRNA expression in both normal and inflammatory conditions ($p < 0.05$). **(D)** Increasing C6 concentrations did not show any significant effects in ApoA-I mRNA expression in both normal and inflammatory conditions. The positive control JQ1(+) and the negative control Thap respectively increased and decreased ApoA-I mRNA expression ($p < 0.001$) in both normal and inflammatory conditions. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against the expression observed in the control condition, which was arbitrarily set at 1. Linear regression for SCFA dose–response effects was performed except for C4, where a quadratic polynomial regression was performed to evaluate the dose–response effects. Changes were considered significant when the regression coefficients were significantly different from zero ($p < 0.05$).

Effects of C4 on KEAP1 and CPT1 mRNA expression and NF-κB transactivation in normal and inflammatory conditions

To understand the possible mechanism underlying how C3, C4 and C5 rescued the reduced ApoA-I mRNA during inflammation, we first explored the effects of C4 on kelch-like ECH-associated protein 1 (KEAP1) and carnitine palmitoyltransferase 1 (CPT1) mRNA gene expressions and nuclear factor kappa b (NF-κB) transactivation. C4 dose-dependently increased KEAP1 mRNA expression ($p < 0.05$) under the inflammatory condition, while KEAP1 mRNA expression was reduced in the normal condition ($p < 0.05$). Furthermore, CPT1 mRNA expression dose-dependently increased ($p < 0.001$) after C4 treatment, both under normal and inflammatory conditions (Figure 4.2). When cells were transfected with

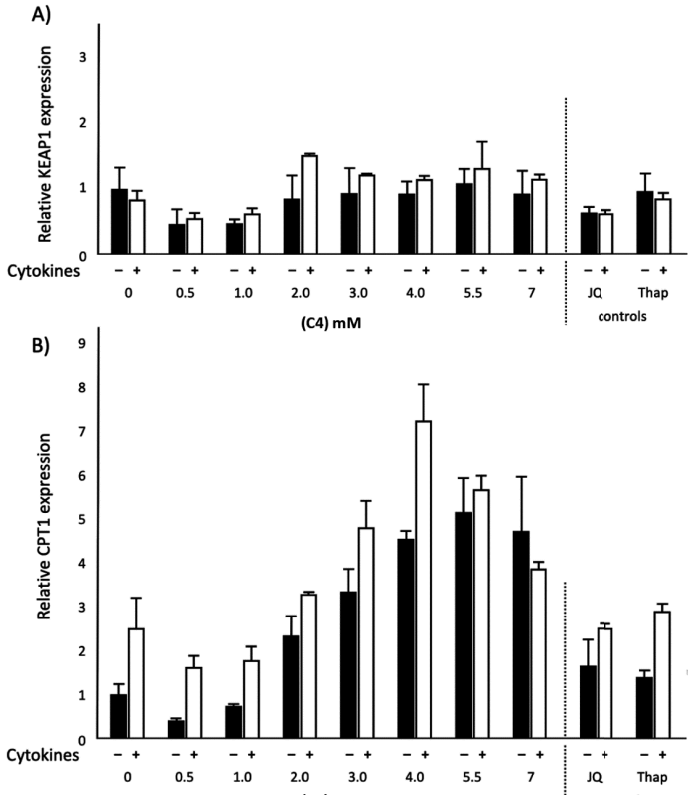

Figure 4.2. Relative kelch-like ECH-associated protein 1 (KEAP1) and carnitine palmitoyltransferase 1 (CPT1) mRNA expressions in HepG2 cells treated with different concentrations of C4, JQ1(+) (3 μM) or thapsigargin (Thap) (0.01 μM). **(A)** Increasing C4 concentrations showed a significant reduction in KEAP1 mRNA expression in the normal condition ($p < 0.05$). Increasing C4 concentrations showed a significant increase in KEAP1 mRNA expression in the inflammatory condition ($p < 0.05$). **(B)** Increasing C4 concentrations showed a significant increase in CPT1 mRNA expression in both normal and inflammatory conditions ($p < 0.001$). All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against the expression observed in the control condition, which was arbitrarily set at 1. A quadratic polynomial regression was performed for C4 dose–response effects. Changes were considered significant when the regression coefficients were significantly different from zero ($p < 0.05$).

the NF-κB reporter, both in normal as well as inflammatory conditions, C4 significantly ($p < 0.05$) decreased NF-κB transactivation (Figure 4.3). Moreover, an inverse correlation was found between CPT1 mRNA expression and NF-κB transactivation in inflamed HepG2 cells after C4 treatment ($r = -0.733$; $p < 0.05$). Finally, looking at the effects of the positive and negative controls, as expected, the bromodomain and extra-terminal (BET) inhibitor JQ1(+) significantly decreased KEAP1 gene expression ($p < 0.05$) both under inflammatory and normal conditions, whereas Thap did not affect KEAP1 gene expression. Furthermore, both JQ1(+) and Thap significantly ($p < 0.05$) increased CPT1 gene expression in both conditions (Figure 4.2).

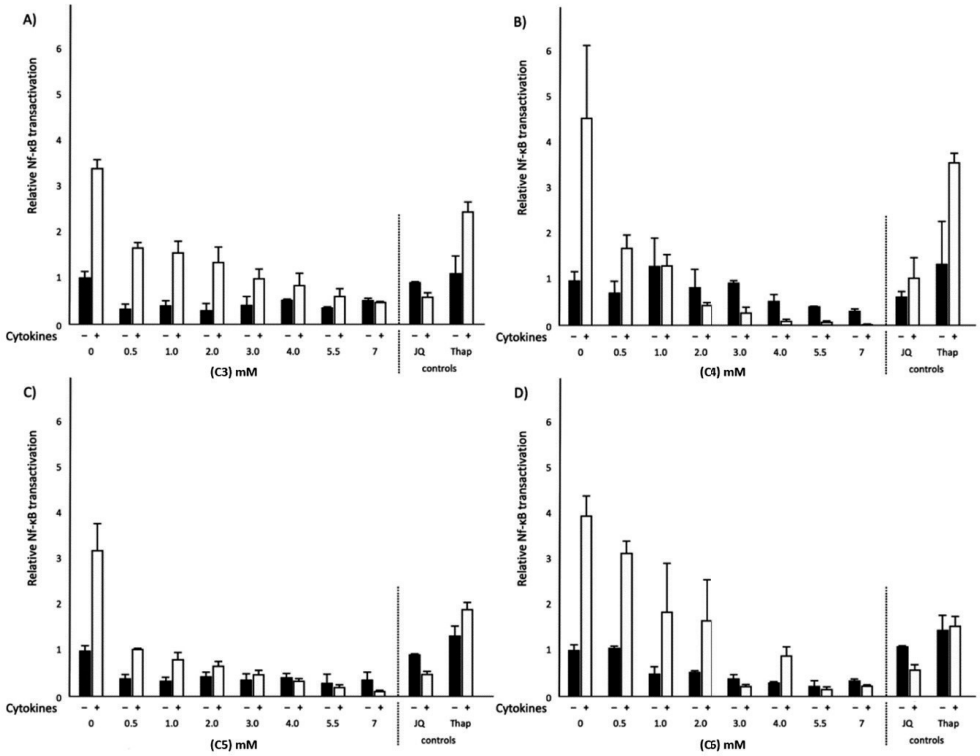

Figure 4.3. Relative nuclear factor kappa b (NF-κB) transactivation in HepG2 cells treated with different concentrations of SCFAs, JQ1(+) (3 μM) or thapsigargin (Thap) (0.01 μM). **(A)** Increasing C3 concentrations showed a significant decrease in NF-κB transactivation in the inflammatory condition ($p < 0.05$). **(B)** Increasing C4 concentrations showed a significant decrease in NF-κB transactivation in both normal and inflammatory conditions ($p < 0.05$). **(C)** Increasing C5 concentrations showed a significant decrease in NF-κB transactivation in the inflammatory condition ($p < 0.05$). **(D)** Increasing C6 concentrations showed a significant decrease in NF-κB transactivation in both normal and inflammatory conditions ($p < 0.05$). All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against the transactivation observed in the control condition, which was arbitrarily set at 1. Linear regression for SCFA dose–response effects was performed except for C4, where a quadratic polynomial regression was performed to evaluate the dose–response effects. Changes were considered significant when the regression coefficients were significantly different from zero ($p < 0.05$).

Effects of different SCFAs on NF-κB transactivation and IL-8 secretion in normal and inflammatory conditions

To extend the observed effects of C4 on NF-κB transactivation, the above-mentioned experiments for C4 were repeated with C3, C5 and C6. In the normal condition, not only C4 but also C6 significantly decreased NF-κB transactivation ($p < 0.05$), whereas C3 and C5 did not have any effects on NF-κB transactivation. In the inflammatory condition, all SCFAs significantly ($p < 0.05$) decreased NF-κB transactivation (Figure 4.3). Moreover, all SCFAs studied did not affect interleukin 8 (IL -8) secretion in normal conditions. On the other hand, in the inflammatory condition, C3, C4 and C5 significantly ($p < 0.05$) decreased IL-8 secretion (Figure 4.4). Interestingly, C6 did not lower IL-8 secretion even

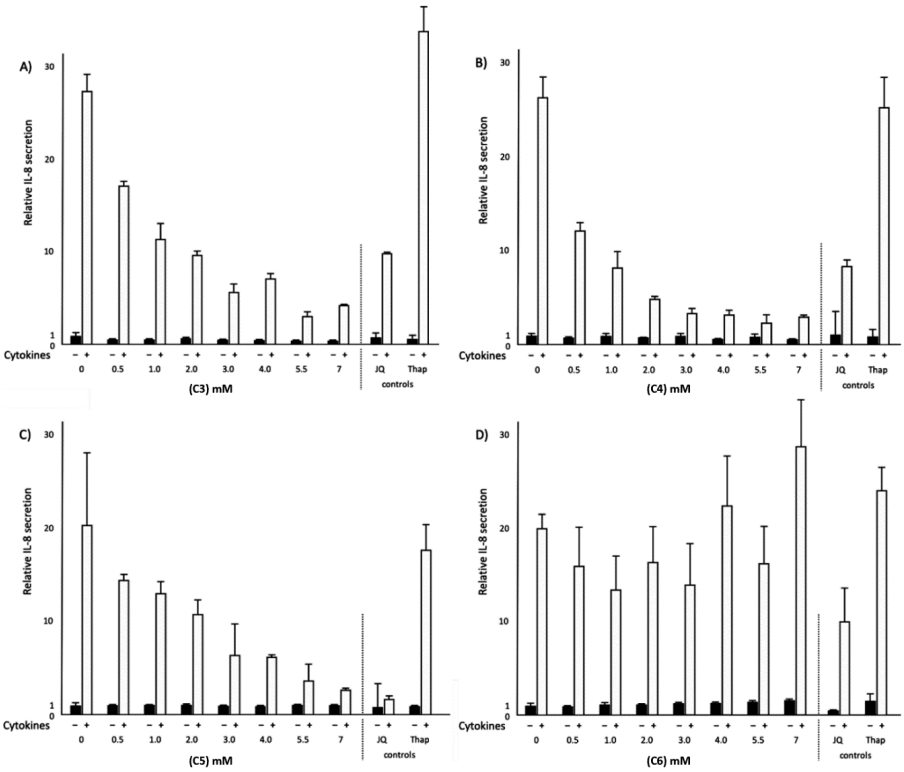

Figure 4.4. Relative interleukin 8 (IL-8) secretion in HepG2 cells treated with different concentrations of SCFAs, JQ1(+) (3 μM) or thapsigargin (Thap) (0.01 μM). **(A)** Increasing C3 concentrations showed a significant decrease in IL-8 secretion in the inflammatory condition ($p < 0.05$). **(B)** Increasing C4 concentrations showed a significant decrease in IL-8 secretion in the inflammatory condition ($p < 0.05$). **(C)** Increasing C5 concentrations showed a significant decrease in IL-8 secretion in the inflammatory condition ($p < 0.05$). **(D)** Increasing C6 concentrations did not show any significant effects on IL-8 secretion in either normal or inflammatory conditions. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against secretion observed in the control condition, which was arbitrarily set at 1. Linear regression for SCFAs dose–response effects was performed except for C4, where a quadratic polynomial regression was performed to evaluate the dose–response effects. Changes were considered significant when the regression coefficients were significantly different from zero ($p < 0.05$).

though C6 lowered NF-κB transactivation. JQ1(+) did not affect NF-κB transactivation, whereas Thap significantly ($p < 0.05$) increased NF-κB transactivation in both conditions (Figure 4.3). Finally, both JQ1(+) and Thap significantly ($p < 0.05$) further increased IL-8 secretion under the inflammatory condition, but these effects were not observed under the normal condition (Figure 4.4).

Effects of different SCFAs on c-Jun and c-Fos mRNA expression in normal and inflammatory conditions

To further explore the role of C3, C4 and C5 in rescuing ApoA-I transcription during inflammation, we also examined – besides NF-κB transactivation – the effects of SCFAs

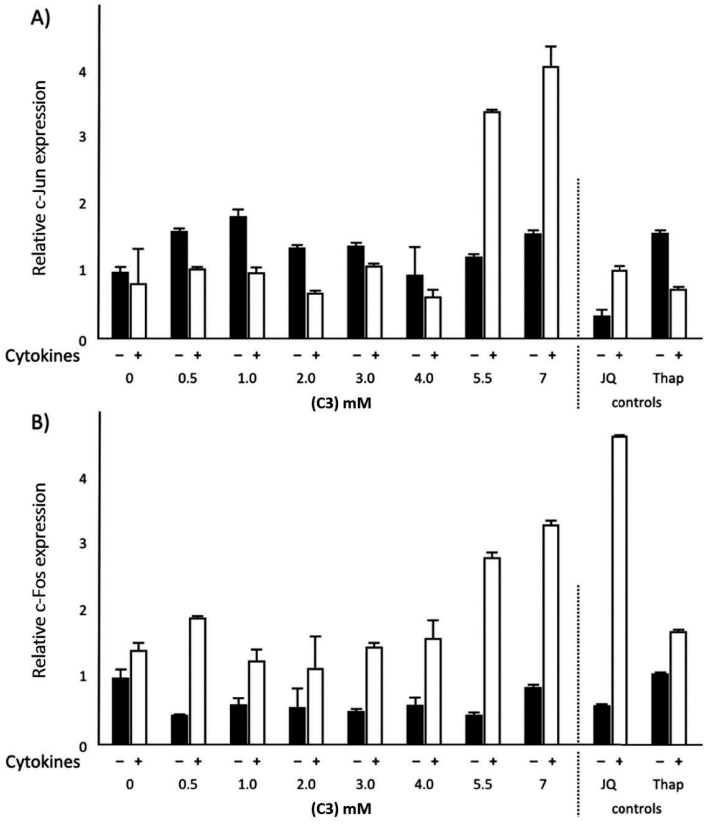

Figure 4.5. Relative c-Jun and c-Fos mRNA expressions in HepG2 cells treated with different concentrations of C3, JQ1(+) (3 μM) or thapsigargin (Thap) (0.01 μM). **(A)** Increasing C3 concentrations showed a significant increase in c-Jun mRNA expression in the inflammatory condition ($p < 0.01$). **(B)** Increasing C3 concentrations showed a significant increase in c-Fos mRNA expression in the inflammatory condition ($p < 0.01$). All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against the expression observed in the control condition, which was arbitrarily set at 1. A linear regression analysis to evaluate C3 dose–response effects was performed. Changes were considered significant when the regression coefficients were significantly different from zero ($p < 0.05$).

on activation of the activator protein (AP-1) pathway by analyzing potential changes in c-Fos and c-Jun mRNA expression. If the AP-1 pathway was involved, we would expect a reduction in AP-1 activation as translated into lower c-Fos and c-Jun expression. In normal conditions, all SCFAs (except C3) significantly ($p < 0.01$) increased c-Jun mRNA expression. In the inflammatory condition, all SCFAs increased ($p < 0.01$) c-Jun mRNA expression. Furthermore, all SCFAs did not change c-Fos mRNA expression in the normal condition, except C4, which significantly increased ($p < 0.001$) c-Fos mRNA expression. Moreover, all SCFAs increased ($p < 0.01$) c-Fos mRNA expression in the inflammatory condition, except C6, which significantly decreased ($p < 0.05$) c-Fos mRNA expression. Effects of C3 are shown in (Figure 4.5), whereas the effects of all SCFAs are shown in Supplementary Table S4.1.

Discussion

The SCFAs propionic acid (C3), butyric acid (C4), valeric acid (C5) and hexanoic acid (C6) are produced after the fermentation of dietary fibers and resistant starches by the microbiota in the cecum and colon (9). An increasing number of functions and beneficial effects of SCFAs on human metabolism have been described (14). Amongst others, SCFAs have anti-inflammatory effects and modulate different processes including cell proliferation, hormone secretion and immune responses (16). SCFAs are either absorbed and utilized by gut epithelial cells or transported directly to the liver via the portal vein (10). Despite different reports describing the beneficial effects of SCFAs in HepG2 cells, the effects of SCFAs on human liver cells under inflammatory conditions have not been studied. We recently described a favorable effect of SCFAs on ApoA-I mRNA transcription (11), but these effects were observed in normal (non-inflamed) HepG2 cells. Exposure to a cocktail of inflammatory cytokines of tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) has already been shown to decrease ApoA-I mRNA levels in HepG2 cells (17). Here we report the effects of SCFAs in HepG2 cells exposed to these cytokines, in which ApoA-I mRNA transcription was indeed lower due to the inflammatory response. This lower ApoA-I transcription in cytokine-exposed cells is in line with the known effects of acute phase responses on lipoprotein metabolism (18, 19).

We observed that all four SCFAs studied here have anti-inflammatory effects as shown by a lower NF-κB transactivation, which in turn (except for C6) rescued the inflammation-induced reduced hepatic ApoA-I mRNA expression. This seems to be a logical response since the ApoA-I gene contains several NF-κB binding sites in the promoter region in hepatocytes (20, 21). The anti-inflammatory effects of C3, C4 and C5 were confirmed by a

reduction in IL-8 secretion into the supernatant of the cells. However, C6 did not affect IL-8 secretion in the inflamed hepatic cells, which was remarkable since C6 did lower NF-κB transactivation just like the three other SCFAs.

These findings for these SCFAs were in line with previous studies which also evaluated the relation between SCFA exposure and inflammation in other cells types. For example, Qiao et al. (22) showed that C4 inhibited TNF-α, IL-6 and myeloperoxidase activity by preventing NF-κB transactivation in the liver cells of Sprague–Dawley rats. Another study showed that both C4 and C3 reduced IL-6 and IL-8 levels in human umbilical vein endothelial cells that were stimulated with lipopolysaccharide (LPS) and TNF-α (23). Moreover, Usami and coworkers showed that C3 and C4 both reduced TNF-α production and downregulated NF-κB transactivation in peripheral blood mononuclear cells (24). Here we extend these observations of C3 and C4 to C5 in HepG2 cells and link this interesting finding to the rescued expression of ApoA-I during inflammation.

To investigate the mechanisms underlying the rescued ApoA-I transcription by SCFA treatment in relation to the observed reduced NF-κB transactivation in more detail, changes in markers for BET inhibition and PPARα transactivation were analyzed. This analysis was conducted via testing BET and PPARα target gene expression (KEAP1 and CPT1, respectively). In both normal and inflammatory conditions, C4 treatment increased CPT1 mRNA transcription, whereas KEAP1 mRNA transcription was decreased in the normal condition only. Furthermore, we found a significant negative correlation between CPT1 mRNA expression and NF-κB transactivation associated with C4 treatment in the inflammatory condition. Consequently, this finding suggests the ability of PPARα to reduce inflammation by inhibiting NF-κB transactivation. All together, these results of C4 suggested that PPARα transactivation might be involved in the effects of SCFAs on ApoA-I expression in normal and inflammatory conditions. This is also in agreement with our previous study in which we found a clear role for PPARα transactivation on ApoA-I mRNA transcription in non-inflamed HepG2 cells (15). Moreover, it seems that BET inhibition does not contribute to the effects of SCFAs on ApoA-I during inflammatory conditions, since KEAP1 was not reduced in inflamed HepG2 cells.

