The anti-inflammatory effects of short chain fatty acids on human endothelial and lung epithelial cells

- Ferment your fruits and vegetables to battle NCDs!

Meng Li

(李梦)

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Science is not and never will be a book. Each of the major achievements will bring new problems. Any development with the passage of time there will be new and serious difficulties.

-- Albert Einstein

科学决不是也永远不会是一本写完了的书。每一项重大成就都会带来新的问题。任何一个发展随着时间的推移都会出现新的严重的困难。

--爱因斯坦

The anti-inflammatory effects of short chain fatty acids on human endothelial and lung epithelial cells

- Ferment your fruits and vegetables to battle NCDs!

De anti-ontstekingseffecten van korte-keten vetzuren

op endotheelcellen en luchtwegepitheel

- Fermenteer je fruit en groenten voor het bestrijden van niet overdraagbare ziekten!

(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1

Thesis aims and outline

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Our body is attacked continuously by a variety of dangerous signals and an effective balanced immune system is essential to recognise and respond to these. Unbalanced immune reactivity plays a key role in so-called non-communicable diseases (NCDs). NCDs are medical conditions or diseases not caused by infectious agents (non-infectious or non-transmissible). NCDs can refer to chronic diseases which may last for long periods of time and progress slowly and may be linked to aging and the result of a combination of genetic, physiological and environmental factors. The main types of NCDs are cardiovascular diseases, chronic respiratory diseases such as chronic obstructive pulmonary disease (COPD), asthma and respiratory allergy, cancers and diabetes [1]. According to the World Health Organization (WHO), the growing burden of NCDs currently poses the greatest threat to global health in both developed and developing countries. NCDs related diseases were recently estimated to be responsible for >60% of deaths worldwide, indicating their importance in the wider public health agenda. As mentioned above, the reasons for the increase in NCDs are multifactorial and include changes in lifestyle, diet, hygiene, microbiome, industrial technologies, infections, *etc.* which together have altered our exposome. Given the aim of WHO global health task force to decrease 25% in NCDs associated mortality before 2025, we need a better understanding of the underlying mechanisms of NCDs.

Since chronic low-grade inflammation is a common feature of all NCDs, reducing the risk of inflammatory responses might be a promising approach regarding both prevention and treatment [2]. Currently, one highly promising candidate to influence the immune system and manage inflammatory processes in a proper way is microbiome modulation. Microbiome modulation can be achieved via different approaches such as the addition of probiotics (defined as viable microorganisms, in sufficient amounts to reach the intestine in an active state and thus exert positive health effects), prebiotics (defined as a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastro-intestinal

microflora that confers benefits upon host well-being and health), synbiotics (defined as a synergistic combination of pro and prebiotics) and more recently postbiotics (the metabolites or fragments produced and/or released by microbes) [3]. Short chain fatty acids (SCFA), fermentation products of non-digestible dietary fibre by microbiota in the intestine, are recognized as an example of postbiotics and receive attention for their anti-inflammatory and immune modulatory properties [4]. This thesis will mainly focus on the effects of SCFA (which regulate inflammatory responses in endothelial cells and modulate the epithelial barrier function in lung epithelial cells in certain NCDs) on cardiovascular diseases and chronic respiratory diseases.

The estimated annual mortality attributable to cardiovascular diseases will approach 25 million worldwide by 2030 [5]. One possible contributor to cardiovascular diseases is localized infection, leading to increased systemic inflammation and impaired endothelial function [6, 7]. Endothelial cells are involved in maintaining homeostasis [8] and can be activated by endogenous cytokines (such as TNFα) or endotoxins (LPS) [9]. Endothelial cell activation is a central component of the vascular inflammatory process that promotes the expression of leukocyte adhesion molecules (such as ICAM-1 and VCAM-1), the secretion of pro-inflammatory cytokines (such as IL-6), chemokines (such as IL-8) and increased immune cells adhesion to the endothelial layer [10] which are two important steps in the initiation and development of atherosclerosis [11].

Chronic respiratory diseases especially COPD are incurable and treatments can only control the symptoms, causing more than 3 million deaths each year (6% of all deaths worldwide) [12]. Studies indicate that damage to the barrier function of the airway epithelial layer enhances mucosal permeability in the airways of patients with COPD, asthma and pulmonary hypertension [13, 14]. The airway epithelium is involved in the protection of the host against inhaled pathogens, allergens and other noxious substances. However, the airway epithelium is more than just a structural barrier. Epithelial cells are also the first cells to interact with inhaled antigens and other harmful substances that can result in increased secretion of cytokines and recruitment of immune cells

into the airways [15]. Damage to epithelial cells causes barrier dysfunction, leading to a further increase in their permeability and the development of respiratory diseases [16].

Meanwhile, epidemiological evidence indicates that increased consumption of dietary fiber reduces systemic inflammation and risk of cardiovascular diseases, immune disorders and pulmonary dysfunction [17-19]. This might be due to the production of SCFA. SCFA, mainly acetate, propionate and butyrate, are metabolites of the fermentation of non-digestible carbohydrates by the anaerobic intestinal microbiota [20]. In recent studies, SCFA indeed showed promising potential therapeutic effects in cardiovascular diseases [21]. However, the mechanisms involved in the effects of SCFA are completely unknown. Furthermore, the effect of SCFA, on LPS or TNFα-induced IL-6 and IL-8 production by primary human umbilical vein endothelial cells (HUVEC), is also unknown, and reports of their roles in adhesion molecule expression are contradictory [22-24]. Moreover, although it is known that barrier dysfunction of the airway epithelium contributes to the development of allergies, airway hyperresponsiveness and respiratory diseases, and that SCFA can enhance and restore the barrier function of the gut epithelium, it is not known whether SCFA are able to protect and/or enhance the barrier function of airway epithelium, nor which mechanisms are involved. In general, SCFA are known to (1) activate G-protein coupled receptors (GPCRs, GPR41 and GPR43, also known as FFA3 and FFA2 receptors) and (2) inhibit histone deacetylases (HDACs) [25]. In this thesis, the effects of SCFA were investigated on isolated cells, in particular endothelial and epithelial cells and the mechanisms involved.

Aims

The main aim of this thesis is to get a better understanding of the effects of SCFA on epithelial dysfunction and LPS- and TNFα-induced endothelial activation and the roles of GPR41/43 and HDACs.

The content of each chapter is described briefly below:

Chapters 2 and 3

Chapter 2 provides a general overview on SCFA, including the synthesis, transportation, metabolism and excretion of SCFA and addresses their potential in preventing and treating cardiovascular disorders, including metabolic imbalances, atherosclerosis and heart diseases. Chapter 3 focuses on the role of GPR41/43 and HDACs in the effects of SCFA on stimuli-induced activation of immune and endothelial cells and the possible mechanisms involved.

Chapter 4

Endothelial cell activation can be induced by TNFα or LPS treatment and is characterized by an increased production of pro-inflammatory cytokines and enhanced expression of adhesion molecules [10]. In this chapter, we investigate three important factors involved in the anti-inflammatory properties of SCFA (acetate, propionate and butyrate) on endothelial cell activation, namely (1) different pre-incubation periods with SCFA, (2) different concentrations of SCFA, and (3) different stimulation periods with either TNFα or LPS.

Chapter 5

Two possible targets for SCFA [25] are GPR41/43 and HDACs, and based on the data collected in chapter 4, the optimal experimental conditions were selected to test whether GPR41/43 and HDACs are involved in the anti-inflammatory effects of SCFA on endothelial activation by using specific antagonists for GPR41/43 and an inhibitor of HDAC.

Chapter 6

IL-33, a novel member of the IL-1 family of cytokines, is constitutively expressed in the nuclei of endothelial cells [26] and is modulated by HDACs [27]. Nuclear IL-33 controls inflammatory responses by regulating the activation of NF-κB [28, 29]. However, the role of endogenous IL-33 in the effects of SCFA is still unclear. Therefore, this chapter investigates the role of

endogenous IL-33 in the effects of butyrate and propionate on TNFα-induced endothelial activation.

Chapter 7

Barrier dysfunction of the airway epithelial layer contributes to the development of chronic respiratory diseases [30, 31]. While SCFA are able to enhance and restore the barrier function of intestinal epithelium [32], their effects on lung epithelial cells are unknown. The purpose of this study was to investigate whether SCFA can influence the barrier function of the airway epithelium and can regulate expression of tight junction proteins by modulating MAPK signaling pathways.

Chapter 8

This chapter summarizes the main findings described in this thesis and proposes a future direction of investigations needed for a better understanding of the effects of SCFA on inflammation in the cardiovascular system and its barrier function in the respiratory system. These may influence the development of new dietary concepts and may open new avenues for both protective and therapeutic targets.

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Chapter 2

The effects of short chain fatty acids on the cardiovascular system

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*Adapted from Richards et al. *PharmaNutrition* 2016; 4: 68.

ABSTRACT

The development of cardiovascular diseases is often attributable to loss of endothelial functions of the vascular tissue or to decreased contractile function of the heart muscle. These disturbances are often caused by imbalances in lipid and glucose metabolism. These imbalances can result in a low-grade inflammatory state of affected endothelial tissue, causing macrophages and fat-rich lipoproteins to accumulate in the subendothelial space. Short-chain fatty acids (SCFA) feature a regulatory function in the cell metabolism of fatty acids, glucose and cholesterol in various peripheral tissues, both directly as well as at a genetic level. In addition, the strong expression of SCFA receptor FFA2 on various leukocyte populations facilitates regulatory effects of the fatty acids on various functions of these immune cells. The immune-regulatory effect and influence on lipids, cholesterol and glucose metabolism of SCFA can thus contribute to the development of metabolic conditions that promote preservation or recovery of endothelial functions and thereby reduce the risk of development or aggravation of cardiovascular diseases. The current review addresses the effects of SCFA on the human cardiovascular system and investigates potential novel interventions for prevention and treatment of cardiovascular disorders using these fatty acids.

1. Introduction

Western societies find themselves increasingly confronted with typical nutrition-related diseases, such as obesity and type 2 diabetes mellitus. These diseases often lead to increased risks for the development of several forms of cardiovascular complications [1]. Short-chain fatty acids (SCFA) are saturated fatty acids featuring an aliphatic chain length of at most eight carbon atoms. The fatty acids are formed by the colonic gut flora from dietary fibres, which manage to escape the host's enzymatic digestive systems in the small intestine. Dietary fibres that manage to reach the large intestine are available for several bacterial fermentative reactions. The fermentation of the different dietary fibres leads to an increase in concentrations of several short-chain fatty acids, especially butyrate, propionate and acetate, in the lumen of the proximal regions of the large intestine [2-4]. The fermentative reactions serve to deliver energy for preservation of the bacterial species and facilitate the excretion of strongly oxidised fatty acids. Thereafter the fatty acids can be absorbed by the enterocytes of the intestines, where they can be further oxidised for the production of energy [5-10].

The beneficial effects of SCFA on inflammatory diseases of the intestinal tract and some forms of colon cancer are known for some time. It appears that SCFA are able to regulate the inflammatory reactions that contribute to the manifestation of these diseases. They can therefore counteract or even prevent the development of such disorders [11-13]. SCFA can also be absorbed by the enterocytes forming the intestinal wall, whereupon they can be used for the production of energy [8, 11, 14]. However, a portion of the absorbed fatty acids will not be consumed and can be released via the basolateral membrane to the hepatic portal vein and will reach the systemic circulation after initial liver passage [15, 16]. Lesser known are the beneficial effects SCFA achieve in the prevention and treatment of various cardiovascular diseases after being absorbed in the circulation [17-19]. In spite of a strong concentration reduction of the fatty acids due to the consumption by enterocytes combined with the ability of the liver to clear

large fractions of these acids, even small blood concentrations appear to induce positive effects [20, 21]. The Food and Drug Administration (FDA) recently acknowledged the claims regarding the use of SCFA in the prevention of cardiovascular disease [22].

Cardiovascular events are often the result of loss of endothelial function of the blood vessels or reduced contractility of the cardiac muscle. Disturbance of both functions can often be ascribed to disruptions in lipid and glucose metabolism and generally appear in obese patients or patients who suffer from diabetes [23-25]. After reaching the systemic circulation, SCFA prove to be able to regulate the metabolism of various peripheral tissues both directly and on a genetic level. Additionally, butyrate and propionate appear to have anti-inflammatory effects on several immune cells that could be involved in the development of, for example, atherosclerosis [26-28]. This review illustrates the effects of SCFA on the human cardiovascular system and subsequently gives an inventory of intervention options in the prevention and treatment of cardiovascular diseases.

2. Synthesis of short-chain fatty acids

In people of Western societies, approximately 20 to 60 grams of the daily intake of carbohydrates evades the digestive and absorptive system of the small intestine and manages to reach the colon [2]. The large intestine houses a large and complex bacterial ecosystem containing around 10^{10} up to 10^{11} cfu per gram wet weight. Having wet weights that vary typically between 250 and 750 grams, this implies a total bacterial population close to 10^{13} cfu of the final section of the gastrointestinal tract [29]. The population comprises at least 400 different bacterial species, of which about 99 percent is anaerobic [2, 29, 30].

Nutrients that manage to evade the digestive system of the small intestine and enter the large intestine are potential substrates for the bacterial metabolism in the latter organ. The anaerobic bacteria form SCFA by degrading these substrates by means of anaerobic respiration and fermentative reactions [2-4]. This leads primarily to the production of the

fatty acids butyrate, propionate and acetate. In addition, some gasses are generated, such as methane, carbon dioxide and hydrogen [5, 31, 32]. These reactions are executed by bacteria to generate energy and release carbon to warrant microbial growth and preservation of the bacterial specie. However, a fraction of the end products can also be deployed by the host [5-10]. Especially mammals appear to be able to absorb the synthesized SCFA from the colonal lumen and generate energy by oxidation of these substrates. In western society, the contribution of SCFA to the total energy requirement turns out to be much lower. People that are subjected to a Western pattern diet obtain typically 5 to 10 percent of their total energy need by the oxidation of these fatty acids [33].

In spite of the limited contribution of SCFA to the total energy requirements of humans, the main purpose of the oxidation of SCFA by the colonal mucosa is the production of energy. Herewith, there appears to be a preference for the oxidation of butyrate, followed by propionate and in a lesser extent for acetate [34]. Colonocytes even seem to prefer the degradation of butyrate to that of glucose, as it has essential features for the preservation of the colonocytes themselves [11]. As a matter of fact, the metabolism of butyrate by the colonocytes yields sufficient energy to prevent autophagocytosis; a mechanism involving cell degeneration in order to meet energy requirements [14]. However, a fraction of the SCFA is not used for energy production in the colonocytes. This portion of the fatty acids is transported over the intestinal epithelium towards the hepatic portal vein [15, 16]. The mechanisms of intracellular transport towards the systemic circulation will be addressed in upcoming paragraphs.

2.1 Substrates

Microbial growth and preservation of the bacterial population in the large intestine strongly depend on the supply of suitable substrates for fermentation and anaerobic respiration. As indicated before, substrates need to pass the small intestine that normally decomposes and absorbs a large fraction of these nutrients. Nutrients that are not degraded by the local

digestive system are able to reach the more distal areas of the intestine and can be divided into two different classes [35-37]. The first class comprises of non-digestible nutrients that cannot be degraded as the digestive system lacks endogenous enzymes needed for the decomposition of these substances [38]. The second class of substrates consists of resistant nutrients which manage to escape the digestive system in the host's small intestine [39]. Both nutrient classes are available for the bacterial metabolism in the large intestine [16].

SCFA are mainly produced from poly-, oligo- and fructo-oligosaccharides, proteins, peptides and glycoprotein precursors by anaerobic micro-organisms [2, 40]. The fermentation of carbohydrates turns out to be most important mechanism for the production of SCFA, as the major part of the bacteria in the large intestine is saccharolytical. Therefore, the bacterial population in the large intestine has a large decomposing capability for this type of substrate [16, 37]. As a result of the vast variety of micro-organisms in the colon, a strong competition for these complex, non-digestible carbohydrates exists [3]. This is confirmed by analyses of the genome of several commensal gut bacteria the large intestine houses. Some bacterial species, such as Bifidobacterium longum and Bacteroides thetaiotaomicron which feature a genome consisting of 2.26 and 6.26 megabases respectively, dedicate at least 8 percent of their genome to the transport and the metabolism of complex carbohydrates [41, 42]. When comparing these species with Escherichia coli, which has a genome comprising of 4.64 megabases, B. longum and B. thetaiotaomicron prove to be able to synthesize respectively two times and nine times as much enzymes for the breakdown of carbohydrates [42].

Another factor that can contribute to the fermentation of primarily, non-degraded carbohydrates is the rich supply of the polysaccharide fraction of plant cell wall material. This fraction cannot be decomposed by the host's digestive system, which enables large amounts of this substrate to reach the colon out of the ileum [2, 10, 43].