We observed variations in the ability of SCFAs to increase ApoA-I expression or to reduce IL-8 secretion. In our previous study in non-inflamed conditions (11), we have shown that C6 was the weakest inducer of ApoA-I mRNA expression as compared to the other SCFAs (C3, C4 and C5). Again, in the present study, we found that C6 was the only SCFA that was unable to rescue ApoA-I transcription in the inflammatory condition. Moreover, although C6 reduced NF-κB transactivation just like the other SCFAs, we found that C6

did not inhibit IL-8 secretion like the other SCFAs. This outcome regarding the effects of C6 suggests that the anti-inflammatory effects of SCFAs related to ApoA-I might not be linked only to a blunted NF-κB transactivation but could additionally also be ascribed to other (inflammatory) pathways. Since the observed reduction in IL-8 was associated with increased ApoA-I expression after C3, C4 and C5 treatment, while C6 did not lower IL-8 expression and also did not elevate ApoA-I expression, we speculated that the ApoA-I rescue effects of C3, C4 and C5 could also be the result of another modulating anti-inflammatory signaling pathway underlying IL-8 secretion. The promoter region of the IL-8 gene contains not only functional binding sites for NF-κB, but also for AP-1 and CCAAT/enhancer binding protein β (C/EBP-β) (25). It has been described that PPARα, which is known as a regulatory factor of ApoA-I (15), is also linked to the AP-1 pathway (26). PPARα transactivation had an inhibitory effect on both NF-κB and AP-1, which led to the inhibition of inflammation (26). Although an earlier study showed a significant correlation between SCFA exposure and the regulation of the AP-1 signaling pathway in intestinal cells (27), the relationship between SCFAs and the AP-1 pathway in inflamed liver cells has not been studied before. Consequently, we decided to investigate the anti-inflammatory effects of SCFAs in relation to the AP-1 signaling pathway as an additional candidate besides lowering NF-κB pathway activity. Therefore, the mRNA expression of c-Jun and c-Fos, both target genes of the AP-1 pathway, were evaluated in both normal and inflamed HepG2 cells. Unfortunately, all SCFAs, again except C6, did not inhibit the AP-1 signaling pathway in the inflammatory condition. This means that inhibition of the AP-1 pathway was probably not involved in the anti-inflammatory effects of those SCFAs that were able to elevate ApoA-I mRNA expression. However, since the transcriptional activity of c-Jun is regulated mainly post-transcriptionally via phosphorylation, other types of experiments need to be performed to confirm the absence of AP-1 involvement. Altogether, our findings regarding the relationship between exposure to SCFAs and NF-κB or AP-1 pathway activation allow us to speculate that maybe the third candidate regulator C/EBP-β with a binding site in the IL-8 promoter might be a potential mediator involved in the effects of SCFAs during inflammation. Indeed, Bai and coworkers have shown that overexpression of C/EBP-β increased the expression of cytokines such as IL-8 (28). Furthermore, the ApoA-I promoter has a C/EBP binding site, which indicates that C/EBP-β might be involved in ApoA-I production (29). As a result, we suggest in future experiments to investigate the role of SCFAs in rescuing the inflammation-induced reduction in ApoA-I expression by evaluating their effects on the C/EBP- β signaling pathway.

For now, we conclude that the SCFAs propionic acid, butyric acid and valeric acid can reduce NF-κB mediated pro-inflammatory responses, probably mediated via PPARα

transactivation, which translates into a rescue of ApoA-I transcription in inflamed HepG2 cells. This anti-inflammatory effect of C3, C4 and C5 was confirmed by the inhibition of both NF-κB transactivation and IL-8 secretion.

Material and methods

Materials

Human hepatocellular liver carcinoma cells (HepG2) were kindly provided by Sten Braesch-Andersen (Mabtech, Nacka Strand, Sweden). Cell culture flasks and plates were obtained from Corning (Corning, NY, USA). Minimum Essential Medium (MEM), sodium pyruvate, non-essential amino acids (NEAA), penicillin and streptomycin were all obtained from Thermo Fisher Scientific (Bleiswijk, Netherlands). Fetal bovine serum (FBS) was purchased from PAA (Toronto, ON, Canada). Propionic acid (C3), butyric acid (C4), valeric acid (C5) and hexanoic acid (C6) were bought from Sigma (Uithoorn, Netherlands). JQ1(+) was obtained from Bio-Techne R&D (Minneapolis, MN, USA). Tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), thapsigargin (Thap), dimethyl sulfoxide (DMSO) and Tri-reagent were purchased from Sigma (Uithoorn, Netherlands).

Cell culture and SCFA treatment

HepG2 cells were cultured at 37 °C in a humidified atmosphere of 5% carbon dioxide (CO_2) in MEM containing 10% heat inactivated FBS, 1% sodium pyruvate, 1% NEAA and 1% of penicillin–streptomycin mixture. For all experiments, cells were seeded in a 24-well plate at a density of 200,000 cells per well. Cell viability was inspected daily by microscope and when cells reached a density of 80–90%, they were incubated for 48 h in the medium (MEM without FBS) plus a concentration range of 0–7 mM SCFAs (C3, C4, C5 or C6) or 3μM JQ1(+) with or without a cytokine cocktail (TNF-α 100 ng/mL and IL-1β 5 ng/mL). A positive control JQ1(+), a BET inhibitor, was included in each experiment to ensure that cells were responsive and produced sufficient amounts of ApoA-I mRNA. Thapsigargin (Thap), an endoplasmic reticulum (ER)stress inducer, was used as a negative control. All SCFAs, JQ1(+) and Thap were dissolved in dimethyl sulfoxide (DMSO, cell culture tested) and effects were expressed relative to those of the carrier control (DMSO only). The final DMSO concentration was always 0.2%. Culture medium was collected for the analysis of IL-8 concentrations and cells were harvested for the analysis of mRNA expression after lysing with Tri-reagent. Both culture medium and lysed cells were snap frozen in liquid nitrogen and stored at −80 °C until further analysis.

Quantification of gene mRNA transcription

To evaluate effects of SCFAs on mRNA expression levels of ApoA-I, KEAP1, CPT1, c-Jun and c-Fos, total RNA was isolated from HepG2 cells using Tri-reagent according to the manufacturer's instructions. The RNeasy Mini Kit (Qiagen, Hilden, Germany) was used for RNA purification. For cDNA synthesis, 350 ng of total RNA was reverse-transcribed using RNAse inhibitor, dNTPs, random hexamers, moloney murine leukemia virus (MMLV) reverse trans, Dithiothreitol (DTT) and 5x reverse transcriptase (RT) buffer (Thermo Fisher Scientific, Bleiswijk, Netherlands). The resulting cDNA was used for real-time quantitative PCR using TaqMan Gene Expression Assays using cyclophilin A (Hs99999904) as a housekeeping control. To quantify ApoA-I, KEAP1, CPT1, c-Jun and c-Fos, the TaqMan gene expression assays Hs00163641, Hs00202227, Hs00912671, Hs00277190 and Hs00170630 were used. Values are presented as relative gene expressions based on the cycle threshold (Ct) values, normalized for the internal control cyclophilin A, and compared to the control conditions.

Luciferase assay

To investigate effects of SCFAs on NF-κB transactivation, HepG2 cells were transfected with X-treme gene 9 DNA transfection reagent (Sigma, Uithoorn, Netherlands) and the plasmids pcDNA3.1, pGL3 and NF-κB pGL3 following the manufacturer's instructions. The pcDNA3.1 (empty vector) and the empty pGL3 luciferase reporter (without NF-κB elements) were used as a negative control. Following transfection and 48 h SCFA treatment with or without the cytokine cocktail, cells were lysed in luciferase lysis buffer (Promega, Madison, WI, USA) and measured for luciferase activity, reflecting NF-κB transactivation, using a GloMax® 96 Microplate luminometer, according to the manufacturer's manual (Promega, Madison, WI, USA).

Quantification of IL-8 secretion levels in the culture medium

To investigate IL-8 secretion by HepG2 cells, IL-8 protein concentrations in culture medium were measured by an enzyme-linked immunoassay (ELISA; Hycult Biotechnology, Uden, The Netherlands) following the manufacturer's instructions.

Statistical analysis

All independent dose–response experiments with the SCFAs were performed in duplicate and each experiment was repeated three times. Six biological (12 technical) replicates were performed for every single SCFA dose. Regression analysis was used to examine the relationships between SCFAs and the parameters. First, we modeled via quadratic polynomial

regression, which fits with a parabolic relationship as seen for C4. In case this did not reach significance, a linear relationship (i.e., as seen for C3 and C5) was examined. Where mentioned, Spearman correlations were calculated. The regression coefficients and Spearman correlation coefficients were considered to be statistically significant when different from zero at $p < 0.05$. Effects of the positive JQ1(+) and negative (Thap) controls were statistically evaluated by Mann–Whitney U test, in which a p-value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS v.25 (IBM Corp., Armonk, NY, USA).

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Supplementary Table S4.1. Relative c-Jun and c-Fos mRNA expressions in HepG2 cells treated with different concentrations of SCFAs. Increasing C4, C5 and C6 concentrations showed a significant increase in c-Jun mRNA expression in both normal and inflammatory conditions ($p < 0.01$). Increasing C4 concentrations showed a significant increase in c-Fos mRNA expression in the normal condition ($p < 0.001$). Increasing C4 and C5 concentrations showed a significant increase in c-Fos mRNA expression in the inflammatory condition ($p < 0.01$). Increasing C6 concentrations showed a significant reduction in c-Fos mRNA expression in the inflammatory condition ($p < 0.05$). All results are presented as the mean, while SD indicate standard deviations. Data were normalized against expression of the control condition, which was arbitrarily set at 1. Linear regression for SCFAs dose-response effects was performed except for C4, where a quadratic polynomial regression was performed for the dose-response effects. Changes were considered significant when the regression coefficients are significantly different from zero ($p < 0.05$).

		mRNA c-Jun				mRNA c-Fos			
		Normal condition		Inflammatory condition		Normal condition		Inflammatory condition	
SCFAs	mM	Fold change	SD	Fold change	SD	Fold change	SD	Fold change	SD
C4	0	1.00	0.87	0.93	0.11	1.00	0.25	0.94	0.55
	0.5	1.31	0.09	0.51	0.13	0.94	0.08	0.93	0.78
	1.0	1.30	0.53	0.30	0.04	0.66	0.30	4.36	1.88
	2.0	1.80	0.39	1.54	0.11	1.23	0.12	8.61	8.19
	3.0	6.93	2.58	4.36	2.00	4.45	0.54	28.42	1.19
	4.0	5.85	0.88	6.17	1.12	8.94	0.95	22.82	2.15
	5.5	8.03	2.50	6.38	0.98	17.15	8.66	43.45	n/a
	7.0	6.25	0.32	5.85	1.53	14.57	0.48	56.86	n/a
C5	0	1.00	0.19	1.42	0.24	1.00	0.15	0.73	0.39
	0.5	1.34	0.24	1.57	0.11	0.85	0.27	0.58	0.06
	1.0	1.18	0.22	0.85	0.17	2.30	2.43	0.52	0.38
	2.0	0.82	0.24	0.71	0.02	0.36	0.05	0.34	0.10
	3.0	1.78	0.15	2.35	1.28	3.26	1.68	0.34	0.28
	4.0	0.87	0.18	5.52	0.96	0.64	0.54	1.46	0.54
	5.5	5.87	0.29	10.76	0.22	3.44	0.07	3.15	0.52
	7.0	8.87	1.94	12.39	1.68	3.32	1.46	5.58	0.54
C6	0	1.00	0.48	1.95	0.51	1.00	0.24	1.92	1.14
	0.5	1.05	0.12	2.27	0.85	0.87	0.15	3.24	0.00
	1.0	1.08	0.19	2.71	0.80	0.49	0.11	1.52	0.90
	2.0	1.46	0.28	2.56	0.59	0.73	0.41	1.55	0.72
	3.0	1.58	0.66	3.85	0.46	0.62	0.31	0.84	0.25
	4.0	2.37	0.11	3.98	0.28	0.36	0.10	0.62	0.10
	5.5	2.77	0.64	5.52	0.31	0.49	0.26	0.72	0.15
	7.0	4.02	0.83	4.65	1.14	0.33	0.08	0.55	0.05

5

Butyric acid added apically to intestinal Caco-2 cells elevates hepatic ApoA-I transcription and rescues lower ApoA-I expression in inflamed HepG2 cells co-cultured in the basolateral compartment

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Abstract

Apolipoprotein A-I (ApoA-I) concentrations are decreased during inflammation, which may reduce high-density lipoprotein (HDL) functionality. Thus, rescuing ApoA-I concentrations during inflammation might help to prevent atherosclerosis. Recent studies have shown that butyric acid (C4) has anti-inflammatory effects and rescues ApoA-I production. However, whether intestinal short chain fatty acids (SCFAs) are able to influence hepatic processes is unknown. Therefore, we investigated C4 anti-inflammatory effects on ApoA-I transcription in the intestine-liver co-culture model. C4 dose-response experiments in the presence or absence of cytokines were performed in a co-culture system including Caco-2 cells, HepG2 cells, or both. Changes in ApoA-I transcription in Caco-2 cells and HepG2 cells were analyzed using qPCR. C4 increased ApoA-I expression in HepG2 cells that cultured alone. When both cells were cultured together, C4 decreased ApoA-I expression in Caco-2 cells and increased ApoA-I expression in HepG2 cells. However, adding C4 to apical Caco-2 cells resulted in a smaller effect in HepG2 cells compared with adding C4 directly to the hepatocytes. Moreover, C4 rescued ApoA-I expression in inflamed HepG2 cells. These findings suggests that intestinal SCFAs can affect hepatic processes. However, the smaller effect in the co-culture experiment indicates cross-talk between intestine and liver.

Introduction

A higher production of short chain fatty acids (SCFAs) in the intestinal lumen may result in various health benefits (1). Butyric acid (C4), the most studied SCFA, is mainly produced by colonic microbiota through the fermentation of water-soluble dietary fibers like pectin (2). In the past, it has been shown that C4 plays a local role in the physiology of the intestinal mucosa, since amongst other functions it is the major oxidative substrate for colonocytes (3). In addition, more recent studies have shown that C4 has local anti-inflammatory effects in human colonic epithelial cells (4). In fact, C4 was reduced in intestinal biopsies of Crohn's disease patients' mRNA expression and production of pro-inflammatory cytokines, such as TNFα, by the inhibition of nuclear factor kappa B (NF-κB) transactivation (5). Beside these local benefits, SCFAs also exert systemically beneficial effects, such as lowering inflammation in macrophages and endothelial cells through the inhibition of inflammatory cytokine production (6) and abating the development of atherosclerosis (7). These latter anti-atherosclerotic effects are thought to be mediated by modulating pro-inflammatory cytokine production, endothelial dysfunction and oxidative stress (8). Additionally, we have recently postulated that SCFAs may also affect atherosclerotic risk via elevating hepatic Apolipoprotein A-I (ApoA-I) transcription (9). To be effective systemically, SCFAs first need to be absorbed from the intestinal lumen by the enterocytes and transported into the circulation via the portal vein (10). This makes it tempting to suggest that SCFAs will most likely affect hepatic (patho)physiology, as shown, for example, in previous studies using liver (HepG2) cells (9, 11). In particular, the observation that C4 could rescue the inflammation-induced reduction in ApoA-I transcription (12) is highly relevant, since ApoA-I is a negative acute phase protein, and its production is significantly lowered during inflammation (13). This consequent reduction in serum ApoA-I concentrations seems undesirable, especially during inflammation when the numerous beneficial effects of the ApoA-I protein itself (14, 15) are needed. However, in our earlier experiments mimicking hepatic inflammation, C4 was added directly to the medium of the HepG2 cells, while as mentioned earlier, SCFAs are formed in vivo in the intestinal lumen and need to be transported to the liver before any hepatic effects can be expected. However, various SCFAs do not reach the liver in similar concentrations since amounts produced and oxidation within the enterocytes might differ (16). To the best of our knowledge, the effects of butyrate on ApoA-I gene expression in the intestinal cells and the possible existence of cross-talk between enterocytes and hepatocytes on ApoA-I transcription are unknown. Theoretically, intraluminal-produced butyrate that is taken up by enterocytes might have a direct effect on hepatocytes when it reaches the liver (17). However, butyrate may also change the secretion of certain factors

by intestinal cells (18), which affect the transcription of ApoA-I in liver cells. Therefore, to understand this in more detail, we here investigated changes in ApoA-I transcription after adding C4 to Caco-2 cells or HepG2 cells directly and compared these changes to the effects of adding C4 to the apical surface of Caco-2 cells, while analyzing ApoA-I expression in HepG2 cells cultured in the basolateral compartment in our co-culture model. These co-culture studies were performed in normal as well as under inflamed conditions.

Material and methods

Materials

Human epithelial colorectal adenocarcinoma (Caco-2) cells were obtained from ATCC (Molsheim, France). Human hepatocellular liver carcinoma cells (HepG2) were kindly provided by Sten Braesch-Andersen (Mabtech, Nacka Strand, Sweden). Cell culture flasks, plates and polyester membrane inserts (12 mm diameter, 0.4 μm pore diameter) were obtained from Corning (Cambridge, MA, USA). Dulbecco's modified eagle medium (DMEM), Minimum essential medium (MEM), and sodium pyruvate and non-essential amino acids (NEAA) were all obtained from Thermo Fisher Scientific (Bleiswijk, Netherlands). Fetal bovine serum (FBS) was purchased from PAA (Toronto, Canada). Butyric acid (C4) was bought from Sigma (Uithoorn, Netherlands). The BET inhibitor JQ1(+) was obtained from Bio-Techne - R&D (Minneapolis, MN, USA). Tumor necrosis factor-alpha (TNFα), interleukin-1beta (IL-1β), dimethyl sulfoxide (DMSO), and Tri-reagent were all purchased from Sigma (Uithoorn, Netherlands).

Cell culture and C4 treatment

Caco-2 cells and HepG2 cells were both cultured in T75 flasks (Corning, Cambridge, MA, USA) at 37 °C in a humidified atmosphere of 5% carbon dioxide (CO_2) in medium (DMEM for Caco-2 and MEM for HepG2). Cell media contained 10% heat inactivated FBS, 1% sodium pyruvate, 1% NEAA and 1% penicillin-streptomycin mixture. For all experiments, Caco-2 cells were seeded to the inside of transwell inserts (1.5 mL/well) at a density of 300,000 cells/mL. For the first 21 days, the inserts were placed in 6-well plates. During these 21 days, the Caco-2 cells were allowed to differentiate to their small intestinal phenotype (19). Next, the inserts were placed in new 6-well plates to determine whether the Caco-2 cell layers were confluent by examining phenol red leakage from the apical to the basolateral compartment (20). For this, MEM with phenol red was added to the apical compartment, while MEM without phenol red was added to the basolateral compartment for 48 h at 37 °C

in a humidified atmosphere of 5% CO_2. The optical density (OD) of the medium solution in both compartments was measured at 450 nm using a spectrophotometer (Beckman, Pasadena, CA, USA) (Figure 5.1). Only confluent Caco-2 monolayers (OD 450 nm of the basolateral medium less than 0.02) were used in the experiments. In addition, HepG2 cells were seeded in 6-well plates (2.6 mL/well), at a density of 300,000 cells/mL, and cultured for 48 h. Three different conditions were used (Figure 5.2). First, Caco-2 cells were seeded in the inserts, without HepG2 cells in the lower compartment. Second, HepG2 cells were seeded in the lower compartment without Caco-2 cells on the insert above. Finally, Caco-2 cells were seeded on the insert in addition to HepG-2 cells in the lower compartment, and both of them were incubated together in a co-culture system to mimic the intestine–liver interaction. The three different setups of the transwell system were allowed to equilibrate for 48 h before the start of the experiments.

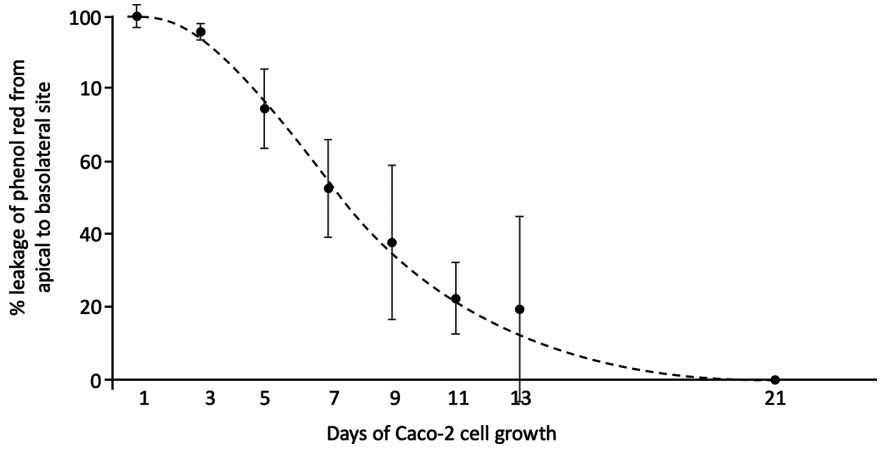

Figure 5.1. The percentage of phenol red leakage from the apical to the basolateral compartment in the transwell system. Medium solution (MEM) with phenol red was added to the apical compartment, while MEM without phenol red was added to the basolateral compartment. The optical density (OD) of the medium solution in both compartments was measured across the different days of Caco-2 cell growth. All results are presented as the mean, while error bars indicate standard deviations.

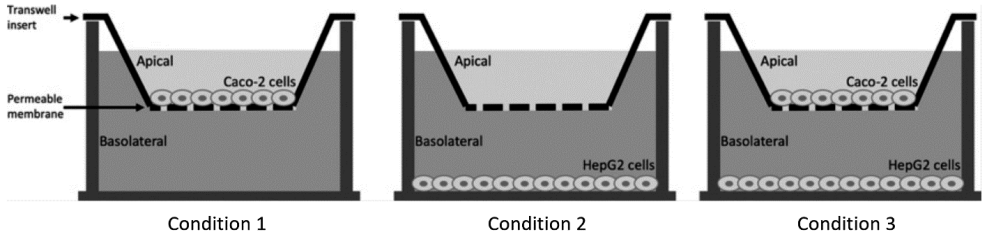

Figure 5.2. Schematic overview of different experimental conditions were performed in the transwell system.

Several experiments were performed. In condition 1, C4 was added to the apical surface of Caco-2 cells in a concentration range of 0, 1, 2, 4, and 6 mM. In condition 2, C4 was added in the same concentration range to HepG2 cells. In condition 3, C4 was added to the apical surface of Caco-2 cells, again in the same concentration range, but this time HepG2 cells were present in the basolateral compartment. In this co-culture condition, we also evaluated the effect of an inflammatory component. For this, a cytokine cocktail of TNFα (with final concentration of 100 ng/mL) and IL-1β (with final concentration of 5 ng/mL) was added either only apically to the Caco-2 cells, or only to HepG2 cells in the basolateral compartment, or simultaneously to both the apical and basolateral compartments. JQ1(+), a BET inhibitor, was included as a positive control in a separate well to ensure that cells were responsive to the treatments and produced sufficient amounts of ApoA-I mRNA (21). For this, JQ1(+) was added in final concentrations of 3 μM. Both C4 and JQ1(+) were dissolved in dimethyl sulfoxide (DMSO, cell culture tested), and effects were expressed relative to those of the carrier control (DMSO only). The final DMSO concentration was 0.2% in all samples. In all experiments, both Caco-2 and HepG2 cells were harvested for analysis of ApoA-I mRNA expression after lysing with Tri-reagent, as described (21) and stored at −80 °C until further analysis.

Quantification of gene mRNA transcription

To evaluate the effects of C4 on ApoA-I mRNA expression, total RNA was isolated from the Caco-2 and HepG2 cells using Tri-reagent and the RNeasy mini kit (Qiagen, Hilden, Germany) according the manufacturer's instructions. For cDNA synthesis, 350 ng of total RNA was reverse transcribed using moloney murine leukemia virus (MMLV) reverse trans, dNTPs, random hexamers, dithiothreitol (DTT) and a 5xFS buffer supplemented with RNAse inhibitor (Thermo Fisher Scientific, Bleiswijk, Netherlands). The resulting cDNA was used for real time quantitative PCR using TaqMan gene expression assays using Cyclophilin A (Hs99999904) as a housekeeping control. To quantify ApoA-I, the TaqMan gene expression assays (Hs00163641) was used. Values are presented as relative gene expressions based on Ct values, normalized for the internal control Cyclophilin A and compared with control conditions.

Statistical analysis

All independent dose-response experiments with C4 were performed in duplicate, and each experiment was repeated three times. Six biological (12 technical) replicates were performed for every single C4 dose. Regression analysis was used to examine dose-response relationships between the concentration of added C4 and the mRNA expression of ApoA-I.

For a dose-response relationship, the regression coefficients had to be significantly different from zero at ($p < 0.05$). Effects of the positive control JQ1(+) in Caco-2 or HepG2 cells when cultured alone were statistically evaluated versus the control condition determined by a Mann–Whitney U test, in which a p-value < 0.05 was considered statistically significant. When evaluating the effects of JQ1(+) on ApoA-I mRNA expression in the different pro-inflammatory cytokines conditions in HepG2 or Caco-2 cells, the Mann–Whitney U test was used with a correction for multiple comparisons, in which a p-value < 0.008 was considered statistically significant. In addition, when evaluating the effects of adding no cytokines versus cytokines added apical, basolateral, or to both compartments side-by-side, the Mann–Whitney U test was also used with a correction for multiple comparisons (requiring $p < 0.008$ for significance). All statistical analyses were performed using SPSS v.25 (IBM Corp., Armonk, NY, USA).

Results

Effects of C4 on ApoA-I mRNA expression in Caco-2 or HepG2 cells (conditions 1 and 2)

To evaluate the effects of C4 on Caco-2 cells (Condition 1, Figure 5.3A), different doses of C4 were added to the apical surface of the Caco-2 cells on the insert, without HepG2 cells in the lower compartment. C4 did not change ApoA-I mRNA expression in the Caco-2 cells.

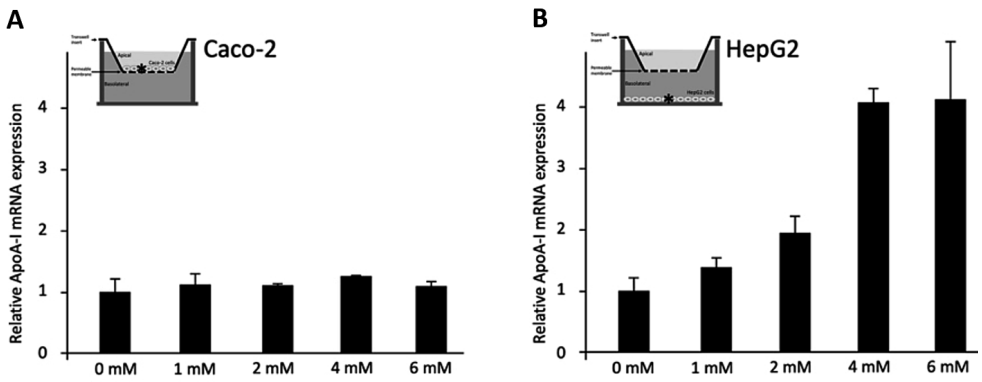

Figure 5.3. Relative Apolipoprotein A-I (ApoA-I) mRNA expressions in Caco-2 and HepG2 cells treated with different concentrations of C4. **(A)** Increasing C4 concentrations did not have any significant effects on ApoA-I mRNA expression in Caco-2 cells. **(B)** Increasing C4 concentrations showed a significant increase in ApoA-I mRNA expression in HepG2 cells ($p < 0.001$). All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against the expression observed in the control condition, which was arbitrarily set at 1. A linear regression was performed for C4 dose-response effects. Changes were considered significant when the regression coefficients were significantly different from zero ($p < 0.05$). ApoA-I, apolipoprotein-I; mRNA, messenger RNA.

When C4 was added to HepG2 cells in the lower compartment without the Caco-2 cells on the insert above (Condition 2, Figure 5.3B), ApoA-I mRNA expression dose-dependently increased ($p < 0.001$), with a maximum of a 4.2-fold at a 6 mM concentration of C4.

Effects of C4 added to the apical surface of Caco-2 cells on ApoA-I mRNA expression in Caco-2 and HepG2 cells (condition 3)

To explore the effects of C4 on Caco-2 and HepG2 cells co-cultured in a transwell system, C4 was added to the apical surface of Caco-2 cells cultured on inserts placed on top of the HepG2 cells in the lower compartment (Condition 3). In contrast to the condition without HepG2 cells (Figure 5.3A), ApoA-I gene expression in Caco-2 cells co-cultured with HepG2 cells significantly ($p < 0.01$) decreased after adding C4 (Figure 5.4A), whereas at the same time, ApoA-I mRNA expression in HepG2 cells was dose-dependently increased ($p < 0.001$) (Figure 5.4B). When cytokines were added to the apical side of the Caco-2 cells, ApoA-I mRNA concentrations were significantly ($p < 0.008$) lowered in both Caco-2 and HepG2 cells. Also, a significant decrease ($p < 0.008$) was observed in both cell lines when cytokines were added to the basolateral compartment instead of the apical side. When cytokines were added to the apical side and basolateral compartment simultaneously, there was even a further reduction ($p < 0.008$) in ApoA-I mRNA in both cell lines (Figure 5.4C-D). When C4 was added apically to the Caco-2 cells in the presence of cytokines (either apical, basolateral or both), C4 did not change ApoA-I mRNA expression in the Caco-2 cells (Figure 5.4C). In HepG2 cells, ApoA-I mRNA expression dose-dependently increased after adding C4 apically to the Caco-2 cells ($p < 0.01$), also in the presence of cytokines either added apical, basolateral or in both compartments (Figure 5.4D). In other words, apical C4 was able to rescue the reduced ApoA-I mRNA expression in HepG2 cells exposed to pro-inflammatory cytokines but not in intestinal Caco-2 cells.

Effects of the positive control JQ1(+) on ApoA-I mRNA expression in Caco-2 and HepG2 cells

The positive control JQ1(+) increased ApoA-I mRNA expression ($p < 0.05$) in Caco-2 or HepG2 cells when cultured alone (Figure 5.5). As mentioned before and shown in Figures 5.4C and 5.4D, ApoA-I mRNA expression in cells exposed to pro-inflammatory cytokines was lower compared with mRNA expression in non-inflamed cells. Effects of JQ1(+) treatment in these inflammatory conditions are shown in Figure 5.5. JQ1(+) did not affect ApoA-I gene expression in Caco-2 cells (co-cultured with HepG2 cells) without cytokines or when cytokines were added to either apical, basolateral or both compartments. Moreover, JQ1(+) significantly ($p < 0.008$) increased ApoA-I gene expression in HepG2 cells (co-

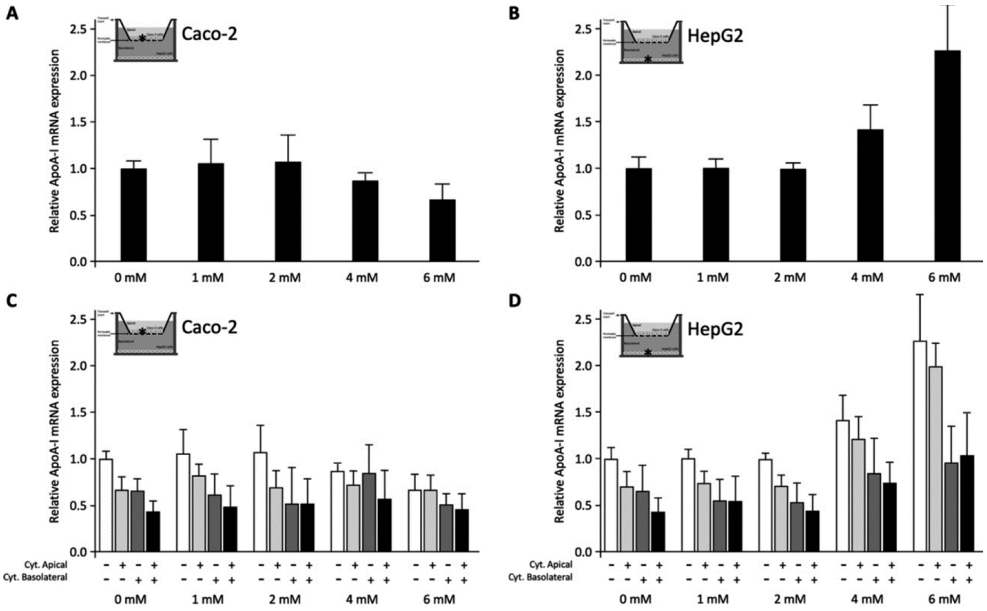

Figure 5.4. Relative Apolipoprotein A-I (ApoA-I) mRNA expressions in Caco-2 and HepG2 cells were cultured in a transwell system and treated with different concentrations of C4. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against the expression observed in the control condition, which was arbitrarily set at 1. A linear regression was performed for C4 dose–response effects. Changes were considered significant when the regression coefficients were significantly different from zero ($p < 0.05$; **A–D**). When evaluating the effects of adding no cytokines versus cytokines added apical, basolateral or to both compartments side-by-side, the Mann–Whitney U test was used with a correction for multiple comparisons in which a p-value < 0.008 was considered statistically significant **(C-D)**. **(A)** Increasing C4 concentrations showed a significant reduction in ApoA-I mRNA expression in Caco-2 cells that were cultured alone ($p < 0.01$). **(B)** Increasing C4 concentrations showed a significant increase in ApoA-I mRNA expression in HepG2 cells that were cultured alone ($p < 0.001$). **(C)** Increasing C4 concentrations did not show any significant effects on ApoA-I mRNA expression in Caco-2 cells co-cultured with HepG2 cells in the presence of cytokines (either apical, basolateral or both compartments). **(D)** Increasing C4 concentrations showed a significant increase in ApoA-I mRNA expression in HepG2 cells co-cultured with Caco-2 cells in the presence of cytokines (either apical, basolateral or both compartments) ($p < 0.01$). When cytokines were added to the apical side of the Caco-2 cells, ApoA-I mRNA expression significantly ($p < 0.008$) decreased in both Caco-2 and HepG2 cells **(C-D)**. A significant reduction in ApoA-I mRNA expression ($p < 0.008$) was observed in Caco-2 and HepG2 cells when cytokines were added to the basolateral compartment instead of the apical side. When the cytokines were added to both the apical and basolateral compartments, there was an even further reduction ($p < 0.008$) in ApoA-I mRNA expression in Caco-2 and HepG2 cells. The presence of cytokines was indicated with (+), while the absence of cytokines was indicated with (-). ApoA-I, apolipoprotein-I; mRNA, messenger RNA.

cultured with Caco-2 cells) with or without cytokines added to the apical compartment. Furthermore, JQ1(+) did not affect ApoA-I gene expression in HepG2 cells (co-cultured with Caco-2 cells) with cytokines added basolaterally or to both compartments (Figure 5.5). Taken together, the cells responded to JQ1(+) as expected (21).

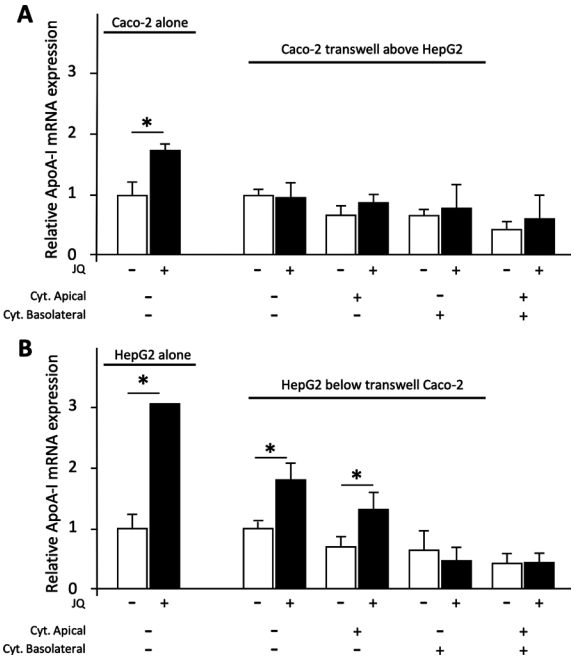

Figure 5.5. Relative Apolipoprotein A-I (ApoA-I) mRNA expressions in Caco-2 and HepG2 cells were cultured in a transwell system and treated with the positive control JQ1(+) (3 μM). **(A)** JQ1(+) significantly increased ApoA-I mRNA expression in Caco-2 cells that were cultured alone ($p < 0.05$). JQ1(+) did not affect ApoA-I gene expression in Caco-2 cells co-cultured with HepG2 cells without cytokines or when cytokines were added to either apical, basolateral or both compartments. **(B)** JQ1(+) significantly increased ApoA-I mRNA expression in HepG2 cells that were cultured alone ($p < 0.01$). JQ1(+) significantly increased ApoA-I mRNA expression in HepG2 cells co-cultured with Caco-2 cells without cytokines or when cytokines were added to apical compartments ($p < 0.008$). JQ1(+) did not affect ApoA-I gene expression in HepG2 cells co-cultured with Caco-2 cells when cytokines were added basolaterally or to both compartments. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against the expression observed in the control condition, which was arbitrarily set at 1. A Mann–Whitney U test was performed to evaluate JQ1(+) effects in Caco-2 or HepG2 cells that were cultured alone versus control conditions, in which a p-value < 0.05 was considered statistically significant. When evaluating the effects of JQ1(+) on ApoA-I mRNA expression in different pro-inflammatory cytokines conditions in HepG2 or Caco-2 cells, the Mann–Whitney U test was used with a correction for multiple comparisons, in which a p-value < 0.008 was considered statistically significant. Changes are indicated with * when the effect of JQ1(+) is significantly different from the control. The presence of cytokines was indicated with (+), while the absence of cytokines was indicated with (-). ApoA-I, apolipoprotein-I; mRNA, messenger RNA.