2.2 Major fermentation products

Acetate, propionate and butyrate are the main end products of fermentative reactions. Even though other SCFA are produced, they are generally involved in cross-feeding that eventually results in the conversion of these fatty acids into one of the three earlier mentioned main end products [44].

2.2.1. Acetate formation

Two different pathways exist for the formation of acetate. First of all, acetate can be generated by decarboxylation of pyruvate to acetyl-CoA, which is then converted to acetate by means of hydrolysis [5]. Secondly, the Wood-Ljungdahl pathway can also be utilized for the production of acetate. In the latter pathway carbon dioxide is reduced to carbon monoxide, after which the resulting carbon monoxide reacts with both a methyl group and a coenzyme A molecule to form acetyl-CoA. Hereafter, similar to the first pathway, hydrolysis can be used to form acetate from acetyl-CoA [45].

2.2.2. Propionate formation

Propionate can be synthesized following two different pathways. The first pathway is via the use of a primitive anaerobic electron transport chain. At low carbon dioxide partial pressures, succinate is converted into methylmalonate and then split into prorpionate and carbon dioxide. The generated carbon dioxide can then be reused for the carboxylation of phosphorenolpyruvate [46, 47].

The second mechanism is based upon the acrylate pathway where pyruvate is firstly reduced to L-lactate by the amphibolic lactate dehydrogenase. It concerns a reversible reaction. In the reduction one molecule NADH is oxidised to NAD^+, after which L-lactate can subsequently be reduced to lactyl-CoA, acrylyl-CoA, propionyl-CoA and finally propionate [48].

It has to be noted that both pathways lead to more re-oxidation of NADH to NAD^+ when compared to singular reduction of pyruvate to lactate.

2.2.3. Butyrate formation

The production of butyrate starts with the condensation of two acetyl-CoA molecules resulting in acetoacetyl-CoA. This molecule is then subsequently reduced to L(+)-hydroxybutyryl-CoA, crotonyl-CoA and finally butyryl-CoA [5]. From this molecule butyrate can be synthesized in two different ways. The first mechanism implies direct conversion of butyryl-CoA to butyrate using the enzymes phosphotransbutyrylase and butyrate kinase [49].

The alternative mechanism involves the use of butyryl-CoA and exogenously produced acetate that are converted into butyrate and acetyl-CoA by butyryl-CoA: acetate-CoA transferase [50].

3. Transport of short-chain fatty acids

Once SCFA are synthesized by the microbial gut flora, these products become available for absorption by the intestinal epithelium. SCFA are subject to oxidation by colonocytes for energy production. Physiological studies of ruminants have shown that SCFA can be readily absorbed by the epithelial cells of their intestines [51]. Investigation of the absorption of acetate, propionate and butyrate in human epithelial cell lines also confirms that colonocytes can easily absorb these fatty acids across their apical membrane, after which a decrease of the intracellular pH can be observed. This is followed by a sodium-independent pH recovery [35, 43, 52-54]. Subsequently, the absorbed SCFA are metabolized into glucose, ketone bodies and amino acids [11, 55]. In this process the substrates undergo multiple oxidations forming strongly reduced compounds. Colonocytes appear to prefer the oxidation of butyrate into CO_2 to the oxidation of propionate and acetate, independently of the presence of other SCFA [34]. However, epithelial metabolization of the SCFA is incomplete, which implies that some of these fatty acids are able to pass through the basolateral membrane, whereupon they can reach the liver and the systemic circulation [11, 21, 55, 56].

In the past 35 years a lot of research has been performed regarding colonic transport mechanisms of short-chain fatty acids. Charting these mechanisms is hampered by two different aspects of the fatty acids. Firstly, SCFA form a major substrate for the metabolism of colonocytes, which complicates investigation of applicable transporters. To circumvent this problem, many studies use SCFA that are metabolized to a lesser extent, such as propionate and isobutyrate. Another problem is the existence of equilibria between the luminal, basolateral and intracellular compartments of the intestinal epithelium. SCFA are weak acids, implying equilibria between the protonated and deprotonated form in all these compartments. This means that small changes in pH strongly affect the relative distribution of SCFA and consequently hinders investigation of absorption mechanisms [51].

In the next paragraphs, the mechanisms underlying the transport of SCFA across cell membranes of the colonocytes will be addressed.

3.1 Apical transport

In vivo research has shown that SCFA are easily absorbed at the apical membrane, in which the absorption approximates the colonic sodium absorption (300 mmol), hence presumably nearing the maximum absorptive capacity for SCFA [51]. It is assumed that two separate transport mechanisms are involved in the apical absorption of these acids. The fatty acids are weakly acidic compounds featuring a pKa value of approximately 4.8. The pH value in the lumen of the large intestine ranges from 5.5 to 6.5. This implies the existence of an equilibrium between the protonated and deprotonated state of the SCFA, wherein 90 to 99 percent of the compounds will be present in the deprotonated form. The protonated form is uncharged and non-polar allowing easy diffusion through colonic cell membranes. The deprotonated form carries a negative charge and is therefore unable to pass the lipophilic cell membranes, thus requiring absorption by transporters [51]. Research has found that the neutral, protonated form is subject to non-ionic diffusion in the weakly acidic environment of the lumen [57-60].

The second transport mechanism deploys integral transmembrane proteins, which act as transporters for the negatively charged, deprotonated fatty acids. Several research groups were able to identify various anion transporters for the absorption of SCFA involving carrier-mediated transport from the lumen [60-63].

3.2 Non-ionic diffusion

As has been indicated before, SCFA are weak acids featuring a carboxyl group with a pKa value of about 4.8. This means that only a small fraction will be protonated and thus will be available for non-ionic diffusion in the lumen of the large intestine. For this type of transport it is essential that fatty acids are present in the neutral, protonated form in order to diffuse through the lipophilic, apical membrane of the colonocytes [51]. This also means that the absorption of the protonated SCFA improves with an increase of the chain length these fatty acids as the non-polar fraction of these molecules increases correspondingly [64]. In spite of the strong concentration gradient that exists between the lumen and the serosa of the colon, paracellular transport hardly occurs. For this reason, research was mainly conducted to the mechanisms concerning transcellular transport by means of non-ionic diffusion [51]. In investigations using acidified mucosa samples from sheep and rabbits a larger absorption of SCFA could be observed. Therefore, it appears that SCFA predominantly diffuse to compartments in which they are more ionized due to higher pH values [59].

Hence, transcellular transport by means of non-ionic diffusion requires SCFA molecules to be present in the protonated form. The intestinal epithelium is able to deliver protons to the lumen of the large intestine enabling protonation of the fatty acids. The proximal region of the colon is rich with sodium-hydrogen antiporters that facilitate the epithelial release of protons. However, protons can also be generated by the epithelium via hydration of CO_2 into H_2CO_3, a weak acid, using the carbonic anhydrase enzyme [35].

It appears that an increase of released protons by the intestinal epithelium can result in larger absorption of fatty acids. Accordingly, stimulation of the

sodium-hydrogen antiporters in the proximal region of the intestine results in an increased acidity of the lumen as well as a larger absorption via non-ionic diffusion. Also, an opposite effect was observed in the research. Inhibition of colonic sodium-hydrogen antiporters with amiloride or theophylline leads to a reduced absorption of SCFA [65]. A similar relation could be observed with stimulation or inhibition of the H^+/K^+ ATPase in the distal regions of the colon [63].

3.3 Transporters

In addition to the uptake through non-ionic diffusion, the colonocytes are also capable of absorbing the deprotonated and thus negatively charged fraction of SCFA by using transmembrane proteins [60-63]. In molecular characterization studies particular plasma membrane vesicles from both the apical and basolateral membrane were investigated [66, 67]. From these studies it was possible to identify three different transmembrane protein families, which contribute to the uptake of anionic short-chain fatty acids.

3.3.1. $SCFA^-/HCO3^-$ transporter

In perfusion studies of the colon, wherein SCFA were administered, a relation was found between luminal secretion of bicarbonate (HCO_3^-) and the administration of these fatty acids [35, 68-70]. Further research into the SCFA-dependent bicarbonate secretion revealed that it concerns most likely an apical $SCFA^-/HCO_3^-$-exchange, that predominantly takes place in the superficial cells of the colon [71, 72]. This first anion-transporter family has been further examined using apical membrane vesicles and was shown to occur on the apical membrane of the human ileum and colon. The transporters are antiporters that link the uptake of SCFA to the secretion of bicarbonate into the lumen of the large intestine[66, 67]. The absorption of SCFA by the antiporter was shown to be independent of the Cl^-/HCO_3^- antiporter of the colonocytes and of both the luminal and intracellular sodium concentration [62, 63, 66, 67, 73]. In particular propionate and butyrate were found to be taken up well by a Na^+-independent $SCFA^-/HCO_3^-$ transporter [66, 67].

3.3.2. Monocarboxylate transporter

The second type of transporter constitutes a family of transporters for monocarboxylates and is synthesized from the SLC16 gene family [74, 75]. The uptake of SCFA from the lumen is possible as the transporter catalyses co-transport with hydrogen ions (H^+). The monocarboxylate transporter (MCT) acts as a symporter and enables electroneutral absorption of the negatively charged, deprotonated fatty acids as the negative charge is compensated by co-transport with this cation [76]. Apart from SCFA, these transporters can also transport lactate and pyruvate [77, 78]. To date, fourteen isoforms of the monocarboxylate transporter have been identified [79].

3.3.3. Sodium-dependent monocarboxylate transporter

The final transporter that contributes to the apical uptake of SCFA is SMCT1. In 2004, it was discovered that gene SLC5A8, which belongs to the solute-linked co-transporters SLC5 gene family, encodes for a Na^+-coupled symporter for SCFA lactate, pyruvate, acetate, propionate and butyrate [80-83]. Gen products belonging to this family transport substances including glucose, myo-inositol, iodide, choline and B-vitamin complexes [84]. The substrate specificity for SLC5A8 shows a strong resemblance to that of MCT36, but in contrast to the latter transporter, SMCT1 co-transport involves sodium ions rather than protons. This difference has implied that the protein product encoded by the SLC5A8 gene is known as the sodium-dependent monocarboxylate transporter 1 (SMCT1) [85].

3.4 Basolateral transport

The fraction of the SCFA that is not metabolized by the colonocytes after apical absorption can leave the colonocytes at the basolateral side and reach the portal vein [11, 21, 55, 56]. Because the intracellular pH-value is much higher than the pH-value that prevails in the lumen of the large intestine, absorbed SCFA, having carboxylic acid group featuring a pKa value of around 4.8, are present in the deprotonated, negatively-charged form. Since the

molecules in this form are strongly polar, no diffusion can take place through the lipophilic membrane, which implies that basolateral transport of the fatty acids requires the use of transporters [51].

Research using basolateral membrane vesicles from rat colonocytes has demonstrated the existence of a basolateral $SCFA^-/HCO_3^-$ transporter [86]. The transport process of the anion symporter is dependent on the pH gradient and the intracellular bicarbonate concentration and shows a transport direction opposite to that of the apical $SCFA^-/HCO_3^-$ transporter [56] [51]. Research indicates that the receptor can be inhibited by 1 mM nuflumic acid, but is insensitive to inhibition by stilbene derivatives, DIDS, SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate and acetazolamide [56]. However, these results are not consistent; research on this transporter by Reynolds *et al.* [86] did actually show inhibition of the transporter on the basolateral membrane vesicles when applying 1 mM DIDS and acetazolamide. Possible explanations for the inconsistent results are differences in the cell lines used or the occurrence of MCTs on the basolateral membrane that are insensitive to inhibition by DIDS [87].

Also, it is likely that the $SCFA^-/HCO_3^-$ transporter found on the basolateral membrane differs from the transporter that is present on the apical membrane of the colonocytes. Kinetic analysis of the uptake activity of butyrate by the two transporters using a Lineweaver-Burk plot provided K_m values of the apical and basolateral transporter being 1.5 mM ± 0.2 mM and 17.5 mM, respectively [56]. Further characterization studies are required in order to identify molecular differences.

Using immunoblotting, the presence of MCT isoforms 3 (SLC16A8), 4 (SLC16A3), and 5 (SLC16A4) could be demonstrated on the basolateral membrane of colonocytes. However, MCT3 is only present in such low concentrations on the basolateral membrane that is unlikely that this isoform significantly contributes to the transport of SCFA into the portal vein [88].

Fatty acids and protons are transported by MCT4 on the basolateral membrane in the opposite direction relative to that of MCT1. Similar to MCT1

on the apical membrane, this transporter uses electroneutral transport and thereby depends on cationic hydrogen ions for co-transport. However, this transporter has a lower affinity for these substrates than MCT1 [140].

MCT5 also appears on the basolateral membrane of colonocytes, but it is unclear whether this transporter is capable of transporting SCFA through this membrane. For clarification further molecular identification of this isoform is necessary [78, 88].

4. Metabolism and excretion of SCFA

SCFA are metabolized at three different locations in the body [89]. First of all, the fatty acids that are absorbed by the colonocytes are partly used for the production of energy [89] [51]. However, the metabolism of these cells is incomplete and a fraction of the fatty acids will therefore have the opportunity to pass through the basolateral membrane in unaltered form [11, 21, 55, 56, 90]. After passing this membrane, these substances will enter the portal vein, which flows into the liver. Entry of the SCFA into systemic circulation requires passage through the liver in unmetabolized form. Then, the peripheral muscle tissue can metabolize these unaltered fatty acids for energy production [89].

However, the liver is specialized in converting endo- and exogenous substances into easily excretable compounds. To this end, two different reactions occur in the hepatocytes of this organ. The first type of reaction is known as Phase I reactions and makes use of a highly diverse and complex system of cytochrome P450 enzymes. This enzyme system handles the biotransformation of a wide variety of substances [91, 92].

The second type is known as phase II reactions and is based on the conjugation of, whether or not biotransformed, conjugates with anionic substrates, such as sulphate, glutathione, glucuronate, and other anionic, polar groups. The transformed compounds can then be released by the liver into the bile, whereafter they leave the body through the stool. Another

option is the release of the metabolites into the systemic circulation, after which they become available for excretion through the kidneys [91, 92].

First, the substrates must be absorbed by the hepatocytes in order to facilitate these metabolic reactions. To this end, they must pass through the sinusoidal membrane of these cells [91, 92]. Recently, two different transporters have been identified on this membrane which facilitate the absorption of fatty acids propionate and butyrate; OAT2 and OAT7 respectively [21, 93]. Both transporters belong to the sodium-independent, multi-specific organic anion transporters (OAT), a subgroup consisting of eight isoforms of the solute carrier family 22 (SLC22) gene family [94, 95]. The SLC22 gene family comprises several plasma membrane proteins, including organic cation transporters (OCT), organic zwitterion / cation transporters (OCTN), wherein in particular the organic anion transporters are involved in the hepatic and renal excretion. Additionally, the OAT-subfamily plays a major role in renal reabsorption and distribution of organic anions through the body, in drug-drug interactions and toxicity of anionic substances, such as uremic toxins and nephrotoxines [21, 96].

It should be noted that there is still a lot of research conducted into the transporters involved in the uptake of SCFA. Only recently the two aforementioned plasma membrane proteins have been identified [21, 93]. Other research shows that fatty acid acetate may be absorbed from the portal vein by the hepatocytes, but the mechanism has not been elucidated as of yet [97].

4.1. Metabolism by the liver

The three SCFA that can evade the metabolism of the colonocytes of the large intestine to a significant degree and reach the portal vein are acetate, propionate and butyrate. Subsequently, these fatty acids will have to pass through the liver in order to enter the systemic circulation. During the passage of the liver, these substances are metabolized to various degrees by the hepatocytes.

Acetate in the systemic circulation has two origins. Exogenous acetate is formed in the fermentation of dietary fibre by the bacteria present in the colon. Moreover, endogenous generation of acetate occurs in different tissues and organs, after which the produced acetate is released into the blood [98, 99]. Research has shown that the liver is capable of metabolizing approximately 70% of the supplied acetate to other substances [97]. The hepatocytes use the acetate not only as an energy source, but also as a substrate for the formation of acetyl-CoA from acetoacetate, long chain fatty acids and β-hydroxybutyrate [100]. It is also used as a co-substrate for the formation of glutamate and glutamine. The remaining acetate is oxidised in the periphery, such as the adipose tissue, the heart, the kidneys and muscle tissue [97, 99]. In the presence of ethanol in the blood, it appears that less oxidation of acetate occurs in the hepatocytes and degradation mainly takes place in the peripheral tissues [101].

In order to avoid high, possibly toxic concentrations of propionate and butyrate in the systemic circulation, the liver clears a large portion of these fatty acids [102, 103]. Concentration measurements of propionate in the portal and hepatic vein indicate that approximately 30% of the propionate is taken up by the liver. The unaltered portion is then extensively metabolised in the periphery. Assessments of the propionate concentrations in the peripheral veins yielded values that were found to be 23% lower than the concentrations that were measured in the hepatic veins [15, 97]. Propionate features an odd number of carbon atoms and is therefore oxidised to propionyl-CoA by propionyl-CoA synthetase in hepatocytes. This oxidation product is then subsequently converted in three steps to succinyl-CoA by proprionyl-CoA carboxylase, methylmalonyl-CoA epimerase and methylmalonyl-CoA mutase. Succinyl-CoA can then participate in the citric acid cycle, in which oxaloacetate is formed. Oxaloacetate can be converted by the liver into glucose and can therefore contribute to gluconeogenesis in this organ as a precursor molecule [20, 21, 104, 105].