Discussion

In earlier experiments, we have shown that C4 treatment increased ApoA-I mRNA expression in HepG2 cells in both normal and inflammatory conditions (9, 12). These positive effects on hepatic ApoA-I transcription were observed when C4 was added directly to HepG2 cells. Here, we have evaluated whether these effects were still evident when C4 was added to the apical surface of enterocytes, which means that it first had to be taken

up by Caco2 cells and transported towards HepG2 cells in the basolateral compartment. This also made it possible to evaluate the effects of C4 on ApoA-I expression in Caco2 cells, which also contribute to ApoA-I concentrations in the circulation (22). Although C4 is mainly produced by microbiome in the colon, we decided to examine the effects of C4 on ApoA-I transcription in small intestinal enterocytes since ApoA-I is mainly produced in the duodenum and jejunum (23, 24). However, although SCFAs as end products of bacterial fermentation are present in higher concentrations in the colon, SCFAs are also present in the proximal intestines (23, 25). We did not find any effect of C4 on ApoA-I gene expression in Caco2 cells when cultured alone (condition 1). In contrast to the effects in Caco-2 cells, but in line with our earlier findings (9), C4 again significantly increased ApoA-I expression in HepG2 cells (condition 2). Interestingly, in the co-culture model (condition 3), we found that ApoA-I mRNA expression in Caco-2 cells was even lowered after adding C4 in the presence of HepG2 cells, whereas effects of C4 on HepG2 cells remained positive, showing a significant increase in ApoA-I gene expression. This illustrates the cell-specific response of enterocytes and hepatocytes in a co-culture model in response to C4 exposure. Moreover, in the presence of cytokines either added to apical, basolateral or to both cell compartments, ApoA-I mRNA levels in HepG2 cells were rescued by C4. However, the effects of C4 on elevating ApoA-I mRNA expression in HepG2 cells when added to the apical side of Caco-2 cells in the transwell were lower compared with the effects of C4 when added directly to HepG2 cells. The question is how the lower hepatic effects of C4 in the co-culture experiments can be explained. Theoretically, decreased bioavailability (i.e., the amount of SCFAs that are transported from the apical to the basolateral side), which translates into lower hepatic exposure to C4, seems the most logical explanation. It is well-known that SCFAs are used as fuel by the intestinal cells (26). Therefore, once SCFAs are metabolized by the intestinal cells, they will not be available (in their original concentrations) in the basolateral compartment, hence explaining the lower bioavailability. Moreover, we also observed that the inhibitory effect on hepatic ApoA-I expression was larger when cytokines were added directly to HepG2 cells. This finding, in the case of cytokines, could also attributed to the bioavailability of cytokines. However, in contrast to C4, cytokines are not used for fueling enterocytes, which again raises the question of why cytokines' effects on HepG2 cells were smaller when they were added apically in the transwell system. An alternative explanation for the lower effects of both C4 and cytokines on HepG2 cells after adding them to Caco-2 cells could also theoretically be a cross-talk phenomenon between Caco-2 cells and HepG2 cells. For example, the secreted factors from the Caco-2 cells into the basolateral compartment might influence the effects of C4 on HepG2 cells. In theory, such cross-talk between intestine and liver may be critical for human health since several liver diseases result from alterations in the intestinal barrier (27). Moreover, such interactions between the intestine, including

its microbiota, and the liver can also be regulated by exposure to dietary compounds (27). A recent study investigated the effects of SCFAs on the gut-liver axis in interconnected human micro-physiological systems (MPS) (28). Adding SCFAs to the apical enterocyte side favorably modulated the gut-liver axis in MPS by innate immune inactivation. Moreover, Trapecar et al. also showed, by using the same MSP system, that apical addition of SCFAs to enterocytes not only inhibited gut inflammation but also increased liver metabolic function, improved lipid metabolism and enhanced hepatic bile acid secretion (28). Altogether, this illustrates that hepatic effects exist after intestinal exposure to SCFAs. However, this can still be simple response to SCFAs transferred from the intestine to the liver, or cross-talk of the liver responding to factors secreted via intestinal cells. To the best of our knowledge, there is so far no known intestinal factor that is secreted in response to SCFAs exposure, influencing hepatic physiology. However, our data may suggest the existence of such a factor. We showed that Caco-2 cells responded in the opposite direction to C4 compared with HepG2 cells. To elaborate, treating Caco-2 cells with C4 had no effects on ApoA-I mRNA when the Caco-2 cells were cultured alone and even decreased intestinal ApoA-I mRNA expression in the co-culture model. The question now is whether we should expect effects on the intestine and liver to go hand-in-hand or whether there are other known conditions where effects on the liver and intestine were also in the opposite direction. A potential difference in ApoA-I mRNA response between the intestinal and hepatic cells could be explained by the need of some coactivators to produce ApoA-I, which is absent in Caco-2 cells. For example, the synergy between the ApoA-I promoter and the ApoCIII enhancer is essential to induce intestinal ApoA-I transcription, whereas the induction of hepatic ApoA-I gene expression seems independent of the ApoCIII enhancer (29). However, although the absence of such coactivators might explain why C4 had no effects on Caco-2 cells when cultured alone, we have now even observed inhibition of ApoA-I expression in Caco-2 in the co-culture model after C4 exposure. This suggests that effects of C4 on Caco-2 cells might activate different pathways that not only translate into lower ApoA-I transcription but could also induce the production of a currently unknown factor. These factors might not only affect ApoA-I transcription in the enterocyte itself but also influence hepatic function, which is evident in our studies as a lower hepatic ApoA-I transcription. Identification of such a factor would be highly informative and deserves further studies. Finally, although we acknowledge that the various SCFAs have different systemic concentrations, we expect other SCFAs to behave identically (1). On the other hand, as explained, it could be that there are differences regarding intestinal cells metabolism between different SCFAs, which might consequently result in different basolateral concentrations and different effects on liver cells. However, since the concentrations of acetate and propionate in vivo are likely to be higher than those of butyrate, it seems "safe" to speculate that comparable effects will be observed for other

SCFAs.

In conclusion, our findings indicate that in co-culture experiments, adding C4 apically to intestinal cells does not increase the lower ApoA-I mRNA level in inflamed Caco-2 cells. Furthermore, C4 added apically to Caco-2 cells elevates hepatic ApoA-I transcription and rescues the lower ApoA-I expression in inflamed HepG2 cells. As we found previously that all SCFAs (in line with C4) were able to enhance hepatic ApoA-I expression when directly added to HepG2 cells (9), we suggest further exploration of the effects of other SCFAs in the intestine-liver co-culture model. Moreover, the effects of adding C4 to the apical side of enterocytes translate into a smaller effect on HepG2 cells compared with adding C4 to hepatocytes directly. We speculate that this could be due to lower bioavailability, but it could also indicate cross-talk between intestine and liver. Identification of such an enterocyte-derived inhibitory factor warrants further studies.

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6

Differential effects of individual amino acids on PPARα transactivation, ApoA-I transcription and (pro)-ApoA-I secretion

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To be submitted

Abstract

The concentration of apolipoprotein A-I (ApoA-I), the major component of HDL, is associated with increased HDL functionality and reverse cholesterol transport (RCT). Thus, a promising strategy to prevent CVD is to accelerate RCT by increasing de novo ApoA-I production. Several experimental animal models have suggested effects of specific amino acids on hepatic lipid and lipoprotein metabolism. Therefore, we here examined the effects of different individual amino acids on hepatic ApoA-I transcription and secretion. Human hepatocytes (HepG2) were exposed to increasing doses of six different amino acids (glutamine, leucine, proline, histidine, glutamic acid and tryptophan) for 48 hours. ApoA-I messenger RNA (mRNA) transcription and secreted pro-ApoA-I and ApoA-I protein concentrations were analyzed using quantitative polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assays (ELISA), respectively. To study underlying mechanisms, changes in mRNA expression of CPT1 and KEAP1 were analyzed. Leucine, glutamic acid and tryptophan dose-dependently increased ApoA-I mRNA transcription as well as CPT1 mRNA expression. In addition, CPT1 mRNA expression was significantly correlated with ApoA-I mRNA expression in response to tryptophan treatment. In contrast, the amino acids glutamine, proline and histidine increased pro-ApoA-I concentrations without any changes in ApoA-I mRNA expression. Lastly, none of the amino acids increased mature ApoA-I protein concentrations. In conclusion, the addition of individual amino acids to human HepG2 cells has differential effects on ApoA-I transcription or (pro)-ApoA-I secretion. Based on our results, it is tempting to suggest that PPARα transactivation is involved in the effects of leucine, glutamic acid and tryptophan on ApoA-I mRNA expression, while others (glutamine, proline and histidine) increased pro-ApoA-I protein secretion.

Introduction

Numerous studies have consistently demonstrated a relationship between serum lipid and lipoprotein concentrations with cardiovascular diseases (CVD) (1-3). The best characterized risk factor for CVD is an elevated serum concentration of low-density lipoprotein (LDL) cholesterol (4). Several highly effective therapies are nowadays used to reduce serum LDL cholesterol concentrations such as the well-known statins (HMG-CoA reductase inhibitors) (5). Although statins are widely used for CVD treatment, the prevalence of CVD remains high. In addition, some patients treated with maximum doses of statins do not always reach their target serum LDL cholesterol values (6, 7). Therefore, strategies to further lower serum LDL cholesterol concentrations by additional treatments such as PCSK9 inhibitors and NPC1L1 inhibitors were introduced (8, 9). On the other hand, high-density lipoprotein (HDL) cholesterol levels are well-known for their inverse relation with CVD. This association is most likely related to the functionality of the HDL particle, a determinant of its cholesterol efflux capacity which is the first step of reverse cholesterol transport (RCT) (10). The concentration of apolipoprotein A-I (ApoA-I), the major component of HDL, is associated with increased cholesterol efflux capacity and RCT (11). Thus, besides lowering LDL cholesterol, a promising additional strategy to prevent CVD is to accelerate RCT by increasing de novo ApoA-I production (12, 13).

Besides pharmacological treatment, preventing dyslipidemia at an early stage receives nowadays more and more attention by incorporating a healthy lifestyle such as losing body weight, implementing a less sedentary behavior and optimizing dietary intake. To increase HDL functionality and ApoA-I concentrations, there has been a strong interest on the effects of increasing the intake of specific dietary fats or fatty acids at the expense of carbohydrates (14-16). Effects of dietary proteins or individual amino acids on serum HDL cholesterol and ApoA-I concentrations have so far not be considered into great detail. However, various animal models have suggested an association between amino acid intake and hepatic lipid metabolism (17, 18). For example, citrulline supplementation lowered hypertriglyceridemia and reduced hepatic fat accumulation in rats with non-alcoholic fatty liver (NAFLD) (17). In ApoE null mice, leucine supplementation improved plasma lipid profiles, diminished systemic inflammation and markedly attenuated atherosclerosis (19). Finally, oral administration of glutamate and aspartate increased serum HDL cholesterol concentrations, maintained plasma ApoA-1 concentrations and reduced fatty streak formation in rabbits fed a high cholesterol diet (20). Based on these results, we hypothesized that amino acids affect hepatic ApoA-I production. Therefore, we examined the effects of six different amino acids (glutamine, leucine, proline, histidine, glutamic acid (glutamate) and tryptophan) on ApoA-I transcription, pro-ApoA-I and ApoA-I protein secretion in

HepG2 cells, which were selected based on different physical and chemical characteristics. Effects on peroxisome proliferator-activated receptor alpha (PPARα) transactivation, and bromodomain and extra-terminal domain (BET) inhibition were also examined as potential regulatory pathways of ApoA-I transcription (21, 22).

Material and methods

Materials

Human hepatocellular liver carcinoma cells (HepG2) were kindly provided by Sten Braesch-Andersen (Mabtech, Nacka Strand, Sweden). Cell culture flasks and plates were obtained from Corning (Cambridge, USA). Minimum Essential Medium (MEM), sodium pyruvate, non-essential amino acids (NEAA), penicillin and streptomycin were all obtained from Thermo Fisher Scientific (Bleiswijk, Netherlands). Fetal bovine serum (FBS) was purchased from PAA (Toronto, Canada). Glutamine (Gln), leucine (Leu), proline (Pro), histidine (His), glutamic acid (Glu), tryptophan (Trp), dimethyl sulfoxide (DMSO) and Tri-reagent were all bought from Sigma (Uithoorn, Netherlands). The BET inhibitor JQ1(+) was obtained from Bio-Techne - R&D (Minneapolis, USA). Chemical characteristics of the six amino acids are shown in Supplementary Table S6.1.

Cell culture and amino acids treatment

HepG2 cells were cultured at 37 °C in a humidified atmosphere of 5% carbon dioxide (CO_2) in MEM containing 10% heat inactivated FBS, 1% sodium pyruvate, 1% NEAA and 1% of penicillin-streptomycin mixture. For all experiments, cells were seeded in a 24-well plate at a density of 200,000 cells per well. Cell viability was daily inspected by microscope and when cells reached a density of 80–90%, they were incubated for 48 h in experimental medium (MEM without FBS and without NEAA) enriched with a concentration range of 0-10 mM amino acids (Gln, Leu, Pro, His, Glu or Trp) or 3 μM JQ1(+). Effects of the amino acids were expressed relative to those of the non-enriched background medium (MEM without FBS and NEAA). The positive control JQ1(+), a BET inhibitor, was included in each experiment to ensure that cells were responsive and produced sufficient amounts of ApoA-I (22). JQ1(+) was dissolved in dimethyl sulfoxide (*DMSO, cell culture tested*) and effects of JQ1(+) were expressed relative to its carrier control, DMSO. The final DMSO concentration was always 0.2%. Culture medium was collected for analysis of pro-ApoA-I and ApoA-I protein concentrations and cells were harvested for analysis of mRNA expression after lysing with Tri-reagent as described before (22). Both culture medium and lysed cells were snap frozen in liquid nitrogen and stored at −80 °C until further analysis.

Quantification of gene mRNA transcription

Total RNA was isolated from the HepG2 to evaluate the effects of amino acids on mRNA expression of ApoA-I, the PPARα target CPT1, and the BET inhibition target KEAP1, using Tri-reagent and the RNeasy mini kit (Qiagen, Hilden, Germany) according the manufacturer's instructions. For cDNA synthesis, 350 ng of total RNA was reverse-transcribed using MMLV reverse trans, dNTP's, random hexamers, DTT and 5xFS buffer supplemented with RNAse inhibitor (Thermo Fisher Scientific, Bleiswijk, Netherlands). The resulting cDNA was used for real time quantitative PCR using TaqMan Gene Expression Assays using Cyclophilin A (Hs99999904) as a housekeeping control. To quantify ApoA-I, CPT1 and KEAP1 the TaqMan Gene Expression Assays Hs00163641, Hs00912671 and Hs00202227 were used. Values are presented as relative gene expressions based on the Ct values, normalized for the internal control Cyclophilin A, and compared to the control conditions.

Quantification of pro-ApoA-I and ApoA-I protein levels in the culture medium

Pro-ApoA-I and ApoA-I protein concentrations in culture medium of HepG2 cells were measured by an enzyme-linked immunoassay (ELISA). The pro-ApoA-I ELISA was performed as described (13). The ApoA-I ELISA was obtained from Mabtech (Nacka Strand, Sweden) and analyses were performed according the manufacturer's instructions with slight modifications, e.g. BSA 10% (Thermo Fisher Scientific, Bleiswijk, Netherlands) was added to the block buffer and to the dilution buffer at final concentrations of 1% and 0.1%, respectively. For both pro-ApoA-I and ApoA-I ELISA measurements, the absorbances were determined at 450 nm using a multi-scan microplate reader (Thermo Fisher Scientific, Bleiswijk, Netherlands). Values are presented as relative fold changes compared to the control conditions.

Statistical analysis

All independent dose-response experiments with amino acids were performed in quadruplicate and each experiment was repeated two times. Thus, eight biological (16 technical) replicates were performed for each single amino acid dose. Regression analysis was used to examine dose-response relationships between the concentrations of added amino acids and the respective parameters. For the correlations between CPT1 mRNA expression and ApoA-I mRNA expression, Spearman correlations were calculated. The regression coefficients and Spearman correlation coefficients were considered to be statistically significant when different from zero at $p < 0.05$. The effects of the positive control JQ1(+) was statistically evaluated versus control conditions by a Mann–Whitney

U test. Again, a p-value < 0.05 was considered to be statistically significant. All results are presented as the mean, while error bars indicate the standard deviations (SD). All statistical analyses were performed using SPSS v.25 (IBM Corp., Armonk, NY, USA).

Results

Effects of different amino acids on ApoA-I mRNA expression

The positive control JQ1(+) increased ApoA-I transcription ($p < 0.001$) indicating that the HepG2 cells responded to external stimuli (Figure 6.1) (22). ApoA-I mRNA expression increased dose-dependently ($p < 0.05$) when Leu, Glu or Trp were added to the medium. At concentrations of 10 mM, ApoA-I mRNA expression increased by 32% for Leu and by 21% for Glu. The most pronounced increased of 47% for Trp was found at a concentration of 5.0 mM. The other amino acids (Gln, Pro and His) did not change ApoA-I mRNA expression (Figure 6.2).

Effects of different amino acids on pro-ApoA-I and ApoA-I protein secretion in culture medium

In line with mRNA expression, the positive control JQ1(+) increased pro-ApoA-I and slightly increased ApoA-I protein concentrations in culture medium ($p < 0.05$) (Figure 6.1). For the individual amino acids, the patterns for pro-ApoA-I protein concentrations

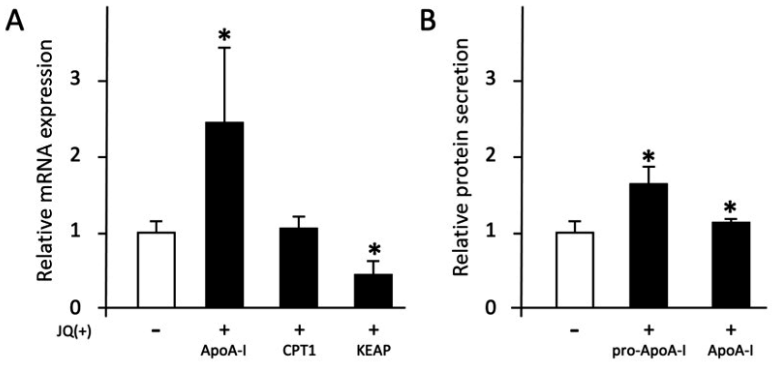

Figure 6.1. Relative ApoA-I, CPT1 and KEAP1 mRNA expressions or pro-ApoA-I and ApoA-I protein secretion in HepG2 cells treated with the positive control JQ1(+) (3 μM). **(A)** JQ1(+) significantly increased ApoA-I mRNA expression and decreased KEAP1 mRNA expression, while did not show any significant effect in CPT1 mRNA expression. **(B)** JQ1(+) significantly increased pro-ApoA-I and ApoA-I protein secretion. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against the expression observed in the control condition, which was arbitrarily set at 1. Mann–Whitney U test was performed to examine JQ1(+) effects on the mRNA expressions or protein secretion, in which a p-value < 0.05 was considered statistically significant.

were opposite to those of ApoA-I mRNA expression. Gln, Pro and His increased dose-dependently pro-ApoA-I protein concentrations ($p < 0.05$), whereas Leu and Glu treatment did not affect pro-ApoA-I protein concentrations (Figure 6.3). Interestingly, pro-ApoA-I

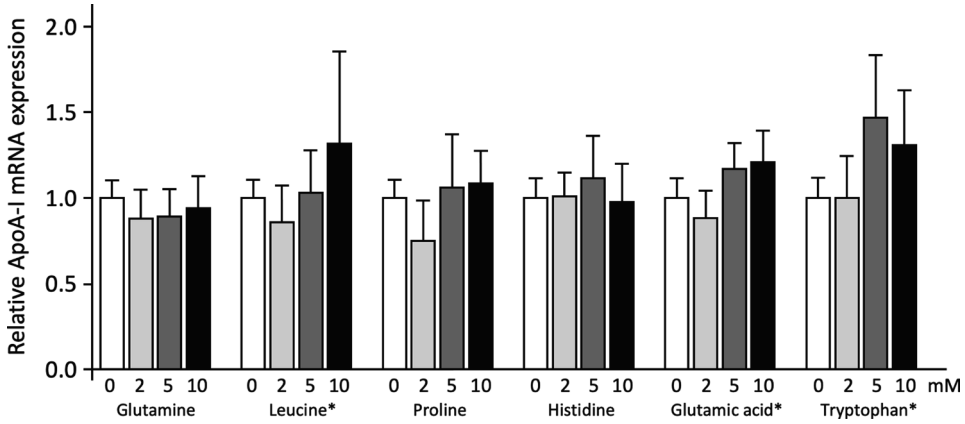

Figure 6.2. Relative ApoA-I mRNA expression in HepG2 cells treated with different concentrations of amino acids. Increasing Leu, Glu and Trp concentrations significantly increased ApoA-I mRNA expression, while increasing Gln, Pro and His concentrations did not. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against the expression observed in the control condition (0 mM), which was arbitrarily set at 1. Linear regression analysis was performed to examine the amino acids effects on ApoA-I mRNA expression. Changes were considered significant when the regression coefficients were significantly different from zero ($p < 0.05$).

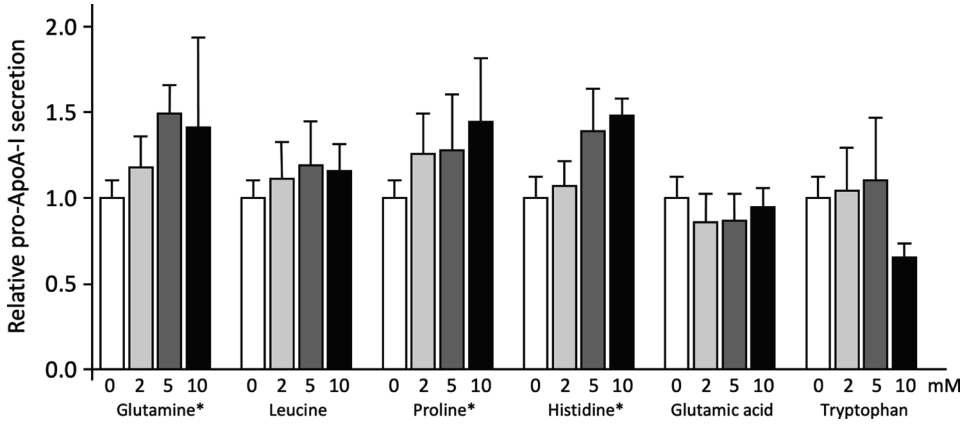

Figure 6.3. Relative pro-ApoA-I secretion in HepG2 cells treated with different concentrations of amino acids. Increasing Gln, Pro and His concentrations significantly increased pro-ApoA-I secretion, while increasing Trp concentrations significantly decreased pro-ApoA-I secretion in HepG2 cells. Leu and Glu did not show any significant effect in pro-ApoA-I secretion. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against the expression observed in the control condition (0 mM), which was arbitrarily set at 1. Linear regression analysis was performed to examine the amino acids effects on pro-ApoA-I secretion. Changes were considered significant when the regression coefficients were significantly different from zero ($p < 0.05$).

protein concentrations even decreased after Trp treatment ($p < 0.001$). Gln, Leu, His, Pro and Trp reduced ApoA-I protein concentrations ($p < 0.05$), while Glu did not have any effects (Figure 6.4).

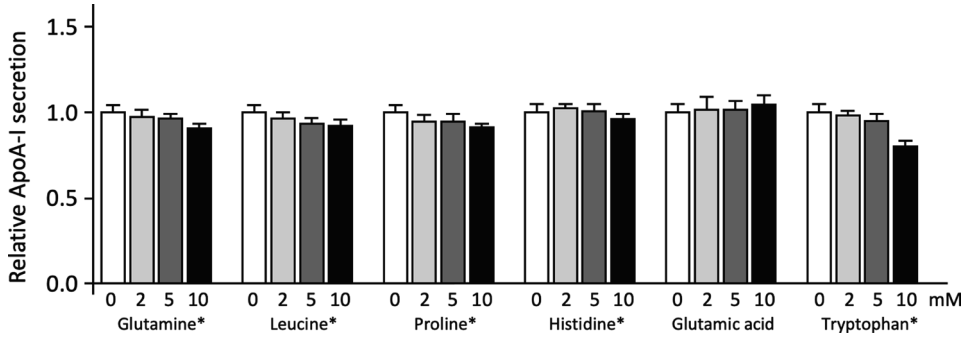

Figure 6.4. Relative ApoA-I protein secretion in HepG2 cells treated with different concentrations of amino acids. Increasing Gln, Leu, Pro, His and Trp concentrations significantly decreased ApoA-I protein secretion, while increasing Glu concentrations did not. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against the expression observed in the control condition (0 mM), which was arbitrarily set at 1. Linear regression analysis was performed to examine the amino acids effects on ApoA-I protein secretion. Changes were considered significant when the regression coefficients were significantly different from zero ($p < 0.05$).

Effects of different amino acids on CPT1 and KEAP1 mRNA expression

Effects of the different amino acids on CPT1 and KEAP1 mRNA expression were analyzed to explore possible pathways underlying the effects on ApoA-I mRNA expression and protein secretion. Interestingly, HepG2 cells exposed to Gln, Leu, His, Glu and Trp consistently showed a significant ($p < 0.01$) increase in mRNA expression of the PPARα target gene CPT1, while Pro did not affect CPT1 mRNA expression (Figure 6.5). As the ApoA-I promotor contains PPAR response elements, the correlation between CPT1 mRNA expression with ApoA-I mRNA expression was calculated. Interestingly, a significant correlation was found after Trp treatment ($r = 0.538$; $p < 0.05$). Such a correlation was not observed for the two other amino acids (Leu and Glu) that increased ApoA-I mRNA expression. Furthermore, mRNA expression of the BET inhibition target gene KEAP1 dose-dependently decreased after Gln treatment ($p < 0.001$), while Trp dose-dependently increased ($p < 0.001$) KEAP1 mRNA expression. Leu, Pro, His and Glu did not significantly change KEAP1 mRNA expression (Figure 6.6). As expected, the positive control JQ1(+), which is a BET inhibitor, significantly decreased KEAP1 gene expression ($p < 0.001$), whereas JQ1(+) did not affect CPT1 gene expression (Figure 6.1).

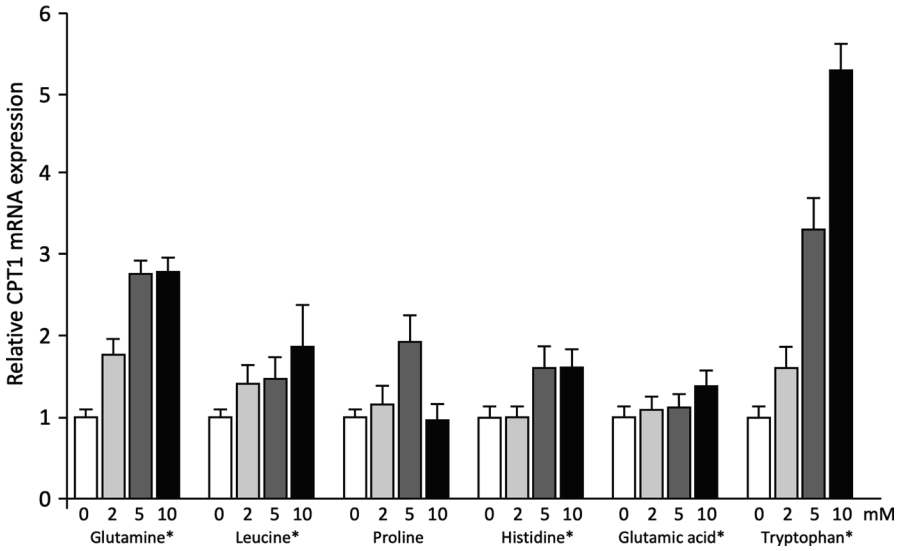

Figure 6.5. Relative CPT1 mRNA expression in HepG2 cells treated with different concentrations of amino acids. Increasing Gln, Leu, His, Glu and Trp concentrations significantly increased CPT1 mRNA expression, while increasing Pro concentrations did not. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against the expression observed in the control condition (0 mM), which was arbitrarily set at 1. Linear regression analysis was performed to examine the amino acids effects on CPT1 mRNA expression. Changes were considered significant when the regression coefficients were significantly different from zero ($p < 0.05$).

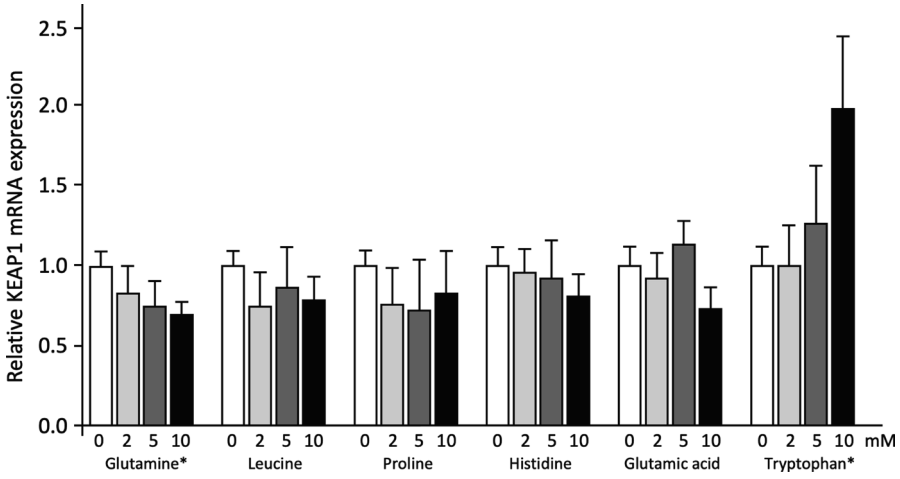

Figure 6.6. Relative KEAP1 mRNA expression in HepG2 cells treated with different concentrations of amino acids. Increasing Gln concentrations significantly decreased KEAP1 mRNA expression, while increasing Trp concentrations significantly increased KEAP1 mRNA expression. Leu, Pro, His and Glu did not show any significant effect in KEAP1 mRNA expression. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against the expression observed in the control condition (0 mM), which was arbitrarily set at 1. Linear regression analysis was performed to examine the amino acids effects on KEAP1 mRNA expression. Changes were considered significant when the regression coefficients were significantly different from zero ($p < 0.05$).

Discussion

Since data from several experimental animal models have suggested effects of proteins or even specific amino acids on hepatic lipid and lipoprotein metabolism, we here explored the effects of different individual amino acids on hepatic ApoA-I transcription and secretion in HepG2 cells. We showed that the amino acids Leu, Glu and Trp enhanced ApoA-I mRNA expression, while Gln, Pro and His were associated with elevated pro-ApoA-I protein concentrations in the culture medium. In addition, both CPT1 (a PPARα target gene) and ApoA-I mRNA expressions were increased after treatment with Leu, Glu and Trp. Furthermore, CPT1 mRNA expression was significantly correlated with ApoA-I mRNA expression in response to Trp treatment. This association was not found for the other amino acids suggesting that Trp could be the most potent PPARα activator, which coincides with the largest elevation in ApoA-I mRNA expression. Based on these data it is tempting to suggest that PPARα transactivation might be involved in the effects of certain amino acids on hepatic ApoA-I expression. The link between PPARα and ApoA-I expression was also found in our previous studies with short chain fatty acids and is supported by the presence of a PPARα binding site for fatty acids in the promotor region of ApoA-I (23, 24). To the best of our knowledge, the link between amino acids and PPARα transactivation has not been evaluated into great detail. The only dietary components known so far to stimulate PPARα transactivation are fatty acids and its metabolites (24). Further research is therefore needed to understand how these amino acids may stimulate PPARα transactivation. Interestingly, the possible link between amino acids and PPARα transactivation was also observed in previous studies using other experimental settings. For example, leucine significantly increased PPARα expression in skeletal muscle myotubes of mice (25). Also, leucine, glutamine and proline all reduced IL-8 production in HepG2 cells, probably via NF-κB inhibition (26), a process in which PPARα is also involved (27). However, our findings were not fully consistent, since Pro did not affect PPARα transactivation, but did increase pro-ApoA-I protein concentrations. This indicates that Pro may modulate ApoA-I production via another, PPARα-independent, mechanism. However, also BET inhibition was not involved, since KEAP1 (a BET target gene) expression was not consistently reduced by the amino acids that were explored. A possible pathway that we did not explore and plays a role in ApoA-I production is modulation of ER stress (22, 28). Lastly, chemical characteristics of the amino acids may have played a role in explaining the selectivity regarding effects on ApoA-I mRNA expression, as the three amino acids that increased ApoA-I expression (Leu, Glu and Trp) were more hydrophobic than the other amino acids that did not (Gln, Pro and His) (29).

Besides effects on PPARα transactivation and ApoA-I transcription, a second finding was that a transcriptional increase of ApoA-I mRNA did not necessarily translate into a similar

change in pro-ApoA-I protein concentrations. In fact, the amino acids Gln, Pro and His did not affect ApoA-I mRNA expression but increased pro-ApoA-I protein concentrations. The question is how these apparent controversial findings can be explained. It can be speculated that the amino acids have different effects on ApoA-I mRNA stability, which affect the translation capacity of ApoA-I mRNA (30). Another explanation may relate to the amino acid deprivation in HepG2 cells, as we did not add serum and NEAA to our cell culture medium prior to the experiments to exclude possible influence of the "background" NEAA on ApoA-I expression or secretion. In a recent study, Georgila et al. showed that amino acid deprivation changed the mTORC1 signaling pathway, which plays a major role in the regulation of ApoA-I protein secretion in HepG2 cells by maintaining the balance between ApoA-I synthesis and autophagy (i.e. protein breakdown) (31). It has been shown that a low availability of amino acids inhibited the mTORC1 pathway, which subsequently induced the autophagy process, while supplementation with amino acids activated the mTORC1 pathway and restored ApoA-I production (31). Interestingly, ApoA-I mRNA concentrations were not sensitive to changes in the mTORC1 pathway (31). Thus, these findings illustrate that ApoA-I protein levels are sensitive to amino acid depletion, while ApoA-I mRNA expression is not. Therefore, ApoA-I mRNA and protein synthesis are not necessarily linked. However, to verify if the individual amino acids differently affect the mTORC1 pathway, further investigation is required.

The third interesting observation in our study was that increases in pro-ApoA-I protein concentrations and ApoA-I mRNA expression in response to amino acids treatment were not reflected by changes in mature ApoA-I protein concentrations. Obviously, the elevated concentrations of secreted pro-ApoA-I protein after incubation with Gln, Pro and His were not further processed into the mature ApoA-I protein since the concentrations were even lowered. To explain this unexpected discrepancy, it is important to consider that our experiments in HepG2 cells were performed in culture medium that lacked NEAA and FBS. However, this experimental condition might have influenced the results in different ways. First, the lack of NEAA and FBS in the culture medium might have influenced the secretory machinery of the cells to secrete proteins (32). However, the cells can still secrete pro-ApoA-I protein as observed for the Gln, Pro and His conditions. The fact that the amino acid treatment did not increase the pro-ApoA-I cleavage into mature ApoA-I protein might relate to the absence of the procollagen C-proteinase enhancer-2 (PCPE2) enzyme that supports the cleavage of the pro-segment in pro-ApoA-I (33) which was absent, because we did not add FBS to the culture medium prior to amino acids treatment. However, the positive control JQ1(+) did increase both pro-ApoA-I and ApoA-I protein concentrations, also in absence of FBS and NEAA, which would argue against the limitation of essential

components for pro-ApoA-I secretion and cleavage. Moreover, in our previous studies (23) we found that JQ1(+) cultured with NEAA increased mature ApoA-I concentrations on average by 25%, while in this current study the elevation in ApoA-I concentrations with the JQ1(+) condition was less than 15%, which could be interpreted as an inhibition. Secondly, the absence of NEAA might have induced cellular stress (34, 35), which subsequently leads to abnormal protein folding (36), which could have resulted into a mature ApoA-I protein that was not recognized by our antibodies. However, again this implies that the positive control JQ1(+) which was also used in conditions without NEAA and FBS did not suffer from this stress-induced protein folding phenomenon. Therefore, this also might not be the probable explanation, which means that further investigations are needed to determine the reasons behind this inability of amino acids treatment to increase the mature ApoA-I protein level.

In conclusions, the amino acids leucine, glutamic acid and tryptophan may increase PPARα transactivation as suggested by the elevation of CPT1 expression and the increase in ApoA-I mRNA expression in HepG2 cells. To explain this novel finding into more detail, future studies focusing on a detailed analysis on the PPARα pathway are warranted. We also showed that the increase in ApoA-I mRNA by leucine, glutamic acid and tryptophan did not translate into elevated pro-ApoA-I concentrations. In contrast, the amino acids glutamine, proline and histidine increased pro-ApoA-I concentrations without any changes in ApoA-I mRNA expression, which may indicate involvement of the mTORC1 pathway. Therefore, we suggest to investigate in future experiments into more detail the effects of different amino acids not only on ApoA-I mRNA expression, but also on mTORC1 pathway, autophagy and other mechanisms such as ER stress that are known to regulate ApoA-I production.

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Supplementary Table S6.1. The Chemical characteristics of different amino acids that were selected in this study

Amino acid	Chemical characteristics
Glutamine	Polar, hydrophilic
Leucine	Non-polar, hydrophobic
Proline	Non-polar, neutral
Histidine	Polar, hydrophilic
Glutamic acid	Polar, Acidic
Tryptophan	Non-polar, hydrophobic

7

General discussion

The protective role of HDL functionality on CVD

Although mortality rates of cardiovascular diseases (CVD) have been decreasing over the last five decades, CVD remain the most common cause of death in Europe and around the world (1). Statistics on CVD show that they account for 47% of all deaths in women and 39% of all deaths in men in Europe (1). A process underlying the development of CVD is atherosclerosis, which is a progressive process characterized by lipid accumulation in the arterial wall (2). Both an increased plasma LDL cholesterol concentration and/or reduced HDL cholesterol concentration are known as major risk factors for atherosclerosis (3). For more than thirty years, elevated LDL cholesterol concentrations were the primary target for atherosclerosis treatment. Statins, HMG-CoA reductase inhibitors, have been used to reduce LDL cholesterol concentrations and consequent CVD risk (4). Several studies have showed that statins decreased the CVD risk by 35%, even by using high doses of statins. However, around 65% from the patients which were treated with statins still developed unfavorable CVD complications. Thus, additional therapeutic strategies beyond statins are needed to further reduce CVD risk (5). Many epidemiological studies have shown that HDL cholesterol concentrations were inversely related to CVD, even at low levels of LDL cholesterol in some cases (6, 7). However, some clinical studies found that an increase in HDL cholesterol concentrations did not reduce CVD risk (8). Over the years, it became evident that HDL functionality plays a more important role in the protection against atherosclerosis development than just the HDL cholesterol concentrations (9). HDL functionality is mainly related to its ability to obtain cholesterol and phospholipids from macrophages, a process called the cholesterol-efflux capacity (10). Recent studies have linked the cholesterol-efflux capacity to the athero-protective role of HDL and CVD reduction (11, 12). ApoA-I, the main component of HDL, comprises approximately 70% of the HDL protein mass (13). Many of HDL athero-protective properties have been attributed to ApoA-I, which goes even beyond its role in cholesterol efflux capacity (14). ApoA-I is the ligand for ATP-binding cassette transporter A1 (ABCA1), which modulates the cholesterol efflux from macrophages in the artery wall (15). Increasing ApoA-I production can translate into newly produced HDL particles that are highly functional, which eventually enhances the cholesterol efflux capacity (16). Together, increasing HDL functionality by modulating ApoA-I gene transcription might be a promising strategy for CVD prevention. The main focus of this thesis was to understand the effects of a selected number of external stimuli on ApoA-I transcription. In this thesis we focused on the effects of antibiotics, SCFAs and amino acids as external stimuli on ApoA-I modulatory capacity.