As butyrate is preferred by the colonocytes for their metabolism, only a small fraction of the butyrate produced in the colon is able to leave the colonocytes through the basolateral membrane. As a consequence, butyrate is only found in low concentrations of around 28.8 µM in the portal vein [104]. As stated earlier, both butyrate and propionate may be toxic, when they exist in high concentrations in the systemic circulation [102, 103]. The liver appears to be able to absorb and oxidise virtually all of the butyrate from the portal vein, causing low butyrate concentrations in the systemic circulation. In the hepatocytes the butyrate molecules are first converted to butyryl-CoA by butyryl-CoA synthetase and then to acetyl-CoA [20, 21].

The acetyl-CoA molecules, which are produced by the hepatic oxidation of acetate and butyrate, can participate in the citric acid cycle to generate NADH and ATP [21]. During fasting, acetate and butyrate can be used for the formation of ketone bodies and can thus contribute to the ketogenesis in the liver [20, 106].

4.2. Excretion

As discussed above, many of the SCFA are converted in the hepatocytes into precursor molecules of the gluconeogenesis, aerobic respiration and ketogenesis and thereby obtain a new function in the energy metabolism of the body. However, in the peripheral tissues and the colonocytes SCFA are oxidised to generate energy. Here, the SCFA are subject to multiple oxidation reactions to form the metabolic end-product CO_2. To date, research on the excretion of the metabolic end-products of the oxidation by these tissues is not available. Possibly, respiratory excretion CO_2 takes place, involving blood for the transport to the lungs. It is not to be ruled out that a fraction of the CO_2 together with water molecules is converted to the weak carbonic acid (H_2CO_3) by potentially present carbonic anhydrase. The corresponding conjugate base (HCO_3^-) might be cleared by the kidneys.

5. Effect of SCFA on cell metabolism

SCFA appear to have a regulatory function in the cell metabolism of fatty acids, glucose and cholesterol. Recently, it has been discovered that the SCFA can act as ligands for a variety of receptors that mediate these regulatory effects. Besides, SCFA are found to be important for the regulation of immune responses by the immune system in the microbial environment of the large intestine [26-28]. The effects expressed by SCFA on healthy cardiovascular tissue and cardiovascular diseases are largely due to modulation of the metabolism of lipids and glucose. This section discusses applicable receptors and how these regulatory effects are mediated.

5.1. Receptors

The identified receptors, wherein SCFA act as a ligand, belong to the G protein-coupled receptor (GPCR) gene family. GPCRs form on of the biggest gene families that could be identified in the human genome [107]. To date, it was possible to characterize approximately 160 receptors from this gene family, which are synthesized from 125 genes. The receptors of which the function and substrates have not been described as of yet, are called *orphan* GPCRs [82].

Recently, it was possible to chart a subset of these orphan GPCRs. The GPR40 subfamily appears to have four members, whose coding genes are all located on chromosome 19q13.1 locus [82]. They appear to feature at least 30% similarity in amino acid sequences [108]. Studies with BLAST searches of the DNA and protein sequence revealed that this receptor subfamily shows a strong resemblance to the protease-activated receptor GPCR subfamily. However, the long N-termini, which serve as substrates for the proteases for activation and that are characteristic of these receptors, are absent in the four members of the GPR40 subfamily. Therefore, it is plausible that these receptors, GPR40 to GPR43, are activated by a different mechanism [108]. Using ligand fishing strategy, wherein the orphan GPCRs are expressed in yeasts, it was shown that GPR41 and GPR43 were activated by short-chain carboxylates in a specific an dose-dependent manner [108, 109]. Structure-

activity studies also revealed that the carboxyl group of the SCFA is required for the activation of these receptors. Aldehydes, ketones, alcohols and esters of fatty acids were found to be totally ineffective in this respect. The activation of the GPCRs requires that the carboxyl group of the SCFA is positioned at the end of the aliphatic chain. Furthermore, this chain must be composed of no more than six carbon atoms [110]. The optimum chain length for the activation of GPR43 appears to span two to three carbon atoms (acetate and propionate) and ranges from three to five atoms (propionate and butyrate) in the case of GPR41. SCFA featuring deviating lengths up to six carbon atoms are also able to activate these receptors; however, activation occurs to a lesser extent [108, 110]. The presence of two or more carboxyl groups in the molecule causes the activity to diminish, regardless of the chain length, as was found from studies using various carboxylates including oxalate, malonate, succinate, glutamate and citrate [110].

After the discovery of these receptors, GPR43 was renamed to free fatty acid 2 (FFA2) receptor and GPR41 to FFA3 receptor. Also, research has been conducted into the intracellular signaling pathways that are activated by ligand binding to FFA2 and FFA3 receptors. It was found that the intracellular routes of both receptors could be inhibited with pertussis toxins, which implies that the intracellular sides of the receptors are coupled to $G_{i/o}$ proteins. In addition to $G_{i/o}$ proteins, FFA2 receptor appears to be coupled to G_q proteins in a similar way. Activation of the intracellular signal transduction pathways by the receptors led in both G-protein families to an increase in inositol 1,4,5-triphosphate (IP_3) synthesis, an increase in intracellular calcium concentration, activation of ERK1 and 2 (extracellular signal-regulated kinases; MAPK1 and MAPK3 respectively) and inhibition of the formation of cAMP [110].

The SCFA receptors also differ with respect to tissue distribution. Various recombinant immune cell models revealed that strong expressions of FFA2 receptors are observable on leukocytes; particularly on monocytes, B lymphocytes and granulocytes. Also, a lower mRNA expression of this

receptor was observed in white and brown adipose tissue, the pancreas, the spleen, bone marrow and the large intestine [108, 110-112]. The FFA3 receptor has a more comprehensive expression pattern, wherein the highest mRNA expression levels can be detected in the adipose tissue. High mRNA expression levels can also be found in the spleen, pancreas, lymph nodes, bone marrow and to a lesser extent, in granulocytes, cardiomyocytes and vasculature [108, 110, 112-114].

The strong expression of FFA2 receptors on various leukocyte populations suggests that SCFA are capable of influencing the immune responses of these immune cells [115-118]. This assumption is further supported by the finding of a higher mRNA expression of the receptor during haematopoiesis (differentiation of the leukocytes) and the activation of these cells [108, 119]. SCFA exert several effects on the immune cells. Changes in cytoplasmic pH values, intracellular calcium concentrations, oxygen consumption, phagocytosis, rate of cell proliferation, actin distribution for the formation of the cytoskeleton, motility and chemotaxis could be observed in studies in which SCFA were administered to leukocytes [115, 120-122]. SCFA are thus capable of inducing a large number of immunological functions of leukocytes, with the exception of the cell adhesion [123]. The inducing effects are likely to be caused through several mechanisms, wherein at least one signal transduction pathway is activated by the G-proteins that are linked to FFA2 receptor [120, 121].

The physiologically relevant site for the immunoregulatory effect of the SCFA is the gastrointestinal tract. Firstly, because of the presence of the enteric microbiota, which makes this environment highly immunogenic and able to exert great influence on the immune system. As a result, there is a great need for regulation of the immune cells in the large intestine [26-28]. Secondly, because the intestinal microflora synthesizes the SCFA in sufficiently high concentrations to be able to regulate the leukocytes in this organ effectively [116]. SCFA are formed in the distal regions of the intestinal tract, where the concentrations of the individual fatty acids may rise from 70 to 100 mM and

are sufficient to cause the immunoregulatory effects [51]. These effects will occur less in other organs and tissues, as the SCFA are present at much lower concentrations in the systemic circulation. As discussed previously, the SCFA are preferred for the production of energy in the colonocytes of the intestinal wall and the liver is capable of absorbing a large portion of the SCFA from the portal vein. Therefore, the fatty acids have a large first pass effect. Accordingly, the concentrations of acetate, propionate and butyrate in the peripheral blood amounts to 100 to 150, 4 to 5 and 1 to 3 µM respectively and thus are likely to be too low to regulate the immune system in the periphery on a large scale [110, 124]. However, the concentration of acetate is found to be able to increase tenfold when alcohol is consumed and could potentially reach effective concentrations for the regulatory action on the leukocytes [125, 126].

5.2. Lipid metabolism

SCFA are capable of inhibiting the fatty acid synthesis and lipolysis and stimulating the oxidation of fatty acids and thermogenesis in the body. Furthermore, SCFA cause an increase in mitochondrial function by stimulating the biogenesis of this organelle which, in conjunction with the three previously mentioned effects, leads to induction of the oxidative phosphorylation of SCFA through β-oxidation in adipose tissue, the liver and muscles. Therefore, SCFA provide a transition from the production of energy-bearing molecules to the consumption of these molecules in these tissues, causing the plasma concentrations of fatty acids to decrease [127-129].

The regulatory effects of short-chain fatty acids on the metabolism of these tissues are established with the aid of the FFA2 and FFA3 receptors present on the involved tissues [127]. Moreover, the fatty acids appear to contribute to the activation of 5'-AMP-activated protein kinase (AMPK) in the liver and muscle tissue. The activation of AMPK allows for an increased expression of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α), a regulator of the transcriptional activity of different transcription factors that are important for the energy metabolism of cells, and can

contribute to the effects of the short-chain fatty acids on the stimulation of the catabolism [130].

Recently, it has been discovered that FFA2 and FFA3 receptors both can stimulate the release of leptin through the white adipose tissue [131]. Propionate as well as butyrate was found to stimulate the secretion of leptin by the adipose tissue, causing the plasma concentration of leptin to increase. Studies with mice in which sodium propionate was administered, it was possible to detect an increase in the concentration of leptin in the blood plasma after approximately seven hours after administration. FFA2 receptors also appear to affect the absorption and storage of lipids in the white adipose tissue. FFA2 receptors are able to stimulate the consumption of lipids in other tissues, whilst inhibiting the storage of fat in white adipose tissue [127].

5.3. Glucose metabolism

In addition to the regulation of the metabolism of lipids, SCFA can also affect the metabolism of glucose. Several studies with various animal and cell models revealed that the fatty acids can reduce the glucose concentration in the blood. The observed reductions are caused by several mechanisms which are mediated by FFA2 and FFA3 receptors and AMPK. In addition, some of the effects are caused by the incretins glucagon-like peptide-1 (GLP-1) and peptide YY (PYY).

Another effect that can contribute to the decrease in the plasma glucose level in the blood is the stimulation of fatty acid oxidation in the liver, muscle and the brown adipose tissue and inhibition of lipolysis in the white adipose tissue by SCFA. High plasma concentrations of fatty acids can reduce the sensitivity to insulin of different tissues and hinder the absorption of glucose. Stimulation of the oxidation of fatty acids in the three previously mentioned tissues causes a decrease in the free fatty acid concentration in the blood plasma, which leads to an increase in the sensitivity to insulin and thus to an enhanced absorption of glucose [132-136]. As a result of the stimulation of the oxidation of fatty acids by the SCFA, the glucose absorbed by the tissues is converted to glycogen [137, 138].

5.4. Cholesterol metabolism

Several studies show that the administration of SCFA or dietary fibre to both rats and humans causes a decline in the plasma concentrations of cholesterol [139-146]. Furthermore, a daily administration of SCFA during a month resulted in a significant, dose-dependent decrease in body weight in subjects with obesity [142]. Cholesterol has a multitude of important functions for the survival of vertebrate organisms. For instance, the molecule is important for the preservation of the structure of cell membranes and for a variety of cell functions. Additionally, cholesterol is an important precursor for the synthesis of bile acids, steroid hormones and oxysterols, which are all important for the maintenance of the organism [147].

6. Effect of SCFA on the cardiovascular system

Research over the past twenty years has demonstrated that the endothelial layer of the cardiovascular system is more than a single layer of cells to cover the muscle tissue of the blood vessels. For example, the endothelium appears to play a major role in the regulation of blood pressure and the free flow of blood. In addition, it prevents bleeding, can inhibit or stimulate inflammatory reactions and can affect the function of nearby vascular muscle tissue in several ways [148].

Disorders of the cardiovascular system are generally caused by disturbances of the functions of the endothelium. The main causes of these disturbances can be traced back to metabolic imbalances and their consequences. Several studies revealed that hyperglycaemia, insulin resistance and dyslipidemia can all cause endothelial dysfunction and low-grade inflammation in this tissue [23-25]. The aforementioned metabolic imbalances can be caused by a variety of disorders, such as obesity, hypertension and type 2 diabetes mellitus and therefore constitute risk factors for the development of cardiovascular diseases [1, 23, 149]. Various studies have found that diabetic patients are two to four times more likely to die from cardiovascular disease than non-diabetics [150-152].

Loss of endothelial function by these diseases leads to accumulation of lipids and inflammatory cells in the subendothelial space, causing reduction of the inner diameter of the blood vessel. The protraction of this phenomenon leads to atherosclerosis; the clogging and hardening of the arteries. In this process a plaque is built at the inside of a blood vessel, which hinders the blood flow. When the blood vessel is completely obstructed and the downstream tissues are no longer provided with nutrients and oxygen, this will lead in an ischemic attack and may cause necrosis in these tissues. Additionally, rupture of an atherosclerotic plaque can occur, leading to the release of a large amount of coagulation factors which initiate the coagulation cascade. As a result, clogging of blood vessels elsewhere in the body can occur. Common sites for the occurrence of an embolism are the coronary arteries, which supply the heart with oxygen, and the cerebral blood vessels. Furthermore, in the case of hypertriglyceridemia, accumulation of fat in the heart muscle can take place, causing the heart to enlarge and making it less able to contract [149, 153-155].

As described in the previous chapter, SCFA affect several metabolic processes in the body and therefore appear suitable for the prevention and treatment of endothelial dysfunction and its effects. Besides the effects on metabolic processes, the fatty acids have an anti-inflammatory effect, which allows inhibition of the release of inflammatory mediators and the influx of immune cells to the site of inflammation.

In the rest of this chapter the physiological role of healthy cardiovascular tissue and how different risk factors can disrupt endothelial functions will be discussed. Then, it will be discussed how this loss of function leads to the development of atherosclerosis and how a high lipid concentration in the blood may result in the accumulation of fat in the heart muscle. In the remainder of the chapter, the ways in which SCFA can contribute to the prevention and treatment of the loss of endothelial function will be addressed.

6.1. Physiological functions of healthy endothelial tissue

Endothelial tissue appears to have a number of important regulatory functions in ensuring an unhindered blood circulation. For the regulation of the flow of blood to the various tissues and organs, the endothelium releases various chemical mediators. In order to achieve vasodilation, the endothelial cells produce nitric oxide by converting the amino acid L-arginine to L-citrulline by endothelial nitric oxide synthase (eNOS); an enzyme which is present in these cells. The cells produce picomolar concentrations of nitric oxide, which are delivered to adjacent smooth muscle of the blood vessel. In the muscle cells, nitric oxide activates the enzyme guanylate cyclase, which leads to an increase in the intracellular cGMP concentration that causes relaxation of the muscle cells [156]. Vasoconstriction is achieved through the release of endogenous produced endothelin to the muscle cells by the endothelium [157]. Under physiological conditions, there is a balance between the endothelium-derived relaxing and contracting factors, but this balance appears to be shifted in diabetic patients and atherosclerosis in patients, which may lead to loss of the endothelial functions [148, 158, 159].

Moreover, the endothelium regulates inflammatory reactions and the blood coagulation. Healthy endothelial tissue appears to be able to inhibit the adhesion of platelets and leukocytes to the vascular surface and a balance between the coagulation and fibrinolytic systems is maintained. Apart from a physiological barrier between the blood circulation and the vascular muscle tissue, the endothelium also prevents the development of atherosclerosis in the vascular and thrombus formation in the blood by limiting vascular adhesion [156, 160-163].

Finally, the endothelium also has a role in the regulation of the proliferation and differentiation of the vascular muscle tissue. To this end, the endothelium produces a number of mediators, which are then delivered to the adjacent muscle cells via diffusion [162, 164-167].

6.2. Metabolic imbalances

As mentioned previously, hyperglycaemia, insulin resistance and dyslipidemia are the main forms of metabolic imbalance that can lead to disturbance of the endothelial functions [23-25]. They frequently occur in patients with obesity or type 2 diabetes mellitus. The different metabolic imbalances lead, together and separately, to the disruption of endothelial functions and thus to an increased risk of the manifestation of cardiovascular disease.

Insulin resistance is a common phenomenon in patients with obesity [168]. The relatively high mass of the white adipose tissue in these patients causes a lesser sensitivity to insulin, which leads to a reduced storage capacity of new fats in this tissue. As a result, the adipose tissue enhances lipolysis, allowing more triglycerides to be hydrolysed to individual fatty acids, which are then released into the circulation [158, 169, 170]. Normally, insulin inhibits the lipolysis in the adipocytes of this tissue, but due to the increased mass of the tissue and insulin resistance, this takes place to an insufficient extent [171]. The increase in plasma concentration of the free fatty acids has effects on different tissues. This increase in plasma concentration is accompanied by a decrease in the insulin sensitivity of the muscle and liver. In the liver, insulin normally causes an increase in the gene expression for triglyceride synthesizing enzymes, but simultaneously inhibits the production of very low-density lipoprotein (VLDL) and apolipoprotein B (Apo B), which are involved in the transport of triglycerides, cholesterol and fatty acids into the adipocytes of the adipose tissues [168, 172-175]. Moreover, insulin appears to be able to induce the hepatic catabolism of Apo B [176]. In the situation outlined above it appears that both triglyceride and glucose synthesis and storage as well as the VLDL production are stimulated, causing the release of large amounts of triglyceride-containing VLDL particles by this organ [177]. In addition to triglycerides, the core of the VLDL particles also contains approximately 600 molecules of cholesterol and 1600 molecules of cholesterol esters [178]. Stimulation of the production and secretion of VLDL

particles comes at the expense of the hepatic production and secretion of HDL particles, which are involved in reverse cholesterol transport and thus are important for the breakdown and excretion of cholesterol and lipids in the body. Peripheral insulin resistance is therefore mainly characterized by an increased concentration of LDL and a decrease in the HDL concentration in the blood [179-181]. Additionally, the glucose absorption in the muscles is reduced due to the increased resistance to insulin [132, 169, 182].

Besides the increase in triglyceride synthesis, the production and release of glucose by the liver is also enhanced. In combination with the increased concentration of fatty acids in the blood plasma, this leads to stimulation of the pancreas to release more insulin. However, due to the increasing resistance the glucose and fatty acid levels in the blood remain high, which results in hyperinsulinemia [183, 184]. Research shows that an intravascular injection of insulin has a vasodilatory effect and causes a reduction in blood pressure [185]. This effect disappears later as the protein is capable of stimulating the reabsorption of sodium in the kidney and thereby can increase the osmolarity of the blood. Consequently, the reabsorption of water increases as well, causing the blood volume to increase and the blood pressure to rise again [186]. However, in the case of insulin resistance it appears that the vasodilatory effect is absent, while the stimulatory effect on the sodium reabsorption is maintained [187, 188]. In addition, insulin appears to be capable of activating the sympathetic nervous system and causing vasoconstriction [189]. It is suspected that this effect is also preserved in the case of insulin resistance [190]. Altogether, in the case of insulin resistance hyperinsulinemia may lead to the emergence of hypertension [191-195].

Next to the mechanisms described above, it is found that the adipose tissue of obese patients also exhibits an increased production of pro-inflammatory mediators. Increased plasma concentrations of both IL-6 and TNFα could be observed, which contribute to an increased lipolytic activity in the white adipose tissue and further strengthen the insulin resistance of adipose tissue.

In addition, increased secretion of these inflammatory mediators into the circulation occurs, causing the insulin resistance of other tissues to increase even further [196-198]. Moreover, the secretion of IL-6 results in an enhanced hepatic glucose production and thus reinforces the hypertensive effects of insulin by indirect stimulation of insulin secretion by the pancreas [199]. The cytokine also stimulates the production and secretion of VLDL, at the expense of HDL production in hepatocytes [200, 201]. Another effect exerted by both cytokines is the stimulation of the release of plasminogen activator inhibitor-1 (PAI-1) by the liver and the adipose tissue and the release of fibrinogen by the liver. The release of these substances leads to an increased tendency of blood coagulation in the cardiovascular system and thereby increases the risk of the formation of an embolus [202, 203]. Furthermore, obesity seems to lead to a further strengthening of hyperinsulinemia, increased plasma concentrations of VLDL, glucose and fatty acid and a decrease in HDL concentration [134, 200, 201, 204-208]. Also, the release of pro-inflammatory cytokines by the liver and the adipose tissue leads to an increased inflammatory state of the cardiovascular system, as demonstrated by an observable increase in the plasma concentration of inflammatory marker C-reactive protein [206, 209-211].

The hyperglycemic conditions, which occur as a result of insulin resistance and increased hepatic glucose production from fatty acids, prove detrimental to the cells of the endothelium. The damage suffered is the result of an increased production of intracellular free radicals by induction of oxidative stress and modulation of different signal transduction pathways, including signaling cascade of protein kinase C by the increased glucose level in the blood [212-215]. Research involving the administration of vasodilator substances to endothelial tissue revealed that there is a reduced vasodilatory effect under hyperglycemic conditions [216]. In addition, a decreased effect of the endothelium-dependent vasodilation can be observed. This is caused by increased inactivation of nitric oxide by the formation of superoxide as a result of increased oxidative stress [156, 217]. While at low concentrations, free radicals can act as signaling molecules in the regulation of cell adaptation

and cell growth, high concentrations of these oxidizing compounds may lead to damage and apoptosis of cells. In the case of hyperglycaemia the oxidative stress of endothelial and vascular muscle tissue is induced, which increases the risk of suffering damage in these tissues that could lead to the disruption of endothelial functions [156].

Furthermore, the increased oxidative stress caused by protein kinase C activation due to the hyperglycermic conditions, results in the synthesis of advanced glycation end products (AGE) in the endothelial cells [156]. The AGE molecules are formed by upregulation of the non-enzymatic post-translational modifications of synthesized proteins, making them highly glycosylated [148]. Then, the glycosylated proteins are secreted and can cause damage to the endothelial cells in various ways. First of all, the AGE molecules are capable of stimulating the production of the radicals in the endothelial cells by binding to RAGE (Receptor for Advanced Glycation Endproducts), thereby further increasing the risk of cell damage and apoptosis [218]. Moreover, AGE can interact with different components of the vessel wall, thereby increasing the vascular permeability and expression of adhesion molecules on the endothelium. As a result of the increased endothelial permeability and an increased adhesion of LDL particles and monocytes on the endothelium, an enhanced accumulation of these particles and cells occurs in the subendothelial space underneath the endothelial layer. Normally, VLDL and LDL particles are not harmful to the endothelium, but due to the elevated free radical formation and release of AGE molecules, these particles can be oxidised. Accumulation of these oxidised particles in the subendothelial space can then lead to atherosclerosis. AGE molecules also prove to be able to induce the production of procoagulants by the endothelium, which results in an even stronger coagulation tendency of the blood at this site of the cardiovascular system. In diabetic patients an even stronger increase in the blood sugar level can be observed, resulting an even stronger enhanced production of AGE molecules and increased oxidation of LDL particles. Due to the increase in influx and uptake from the circulation

and oxidation of the LDL particles in the subendothelial space, these patients are at increased risk for the development of atherosclerosis [219-223].

Hyperglycemia also appears able to stimulate the production of the hormone endothelin-1 in endothelial cells. Release of this hormone by these cells can cause vasoconstriction and induce the proliferation and remodeling of vascular smooth muscle. In addition, the secretion leads to an increased pro-inflammatory cytokine production. The release of these cytokines by the endothelium can contribute to the low-grade inflammation state of the tissue [157]. Also, hyperinsulinaemia appears to be able to contribute to an increase in blood pressure through inhibition of eNOS, which causes inhibition of the production of the vasodilatory nitric oxide. In addition, cultivation studies, in which endothelial cells were exposed to an insulin-rich medium, revealed that insulin is able to stimulate the expression of various adhesion molecules and an enlarged transendothelial migration of neutrophils could be observed. Both of these effects thereby increase the risk for the development of atherosclerosis [224, 225].

The development of hypertension can be associated with an increase in angiotensin II formation and activity in the vascular tissue. Angiotensin II is produced by the endothelial cells and released locally. The hormone has a regulatory effect on a number of functions of the vascular muscle tissue, such as contraction, growth, proliferation and differentiation of these cells [226, 227]. An enlarged angiotensin II activity provides an increased expression of the adhesion molecule intercellular adhesion molecule-1 (ICAM-1) and an enhanced release of the monocyte chemotactic protein 1 (MCP-1) by the endothelial cells and can thus promote the influx to and uptake of monocytes into the subendothelial space and thus contribute to development or exacerbation of atherosclerosis [228-230].

In summary it can be stated that the combined and separate influences of the risk factors for the development of cardiovascular diseases lead to disruption of the endothelial functions by increasing the blood pressure, the induction of inflammatory mediators, the stimulation of the blood clotting or

induction of the expression of chemotactic substances and adhesion molecules on the endothelium [231-233].

6.3. Atherosclerosis

The previously described metabolic imbalances bring the endothelial tissue in a low-grade inflammatory state, in which various pro-inflammatory cytokines and free radicals are produced by this tissue. The presence of these cytokines causes an increased expression of several adhesion molecules on the endothelial layer, including the lectin-type oxidised LDL receptor 1 (LOX-1). This receptor is responsible for the absorption of LDL particles from the circulation, after which, under the influence of the oxidative stress induced by the metabolic imbalances, oxidises these particles. The oxidised LDL particles (ox-LDL) can then be released into the subendothelial space, but also have several intracellular effects in the endothelium [153, 234-236].

Research revealed that ox-LDL can promote the intracellular radical formation and induce the activation of transcription factor NF-kB [528–530]. The transcriptional activity of NF-kB is based on translocation to the nucleus and requires the dissociation of IκB from the heterotrimer. Ox-LDL as well as the pro-inflammatory cytokines stimulate the phosphorylation of IκB, which subsequently causes the heterotrimer to dissociate and the p65/p50 heterodimer to be activated. The free IκB is then degraded in the proteasome. The dimer then moves to the nucleus and affects the gene expression of many proteins that could contribute to the pathological process of atherosclerosis [181]. In this way, ox-LDL stimulates the expression and release of pro-inflammatory cytokines TNFα, IL-1β, IL-6, and macrophage colony-stimulating factor (M-CSF).

Oxidised LDL itself as well as the pro-inflammatory cytokines and the increase in AGEs then induce the expression of various adhesion molecules, including leukocyte adhesion molecule-1 (LAM-1), ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), P-selectin and E-selectin [235, 237-244].

In addition, ox-LDL stimulates the production and release of monocyte chemotactic protein 1 (MCP-1), a chemokine that attracts monocytes, to the bloodstream by the endothelial tissue [245]. Furthermore, ox-LDL enhances the blood coagulation by suppressing the expression and activation of a number of fibrinolytic proteins, thereby increasing the coagulation tendency. This is achieved by inhibiting the expression of thrombomodulin and the activation of the anti-coagulation factor protein C and limiting the release of tissue factor pathway inhibitor and tissue-type plasminogen activator (tPA). The blood coagulation is even further induced by ox-LDL by increasing the production and release of plasminogen activator inhibitor-1 (PAR-1) and tissue factor, a co-activator of blood clotting protein factor VII, in the subendothelial space by the endothelium [246-252].

Both the stimulation of the endothelial production of cytokine TNFα by the metabolic imbalances and the stimulation by ox-LDL, lead to an induction in the expression of the receptor LOX-1 on the endothelial cells [119, 253, 254]. The induction thereby causes for an enhanced absorption of LDL particles from the circulation into the subendothelial space, where they are oxidised to ox-LDL due to the oxidative stress of the tissue. Consequently, a feed-forward loop is created, wherein the induction by ox-LDL leads to a stimulation of oxidation and absorption of other LDL particles. This causes accumulation of oxidised LDL in the subendothelial space and resulting in an enhanced production of various inflammatory mediators, procoagulent and fibrinolytic factors and adhesion molecules, which lead to aggravation of the endothelial dysfunction, stimulation of oxidative stress and the inflammatory state of this tissue [119].

A subsequent significant step in the development of atherosclerosis is the influx of immune cells into the affected endothelial tissue. Due to the inflammatory state, the upregulation of the expression of adhesion molecules on the endothelial tissue and the increased vascular permeability, a large influx of monocytes into the affected tissue can be observed [255-257]. Besides these factors the release of chemokine MCP-1 also promotes

the migration of monocytes. The monocytes feature C-C chemokine receptor type 2 receptors, which are able to bind the chemokine and provoke chemotaxis. The monocytes may then adhere to the adhesion molecules on the endothelium, after which extravasation to the subendothelial space takes place [258]. Due to the high concentration of oxidised LDL particles, the monocytes differentiate into macrophages that are able to incorporate the LDL particles and processing these lipoproteins. After processing, the end products are delivered to HDL particles. In this manner, the macrophages constitute a clearance mechanism for the accumulation of triglycerides and cholesterol and try to protect the tissue from the harmful effects of ox-LDL [149].

For the absorption of the oxidised lipoproteins the macrophages express various scavenger receptors, such as CD36 and scavenger receptor type 1 (SR-A1) [259, 260]. The affinity of CD36 for ox-LDL is approximately three times higher than for regular LDL [259]. Therefore, CD36 appears to be the primary route of ox-LDL absorption by the macrophages [261]. Absorbed cholesterol esters are hydrolysed in the lysosome to individual cholesterol molecules that can be incorporated may be converted into the cell membrane of the macrophage or converted again to a variety of ester derivatives by acyl-CoA cholesterol acyltransferase-1 (ACAT1) in the endoplasmic reticulum. The reaction products of this enzyme are then, together with triglycerides, phospholipids and other esterified sterols, stored in lipid droplets within the cytoplasm of the macrophage. In this process, a fraction of these lipids is also delivered to HL particles using the ATP-binding cassette transporter-1 (ABCA-1), which is expressed on the cell membrane. Then, HDL-particles filled with lipids are transported to the liver to be degraded. The concentration of cholesterol present in the cell membrane is an indicator for the utilized storage capacity of the macrophage [262, 263].

However, in the subendothelial space an imbalance is formed between the absorption of lipids from ox-LDL and the release of lipids to HDL particles. First of all, the metabolic imbalances cause a decreased HDL concentration

in the blood plasma, resulting in a reduced secretion capacity of lipids. Secondly, research has revealed that ox-LDL is capable of downregulating ABCA1 by inhibiting the activation of transcription liver X receptor (LXR) in a dose-dependent manner [264]. Consequently, less delivery of the lipids to the HDL particles takes place. Furthermore, ox-LDL stimulates the production and release of the cytokine M-CSF, which is able to induce the expression of SR-A1 on the macrophages, whereby the absorption capacity of the macrophages is enhanced [180]. This shift in the balance between the absorption and secretion of lipids results in the loss of the clearance function of the macrophages. Hence, lipids continue to accumulate in the form of lipid droplets in the cytoplasm of these immune cells. This severe continued accumulation causes the macrophages in the subendothelial space to transform to lipid-rich foam cells and form a plaque inside the vascular wall [149].

In addition to stimulation of the adhesion molecules, receptors and pro-inflammatory mediators, ox-LDL also induces the expression and release of basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) to the subendothelial space by the endothelium as well as macrophages and smooth muscle of the vascular system. In this way proliferation and differentiation of the smooth muscle cells is stimulated, allowing them to migrate into the subendothelial space [265-267]. Moreover, the activation of this tissue by bFGF and PDGF leads to the production of pro-inflammatory cytokines, procoagulents and metalloproteinases. After migration into subendothelial space, the differentiated smooth muscle cells stabilize the atherosclerotic plaque by forming a fibrous network around the foam cells and ox-LDL particles. In this way, the so-called fibrous cap is assembled [268, 269].

The progressive growth of the plaque leads to a reduced dilatory ability of the blood vessel and, together with the simultaneous volume increase of the subendothelial space, leads to narrowing of the blood vessel, which can result in the occurrence of several clinical complications [270, 271].

Consequently, ischemia can arise as the resulting obstruction of blood flow leads to shortages of oxygen and nutrients and the accumulation of waste products in the downstream tissues. This complication is called peripheral artery disease and can cause necrosis in these tissues [272].

A second complication develops when the plaque ruptures, causing the accumulated procoagulents and tissue factor to be released into the blood stream and results in the formation of a thrombus. Furthermore, the inhibition of the fibrinolytic systems by dysfunction of the endothelium further enhances this formation. Subsequently, the thrombus can be carried away by the blood flow and cause embolisms and ischemic attacks in remote parts of the body. Most frequently this occurs in the coronary arteries or one of the cerebral vessels. In the first case this leads to the development of a myocardial infarction, while in the second case a transient ischemic attack is developed [153-155, 273].

6.4. Lipotoxic heart disease

Besides cardiovascular complications that may occur as a result of the disruption of various endothelial functions, disturbance of the lipid metabolism in the heart muscle itself may also lead to complications. Several experiments have found that storage of triglycerides in the myocytes of the heart as a result of high concentration of VLDL particles in the blood, can lead to damage of the myocardium [274]. The heart consumes about ten times its own weight in molecules of ATP per day, and thus has a very high energy requirement [75]. Accordingly, a sufficient amount of energy has to be available in the myocytes at all times in order to comply with changes in the cardiac energy metabolism [275].