Effects of antibiotics on HDL cholesterol in humans

Despite the fact that antibiotics such as amoxicillin are generally considered as safe and well-tolerated highly essential drugs in certain conditions, they have also been linked to several side effects (17, 18). For example, many antibiotics cause nausea, vomiting, diarrhea, allergic reactions and hepatotoxicity (19-21). However, the interaction of antibiotics with lipid and lipoprotein metabolism has not been evaluated in a great detail. In a recent clinical trial, we found that amoxicillin intake for seven days significantly decreased serum HDL cholesterol concentrations in healthy subjects (22). Of course, we do not by any means want to say that we should not treat a patient with antibiotics because HDL cholesterol concentrations may become reduced, since the disease for which the antibiotics are prescribed is for sure more damaging for the patient. It is just a possibility to understand in more detail which factors are involved in regulating HDL cholesterol and/or ApoA-I metabolism. Despite the observation in the above-mentioned intervention study with amoxicillin, it is unknown whether amoxicillin reduced HDL levels via a direct or an indirect effect. Antibiotics might influence lipoprotein metabolism directly by modulating ApoA-I gene expression, but also this effect on lipoprotein metabolism could be the indirect result from alterations in microbiota composition (Figure 7.1). In this thesis we focused on both of these possibilities, first by testing the direct effects of antibiotics on ApoA-I transcription and protein secretion. Secondly, we have also investigated the indirect effect by evaluating possible SCFAs dose-response effects on ApoA-I gene expression.

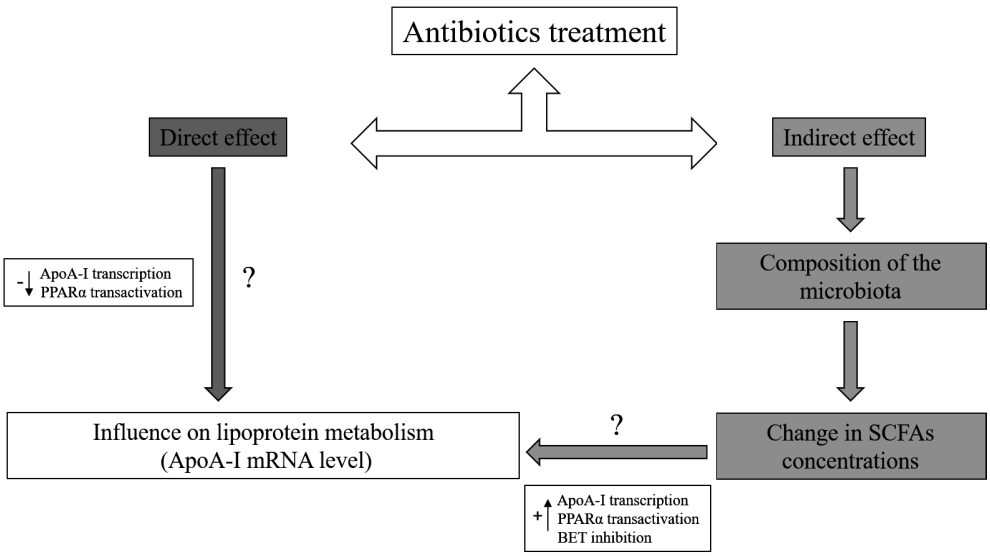

Figure 7.1. Schematic representation of the research questions. The lines with the question marks are unknown relationships and relate to the research questions of our experiments in chapter 2 and chapter 3.

The direct effect of antibiotics on HDL

In our study (**chapter 3**), we evaluated the direct effect of several antibiotics with different working mechanisms on the mRNA expression and protein secretion of genes involved in HDL metabolism in hepatocytes (HepG2 cells) and enterocytes (Caco-2 cells). We found that antibiotics i.e. amoxicillin significantly decreased ApoA-I mRNA expression and protein secretion in both cell lines. In this case, the antibiotic effect on lipoprotein metabolism was a direct effect without interference of a change in microbiota composition, which support our hypothesis that antibiotics elicit direct effects on ApoA-I (HDL) metabolism. Our finding was in line with several studies which had shown that antibiotics induced dyslipidemia in animals and humans. In a recent study, 5 days of amoxicillin treatment significantly reduced HDL cholesterol concentrations by 63% in rats (23). Furthermore, also in tetracycline-treated rats, a significant decrease in HDL cholesterol levels was observed (24). Moreover, Kesaniemi et al. reported that administration of neomycin decreased serum HDL cholesterol concentrations by 16% in slightly hypercholesterolemic men (25). Altogether, these results reveal the association between antibiotics and disturbances in HDL metabolism. However, we cannot confirm here that the antibiotic effects were only a direct (independent of microbiota composition) effect since these findings were obtained from animals or humans. Thus, to exclude the potential impact of microbiota, the direct effect of antibiotics on lipoproteins metabolism could only be observed in cells studies. In agreement with our observation about ApoA-I, antibiotics were found to modulate the expression of genes involved in lipid metabolism in vitro (26). For example, rapamycin treatment downregulated expression of sterol regulatory element-binding protein 1 (SREBP1) and lipin1 in human adipose tissue (26). The underlying processes of these antibiotic-induced HDL lowering effects remained to be determined. Increased cholesteryl ester transfer protein (CETP) activity during amoxicillin treatment has been suggested as a possibility, as this would have resulted in decreased HDL cholesterol concentrations (27). In addition, amoxicillin treatment was associated with induction of cholesterogenesis and phospholipidosis in rat tissues (28), which consequently might result in dyslipidemia. Interestingly, our data added to the current knowledge, that PPARα transactivation was reduced after antibiotic treatment. PPARα, which is known as a target to elevate ApoA-I expression (29), is probably involved in the antibiotic effects on ApoA-I expression and secretion. Further studies are warranted to confirm the role of PPARα in more details, since the link between antibiotics and PPARα has not been studied before.

The indirect effect on HDL via SCFAs alterations

During the last decades, the gut microbiota has been identified as an important factor contributing to metabolic abnormalities in health disorders such as obesity and CVD (30, 31). The gut microbiota composition can be influenced by different factors including dietary composition and antibiotic treatment (32, 33). Several studies have evaluated the potential link between gut microbiota composition with lipid and lipoprotein metabolism. For example, the gut microbiota modulates lipid metabolism and elevates triglyceride levels in adipose tissue of conventionally raised mice (34). Moreover, the gut microbiota is responsible for the conversion of cholesterol to coprostanol in humans (35). The cholesterol conversion to coprostanol and the deconjugation of bile acids by gut microbiota suggest that gut microbiota could affects hypercholesterolemia (35). Therefore, targeting the gut microbiota has been proposed as a promising strategy for lipid disorders treatment (36). Antibiotics affect the density and diversity of the microbiota composition (37). For example, vancomycin administration selectively reduced gram-positive bacteria in mice (38), while amoxicillin treatment also altered the composition of gut microbiota in mice and depleted the gram-positive bacteria (39). The effects of antibiotics on gut microbiota composition in animals and humans also lead to alterations in microbial-derived gut metabolites i.e. SCFAs concentrations (40-42). Reijnders et al. have shown that vancomycin treatment significantly lowered butyrate levels in plasma and feces of obese humans, whereas valerate and hexanoate concentrations were reduced in the fecal samples (43). Furthermore, cefoperazone administration significantly decreased butyrate and propionate concentrations in the cecum of treated mice (44). Altogether, antibiotics influence gut microbiota and SCFAs concentrations. Thus, we speculated that altered SCFAs levels might also play a role in the observed effects of antibiotics on HDL cholesterol concentrations. In our studies using HepG2 cells we evaluated the effects of different SCFAs on ApoA-I transcription (**chapter 2**) to mimic the possibility of indirect effects by antibiotics on lipoprotein metabolism. We found a significant increase in ApoA-I mRNA levels in HepG2 cells after SCFAs treatment. Our findings support the idea that modulation of microbiota composition, which theoretically also changes SCFA levels, could also regulate HDL production. In line with our studies, similar effects of SCFAs were observed in a recent study, in which hamsters received SCFA supplementation, which increased HDL cholesterol levels. To the best of our knowledge, there are no previous studies that evaluated changes in ApoA-I production in response to SCFAs treatment. Therefore, further studies are needed to identify the effects of SCFAs on ApoA-I production in human interventions.

The relation between the dietary fibers and HDL concentrations

SCFAs are generated during fermentation of dietary fibers in the colon (45). One strategy that may elevate SCFA production is via increasing fermentable dietary fiber intake, which may as such be regarded as a novel approach for managing CVD (46). Numerous nutritional studies have explored the relationship between dietary fibers intake and health disorders such as obesity, hypertension and dyslipidemia (47-49). In a cross-sectional study, Zhou et al. showed a dose-response relationship between HDL cholesterol concentrations and dietary fiber intake in human subjects (46). HDL cholesterol concentrations were 10.1% higher, when the daily dietary fiber intake was higher than 30 g/day as compared to fibers intake of less than 18 g/day (46). In an intervention study, Singh et al. showed a significant increase in HDL cholesterol by 8% in hypertensive patients after 12 weeks of guava fruit substitution intake (50). Guava (Psidium guajava L.) is a fruit species rich in fibers, in particular the soluble fiber pectin (51). In another more recent study, Maryanato et al. found that red guava significantly increased HDL cholesterol concentrations by 18% in hypercholesterolemic rats (52). In addition, the red guava reduced serum total cholesterol, LDL cholesterol and triglyceride concentrations (52). Fermentation of the soluble fiber pectin from red guava caused SCFAs production in the colon. For example, propionic and butyric acid concentrations in the caecum were increased after red guava intake in hypercholesterolemic rats (52). Also, pectin fermentation by gut microbiota of Korean individuals induced changes in the microbiota composition, which resulted in an elevated butyrate production (53). Furthermore, Fernando et al. indicated that supplementation of soluble fibers (e.g. pectin) stimulated the formation of acetate in human gut, which could in turn also result in a higher butyrate colonic level (54). However, the question is whether HDL cholesterol or ApoA-I was also increased in response to fibers intake. Unfortunately, several studies showed that high fiber diet did not affect ApoA-I concentrations in humans (55-57). The discrepancy in the effects of dietary fibers on SCFAs versus HDL and ApoA-I production could be attributed to the type of fibers used. Previous studies investigated the effect on ApoA-I of only a limited number of fiber types (16). Therefore, more nutritional human intervention studies to evaluate the effects of other types of dietary fibers on ApoA-I production are required.

The anti-inflammatory effects of SCFAs via NF-κB inhibition

It is well known that inflammation lowers ApoA-I concentrations, since ApoA-I is a negative acute phase protein (58). Therefore, it is challenging to explore the effects of SCFA on ApoA-I expression in HepG2 cells under inflammatory conditions. Thus, we

evaluated the effects of 4 different SCFAs in inflamed HepG2 cells that were simultaneously incubated with pro-inflammatory cytokines (**chapter 4**). It was expected that the pro-inflammatory cytokines would lead to a reduction in the hepatic ApoA-I levels (59) and we hypothesized that SCFAs could rescue the reduced levels of ApoA-I expression during this inflammatory condition. Our study revealed that all SCFAs (except C6) were indeed as hypothesized able to fully recover the decreased ApoA-I levels in the presence of the pro-inflammatory cytokines. In addition, all SCFAs dampened the inflammatory pathway by the inhibition of NF-κB transactivation. In line with the normal condition, the ability of PPARα to enhance ApoA-I mRNA levels was observed also in the inflammatory condition. Previous studies also showed a reduction of NF-κB transactivation after SCFAs treatment. For example, Usami et al. found that SCFAs lowered TNFα production and downregulated NF-κB activation in human peripheral blood mononuclear cells (60). Also, animal studies reported that C4 reduced TNF-α production via preventing NF-κB transactivation in the liver cells of Sprague-Dawley rats (61). The inhibition of histone deacetylase (HDAC) was suggested as a possible underlying mechanism behind SCFAs inhibitory effects on NF-κB (62). HDAC inhibitors have for long been studied in clinical research as potential protective compounds against inflammation (63). Also, HDAC inhibitors have been shown to produce a positive effect on atherosclerosis and CVD (64). Interestingly, the SCFAs propionate and butyrate are indeed well known as HDAC inhibitors (65). The acetylation or deacetylation process of NF-κB lysine residues plays a major role in NF-κB regulation. Thus, HDAC inhibitors might attenuate the inflammation response by modulating NF-κB acetylation levels (66). Moreover, HDAC was found to interact with NF-κB subunits (p50 and p65), which consequently modulates NF-κB transactivation (67). For example, HDAC3 was considered as an activator of NF-κB, based on its role in maintaining the deacetylated state of NF-κB (68). This relationship between HDACs and NF-κB transactivity indicates that the anti-inflammatory effects of SCFAs, which were accompanied with NF-κB inhibition, might be mediated by the inhibition of HDAC. In addition, a previous study indicated that PPARα transactivation might results from HDAC3 inhibition by C4 treatment in the liver tissues of mice (69). In the chromatin immunoprecipitation assay, HDAC3 was found to interact with PPARα at the PPRE response element (69), which suggests that HDAC3 can modulates PPARα transactivation. Altogether, this relationship between HDAC3 and PPARα element supports our hypothesis that SCFAs inhibitory effects on NF-κB are mediated via PPARα transactivation (Figure 7.2).

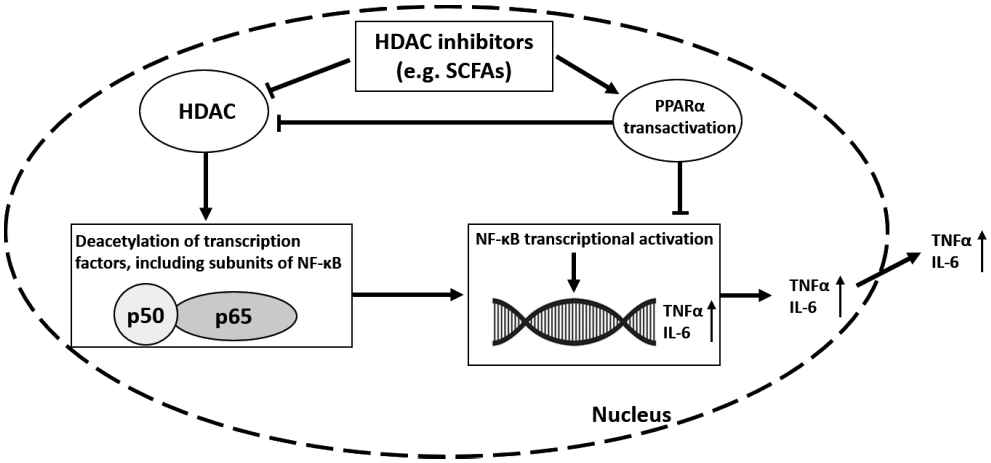

Figure 7.2. Schematic representation of the role of HDAC inhibitors in the regulation of NF-κB transactivity.

The cross-talk between liver and intestine modulates SCFAs effects

SCFAs are physiologically absorbed as nutrients across the intestinal epithelium and then transported into the circulation via the portal system, which explains not only possible intestinal effects but also hepatic and systemic effects (70-72). To verify whether hepatic ApoA-I transcription was modulated via the absorption of SCFAs (i.e. C4) by enterocytes, we investigated the effects of C4 in a co-culture system, including Caco-2 and HepG2 cells (**chapter 5**). Although SCFAs are mainly produced in the colon, it has been shown that SCFAs are also released in the proximal intestine (73). Thus, we decided also to explore the relationship between C4 treatment and intestinal ApoA-I expression in enterocytes when co-cultured with hepatocytes (**chapter 5**). We found that adding C4 to the apical side of the Caco-2 cells increased ApoA-I transcription in HepG2 cells. On other hand, C4 added to the apical side decreased ApoA-I expression in Caco-2 cells. Moreover, the effect of C4 in HepG2 cells in these co-culture experiments was smaller as compared to C4 effects when added directly to the HepG2 cells. We found that the highest effects on ApoA-I expression in the co-culture experiments were observed with nearly double the C4 concentrations that resulted in the optimal ApoA-I expression in single HepG2 experiments. In the co-culture experiment C4 increased ApoA-I mRNA expression up to 2.5 fold at 6 mM, while in the single HepG2 experiment 3 mM of C4 elevated ApoA-I up to 3.0 fold. This observation indicates either a lower bioavailability of C4 that was transported from the enterocytes to liver cells or a cross-talk between intestine and liver cells. Several co-culture studies in line with our study have also shown that compounds added to the apical Caco-2 cells were less

bioavailable at the basolateral compartment. For example, recent Caco-2/HepG2 transwell experiments showed that nitrocatechol concentrations at the basolateral side were lower than apical side concentrations 4 h after of adding the compound to the apical side (74). Moreover, Netzel et al. investigated the transport of carotenes by Caco-2 cell in a transwell culture model (75). Carotenes that were added to the apical culture medium resulted in a lower concentration of carotenes in the basolateral medium compared to the apical medium concentration (75). Together, evaluating effects in the basolateral compartment of co-culture experiments should consider bioavailability of apically added compounds. Interestingly, a human study reported that the physiological concentration of butyric acid in the intestinal lumen was between 1 and 10 mM (76). In our co-culture study, the maximum C4 dose that was added to the intestinal cells was 6 mM, which means that using our SCFAs doses in the clinical application of SCFAs should be feasible. As mentioned before, we speculated that a cross-talk between the intestine and liver might also explain the effects of SCFAs on hepatic ApoA-I. The intestine, as the main site of cholesterol absorption and excretion, plays a major role in the regulation of cholesterol metabolism (77). The imbalance between cholesterol synthesis and excretion requires an extensive cross-talk between intestine and liver (77). Most previous discussions regarding the intestine-liver interaction were focused on the role of bile acids as signal transducing molecules (77, 78). The bile acid could flow from the liver to the small intestine as a result of the high excretion of biliary bile acids after dietary intake (78). Bile acids serve as important messengers between the liver and the intestine, which results in several metabolic consequences (79). However, our co-culture model does not contain bile acids to simulate these effects. Thus, the effect of C4 in our intestine-liver co-culture model should have been modulated by another hypothetical intestinal factor that is secreted in response to SCFAs treatment, as such influencing hepatic physiology. So far, in the previous studies there are no known intestinal factors that are secreted in response to SCFAs exposure. On the other hand, a recent study indicated that a high-fat diet in mice was associated with more transported flagellin from the intestine to the liver (80). Increased hepatic flagellin exposure activated the Toll-like receptor 5 (TLR5) pathway and elevated hepatic ApoA-I transcription (80). In addition, TLR5 activation by flagellin in liver cells modulated ApoA-I production via the NF-κB signaling pathway (80). Further research is warranted to determine whether such intestinal factors (e.g. flagellin) could modulate hepatic ApoA-I expression in response to SCFAs treatment. In line with our previous finding regarding anti-inflammatory effects of SCFAs on ApoA-I (**chapter 4**), we found that C4 also in the co-culture study (**chapter 5**) could rescue the reduced hepatic ApoA-I expression during inflammation. The anti-inflammatory effects of SCFAs in co-culture models were also found in a recent in vitro study using micro-physiological systems (MPS) (81). The apical addition of SCFAs to the enterocytes in a transwell system reduced

the inflammation and improved lipid metabolism in the liver (81). Furthermore, we found that the anti-inflammatory effects of C4 in the co-culture study were significant with the different conditions of added cytokines. The pro-inflammatory cytokines were added to the apical (Caco-2) or to the basolateral (HepG2) or to both compartments, which represent the intestinal or hepatic or systemic inflammation, respectively. For the hepatic ApoA-I, the anti-inflammatory effects of C4 were observed in all of these different inflammatory conditions. In contrast to hepatic ApoA-I, the reduced intestinal ApoA-I expression was not recovered by C4 treatment in all conditions.