This energy is mainly generated through oxidation of fatty acids by the mitochondria of myocytes. When fatty acid oxidation is not sufficient, as can be modulated with the obese Zucker rat, triglycerides accumulate in the myocytes, causing cardiac steatosis, increased retention of fats in this tissue, and can even result in apoptosis [276]. This process is initiated by the infiltration of areas between the myocardial fibres by adipocytes from the

epicardium [277]. Then, accumulation of triglycerides in the myocardium occurs, leading to the development of cardiac steatosis. During the development, the storage of triglycerides in lipid droplets in the cytosol of myocytes can be observed. Over time, this phenomenon leads to a reduced contractility of the cardiac muscle [574]. This is thought to be caused by the conversion of the triglycerides to ceramide, causing inducable NOS (iNOS) to be activated which results in hypertrophy of the muscle cells [274, 278, 279]. This process ultimately leads to apoptosis of muscle cells, causing the contractility to be greatly reduced [278, 280].

Moreover, PGC-1α appears to affect the mitochondrial metabolism of the heart muscle. Most of the energy is obtained from the oxidation of fatty acids. As described previously, PGC-1α is able to stimulate mitochondrial biogenesis, which could result in an increased mitochondrial capacity enabling the heart to respond to increasing load of the muscle [281, 282]. Therefore, stimulation of the PGC-1α activity may be a novel therapeutic opportunity for the treatment of cardiac steatosis and preservation of the heart muscle function. However, in vivo studies involving mice revealed that overexpression of PGC-1α in the cardiac muscle tissue leads to an extremely strong mitochondrial proliferation, causing the myofibrils to be moved. These morphological changes of the heart muscle can consequently trigger the development of cardiomyopathy and congestive heart failure [282]. On the other hand, research has demonstrated that inhibition of PGC-1α with histone deacetylase 5 (HDAC5) and cyclin-T1 can cause mitochondrial dysfunction and heart failure, in the case of an increased working load on the heart muscle [283-286].

This effect of PGC-1α was further confirmed in research involving the administration of the beta-adrenergic agonist dobutamine to PGC-1α KO mice hearts. It was found that the ATP synthesis in these mice was not sufficient to meet the increased labour, while wild-type mice were able to comply with the increasing energy demands. This implies that PGC-1α performs an adaptive role for the heart to be able to comply with changes in

energy demands during spontaneous labour increase, but is not essential for the basal pump activity [287]. Therefore, PGC-1α contributes to the prevention of heart failure by improving the contractile function when present in physiological concentrations [282, 288].

6.5. Preventive effects on heart and blood vessels

SCFA may be able to prevent or counteract a large number of the metabolic disturbances which could lead to loss of endothelial functions and ultimately can result in the development of cardiovascular diseases. From the previously described effects of the fatty acids on the metabolism of lipids and glucose, among others, various preventive mechanisms of action can be derived.

As stated previously, insulin resistance of adipose tissue, which can result of obesity, leads to a reduced fat storage and an increase in the lipolytic activity in this tissue. Due to this increased activity, higher plasma concentrations of free fatty acids arise that reduce the insulin sensitivity in other tissues. In an effort to compensate for the loss of sensitivity to insulin, the pancreas will secrete larger quantities of insulin, which can result in hyperinsulinemia. Subsequently, hyperinsulinemia can cause elevation of the blood pressure and thus contributes to the development of cardiovascular complications.
SCFA prove to be able to inhibit the lipolytic activity in the white adipose tissue. To this end, these fatty acids bind to the FFA2 and FFA3 receptors present on the adipocytes of this tissue and lead to the activation of intracellular G_i proteins that inhibit adenylyl cyclase. Inhibition of this enzyme leads to subsequent inactivation of the cAMP-dependent PKA and hormone-sensitive lipase. Consequently, the triglycerides are no longer hydrolysed to the individual fatty acids. In this way less fatty acids are released into the circulation by white adipose tissue, causing the plasma concentration of the fatty acids to fall [127, 289-291].
Another mechanism which can contribute to a reduction in the plasma levels of fatty acids is the ability of SCFA to induce the fatty acid oxidation in several tissues. SCFA appear to be able to activate AMPK, which is able to suppress

the enzymes involved in the inhibition of the fatty acid oxidation whilst activating the enzymes that induce these oxidations, in both the liver and the skeletal muscles. In addition, AMPK can activate the coactivator PGC-1α which is capable of binding to several transcription factors that are involved in the metabolism of fatty acids. Accordingly, PGC-1α can inhibit the expression of these enzymes at a genetic level, thereby contributing to the stimulation of the fatty acid oxidation. This way PGC-1α can inhibit the expression of these enzymes at the genetic level or stimulating and thus contribute to a stimulation of fatty acid oxidation. Likewise, the kinase and coactivator can inhibit the hepatic synthesis of fatty acids by inactivation and downregulation of acetyl-CoA carboxylases and fatty acid synthetases [130, 139, 292].

Additional stimulation of the fatty acid oxidation by AMPK and PGC-1α can occur by promoting the secretion of leptin by the white adipose tissue. It was established that binding of SCFA to the FFA receptors on this tissue caused an increase in the production and secretion of leptin. Leptin has proved to be able to elevate the AMP: ATP ratio in the liver. As a result, AMPK activation occurs more readily and stimulation of the fatty acid oxidation via this kinase and PGC-1α is further enhanced [131, 293-299]. Moreover, leptin is a potent anorexigenic hormone, allowing the absorption of fatty acids may be limited from the feed, therefore restricting the absorption of fatty acids from the ingesta [300-304].

Both hypertriglyceridemia and the increased concentration of VLDL particles can provoke the pancreas to release more insulin into the circulation, thereby causing the harmful effects of hyperinsulinemia on the endothelial tissue. Additionally, the increased release of VLDL particles cause accumulation of triglycerides in the myocytes of the heart as well as steatosis and heart defects [168, 172-178].

First of all, these effects are counteracted by the above-described lowering and inhibitive effect of the SCFA on the plasma concentration and hepatic synthesis of free fatty acids. Accordingly, fewer amounts of fatty acids are available to be esterified to a glycerol molecule to form triglycerides.

Furthermore, leptin possesses an inhibitory effect on the hepatic triglyceride synthesis, thereby limiting the synthesis of triglycerides even further [293, 305]. It also appears that SCFA are able to inhibit the cholesterol synthesizing enzymes of the liver via the activation of AMPK, thereby reducing the cholesterol production in this organ [139, 306-311]. Besides inhibiting the synthesis of cholesterol, the SCFA stimulate the degradation and excretion of cholesterol by the liver. Firstly, this leads to a reduced cholesterol content and production of VLDL particles by the liver [312-314]. Secondly, cholesterol is also capable of inducing the triglyceride synthesis of the liver. Consequently, inhibition of the formation of cholesterol in this organ will also suppress the synthesis of triglycerides [140, 315]. An additional effect of the limitation of the LDL and triglyceride synthesis is the secretion of larger amounts of HDL particles by the liver. These particles are involved in the reverse cholesterol transport and thus further contribute to the degradation and excretion of this lipid [312-314].

Hyperglycaemia is the result of an increased hepatic production of glucose from absorbed fatty acids and an increased resistance of the tissues to the actions of insulin, causing the glucose content of the blood to remain high [212-215]. The hyperglycemic conditions thereby prove detrimental to the endothelium by provoking the development of oxidative stress in these tissues. The formation of superoxide can result in cell damage and hypertension [156, 217]. This process also contributes to the development of a low-grade inflammatory state of the endothelium. In this way, hyperglycaemia can contribute to the development of atherosclerosis [157]. As with the reduction of VLDL and triglyceride synthesis, restriction of the plasma concentration of free fatty acids results in a reduced availability of substrates for the production of glucose. SCFA are able to activate AMPK by elevating the AMP: ATP ratio [292, 316-320]. Next, the activated AMPK can inhibit enzymes that are involved in the gluconeogenesis, including glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) [321-324]. Phosphorylation of these enzymes, thereby leads to a reduced production and secretion of the glucose formed by the hepatocytes [321-

323]. In addition, SCFA are capable of lowering the glucose levels of the blood even more through the activation of the FFA2 and FFA3 receptors, which are expressed on the enterocytes of the intestinal tract. Stimulation of these cells via the receptors leads to the secretion of PYY and GLP-1 into the portal vein, which is able to reach the systemic circulation after liver passage [325-328]. The secretion of PYY leads to improved insulin sensitivity in adipose and muscle tissue. Consequently, more absorption of glucose occurs, thereby decreasing its plasma concentrations [329]. PYY can also suppress appetite by inhibiting NPY neurons in the arcuate nucleus of the hypothalamus and this anorexigenic effect may further limit the intake of glucose [330]. A positive effect on the level of glucose in the blood is achieved by GLP-1 by reducing the production and release of glucagon. This reduction leads to inhibition of the conversion of stored glycogen to glucose, thereby decreasing the plasma concentration of glucose [331, 332].

SCFA also appear to exert vasodilatory effects. For example, the intravenous administration of acetate leads to an increase of the cardiac output and an increase in the coronary blood flow. These effects appear to be caused by a direct and an indirect mechanism. First of all, the SCFA can activate the FFA3 receptors present on the vasculature, thereby causing vasodilation [333-337]. Secondly, the administration of SCFA can stimulate the production of adenosine. The ribonucleoside is a potent vasodilator and is formed by conversion of AMP by the enzyme 5'-nucleotidase [338-342]. The SCFA induce the ATP consumption in several tissues, resulting in increases of the intracellular AMP concentrations. The increased availability of substrates for 5'-nucleotidase causes enhanced formation of adenosine and therefore reinforces the vasodilatory effects [339, 340]. Due to the reduction of the constriction of blood vessels, the heart is able to pump blood through the vascular system more easily, hence reducing the risk of heart failure [343]. Moreover, a reduction in blood pressure also results in decreased tendency of blood coagulation, thereby reducing the risk of the occurrence of embolism [344-346]. Furthermore, the lowered blood pressure produces a reduced sensitivity of the endothelium for inflammatory responses and thus

lowers the risk for the loss of endothelial functions and the development of atherosclerosis [346-348].

In addition to the vasodilating effects caused by SCFA, these fatty acids are also capable of causing vasoconstrictive effects through activation of the Olfr78 receptors, which are present on the vasculature and the renal afferent arteriole [333, 349, 350]. This effect is achieved by two different mechanisms. First of all, the activation of Olfr78 by acetate or propionate results in the intracellular activation of stimulatory G-proteins. The activation of these proteins antagonises the effects of inhibitory G-proteins and therefore the vasodilatation caused by activation of FFA3 receptors by SCFA. However, it is currently unknown whether both receptors may or may not be co-expressed by one or more subsets of vascular smooth muscle cells. Secondly, activation of the Olfr78 receptors expressed on the renal afferent arteriole induces the release of renin from the juxtaglomerular apparatus, thereby activating the renin-angiotensin-aldosterone system (RAAS). Activation of this system results in an elevation of blood pressure and can therefore counteract the hypotensive effects [113, 350, 351]. Activation of the Olfr78 receptors in the renal afferent arteriole leads to modulation of the blood pressure within a few hours to days, whereas activation of these receptors on vascular smooth muscle cells can alter the vascular tone in a matter of seconds to minutes [351, 352].

However, Olfr78 has a lower sensitivity for acetate and propionate than the FFA3 receptor. Therefore, the compensating effects occur only at higher concentrations of these fatty acids. Several studies report that SCFA are found in the circulation in concentrations ranging from 0.1 to 10 mM [353, 354]. Because of the lower sensitivity of Olfr78 for acetate and propionate when compared to the FFA3 receptor, its compensating effects are only exerted at higher concentrations of these fatty acids. Therefore, the hypertensive effects of Olfr78 feature a buffering character. After all, high concentrations of SCFA may result in extreme and potentially dangerous hypotensive responses via stimulation of the FFA3 receptors. The buffering

action that occurs at higher concentrations may therefore counteract excessive drops in blood pressure [113, 351].

6.6. Treatment of cardiovascular diseases

SCFA can contribute to inhibition of the development or even to curing of cardiovascular diseases through several mechanisms. In addition to the influence on metabolic processes, the fatty acids also exert an anti-inflammatory on leukocytes and can thereby regulate various immune functions of these cells.

In the case of atherosclerosis, the accumulation of oxidised LDL particles, the low-grade inflammatory status of the endothelium and the release of various pro-inflammatory cytokines and chemokines result in an increased extravasation of monocytes from the circulation to the subendothelium [149, 255-257]. At the subendothelium, the monocytes differentiate into macrophages, which can contribute to the uptake, degradation and excretion of the lipids in the ox-LDL particles. To this end, the macrophages express more genes that are involved in the absorption and secretion of lipids. Due to the pro-inflammatory environment the uptake and release of absorbed lipids by macrophages are disturbed, causing the intracellular lipid concentrations to increase and the cells to transform into foam cells [149].

It appears that the altered gene expression of these macrophages can be influenced by various transcription factors, such as the peroxisome proliferator-activated receptors (PPAR) α, -β and -γ. These transcription factors can be activated by activation of PGC-1 [129, 130]. The strong expression of FFA2 receptors on the cell membrane of leukocytes enables binding of SCFA to the macrophages and facilitates the activation of intracellular AMPK [108, 110-112]. AMPK then activates PGC-1α, a co-activator for a wide variety of transcription factors, including PPARs [149, 355]. Research into the effects of PPARs on inflammatory tissue revealed that the transcription factors were able to suppress the expression of several pro-inflammatory response genes by interfering with pro-inflammatory signaling pathways of activator protein 1 (AP-1) and NF-κB [149]. For example,

activation of PPARα leads to inhibition of the induction of adhesion molecules ICAM-1 and VCAM-1 by pro-inflammatory cytokines. This reduces the extravasation of monocytes to affected endothelial tissue, thus reducing accumulation in the subendothelial space [356, 357]. Additionally, PPARα activation leads to reduced lipid uptake and storage. The concentration of cholesterol on the plasma membrane is an indicator of the utilized storage capacity of lipids in the macrophage. To this end, cholesterol stored in the intracellular lipid droplets is transferred to the plasma membrane with the aid of, inter alia, Niemann Pick Type C proteins 1 and 2 (NPC1 and -2) [149, 358, 359]. Activation of PPARα results in stimulation of lysosomal mobilization of cholesterol to the plasma membrane via induction of the expression of NPC1 and NPC2 proteins [360]. The resulting rise in cholesterol levels on the cell membrane of the macrophage causes greater delivery of cholesterol and other lipids to HDL particles. This process is further enhanced through stimulation of the expression of several HDL receptors and the inhibition of ACAT1, which reduces formation and storage of cholesterol derivatives in the macrophages [149].

Apart from the reduced ICAM-1 expression, the activation of transcription factor PPARβ also results in a decreased expression and release of chemokine MCP-1. As a result, a reduced influx and accumulation of monocytes into the subendothelial space could be observed in studies with LDL receptor knockout mice [361].

Research in which monocytes were exposed to PPAR agonists showed that activation of this transcription factor can inhibit both the migration and proliferation of monocytes. PPARγ appears to be able to inhibit the CCR2 expression, thereby suppressing the MCP-1 mediated chemotaxis [362-365]. Moreover, downregulation of the SR-A receptors on macrophages was observed, which resulted into a decreased absorption of lipids [366].

Furthermore, research in which macrophages were incubated with triglyceride-rich lipoproteins has revealed that both PPARα and -γ are able to inhibit the expression of the apoB-48 receptor, thereby reducing the uptake of triglycerides [367]. In addition, both transcription factors induce the β-

oxidation of the macrophages through upregulation of CPT1, which prevents the accumulation of triglycerides, therefore making the macrophages less prone to differentiate into foam cells [149].

Also, both transcription factors were able to inhibit the lateral migration into the subendothelial space as well as the proliferation of smooth muscle tissue. The transcription factors thereby stimulate the expression of cyclin-dependent kinase inhibitor p16, which inhibits the proliferation between the G1 and S-phase of the cell division. In this way, the formation of the fibrous cap is counteracted [368].

Several drugs achieve their effects on macrophages through activation of PPARs. For example, analysis of the CCR2 gene promoter yielded that statins affect the expression of CCR2 on monocytes through the activation of PPARγ. The suppression of the CCR2 expression by simvastatin appears reversible with the synthetic PPAR inhibitor GW9662 [149, 369].

In addition to the anti-inflammatory effects that are achieved through the activation of PGC-1α, both butyrate and propionate feature anti-inflammatory properties [370, 371]. For example, after being absorbed, the fatty acids appear capable of suppressing the production of pro-inflammatory cytokines TNFα, IL-1β and IL-6, whilst stimulating the synthesis and excretion of anti-inflammatory cytokine IL-10 [242, 243, 372-374]. These SCFA also inhibit the expression of VCAM-1 and the chemokine MCP-1, which leads to reduced adhesion and migration of monocytes [181, 375].