The role of amino acids in the metabolic health and ApoA-I production

Enriching our diet with specific functional compounds is an attractive approach to reduce the risk to develop CVD (82). Amino acids as protein building blocks and their dietary sources have earlier been suggested to improve health disorders such as hypercholesterolemia and atherosclerosis (83, 84). Although there is a wealth of data demonstrating the effects of fats and fatty acids on HDL metabolism (85-87), little is known about the contribution of proteins and more specific certain amino acids. In our study (**chapter 6**) we explored the relationship between amino acid supplementation on ApoA-I expression and secretion in HepG2 cells. Interestingly, we found that adding a dose range of amino acids (i.e. leucine, glutamate and tryptophan) was positively associated with ApoA-I mRNA levels. Our observation regarding these positive effects of amino acids on ApoA-I expression was supported by a recent animal study that found that HDL cholesterol concentrations increased by amino acid supplementation. For example, leucine was reported to improve lipid profiles, mainly by increasing HDL concentrations, which attenuated atherosclerosis development in ApoE−/− mice (88). Moreover, in cholesterol-fed rabbits, glutamate and aspartate supplementation also elevated HDL levels and maintained ApoA-1 plasma concentrations (89). Now the question is how to explain these effects of amino acids. In our human liver cells, we showed that all amino acids (except proline) were able to enhance PPARα transactivation which suggest the potential involvement of PPARα-related mechanisms to explain the effects of amino acids on ApoA-I. This was also observed in the previous studies (90). Furthermore, previous study reported that autophagy process can be induced by amino acid deprivation in HepG2 cells (91, 92). Moreover, the amino acid deprivation modulated the mTORC1 signaling pathway, a process regulates ApoA-I synthesis (93). Since our experiments (**chapter 6**) were done during non-essential amino acid (NEAA) starvation in HepG2 cells, this might suggest that mTORC1 signaling pathway

or autophagy process were altered in the HepG2 cells before the tested amino acids were added to the cells. Taken together, we speculated that one option is that amino acid treatment modulated ApoA-I via autophagy inhibition, which actually suggests that the amino acid supplementation effectively diminished the consequences of the absence of NEAA in the medium of the HepG2 cells. Indeed, a recent study confirmed this association between amino acid supplementation and autophagy inhibition in HepG2 cells, which eventually led also to intracellular ApoA-I accumulation (93). Therefore, we suggest to consider these important effects of amino acids deprivation in HepG2 in future experiments. However, this is most likely not the only explanation. Particularly, the fact that not all amino acids increased ApoA-I mRNA or protein concentrations in our hands suggests that it is not only a compensation of the low amino acid conditions. In addition, it is interesting to speculate the possible link between amino acid intake and inflammation. We observed earlier that amino acids reduced IL-8 production and NF-κB activity in HepG2 cells (94). Whether these potential effects of amino acids on the NF-κB pathway, PPARα transactivation and ApoA-I expression are also relevant for the in vivo condition as part of a whole diet approach that already contains a lot of proteins needs further investigation.

The main regulatory pathways of ApoA-I expression and relation to inflammation

In our studies, we evaluated the involvement of pathways that are known to influence ApoA-I transcription (95). We found that the mechanisms of BET inhibition and PPARα transactivation were both involved explaining the positive effects of SCFAs on ApoA-I expression (**chapter 2**), while the PPARα pathway was also associated with the effects of amino acids on ApoA-I (**chapter 6**). Overall, our findings show the pivotal role of PPARα transactivation in ApoA-I expression. PPARs are transcription factors, members of nuclear hormone receptors group which regulate the expression of several genes in metabolic pathways (96). These interactions between the dietary components and PPARα transactivation can be applied clinically in dyslipidemia conditions because of its ability to improve HDL and lipoprotein metabolism (97, 98). PPARα is an important player in lipoprotein metabolism mainly due to the presence of a binding place for PPAR elements in the promoter or enhancer regions of ApoA-I gene (99). When natural ligands such as SCFAs or amino acids bind to PPARα, this forms a heterodimer with the retinoid X receptor (RXR), which results in activation of PPARα and binding to specific response elements (PPRE) within the promoter region of ApoA-I gene (100). This increases the expression of ApoA-I and downregulates the expression of Apo-CIII, which eventually

elevates HDL production (101). Several studies, in line with our experiments, reported a positive correlation between PPARα and SCFAs or amino acids treatment in other models or cell systems. For example, the incubation of SCFA (butyrate) for 24 h increased PPARα expression in human endothelial cells (102). Also, amino acid (leucine) treatment for 24 h has been shown to induce PPARα expression in skeletal muscle myotubes (90). In addition, we also found that PPARα was involved in the anti-inflammatory effects of SCFAs in inflamed HepG2 cells (**chapter 4**). The role of PPARα in the inflammatory process has been discussed in great detail in several reviews (103-105). PPARα exerts anti-inflammatory effects (105, 106) probably via negatively regulating the NF-κB pro-inflammatory pathway (107). This observation supports our findings (**chapter 4**), in which SCFAs treatment increased PPARα transactivation and reduced NF-κB transactivation in HepG2 cells. We also found that SCFAs improved the expression of ApoA-I during inflammation, which goes hand in hand with the inhibitory effects of SCFAs on NF-κB, which reveals the potential link between ApoA-I levels and the NF-κB pathway (108). Morishima et al. found that the inhibition of NF-κB increased ApoA-I secretion in HepG2 cells. This increase was blocked by a PPARα inhibitor (MK886) suggesting that ApoA-I secretion was largely regulated by PPARα transactivation (109). In addition, the plasma ApoA-I level was higher in NF-κB-deficient mice compared to control animals (109). As mentioned before, our findings in inflamed HepG2 cells (**chapter 4**) showed that not only PPARα transactivation, but also BET inhibition was involved in the observed effects on ApoA-I in the inflammatory condition. BET inhibition also elevated PPARα transactivation in inflamed HepG2 cells since JQ1(+), a BET inhibitor, increased CPT1 (a PPARα target gene) expression in the inflammatory condition, while BET inhibition did not affect NF-κB transactivation (Figure 7.3).

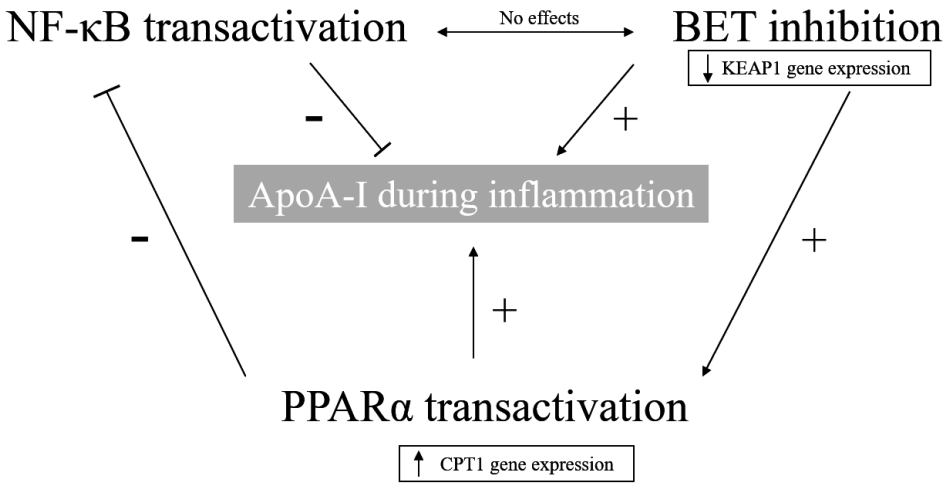

Figure 7.3. Schematic overview of the main regulatory pathways of ApoA-I during inflammation.

Some striking observations regarding ApoA-I expression in HepG2 and Caco-2 cells

SCFAs did not affect ApoA-I expression in differentiated Caco-2 cells

Although SCFAs are mainly produced in the colon, they can also be found in the small intestines (73). Therefore, to discover the effects of SCFAs on intestinal ApoA-I transcription, we performed our SCFAs experiments not only in HepG2 cells but also in the differentiated Caco-2 cells. In contrast to the liver cells, we found that different SCFAs did not affect ApoA-I mRNA transcription in the intestinal cells (Figure 7.4). Theoretically, SCFAs are available in the intestinal lumen in higher concentrations compared to the hepatic portal system (71, 110). Thus, we decided also to test the effects of SCFAs on the intestinal Caco-2 cells in a wider range with higher doses. Despite the fact that we used higher doses (up to 25 mM) in Caco-2 cells experiments compared to HepG2 cells experiments (up to 7 mM), we still did not observe any positive effects on intestinal ApoA-I expression in response to SCFAs treatment. This latter finding in Caco-2 cells was observed also in our co-culture experiments (**chapter 5**), in which C4 treatment also did not increase intestinal ApoA-I

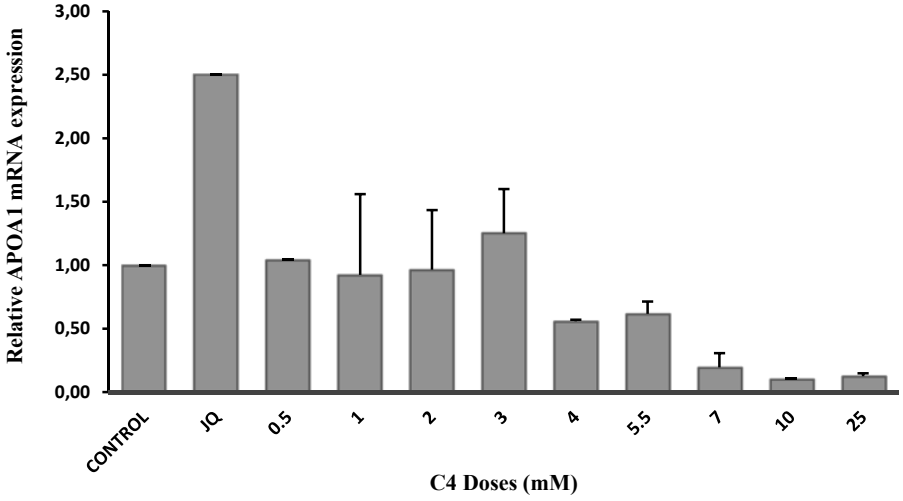

Figure 7.4. Relative ApolipoproteinA-I (ApoA-I) mRNA expressions in Caco-2 cells treated with different concentrations of C4 or with the positive control JQ1(+) (3 μM). Increasing C4 concentrations showed a significant reduction in ApoA-I mRNA expression in Caco-2 cells, the regression coefficients was significantly different from zero ($r = 0.538$; $p = 0.001$). JQ1(+) significantly increased ApoA-I mRNA expression in Caco-2 cells ($p < 0.05$). All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against the expression observed in the control condition, which was arbitrarily set at 1. A linear regression was performed for C4 dose–response effects. Changes were considered significant when the regression coefficients were significantly different from zero ($p < 0.05$). A Mann–Whitney U test was performed to evaluate the effect of JQ1(+) versus control condition, in which a p-value < 0.05 was considered statistically significant.

expression in the intestine-liver co-culture model. The possible explanation of this different response of ApoA-I to SCFAs treatment between hepatic and intestinal cells might be attributed to additional tissue-specific regulatory players like for example the co-activator apolipoprotein CIII (ApoCIII) (111). In transgenic mice (C57BL/6), it was shown that the interaction between ApoA-I promoter and ApoCIII enhancer was needed to induce intestinal ApoA-I expression, while this was not necessary for the hepatic ApoA-I expression (111). Thus, the observed effect of SCFAs on ApoA-I expression in HepG2 cells could be independent of ApoCIII enhancer. On the other hand, the positive control JQ1(+) was able to increase the intestinal ApoA-I expression, which suggests that JQ1(+) simultaneously affects the ApoCIII enhancer.

SCFA incubation without serum in culture medium negatively affects ApoA-I expression

In our in vitro studies, we observed that SCFAs treatment reduced ApoA-I expression when using higher doses (5.5 and 7 mM), even though there was a significant elevation of ApoA-I expression with lower SCFAs doses. We tried to explain the reason behind these inhibitory effects on ApoA-I expression. Previous studies have demonstrated that the composition of the cell culture medium such as the fetal bovine serum (FBS) affects HepG2 cells growth and phenotype characteristics (112, 113). Since we speculated that the effects of SCFAs would be more visible when there were no fatty acids present in the medium, our SCFAs experiments in HepG2 cells were always performed in absence of FBS. Moreover, to test the possible effect of FBS-free medium on ApoA-I expression in HepG2 cells, we evaluated the effects of C4 under two different experimental conditions, with and without added FBS (Figure 7.5). Interestingly, the inhibitory effect that was observed when using high C4 concentrations, largely disappeared when the HepG2 cells were cultured in medium containing FBS. The question now is how to explain this observation. For this, there can be numerous reasons since FBS not only delivers more energy but also contains a wide variety of different compounds (114). Regarding the energy content, it is known that gene regulation is associated with cell energy metabolism (114). Therefore, it could be that the need of HepG2 cells for the energy that is delivered by the serum (FBS) is responsible for the higher levels of ApoA-I in response to the higher doses of SCFAs. Alternatively, it could also be due to a potential interaction between components in FBS and the added SCFAs, which could result in a higher mRNA ApoA-I expression at higher SCFAs concentrations. Taken together, we conclude that the positive effect of SCFA (i.e. C4) on ApoA-I expression was clearly present in absence of FBS, but when applying higher doses of C4, the energy content from FBS or specific elements from FBS are needed to maintain the higher ApoA-I expression.

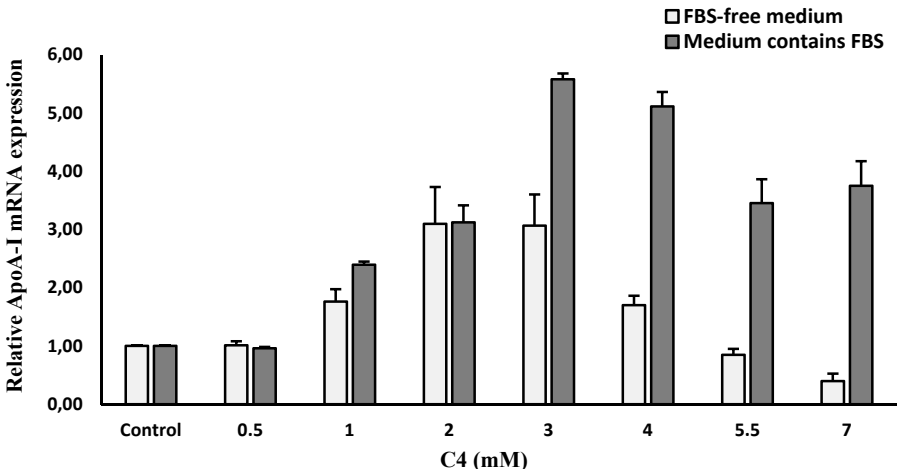

Figure 7.5. Relative ApolipoproteinA-I (ApoA-I) mRNA expressions in HepG2 cells treated with different C4 concentrations. High C4 concentrations (5.5 and 7 mM) showed a significant reduction in ApoA-I mRNA expression when HepG2 cells were cultured with FBS-free medium ($p < 0.01$). High C4 concentrations (5.5 and 7 mM) significantly increased ApoA-I mRNA expression when HepG2 cells were cultured with medium containing FBS ($p < 0.01$). All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against the expression observed in the control condition, which was arbitrarily set at 1. A Mann–Whitney U test was performed to evaluate the effects of C4 higher doses (5.5 and 7 mM) versus control condition, in which a p-value < 0.05 was considered statistically significant.

SCFAs acetic and salt forms produce the same effects on ApoA-I expression

Cell culture experiments using SCFAs can be performed either by using the salt form or the acetic form. In our experiments, we always used the acetic forms of SCFAs. However, also the sodium salt of C4, which is called sodium butyrate, was used in a number of SCFAs studies (115-117). To assure that the positive effects of SCFAs on ApoA-I transcription as observed were also present when the SCFAs were given in their salt form, we compared the effects of both C4 forms on ApoA-I mRNA expression side-by-side. Interestingly, both sodium butyrate as well as our acetic form of C4 significantly increased ApoA-I expression (Figure 7.6). Therefore, our finding indicates that both SCFAs forms are able to modulate ApoA-I transcription in the same direction.

The limitations of this thesis studies

It is good to consider some limitations related to the in vitro studies described in this thesis. First, HepG2 cells are a liver cell line derived from an oncologic patient, and they are different from the primary liver tissue in a number of characteristics, which could have had an impact on function and behavior of the cells (118). Also, we only used one type of

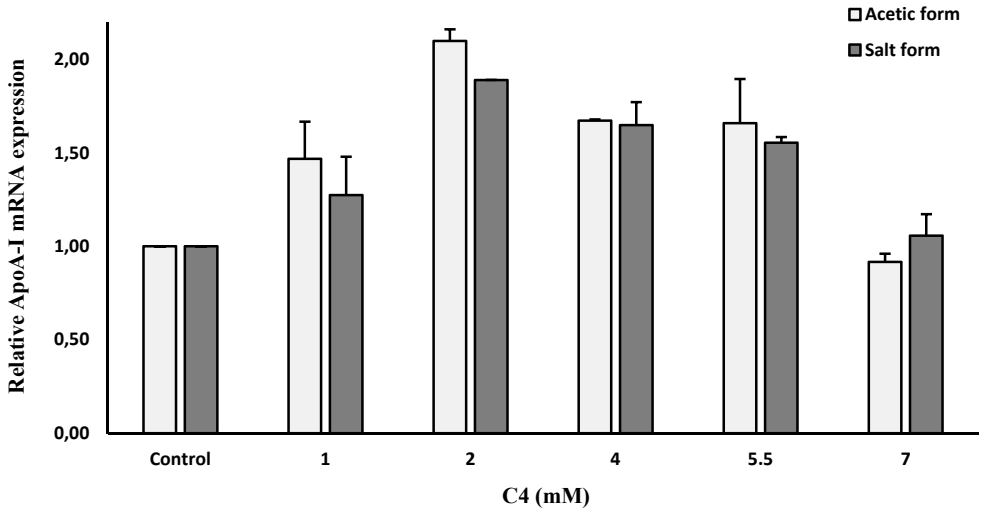

Figure 7.6. Relative ApolipoproteinA-I (ApoA-I) mRNA expressions in HepG2 cells treated with C4 different concentrations. Increasing concentrations of the acetic form of C4 significantly increased ApoA-I mRNA expression, the regression coefficients was significantly different from zero ($r^2 = 0.808$; $p = 0.001$). Increasing concentrations of the salt form of C4 also significantly increased ApoA-I mRNA expression, the regression coefficients was significantly different from zero ($r^2 = 0.795$; $p = 0.001$). No significant differences were observed between the effects of the acetic form of C4 and the effects of the salt form of C4 on ApoA-I mRNA expression. All results are presented as the mean, while error bars indicate standard deviations. Data were normalized against the expression observed in the control condition, which was arbitrarily set at 1. A quadratic polynomial regression was performed to determine dose-response relationships. Changes were considered to be significant when the regression coefficients are significantly different from zero ($p < 0.05$). In addition, when evaluating the effects of the acetic form of C4, versus the salt form of C4, the Mann–Whitney U test was used, in which a p-value < 0.05 was considered statistically significant.

liver cells (HepG2) and one type of enterocytes (Caco-2), while there are also other hepatic and intestinal cells that could have been used to verify the results. Therefore, it is a valid question whether the observations can be translated as such to the human situation. It would have been a possibility to work with organoids which are a bit closer to the human situation. Moreover, FBS was not added to the culture medium in some of our experiments, and cells were depleted in amino acids in some other experiments, which definitely affected the physiological functions of the cells (119). Therefore, although these conditions can be explained by a scientific model system approach, it needs to be verified whether the effects will be identical in vivo. Moreover, data linking high fiber intake as well as protein intake and ApoA-I concentrations in human studies is largely lacking. Although this might relate to the type of fibers and the source of protein used, this data is warranted to make our in vitro findings relevant. Finally, another limitation in our studies is the use of TNFα and IL-1β as a cytokine cocktail to induce inflammation in our cell systems. This means that we can only tell that SCFAs rescue the inflammation induced effects on ApoA-I and not whether

this is generic for all types of inflammation. Altogether, our finding regarding the effects of SCFAs and amino acids on ApoA-I in vitro forms the basis for future in vivo testing to facilitate translation to clinical outcomes.