The anti-inflammatory activity of propionate and butyrate is based on the ability of these agents to inhibit the histone deacetylases (HDAC), causing changes in the expression pattern of various cytokines [376]. Furthermore, the fatty acids interfere with the signal transduction of NF-κB by decreasing the intracellular concentration of p65 as well as by inhibiting the translocation of the p65/p50 heterodimer. Moreover, the SCFA stimulate the expression of p50, resulting in an increased formation of p50 homodimers in the cytosol. The formation of the homodimer causes a decreased transcriptional activity of NF-kB, which leads to an even stronger reduction in pro-inflammatory cytokines [377].

The SCFA also stimulate the expression of anti-inflammatory cytokine IL-10. Research has demonstrated that release of IL-10 results in decreased expression of CD36 on the cell membranes of macrophages. Consequently, this lower expression leads to a reduced ox-LDL absorption capacity, thereby counteracting the formation of foam cells [378-380].

Summarizing it can be stated that the SCFA inhibit the low-grade inflammatory status of the endothelium by reducing the expression of pro-inflammatory cytokines and adhesion molecules, whilst stimulating the production and excretion of anti-inflammatory cytokine IL-10 [181].

As described earlier in this chapter, PGC-1α performs an adaptive role in the case of changes in energy demands of the heart due to spontaneous labour increase. Induction of PGC-1α could improve the contractile activity of the cardiac muscle cells and can therefore prevent heart failure [287]. However, overexpression of the co-activator leads to cardiomyopath [288], but possibly a protective effect occurs at physiological concentrations [287]. As SCFA can stimulate the activation of PGC-1α via the FFA3 receptors expressed on the cardiac muscle tissue, the fatty acids might be used to achieve this protective effect of PGC-1α [114]. Especially diabetics could benefit from treatment with SCFA, since the PGC-1α expression and mitochondrial capacity are found to be reduced in the heart muscle of these patients [381, 382].

7. Conclusions

In summary the following conclusions can be drawn.

- Cardiovascular disorders are mainly caused by a loss of endothelial functions, or by accumulation of triglycerides in the heart muscle due to imbalances in the metabolism of lipids and glucose [149, 153].
- SCFA are able to restore the imbalances in lipid and glucose metabolism, and thus can make a contribution to the prevention and treatment of cardiovascular diseases [23-28].
- Butyrate and propionate have the ability to regulate various functions of immune cells and can counteract the low-grade inflammatory status of

affected endothelial as to minimize the loss of endothelial functions [108, 110-112, 149, 355-357].

- In the case of atherosclerosis, SCFA can inhibit the formation of foam cells, the influx of monocytes as well as reduce the production of pro-inflammatory cytokines by the endothelium, and thus contribute to recovery of endothelial dysfunction [242, 243, 370-374, 376, 377].
- For the development of robust therapies with SCFA or pre- and probiotics based therapies, in which the production of fatty acids is stimulated, some aspects are still insufficiently mapped, making it unclear whether all preconditions for correct treatment can be met [383, 384].

For effective development of interventions for the prevention and treatment of cardiovascular disease based on SCFA the following recommendations apply.

1 Research into the metabolic capacity and the kinetic order of the metabolism of SCFA in both the liver and the colonocytes.

2 The mapping of the widths of the therapeutic windows of the SCFA in order to meet requirements with respect to safety and effectiveness.

3 For the application of pre- and probiotics on based therapies, it is necessary to identify the full phylogeny of the microflora in the large intestine, the associated metabolic properties of each species and the potential interactions between the species.

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Chapter 3

Pro- and anti-inflammatory effects of short chain fatty acids on immune and endothelial cells

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ABSTRACT

In the gastro-intestinal tract, short chain fatty acids (SCFA) have protective effects on epithelial cells. However, their effects on inflammatory cytokine production by endothelial and immune cells and the recruitment of immune cells and their trans-migration across the endothelial layer remain controversial. Both cell types are associated with the initiation and development of inflammatory diseases, such as atherosclerosis and sepsis. SCFA modulate immune and inflammatory responses via activation of free fatty acid (FFA) receptors type 2 and 3 (FFA2 and FFA3 receptors), G protein-coupled receptor 109A (GPR109A) and inhibition of histone deacetylases (HDACs). This review will focus on the effects of SCFA on lipopolysaccharide (LPS)- or tumor necrosis factor-alpha (TNFα)-induced inflammatory response on endothelial and immune cells function, and an overview is presented on the underlying mechanisms of the effects of SCFA on both immune and endothelial cells, including HDACs, FFA2 and FFA3 receptors and GPR109A regulation of nuclear factor-kappa B (NF-κB) activation and mitogen-activated protein kinase (MAPK) signaling pathways.

1. Introduction

The vasculature is the main "organ" for blood supply to our vital organs and mainly consists of three layers. The luminal side of blood vessels is endothelial layer which has multiple roles in keeping the human body in homeostasis by maintaining a stable anti-inflammatory, anti-coagulant and anti-adhesive status and by controlling exchanges between circulating blood components and cells [1]. Endothelial cells also cooperate with immune cells in the regulation of local and systemic inflammation. Both endothelial and immune cells can be activated by lipopolysaccharide (LPS) and tumor necrosis factor-alpha (TNFα) and lead to endothelial and immune cells dysfunction [2, 3]. Endothelial dysfunction-induced by endogenous and external stimuli will effectively induce a systemic state of inflammation and other immune responses by increasing the expression and production of (pro-) inflammatory mediators, adhesion molecules and excessive immune cell adhesion and migration [4, 5]. Immune cell adhesion to endothelial cells is mediated by adhesion molecules expressed on immune cells and endothelial cells, e.g. P-, E- and L-selectins, β_1- and β_2-intergrins, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and chemokines, e.g. monocyte chemo-attractant protein-1 (MCP-1) [6, 7]. The recruitment of monocytes to the endothelial layer facilitates the transmigration to the sites of the lesion, where monocytes differentiate into macrophages which become foam cells after lipid uptake, leading to the development of atherosclerosis [8]. Moreover, the transmigrated immune cells aggravate the inflammatory responses by producing more cytokines, thereby creating a continuous cycle between endothelial cells and immune cells (**Figure 1B**). Excessive cytokine production and immune cell adhesion to the sites of lesion are two important contributors to the development of inflammatory disorders, including atherosclerosis [9] and sepsis [10, 11]. Endogenous cytokines produced by endothelial and/or immune cells enhance the inflammatory response and initiate tissue damage. The levels of TNFα, interleukin-6 (IL-6) and LPS are regarded as diagnostic markers in inflammatory diseases [12, 13]. However, the therapeutic potential of

neutralizing antibodies for these cytokines failed to diminish the outcome of inflammatory diseases [11, 13, 14]. Therefore, new efficient drugs to prevent, combat and cure inflammatory diseases are needed.

Epidemiological evidence indicates that increased consumption of dietary fibers improves cardiovascular function and reduces systemic inflammation, atherosclerosis as well as immune disorders [15, 16], while low fiber diets are associated with increased inflammatory disorders [17]. For example, after two weeks addition of supplementary soluble fiber in the diet, the levels of circulating pro-inflammatory mediators such as TNFα, IL-6 and IL-8 were reduced [18]. Dietary fibers affect host physiology by the production of metabolites, such as short chain fatty acids (SCFA) [19-21]. SCFA, mainly acetate, propionate and butyrate, are fermentation metabolites of carbohydrates produced by the intestinal microbiome. The total SCFA concentration in the lumen of the colon decreases progressively from the proximal to the distal end from 70-140 mmol/l to 20-70 mmol/l respectively [22] with the ratio of acetate, propionate and butyrate in the colon being 60:25:15 [23]. SCFA are absorbed by the colonic epithelial cells, pass the portal vein, but mostly are then metabolized by the hepatocytes in the liver. A small fraction of SCFA can pass the liver and result in a low but measurable concentration in the systemic circulation. Moreover, intravenous administration of SCFA [24], sodium butyrate [25] or oral administration of tri-butyrin (a prodrug of butyrate) [26], increases the systemic SCFA concentrations.

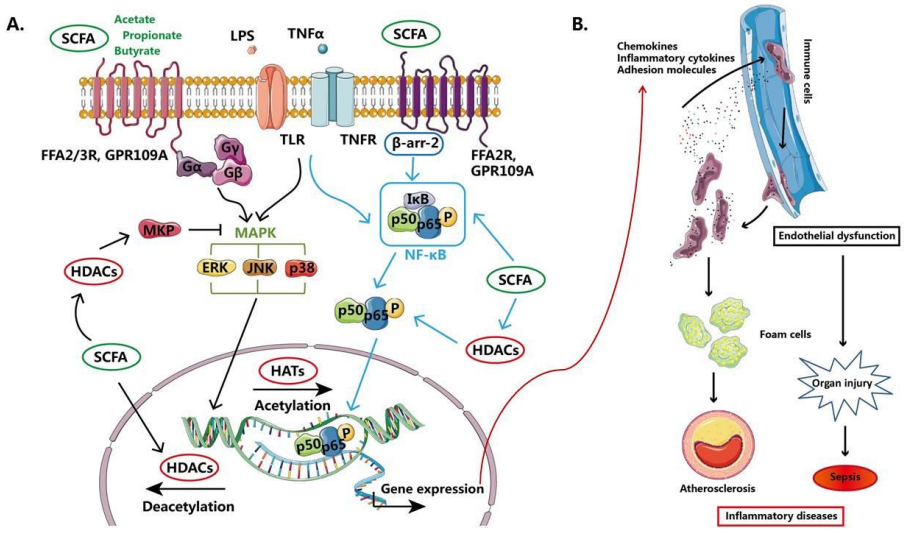

Figure 1. An overview on the effects of SCFA on LPS- or TNFα-induced endothelial dysfunction and immune cell activation. A: LPS or TNFα binds to its receptor and activates MAPK and NF-κB signaling pathways to modulate gene expression including inflammatory cytokines, chemokines and adhesion molecules which are important factors in the development of atherosclerosis and sepsis. SCFA, mainly acetate, propionate and butyrate, can regulate MAPK signaling pathway and activation of NF-κB via activation of FFA2 and FFA3 receptors and GPR109A or inhibition of HDACs activity. **B:** LPS or TNFα-dependent production of inflammatory cytokines, chemokines and adhesion molecules induce immune cell adhesion and recruitment to the inflammation lesion. The recruited immune cells produce excessive cytokines and attract more immune cells which forms a vicious circle which leads to foam cell formation and the development of atherosclerosis. Besides, endothelial dysfunction and activated immune cells are also important in the progression of organ injury and sepsis.

Although there is a clear correlation between high fiber consumption and changes the host immune status, the underlying mechanisms are largely unknown. SCFA form a link between dietary intake and improved health outcomes in Western societies. However, there is insufficient evidence for appropriate clinical or public health interventions with clearly defined outcomes using SCFA formulations in intervention or treatment of cardiovascular diseases. This review aims to offer an integrated view of

recent achievements in understanding the effects of SCFA on LPS- or TNFα-induced endothelial and immune cell dysfunction, in particular, aspects of inflammatory cytokine production and migration and recruitment of immune cells, as well as the main mechanisms involved in the inflammation modulatory effects of SCFA.

2. SCFA as agonists of free fatty acid (FFA) receptors type 2 and 3, G-protein coupled receptor 109A (GPR109A) and inhibitor of histone deacetylases (HDACs)

SCFA appear to have a regulatory function in cardiovascular disorders. The downstream effects of SCFA are mainly ascribed to two pathways: (1) activation of receptors, including FFA2 and FFA3 receptors, and GPR109A, and (2) inhibition of HDACs [27].

2.1 SCFA as agonists of FFA2 and FFA3 receptor and GPR109A

The FFA receptors are G protein-coupled receptors, which are dose-dependently activated by free fatty acids and are involved in various physiological and pathophysiological processes [28, 29]. FFA2 and FFA3 receptors and GPR109A are the main receptors for SCFA. FFA2 and FFA3 receptors are differentially expressed on cells and regulate diverse cellular functions. The FFA2 receptor is mainly expressed on immune cells, including neutrophils, eosinophils, dendritic cells and monocytes, indicating a broad role in inflammatory and immune responses [30]. The FFA3 receptor is mainly expressed on pancreas, spleen and adipose tissue and has been implicated in obesity and other metabolic diseases. However, FFA3 receptors are also expressed on immune cells, but at lower levels compared to FFA2 receptors [30]. The FFA2 receptor is likely the main receptor contributing to the effect of SCFA on inflammatory and immune disorders, but the effects via FFA3 receptor on metabolic disorders have also been widely recognized [31]. SCFA can activate FFA2 and FFA3 receptors and their potencies on activation of FFA2 and FFA3 receptors are different. The optimum chain length for the activation of the FFA2 receptor spans two to three carbon

atoms (acetate and propionate) and for the activation of the FFA3 receptor ranges from three to five atoms (propionate and butyrate), both with half maximal effective concentration (EC_{50}) of around 0.5 mM [29, 32]. Therefore, the potency rank orders of SCFA for the FFA2 receptor is acetate ~ propionate > butyrate, and for the FFA3 receptor is propionate ~ butyrate > acetate [31].

SCFA activate FFA2 and FFA3 receptors and trigger different downstream signaling cascades. The activated FFA3 receptor is coupled to $G_{\alpha i}$ and inhibits adenylyl cyclase, and therefore decreases the levels of cyclic AMP (cAMP). Activation of the FFA2 receptor is linked with both $G_{\alpha i}$ and $G_{\alpha q}$ and decreases cAMP levels and increases cytoplasmic calcium concentrations [29]. The FFA2 receptor also engages an alternative signaling pathway mediated by β-arrestins-2, producing anti-inflammatory effects by inhibition of NF-κB. For example, the pro-inflammatory cytokines IL-6 and IL-1β were down-regulated by activation of FFA2 receptor and knocking-out β-arrestin-2 recovered their expression [33, 34]. There are, as far as we know, no reports linking FFA3 receptors to β-arrestins [33].

GPR109A is classified as an orphan G-protein coupled receptor and known as hydroxyl-carboxylic acid 2 (HCA_2) receptor. GPR109A is expressed primarily on adipocytes and also expressed on immune cells including neutrophils and macrophages [35]. But there is no information indicating its presence on endothelial cells. Interest in GPR109A is growing since its discovery as the receptor for niacin a decade ago, along with deorphanisation as the receptor for endogenous ligand 3-hydroxy-butyrate. SCFA, mainly butyrate, show property to activate GPR109A. Activation of GPR109A is also coupled to the inhibitory G protein G_i/G_o [36] and activation of GPR109A can recruit β-arrestins from the cytosolic compartment to the cell membrane [35]. GPR109A is also linked to the regulation of vascular inflammation in atherosclerosis [35].

Taken together, signaling through FFA receptors could result in different outcomes in different cell types depending on receptor expressions and

binding of different subunits of FFA receptors or β-arrestins. For example, butyrate inhibited reactive oxygen species production in neutrophils in a pertussis toxin (PTX)-sensitive manner, while acetate increased reactive oxygen species production in macrophages in a PTX-insensitive manner [37]. These findings stress the opposing signaling via FFA2-$G\alpha_{q/11}$ and FFA3- $G\alpha_{i/o}$.

2.2 SCFA as HDAC inhibitors

Most of the HDACs are ubiquitously expressed in immune, endothelial and vascular smooth muscle cells. HDACs and histone acetyltransferases (HATs) modulate acetylation of histone protein which is involved in epigenetic DNA modification [38]. HDACs are an evolutionary conserved family of proteins that include four classes: I (HDAC1-3 and 8), II (HDAC4-7 and HDAC9-10), III sirtuins (SIRT1-7) and IV (HDAC11) and are present in the cytoplasm or nucleus of cells [39, 40]. Inhibition of HDACs activity causes an increase in acetylation in histone proteins and decreases positive charge on histones. The decreased positive charge reduces binding of histones to the negatively charged DNA, leading to an open structure of DNA/chromatin, which facilitates the binding of transcription factors, such as signal transducer and activator of transcription3 (STAT3), NF-κB and forkhead box P3 (FOXP3), thereby initiates gene transcription (**Figure 1A**). Although, inhibition of HDACs theoretically results in an increase in gene transcription, HDAC inhibitors can either inhibit or facilitate specific gene expression, depending on the promoter and chromatin status [41]. HDAC inhibitors were originally developed as anti-cancer agents [42]. Now we know that HDACs are also involved in the regulation of inflammatory gene expression, vascular integrity and the development of cardiovascular diseases, including atherosclerosis and sepsis [43-46].