Main conclusions and future recommendations

In summary, this thesis has contributed to an improved understanding of antibiotics, SCFAs and amino acids in relation to ApoA-I mRNA expression in human liver cells. We confirmed that antibiotics, SCFAs and amino acids can modulate ApoA-I, the main protein of HDL, which is inversely associated with the development of atherosclerosis. A summary of our research objectives, methods, main results and corresponding conclusions is given in Table 7.1. We found that amoxicillin reduced ApoA-I mRNA expression and protein secretion in HepG2 and Caco-2 cells. This direct effect of amoxicillin on ApoA-I expression was in line with earlier clinical observations, indicating that antibiotic treatment lower serum HDL cholesterol concentrations. Moreover, our findings demonstrated that the effect of antibiotics on ApoA-I can be also an indirect effect via alterations in SCFA concentrations possibly due to changes in microbiota composition. We showed that ApoA-I mRNA expression was increased in response to SCFAs treatment in HepG2 cells. In addition, PPARα transactivation pathway is probably involved in the positive effects of SCFAs on ApoA-I expression. Furthermore, it is known that ApoA-I expression is lowered in the inflammatory conditions since ApoA-I is a negative acute phase protein. We observed that SCFAs inhibit the NF-κB pro-inflammatory signaling pathway, most likely also mediated via PPARα transactivation, which translates into a rescue of ApoA-I expression in inflamed liver cells. How these findings compare to the human situation, more specific whether fiber consumption affects SCFAs production and consequent HDL concentrations, either in normal or inflammatory conditions, warrant further studies. We recommend to explore in future research in more details which fibers are most efficient in producing SCFAs that affect ApoA-I synthesis in the human situation. Our observation that besides the SCFAs also amino acids have the capacity to elevate ApoA-I mRNA expression and/or pro ApoA-I protein production provides insights for future studies. We provided a novel insight with the finding that amino acids (leucine, glutamic acid and tryptophan) can increase ApoA-I expression in HepG2 cells, again probably via involvement of PPARα transactivation. Further research is needed to fully understand the underlying process, in which the role of mTORC1 signaling pathway seems interesting to be explored in more details.

Table 7.1. Overview of the most important findings and conclusions

	Objectives	Methods	Results	Conclusions
Chapter 2	Evaluate the effects of SCFAs on hepatic ApoA-I expression, secretion, and the underlying pathways	- SCFAs dose-response experiments - qPCR: ApoA-I, PPARα, KEAP1 and CPT1 - PPARα transactivation assay in HepG2 cells - ELISA: ApoA-I	- SCFAs increased ApoA-I, PPARα, CPT1 expression and decreased KEAP1 expression - SCFAs increased PPARα transactivation - SCFAs reduced ApoA-I protein secretion	- SCFAs were found to stimulate hepatic ApoA-I expression - BET inhibition and PPARα transactivation were identified as mechanisms affect ApoA-I expression
Chapter 3	Examine the effects of antibiotics on ApoA-I transcription and secretion, and the underlying mechanisms	- Antibiotics dose-response experiments - qPCR: ApoA-I, KEAP1, CPT1 and CHOP - PPARα transactivation assay in HepG2 cells - ELISA: ApoA-I	- Amoxicillin decreased ApoA-I transcription and secretion - Amoxicillin decreased KEAP1, CPT1, and CHOP expression - Amoxicillin lowered PPARα transactivation	- Amoxicillin was found to reduce ApoA-I transcription and secretion - PPARα transactivation is a potential mechanism behind amoxicillin effects on ApoA-I expression
Chapter 4	Evaluate the effects of SCFAs on the inflammatory pathways in relation to ApoA-I transcription	- SCFAs dose-response experiments in normal and inflamed HepG2 cells - qPCR: ApoA-I, KEAP1, CPT1, c-Jun and c-Fos - NF-κB transactivation assay in HepG2 cells - ELISA: IL-8	- Except for C6, SCFAs increased ApoA-I transcription and lowered IL-8 secretion in both normal and inflammatory conditions - C4 increased CPT1 transcription in both conditions - SCFAs inhibited NF-κB transactivation	- C3, C4 and C5 elicit anti-inflammatory effects which might rescue ApoA-I transcription in inflammatory conditions - SCFAs anti-inflammatory effects induced via PPARα transactivation mediated NF-κB inhibition
Chapter 5	Investigate C4 anti-inflammatory effects on ApoA-I transcription in the intestine-liver co-culture model	- C4 dose-response experiments in the presence or absence of cytokines in a co-culture system including Caco-2 cells and HepG2 cells - qPCR: ApoA-I	- C4 decreased ApoA-I expression in Caco-2 cells - C4 increased ApoA-I expression in HepG2 cells - C4 rescued ApoA-I expression in inflamed HepG2 cells	- C4 added apically to Caco-2 cells elevates hepatic ApoA-I transcription and rescues the lower ApoA-I expression in the inflamed HepG2 cells - Intestinal SCFAs can affect hepatic processes - The smaller effect in the co-culture experiment indicates a cross-talk between intestine and liver
Chapter 6	Explore the effects of amino acids on ApoA-I transcription, pro-ApoA-I and ApoA-I protein secretion, and the underlying mechanisms	- Amino acids dose-response experiments in HepG2 cells - qPCR: ApoA-I, KEAP1 and CPT1 - ELISA: pro-ApoA-I and ApoA-I	- Leucine, glutamic acid and tryptophan increased ApoA-I expression - Glutamine, proline and histidine increased pro ApoA-I concentrations - Except for Proline, all tested amino acids increased CPT1 expression	- Different amino acids (except Proline) modulate ApoA-I expression or secretion, predominantly via PPARα transactivation - The chemical properties of amino acids might modulate their ability to induce ApoA-I mRNA expression

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8

Summary

Summary

Cardiovascular diseases (CVD) are the leading cause of death in the western countries. Dyslipidemia, one of the modifiable CVD risk factors, is defined as having high plasma low-density lipoprotein (LDL) cholesterol and triacylglycerol concentrations or low levels of serum high-density lipoprotein (HDL) cholesterol. Indeed, several epidemiological studies have shown an inverse relation between HDL cholesterol levels and CVD risk. Surprisingly, recent clinical studies however showed that increasing serum HDL cholesterol concentrations by pharmacological interventions failed to reduce CVD risk. Thus, the focus has switched to improving HDL functionality instead of elevating serum HDL cholesterol concentrations. Increasing evidence suggests that HDL functionality plays a major role in inflammation and atherogenesis. For example, HDL particles promote reverse cholesterol transport (RCT), a mechanism by which accumulated cholesterol is transported from the arterial walls to the liver for excretion, which consequently reduces atherosclerosis development. Apolipoprotein A-I (ApoA-I), the major protein component of HDL particles, has been identified as an important player in explaining HDL functionality and RCT. Therefore, approaches that target to elevate ApoA-I is a promising strategy to reduce CVD risk. In this thesis we therefore focused on the effects of antibiotics, short-chain fatty acids (SCFAs) and amino acids on ApoA-I transcription. In addition, we evaluated the effects of SCFAs on ApoA-I expression in a co-culture model by culturing enterocytes and hepatocytes together in a transwell system.

In an earlier clinal trial, the plasma HDL cholesterol concentration was significantly reduced after antibiotic (amoxicillin) treatment. This finding raised the question whether this HDL cholesterol lowering effect by amoxicillin was a direct effect on lipoprotein metabolism or an indirect effect resulting from alterations in the composition of microbiota and consequent SCFAs concentrations. To investigate the potential direct effect, we examined the effects on ApoA-I transcription when different concentrations of various antibiotics were added to the HepG2 and Caco-2 cells (**chapter 3**). In line with the clinal trial, we found that amoxicillin significantly reduced both ApoA-I mRNA expression and protein secretion in HepG2 and Caco-2 cells. We also examined potential mechanisms underlying these direct effects of antibiotic on ApoA-I expression. We found that amoxicillin treatment inhibited PPARα transactivation, which was confirmed by a lowered mRNA expression of CPT1, a well-known PPARα target gene. Therefore, we concluded that antibiotic treatment can directly affects lipoprotein metabolism via inhibiting ApoA-I transcription and secretion. In **chapter 2**, we examined the potential indirect effect of the antibiotics on HDL metabolism by evaluating dose-response relationships between the different SCFAs with ApoA-I transcription. Interestingly, increasing SCFAs concentrations significantly increased ApoA-I

mRNA expression in HepG2 cells. This increase was associated with the enhanced PPARα transactivation, which indicates that the PPARα pathway is most likely involved in the positive effects of SCFAs on ApoA-I mRNA expression. These findings further suggest that the effects on serum HDL cholesterol concentrations as observed in our human intervention study with amoxicillin could be due to a combination of direct inhibitory effects on ApoA-I transcription as well as indirect effects via reduced SCFAs production. Moreover, previous studies have suggested that ApoA-I concentrations are reduced during inflammation, since ApoA-I is a negative acute phase protein. To evaluate effects of SCFAs on ApoA-I expression in the inflammatory condition, we performed our SCFAs experiments also in inflamed HepG2 cells (**chapter 4**). In line with the normal condition, SCFAs also exerted their beneficial effects on ApoA-I during inflammation. Moreover, SCFAs were able to recover the reduced level of ApoA-I expression in the inflamed HepG2 cells up the normal level, and even increased ApoA-I expression at higher concentrations. In addition, SCFAs treatment inhibited NF-κB transactivation and lowered IL-8 secretion from the inflamed HepG2 cells. Our data suggest that PPARα transactivation also underlies these anti-inflammatory effects of SCFAs in the inflamed HepG2 cells. Although SCFAs are produced mainly by microbiota in the colon, they are also produced in lower amounts in the small intestine. It is known that the intraluminally produced SCFAs are taken up by the intestinal cells and used as energy source. However, a part of the SCFAs is transported to the liver via the portal vein. In addition, their uptake by enterocytes might trigger the secretion of factors that could be transported from the enterocytes to the liver and affect hepatic processes. To study such a possible cross-talk between intestine and liver on hepatic ApoA-I expression, we evaluated the effect of C4 on hepatic ApoA-I mRNA expression in a co-culture system including Caco-2 and HepG2 cells (**chapter 5**). The positive effects of SCFAs on hepatic ApoA-I expression were also observed in these co-culture experiments after adding C4 apically to the intestinal cells. However, the effect of C4 on hepatic ApoA-I expression in this study was smaller than the effect of C4 when it was added directly to the liver cells. This latter finding indeed suggests a lower bioavailability of C4. Also, it might be the result from a cross-talk between the intestine and liver cells. In that situation the enterocytes should secrete an inhibitory factor to dampen hepatic ApoA-I expression. Finally, since some studies have suggested that amino acids influence PPARα transactivation, we explored in **chapter 6** the effects of different amino acids on ApoA-I expression in HepG2 cells. Interestingly, leucine, glutamic acid and tryptophan increased mRNA expressions of both ApoA-I and PPARα. Therefore, amino acids could be interesting targets for further studies to increase ApoA-I production.

In summary, the research in this thesis was performed to get more insights into the effects of external factors like antibiotics, SCFAs and amino acids on hepatic and intestinal ApoA-I

transcription. Besides the negative effect of antibiotic on ApoA-I transcription and secretion, our findings have shown that SCFAs have positive effects on ApoA-I expression in HepG2 cells in both normal and inflammatory conditions. Moreover, C4 treatment also increased hepatic ApoA-I mRNA expression in the intestine-liver co-culture model. Finally, different amino acids were identified as natural compounds that have the potency to transactivate PPARα and consequently increased hepatic ApoA-I expression.

9

Impact paragraph

Social and economic relevance

In this section, the potential impact of our scientific findings for society and economy will be described. This mostly concerns the possible future applications of our findings in specific target groups, i.e. subjects with risk factors for cardiovascular diseases (CVD) such as dyslipidemia. Despite the fact that treatment has improved tremendously over the past decades, CVD remains the leading cause of morbidity and mortality worldwide (1). The most recent world health organization (WHO) report indicates that more than 17.9 million persons die from CVD each year. Currently, the number of newly diagnosed CVD cases in Europe is more than 11 million yearly, which results in a high economic burden and concomitant health care related costs of around €210 billion per year (1). Clearly, there is a need to discover and use additional therapies on top of already existing portfolios to further reduce the risk of CVD development and the high costs of CVD-related medical care. Although some studies showed conflicting results about the link between elevating circulating high-density lipoprotein (HDL) cholesterol concentrations and CVD (2), recent experimental studies have shown that CVD can be reduced by the enhancement of HDL functionality (3). The main function of HDL is to transport the excess cholesterol from peripheral tissues to the liver for excretion, a process called a reverse cholesterol transport (RCT) (4). Recent evidence suggested that increasing RCT process protects against CVD and atherosclerosis (5). Therefore, targeting factors known to stimulate RCT, such as increasing apolipoprotein A-I (ApoA-I) synthesis (6), is a promising strategy to reduce the progression of atherosclerosis development and consequent CVD. Although there are already some pharmaceutical options to increase ApoA-I production in specific high risk patient populations (7), dietary recommendations that aim to increase ApoA-I and HDL functionality may be needed to prevent CVD. Therefore, in our studies we aimed to discover the potential contribution of some (dietary natural) compounds on ApoA-I production with the ultimate aim to lower CVD risk and progression, and the financial costs of current CVD treatment. Thus, the main goal of this thesis was to explore the effects of several external stimuli on ApoA-I transcription and secretion. We focused on the effects of SCFAs, antibiotics and amino acids to understand underlying mechanisms and to generate leads for future CVD treatment and prevention.

Scientific gain of this thesis

The research in this thesis supported our previous observation from a placebo-controlled double blind human intervention trial showing that antibiotic treatment was linked to alterations in metabolic biomarkers, amongst others a reduction in serum HDL cholesterol

concentrations (8). Our in vitro studies described in this thesis were the first showing that antibiotic treatment could directly influence ApoA-I transcription and secretion in human liver and intestinal cells. Moreover, in line with several human and animal studies (9-12), we revealed favorable effects of SCFAs (linking to potential indirect effects of antibiotic treatment) as well as specific amino acids on hepatic ApoA-I transcription. Our findings further provide a mechanistic insight underlying the effects of these compounds on ApoA-I transcription. Sharing these research findings within the scientific community helps to evolve our understanding of the underlying processes and the regulatory factors involved in ApoA-I transcription and to develop strategies targeting ApoA-I transcription via dietary approaches. Therefore, it is important that the data presented in this thesis is available to the (scientific) public through publications in international scientific journals. Also, our preliminary results were presented at national and international conferences that support the development of fundamental knowledge and created the possibility to discuss the findings with several experts from the field. Altogether, this stimulates that the leads we presented here can also be further developed by others. This thesis was funded by a scholarship granted by university of Jeddah, a leading governmental institution of higher education in Saudi Arabia. This implies that the collaboration between the university of Jeddah and Maastricht university contributes to obtain new international scientific perspectives, transfer knowledge between research institutions and to gain new insights into CVD treatment and prevention.

Translation into clinical application

The studies described in this thesis are in vitro experiments using different cell lines, which means that our observations cannot be directly used as clinical applications. First, our findings need to be confirmed in additional (in vivo) experiments. In other words, this research alone is not enough to adapt the dietary guidelines for fiber or protein intake or to recommend any dietary adjustments for the food industry. If our findings could be confirmed in human interventions, i.e. elevating the uptake of specific SCFAs and/or amino acids effectively increased ApoA-I and HDL functionality, this will be of high relevance for nutritional interventions targeting ApoA-I. However, more studies are needed to identify the most effective dietary fibers (that need to be processed into the optimal SCFAs by our microbiota) or proteins (that need to contain the optimal amino acids composition), thereby stimulating similar effect as reported in our in vitro experiments. Into more detail, after the confirmation of these positive findings in human studies, the intake of SCFAs or amino acids could be used by health care professionals as a dietary recommendation to

prevent the development of CVD in general, or for example as adjunct therapy for patients treated with antibiotics or by the food industries for the development of novel functional foods.

To conclude, this thesis provides a first insight into the potential role of SCFAs and amino acids on ApoA-I transcription and secretion in human cell line models. Our finding can be used to design future human interventions to investigate the effects of SCFAs and amino acids in clinical applications. Finally, these positive effects might contribute to the development of new nutritional strategies to improve the HDL functionality and subsequently to reduce CVD risk.

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Abbreviations

ABCA1	ATP-binding cassette transporter A1
AP-1	Activator protein 1
ApoA-I	Apolipoprotein A-I
BET	Bromodomain and Extra-Terminal domain
Caco-2	Human colonocytes
cDNA	Complementary DNA
C/EBP-β	CCAAT/enhancer binding protein β
CETP	Cholesteryl ester transfer protein
CPT1	Carnitine palmitoyl transferase 1a
CVD	Cardiovascular diseases
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked sandwich immunoassay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
HDL	High-density lipoprotein
HDAC	Histone deacetylase
HepG2	Human hepatocytes
IL-1β	Interleukin 1 beta
IL-8	Interleukin 8
KEAP1	Kelch like ECH associated protein 1
LCAT	Lecithin cholesterol acyltransferase
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MEM	Minimum essential medium
mRNA	Messenger RNA
NF-κB	Nuclear factor kappa B
PPARα	Peroxisome Proliferator Activated Receptor Alpha
PPRE	PPAR response element
q-PCR	Quantitative polymerase chain reaction
RCT	Reverse cholesterol transport
RXR	Retinoid X receptor
SCFAs	Short-chain fatty acids
SREBP1	Sterol regulatory element-binding protein 1
Thap	Thapsigargin
TG	Triglyceride
TLR5	Toll-like receptor 5
TNFα	Tumor necrosis factor-α

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Curriculum vitae
List of publications

Curriculum vitae

Jehad Zuhair Tayyeb was born on December 3rd, 1985 in Birmingham, United Kingdom. He was raised in Jeddah, Saudi Arabia and completed his bachelor's (MBBS) degree in medicine and surgery at King Abdulaziz University in 2011. During undergraduate studies he enrolled in several internships in internal medicine, surgery, pediatrics, obstetrics-gynecology, emergency medicine, ophthalmology, psychiatry and radiology in King Abdulaziz University hospital. He continued his education in the Netherlands, where he obtained his master's degree in Biomedical Sciences at Maastricht University in 2016 at the Faculty of Health, Medicine and Life sciences. In 2016, Jehad started his PhD at Maastricht University within NUTRIM School of Nutrition and Translational Research in Metabolism under the supervision of Prof. Dr. Jogchum Plat, Prof. Dr. Ronald Mensink and Dr. Herman Popeijus. The research that was performed during this PhD project is described in this dissertation, entitled "the effects of antibiotics, short-chain fatty acids and amino acids on Apolipoprotein A-I transcription and synthesis in normal and inflamed HepG2 and Caco-2 cells". During his PhD, Jehad has presented his work as oral presentations in national symposiums (The Dutch Nutritional Science Days, NSD from 2016 to 2019, Heeze) and international conferences (European Atherosclerosis Society Congress, EAS 2019, Maastricht).

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