HDAC inhibitors can be structurally classified into at least four classes: hydroxamates, cyclic peptides, aliphatic acids and benzamides [47]. Trichostatin (TSA) and suberoylanilide hydroxamic acid belong to hydroxamates class and are very potent inhibitors with an efficacy at a nanomolar to low micromoloar range. SCFA belong to aliphatic acids class

with effective inhibitors of HDAC enzymes in the millimolar range [48]. These HDAC inhibitors are generally known as broad-spectrum inhibitors. SCFA, mainly butyrate and propionate, inhibit class I and class IIa HDACs, and down-regulate SIRT1 expression [49, 50]. Butyrate and propionate are non-competitive inhibitors of HDACs and specifically inhibit the activity of HDAC1 and HDAC3 [51]. Among the SCFA, butyrate is the most potent inhibitor of HDACs with approximately 80% inhibitory efficiency, while the inhibitory efficiency of propionate is approximately 60% [52]. Most, but not all of the studies indicate that acetate has no HDAC inhibitory activity. For instance, acetate enhanced IL-6, IL-8 and TNFα production in LPS-exposed macrophages accompanied with increased acetylation of pro-inflammatory gene histones [53]. HDAC activity may be inhibited by SCFA after entering the cell via passive diffusion or sodium-coupled monocarboxylate transporters ((SMCT-1)/Slc5a8 and via FFA receptor activation [54]. However, the roles of FFA receptors in inhibiting HDACs are controversial. For example, inhibition of HDACs by activation of FFA3 receptors in Chinese hamster (*Cricetulus griseus*) ovary cell lines suppressed histone acetylation [55]. SCFA-induced inhibition of HDACs in colon tissue was largely FFA2 receptor dependent [56] while acetate may influence the inflammatory process by regulating epigenetic modification in a FFA receptor-independent manner [57]. Furthermore, butyrate and propionate inhibited HDAC activity, independent of FFA2 and FFA3 receptors [58]. It is not completely clear whether this effect is direct or indirect, and further studies are necessary to confirm any causal relationship between FFA receptor activation and HDAC inhibition.

3. The roles of SCFA in the regulation of inflammation in immune cells

The immune system protects the host against pathogens by secreting inflammatory cytokines and mediating the clearance of pathogens. However, excessive production of cytokines will lead to systemic inflammation and pathological diseases [59]. SCFA modulate inflammation by regulating

immune cell cytokine production. For example, butyrate and propionate decrease LPS-induced TNFα and nitric oxide synthase (NOS) expression in monocytes [51]. These effects are mediated by activation of FFA2 and FFA3 receptors and GPR109A or inhibition of HDACs.

3.1 FFA2 and FFA3 receptors and GPR109A mediate the pro- and anti-inflammatory effects of SCFA in immune cells

FFA2 and FFA3 receptors expression is upregulated by LPS stimulation in monocytes and macrophages and this indicates a potential role of these receptors during systemic inflammation [60]. However, it is not clear which of the two FFA receptors is more important or whether they cooperate to induce anti-inflammatory or pro-inflammatory effects, because controversial results are reported depending on ligand, cell type, organ and disease.

SCFA reduced IL-8 production in the airways during airway inflammation by activation of FFA2 and FFA3 receptors on neutrophils and macrophages [61]. Acetate also inhibited LPS-induced TNFα secretion from mice and human mononuclear cells by activating FFA receptor pathways [62]. Administration of propionate to allergic mice reduced inflammatory mediators, such as IL-4, IL-5 and IL-17A in the lungs, through an FFA3 receptor-dependent manner [63]. In macrophages, butyrate had anti-inflammatory effects (decreasing inducible NOS (iNOS), TNFα, MCP-1 and IL-6 production) by activation of FFA3 receptors [64]. These effects were incompletely blocked by PTX since PTX blocked the response to FFA3 receptor activation, but not to FFA2 receptor activation, indicating that the anti-inflammatory effects of butyrate are associated with FFA3 receptors and other non-FFA receptor pathways. These observations indicate that FFA2 and FFA3 receptors act as anti-inflammatory receptors and FFA2 and FFA3 receptor agonists could offer new opportunities for the treatment of inflammatory diseases.

In contrast, pro-inflammatory roles of activated FFA2 or FFA3 receptors are also reported and associated with the activation of MAPK, phosphoinositide 3-kinase (PI3K) or rapamycin (mTOR) signaling pathways [65, 66]. Activation of FFA2 and FFA3 receptors by acetate increased the production of cytokines

(IL-6, CXCL1 and CXCL2) via activation of the extracellular signal-regulated kinases 1/2 (ERK1/2) and p38MAPK signaling pathways. Deletion of FFA2 or FFA3 receptors in mice diminished IL-6 production and delayed the expression of interferon gamma (INFγ) and chemokines [67], thereby protecting these mice against inflammatory tissue destruction. These studies indicate the pro-inflammatory effects of activation of FFA2 and FFA3 receptors and antagonists of FFA2 and FFA3 receptors may have protective effects in inflammatory diseases.

GPR109A expression is upregulated by cytokines such as IFN-γ in macrophages [68], pointing to a role for GPR109A in immunity and inflammation. GPR109A activation and its direct anti-inflammatory potential in the vasculature had also emerged. Activation of GPR109A inhibits TLR4-induced expression and secretion of TNFα, IL-6 and MCP-1and reduces progression of atherosclerosis [69]. Among the SCFA, only butyrate binds to GPR109A with low affinity and activation of GPR109A by butyrate exerts anti-inflammatory effects in colonic inflammation [35, 70]. However, there is a lack of information about GPR109A-mediated effects of butyrate on immune cells in the regulation of cardiovascular function. Since GPR109A shows anti-inflammatory effects, it is worthwhile to investigate GPR109A-mediated effects of SCFA on immune cells in cardiovascular disorders, such as atherosclerosis.

Due to the opposite effects of activation of FFA2 and FFA3 receptors and unravelled roles of GPR109A, it remains unclear whether the use of an agonist or an antagonist of FFA2 and FFA3 receptors would be preferred in clinical settings. In a study in humans, it was demonstrated that GLPG0974, an FFA2 receptor antagonist, did not meet clinical endpoints due to the induction of mild-to-moderate ulcerative colitis in spite of a reduction in neutrophil activation and infiltration [71]. In general, acetate and propionate stimulate while butyrate inhibits immune cell function [72]. This might explain the conflicting data when mixtures of SCFA are used, and may be further complicated when non-receptor mediated anti-inflammatory effects

are involved. FFA receptors may provide a link between diet, gut microbiota and host immune homeostasis, and highlight the importance of FFAs in the regulation of inflammatory and immunological processes.

3.2 HDACs mediate the pro- and anti-inflammatory effects of SCFA in immune cells

Butyrate and propionate showed anti-inflammatory activities by inhibition of HDACs in macrophages and dendritic cells. Butyrate and propionate decreased LPS-induced TNFα production in mononuclear cells via inhibiting NF-κB activation, and the effects of butyrate and propionate were similar to the HDAC inhibitor TSA [73]. In another study, treatment with butyrate and propionate suppressed TNFα production and NF-κB activity, and promoted the production of anti-inflammatory cytokine IL-10 in LPS-activated mononuclear cells and neutrophils by inhibition of HDACs [21, 58, 74]. However, these roles of HDACs in modulating the inflammatory response were demonstrated by non-specific HDAC inhibitors. Specific HDAC inhibitors should be used to investigate the specific role of each HDAC subtype.

HDAC3-deficient macrophages were unable to activate almost half of the inflammatory gene expression program when stimulated with LPS. Especially, the IFNβ-dependent branch of the LPS response was almost completely abrogated because of the reduced basal and LPS-inducible IFNβ expression. These data indicate a central role for HDAC3 in inflammation and may have relevance for the use of selective HDAC inhibitors as anti-inflammatory agents [75].

HDAC5 belongs to class II and is regulated by phosphorylation of serine residues at the N-terminus of the enzyme. Class II HDACs can shuttle between the nucleus and cytoplasm. The capacity to translocate enables the interaction with cytoplasmic non-histone proteins such as NF-κB [76]. Knock-down of HDAC5 significantly reduces the LPS-induced production of TNFα and MCP-1 in murine and human macrophage cell lines, and over-expression of HDAC5 significantly elevated the production of these cytokines as well as

anti-inflammatory IL-10. These effects were accompanied by increased NF-κB activity [76]. Therefore, HDAC5 has a regulatory function in the pro-inflammatory response of macrophages.

HDAC6 is involved in the regulation of inflammatory and immune responses, specifically at the level of the antigen-presenting cells / T cell immune synapse, regulatory T cell function and macrophage responses [77]. HDAC6, as a transcriptional activator, is required for the production of IL-10 by macrophages and inhibition of HDAC6 disrupted the anti-inflammatory STAT3/IL10 axis in macrophages [78]. However, the primarily nuclear protein HDAC11 represses IL-10 gene expression in macrophages [79]. Two different HDACs are recruited to the same gene promoter to dictate divergent transcriptional responses.

Taken together, these data indicate that the HDAC subtypes do have inflammation modulatory properties on immune cells. Hitherto, due to their broad-spectrum inhibition of HDACs, it is not completely clear which specific HDAC mediates the effects of SCFA on stimuli-induced immune cell dysfunction. This might be the reason for the pleiotropic effects of SCFA. Therefore, specific HDAC knockdowns in different cell types and animals are necessary for investigating the roles of the different HDACs and SCFA in cardiovascular function and disease in which (chronic) inflammation plays a pivotal role.

4. The roles of SCFA in regulation of inflammation in endothelial cells

Data on the roles of FFA2 and FFA3 receptors and HDACs in the effects of SCFA on endothelial cells is scarce despite of the fact that SCFA (acetate, propionate and butyrate) attenuate TNFα- or LPS-induced endothelial activation by inhibiting the production of pro-inflammatory cytokines (IL-6 and IL-8) [80] and FFA2 and FFA3 receptors as well as HDACs are expressed on/in endothelial cells [81, 82]. Voltolini *et al.* studied the involvement of FFA2 and FFA3 receptors in the effects of SCFA on regulation of inflammatory cytokine production in endothelial cells and found that the LPS-induced

mRNA expression of FFA2 receptors and inflammatory genes, such as IL-6 and IL-8 was attenuated by sodium propionate [83].

HDAC inhibitors stimulate anti-inflammatory signaling pathways in the endothelium, pointing to a therapeutic potential of HDAC inhibitors in the treatment of inflammatory diseases. SCFA, especially butyrate as a HDAC inhibitor, protect against vascular inflammation and atherosclerosis, thereby modulating endothelial function, pro-inflammatory cytokine production and oxidative stress [84, 85]. Administration of two structurally unrelated HDAC inhibitors, TSA and sodium butyrate, alleviated sepsis-induced lung injury accompanied with LPS-induced IL-6 and cyclooxygenase-2 (COX-2) expression in human intestinal endothelial cells [86]. Based on limited information, FFA2 and FFA3 receptors and HDACs are involved in the anti-inflammatory effects of SCFA. However, the roles of SCFA in regulation of endothelial dysfunction and cardiovascular diseases and the exact roles of FFA2 and FFA3 receptors and HDACs in the effects of SCFA are still an open area for fundamental research.

5. FFA2 and FFA3 receptors and HDACs mediate the effects of SCFA on the recruitment of immune cells to endothelial cells

SCFA not only modulate the production of pro-inflammatory cytokines but also affect migration and recruitment of immune cells to endothelial cells, which is also an important step in the development of inflammatory diseases including atherosclerosis and sepsis (Figure.1 B) [87-89]. These effects are mediated by modulation of adhesion molecules expression on immune and endothelial cells by activation of FFA2 and FFA3 receptors or inhibition of HDACs.

5.1 FFA2 and FFA3 receptors mediate the effects of SCFA on the recruitment of immune cells and adhesion molecule expression in immune cells

The data on FFA2 and FFA3 receptors mediated effects of SCFA on the recruitment of immune cells are still controversial. SCFA induce migration

and recruitment of neutrophils to inflammatory sites by activation of FFA2 receptors following activation of MAPK signaling pathways [54, 90, 91]. On the contrary, some data indicated that SCFA inhibit the recruitment of immune cells. Butyrate suppressed INFγ-induced ICAM-1 and lymphocyte function-associated antigen-3 (LFA-3) expression on monocytes [92]. Propionate and butyrate decreased neutrophil migration and L-selectin expression, whose effects were diminished in FFA2R$^{-/-}$ mice [93]. These discrepancies might be due to the different cell types, the activation status of cells used and the different adhesion molecules investigated.

5.2 HDACs mediate the effects of SCFA on adhesion molecule expression in endothelial cells

Differential effects of SCFA on expression of ICAM-1 and VCAM-1 on endothelial cells were found and HDACs might be involved in the effects of SCFA. Incubation of human umbilical vein endothelial cells (HUVECs) with butyrate or propionate increased ICAM-1 expression, but not VCAM-1 [94, 95]. This was supported by another study in which butyrate increased ICAM-1 expression by inhibition of histone acetylation [86, 95]. These data suggest that SCFA facilitate adhesion molecules expression and the recruitment of immune cells. However, pre-incubation of TNFα-stimulated HUVECs with butyrate or propionate significantly decreased VCAM-1 but not ICAM-1 expression and reduced the adhesion of monocytes and lymphocytes to HUVECs [80, 87, 94, 96]. Butyrate inhibited VCAM-1 in oxLDL-induced EA.hy926 cells (HUVEC-derived cell line) via inhibition of NF-κB. This led to a reduced migration and adhesion of monocytes to the lesion area, indicating that butyrate may have a role in the prevention and treatment of atherosclerosis [88]. Due to the diverse effects of SCFA, different subtypes of HDACs might be involved in the regulation of adhesion molecules expression.

HDAC3 is highly expressed in endothelial cells and is involved in regulating vascular function. However, the role of HDAC3 differs in the regulation of different adhesion molecules. Inflammation-induced down-regulation of HDAC3 is associated with an NF-κB-dependent increase in ICAM-1 expression,

indicating that HDAC3 inhibits ICAM-1 expression [97, 98]. In contrast, HDAC3 knockdown or inhibition represses TNFα-induced monocyte adhesion via inhibition of VCAM-1 expression in HUVEC [99] indicating that HDAC3 facilitates VCAM-1 expression in endothelial cells.

HDAC4 is also involved in the regulation of vascular function by affecting endothelial cells. Kruppel-like factor-2 (KLF-2) is a novel transcriptional regulator of endothelial pro-inflammatory activation that inhibits the expression of pro-adhesive factors, such as VCAM-1, and immune cell adhesion to the endothelial monolayer. KLF-2 expression was significantly reduced by TNF-α via activation of NF-κB and HDAC4 [100]. Therefore, HDAC4 modulates VCAM-1 expression and recruitment of immune cells by regulation of KLF-2.

It is clear that SCFA modulate the migration and recruitment of inflammatory cells to the endothelium by regulating the expression of adhesion molecules. However, their diverse effects on adhesion molecules expression might be due to their broad-spectrum effects on activation of FFA2 and FFA3 receptors and inhibition of different HDACs.

6. Mechanisms involved in the effects of SCFA on regulation of inflammation and recruitment of immune cells

Generally, FFA receptors are phosphorylated and then transduce a signal leading to the internalization and desensitization of FFA receptors. Activation of FFA receptors down-regulates NF-kB downstream genes expression and regulates several intracellular pathways including MAPKs (ERK, c-Jun N-terminal kinase (JNK) and p38MAPK) [101]. Other studies also show that HDACs can modulate NF-κB activation and MAPK signaling pathway [102, 103].

6.1 NF-κB activation

NF-κB mediates the transcription of multiple pro-inflammatory genes and is pivotal in immune and inflammatory responses. Using promoter deletion mutagenesis and reporter gene analysis, it was demonstrated that NF-κB is

crucial for LPS- and cytokine-activated promoter activity of over 200 genes involved in the development of inflammatory diseases. These genes include cytokines (TNFα, TNFβ, IL-1β, IL-2, IL-3, IL-5, IL-8, IL-12, IL-18), chemokines (IL-8, MIP-1α, MIP-2, MCP-1), adhesion molecules (ICAM-1, VCAM-1, E-selection, P-selectin) and enzymes (iNOS, COX-2) [104-106]. Hence, inhibition of NF-κB activation can inhibit expression of multiple pro-inflammatory genes, reduce tissue neutrophil influx and prevent endothelial leakage. The order of potency for the suppression of NF-κB activity is butyrate>propionate>acetate [107], which is similar to the order for the inhibition of HDACs [108].

Traditionally, NF-κB activity is regulated by signal-induced IκB degradation leading to NF-κB activation. However, NF-κB transcriptional activity can also be modulated by acetylation and deacetylation of proteins in the NF-κB pathway and by accessibility of NF-κB target genes. For example, CBP/p300, upstream of NF-κB, interacts with HATs to induce gene expression [109] and subunits of NF-κB (p65 and p50) interact with HDACs to repress transcription [110]. The acetylation status of p65 modulates its binding to IκBα. Acetylated p65 interacts weakly with IκBα, whereas deacetylated p65 by HDAC3 enhances p65 binding to IκBα, which in turn, can result in the export of NF-κB complexes from the nucleus back to the cytoplasm [111]. Moreover, the association of p65 with HDAC1 and HDAC2 inhibits the expression of NF-κB-regulated genes at both basal and induced levels (**Figure 1A**) [110]. HDAC1 directly associates with the Rel homology domain of p65 which modulates NF-κB activation or repression. HDAC2 does not interact with NF-κB directly, but can regulate NF-κB activity via its association with HDAC1. Nuclear NF-κB in unstimulated cells mainly consists of p50 homodimers coupling with HDAC1 bound to DNA and repressing NF-κB dependent gene expression, such as IL-6, IL-8, iNOS and TGFβ. Following activation, p50/p65 heterodimers, containing phosphorylated p65, translocate into the nucleus and displace DNA-bound p50/HDAC1 [109]. These mechanisms ensure that only NF-κB activates transcription in activated cells.

Butyrate and propionate are known as HDAC inhibitors and also shown to modulate NF-κB activity. For example, butyrate upregulated IL-10 production and repressed the production of pro-inflammatory molecules IL-12, TNFα, IL-1β, NO by inhibiting NF-κB activity [73, 112, 113]. Moreover, propionate inhibited the production of NO by macrophages, an effect associated with inhibition of NF-κB activation [73]. However, there is still no direct evidence proving that the effect of butyrate or propionate on inhibition of NF-κB activity is mediated by inhibition of HDACs.

6.2 MAPK signaling pathways

MAPK signal transduction pathways including ERK, JNK and p38MAPK are involved in regulation of multiple cell functions. The ERK signaling pathway is a major regulator of cell proliferation, while JNK and p38MAPK are associated with inflammation processes. MAPK signal transduction pathways can be interfered by HDACs. Acetylation status of mitogen-activated protein kinase phosphatase-1 (MKP-1) enhances its interaction with MAPK substrates, dephosphorylates ERK, JNK and p38 MAPK, and negatively regulates MAPK signaling. Simultaneous inhibition of HDAC1-3 increased MKP-1 acetylation and decreased LPS-induced phosphorylation of p38MAPK in macrophages but not in MKP-1 null macrophages. Finally, inhibition of HDAC1- 3 decreased LPS-induced expression of TNFα, IL-1β, iNOS and nitrite synthesis [102]. However, the effects of HDACs might also be independent of the MAPK signaling pathways. TSA (HDAC inhibitor) treatment strongly inhibited TNFα and IL-6 production in a time- and dose-dependent manner in macrophages. However, TSA did not inhibit ERK1/2 or p38 phosphorylation or NF-κB, c-jun or IRF7 nuclear translocation in these macrophages. Instead, TSA strongly increased Mi-2β, a transcriptional repressor recruited to the IL-6 promoter in macrophages exposed to LPS. TSA also reduced the binding of Mi-2β to the Tnf promoter [103].

Moreover, FFA receptors can be linked to different signaling cascades downstream of MAPK. FFA2 and FFA3 receptors activation induces

phosphorylation of ERK1/2 and FFA2 receptor activation induces phosphorylation of p38MAPK, while FFA receptors can weakly activate JNK [66]. It has been shown that propionate-induced FFA3 receptor activation is followed by ERK1/2 activation, whereas acetate treatment, the FFA2 receptor agonist, resulted in weaker activation. There is a clear link between FFA2/FFA3 receptors/HDACs and NF-κB/MAPK and link between SCFA and FFA2/FFA3 receptors/HDACs, however it is not clear if activation of FFA2 and FFA3 receptors or inhibition of HDACs by SCFA regulates NF-κB or MAPK signaling pathway mediating the effects of SCFA on immune and endothelial cells.

7. Conclusions and future perspectives

SCFA might play an essential role in regulation of inflammation and contextually result in either protective or causative effects, by stimulating or dampening production of inflammatory cytokines, as well as inhibiting or facilitating migration and recruitment of immune cells, which are likely mediated by a combination with cell surface receptors (FFA2, FFA3 receptors and GPR109A) or inhibiting intracellular enzyme activity (HDACs) (**Figure 1A**). Although, the effects of SCFA on regulation of endothelial and immune cell (dys)function are complex, SCFA still show promising therapeutic potential in the treatment of inflammatory and cardiovascular diseases. There are some key research questions that remain to be investigated because the latest clinical trials investigating the effects of receptor agonists or HDAC inhibitors in inflammatory disorders failed [71]. First, more information on FFA2 and FFA3 receptors and HDACs mediated effects of SCFA in endothelial cells in the development of inflammatory diseases is needed. Second, most studies used non-specific receptor agonists and antagonists or HDAC inhibitors which might contribute to the variable effects on regulation of inflammation. Therefore, specific receptor agonists and antagonists and specific HDAC inhibitors are required. Besides, the downstream molecular processes involved in the activation of receptors and/or inhibition of HDACs will not only elucidate the underlying mechanisms, but also offer the explanation for

diverse effects of SCFA. Third, the pleiotropic roles of different SCFA indicate that the effects of acetate, propionate and butyrate treatment alone or in combination should be investigated.

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Chapter 4

Time and concentration dependent effects of short chain fatty acids on lipopolysaccharide- or tumor necrosis factor α-induced endothelial activation

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ABSTRACT

Background and Aim

Endothelial activation is characterized by excessive production of cytokines and chemokines as well as adhesion molecules expression which is involved in the development of atherosclerosis. The aim of our study is to investigate the effects of short chain fatty acids (SCFA) on lipopolysaccharide (LPS) or tumor necrosis factor alpha (TNFα)-induced endothelial activation.

Methods and results

Human umbilical vein endothelial cells (HUVEC) were pre-treated with acetate (10mM), butyrate (0.1mM) or propionate (0.3mM) for 1h, 16h or 24h and then stimulated with LPS (1μg/ml or 10μg/ml) or TNFα (100pg/ml or 1ng/ml) for 6h, 12h or 24h. Cytokines in the supernatant were measured by ELISA. HUVEC were pre-treated with acetate (10mM), butyrate (5mM) or propionate (10mM) for 24h and then stimulated with LPS (1μg/ml) or TNFα (1ng/ml) for 8h. The expression of the adhesion molecules intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) was detected by flow cytometry. The human blood mononuclear cell adhesive level to HUVEC monolayer was measured. LPS and TNFα induced a significant increase in the release of interleukin-6 (IL-6) and IL-8. Acetate, butyrate and propionate reduced IL-6 and IL-8 levels and the magnitude was dependent on the incubation times. LPS or TNFα increased ICAM-1 and VCAM-1 expression. Pre-incubation with acetate had no effect. In contrast, butyrate and propionate decreased VCAM-1 expression in TNFα stimulated cells but showed no effects on ICAM-1 expression. Butyrate significantly inhibited the adhesion of mononuclear cells to an endothelial monolayer and propionate was less effective.

Conclusion

SCFA, including acetate, butyrate and propionate, influenced LPS- or TNFα-induced endothelial activation by inhibiting the production of IL-6 and IL-8, and reducing the expression of VCAM-1and subsequent cell adhesion.

Results were dependent on the concentrations and pre-incubation time of each SCFA and stimulation time of LPS or TNFα.

1. Introduction

Vascular endothelial cells serve as an important barrier to sustain homeostasis by maintaining normal vascular tone and blood fluidity and low production and release of pro-/anti-inflammatory factors. The homeostasis is disturbed by inflammation which leads to endothelial activation [1]. Endothelial activation is a well-established early step to cardiovascular risk factors and is involved in the development of atherosclerosis [2]. Endothelial activation induced by lipopolysaccharide (LPS, endotoxin) and pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) is characterised by increased inflammatory cytokine production and adhesion molecule expression [3] which are two important steps in the development of atherosclerosis [4]. Increased production of cytokines, such as interleukin-6 (IL-6) and IL-8, is regarded as diagnostic markers in inflammatory diseases including atherosclerosis, obesity, heart diseases and sepsis [5-8]. IL-6 has broad impacts on immune cells as a lymphocyte-stimulating factor and is involved in innate and adaptive immunity [9]. IL-6 has pro- and anti-inflammatory effects which is dependent on the receptor that IL-6 binds to and IL-6 exerts pro-inflammatory effects on endothelial cells via binding to sIL-6R [10]. Therefore, in our study, IL-6 induced by LPS or TNFα is regarded as a pro-inflammatory factor. IL-8 is a chemokine secreted by activated macrophages and endothelial cells which stimulates vascular adhesion of neutrophils [7, 11]. Leukocyte transmigration over the endothelium is a multi-step process including capturing, rolling, leukocyte arrest, crawling to sites of exit and transmigration [12] and each step is regulated by distinct endothelial adhesion receptors such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and selectins. Excessive expression of ICAM-1 and VCAM-1 is regarded as an early event of atherosclerosis [13]. The transmigrated inflammatory cells produce more cytokines and form a perpetual cycle between cytokines and cell adhesion. Therefore, attenuation of endothelial activation is a crucial step in preventing and treating cardiovascular diseases including atherosclerosis.

Unbalanced diets may lead to acute or chronic low-grade inflammation which is a risk factor for various pathological conditions including cardiovascular diseases. Based on epidemiological studies, increased consumption of dietary fibres decreases the risk of cardiovascular diseases, such as atherosclerosis, and this may be due to the enhanced production of short chain fatty acids (SCFA) [14]. SCFA, such as acetate, butyrate and propionate, are the major products formed during colonic fermentation of dietary fibres. SCFA are used by local enterocytes as an energy resource or are transported across the gut epithelium into the bloodstream where they can interact with peripheral cells and tissues as signaling molecules. Under physiological condition, the concentrations of SCFA in the peripheral blood are very low due to the hepatic metabolism and the predominant SCFA in circulation is acetate [15]. However, in *in vivo* experiments, administration of the pro-drug of butyrate [16] or SCFA intravenously [17] increases the concentrations of SCFA into millimolar levels in blood and could be clinically used. SCFA, especially butyrate, were originally studied as anti-cancer agents, such as in colorectal cancer [18]. In recent researches, SCFA showed promising potential therapeutic effects in inflammatory bowel diseases [19], obesity, insulin resistance [20] and cardiovascular diseases [14]. However, there are no studies about the effects of SCFA on LPS or TNFα-induced IL-6 and IL-8 production by primary human umbilical vein endothelial cells (HUVEC) and their roles in adhesion molecule expression are contradictory [21-23]. Therefore, we investigated the effect of SCFA (acetate, butyrate and propionate) on LPS or TNFα-induced endothelial activation by regulation the production of IL-6 and IL-8 and expression of ICAM-1 and VCAM-1 under different experimental conditions.

2. Materials and Methods

2.1 Reagents and materials

Sodium butyrate, propionate and LPS (*Escherichia coli* 0111: B4) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Acetate was bought form Merck Millipore (Germany). Cell cytotoxicity detection kit (lactate

dehydrogenase, LDH) was obtained from Roche (Switzerland). Human IL-6 and IL-8 ELISA (enzyme-linked immunosorbent assay) kits were purchased from Invitrogen (Netherlands). Human recombinant TNFα, anti-human CD54 (ICAM-1) PE, anti-human CD106 (VCAM-1) PE and viability fixable dyes were bought from eBioscience (Netherlands). EGM-2 Bulletkit was bought from Lonza (Switzerland).

2.2 Cell culture and treatment

HUVEC from *umbilical vein* were provided by mrs. J.H. van Kats-Renaud (University Medical Center, Utrecht) after informed consent and isolated and cultured by adapting the method of Jaffe *et al* [24]. HUVEC were cultured in EGM-2 (Lonza) containing 2% fetal bovine serum and VEGF for rapid proliferation, and maintained in a humidified incubator at 37°C in 5% CO_2. Medium was changed every 2-3 days. HUVEC were cultured until about 85% confluence and cells of passages 2-7 were used for the experiments.

2.2.1 LPS or TNFα stimulated-HUVEC

To induce an inflammatory response in the HUVEC, two different triggers were used namely LPS and TNFα. The first goal was to find the optimal time and dose of stimulation and to investigate which cytokines were produced. HUVEC were incubated in LPS (1µg/ml or 10µg/ml) and TNFα (100pg/ml or 1ng/ml) for 6h, 12h and 24h.

2.2.2 SCFA treated LPS or TNFα-stimulated HUVEC

Each SCFA was dissolved in EGM-2 and then was filtered over a 0.2µm filter to make it sterile. The stock concentration of each SCFA was 10 times of final concentration and was stored in 4℃. Confluent HUVEC were pre-incubated with pre-warmed stock solution of each SCFA with final concentration of acetate (0.3mM, 1mM, 3mM, 10mM, 30mM or 100mM), butyrate (0.1mM, 0.3mM or 1mM) and propionate (0.1mM, 0.3mM, 1mM or 5mM) or medium only for 1h, 16h or 24h. Next, LPS at 1µg/ml or 10µg/ml, TNFα at 100pg/ml or 1ng/ml or medium only were added for 6h, 12h or 24h.

2.3 Cell cytotoxicity LDH test

The impact of each SCFA on the cell cytotoxicity was assessed by LDH and the procedure described by the manufacturer followed. Briefly, 100µl cell suspension (0.5-2 x 10^4) was added into 96-well plates and medium was refreshed every 2-3 days until confluence. Cells were treated with acetate (1, 10 and 100mM), butyrate (0.1, 0.3 and 1mM) and propionate (0.3, 1 and 5mM) for 48h, and butyrate (5mM) and propionate (10mM) for 32h. After that, the supernatants were collected and put into a new 96-well plate for LDH assays. 100µl of the reaction mixture was added to each well and incubated in the dark at room temperature for 30 min before the absorbance of samples was measured at 490nm. 1% Triton-X treated cells, which were killed 100%, were regarded as a positive control in comparison with other groups in the LDH analysis.

2.4 Enzyme linked immunosorbent assay (ELISA)

After pre-incubation with acetate, butyrate or propionate and LPS or TNFα stimulation, the supernatants were collected and stored at -20℃ for further analysis. The levels of IL-6 and IL-8 in each group were measured by using ELISA according to the manufacturer's instructions.

2.5 VCAM-1 and ICAM-1 expression

After pre-incubation with acetate (10mM), butyrate (5mM) and propionate (10mM) for 24h followed by 8h LPS (1µg/ml) or TNFα (1ng/ml) stimulation, cells were detached from the cell culture plate surface by incubation with 0.05% trypsin/EDTA for 5 min at 37℃. Cell suspensions were centrifuged at 2151g/min at 4℃ for 5 min. Supernatants were discarded and cell pellets were re-suspended in PBS/0.1%BSA (w/v). PE-labelled ICAM-1 and VCAM-1 antibodies were added in a dilution of 1:800 (v/v) and cells were incubated for 30 min on the ice in the dark followed by staining with viability fixable dyes for live and dead cells analysis. Flow cytometry analyses were performed using BD FACS Canto II cytometer and Flowlogic version 7.

2.6 Blood mononuclear cells isolation

Human blood mononuclear cells from healthy donors were isolated from buffy coats (Sanquin, Amsterdam, the Netherlands). Cells were purified using Ficoll-Paque Plus gradient centrifugation.[25] Briefly, mononuclear cells were isolated by centrifugation according to the manufacturer's instructions. Mononuclear cells above the polyester gel were collected, washed with PBS containing 2% FBS by centrifuging at 1200rpm for 5min, re-suspended the cell pellet at a concentration of $2x10^6$cells/ml in RPMI1640 medium without phenol red containing 10% FBS and 1% penicillin-streptomycin. The viability of mononuclear cells was determined by trypan blue staining and cell number was counted.

2.7 Endothelial cell monolayer adhesion experiment

HUVEC were seeded in 96-well plates until they were confluent. Isolated mononuclear cells were washed with warm PBS three times and mononuclear cells ($2x10^6$/mL) labelled with 1µM calcein-AM for 30min according to the manufacturer's instruction. HUVEC were pre-incubated with propionate (10mM) and butyrate (5mM) for 24h, followed by 8h of TNFα stimulation. After stimulation, the medium was exchanged for fresh medium and co-cultured with labelled mononuclear cells ($2x10^5$cells/well) for 30min. The un-adhesive mononuclear cells were then washed away and HUVEC with adhesive mononuclear cells were fixed with 4% paraformaldehyde. The fluorescence image of labelled mononuclear cells was captured by Yokogawa CV7000S imager and relative fluorescence intensity was measured by fluoroskan Ascent™ FL with excitation wavelength 492nm and emission wavelength 518nm.

2.8 Statistical analysis

All data are expressed as mean ± standard deviation (SD). Group comparisons were performed using the one-way ANOVA analysis of variance of the experiments. The method of least-significant difference (LSD) was used as a post hoc test for multiple comparisons, to determine significant difference

between specific treatment groups. In all cases, *P-value* < 0.05 was considered statistical significant.

3. Results

3.1 LPS or TNFα-induced release of IL-6 and IL-8 by HUVEC

Both LPS (1μg/ml or 10μg/ml) and TNFα (100pg/ml or 1ng/ml) significantly enhanced the IL-6 and IL-8 release by HUVEC after 6h, 12h and 24h stimulation (**supplementary Figure 1**). Based on these experiments, LPS and TNFα were respectively used at concentrations of 1μg/mL and 1ng/mL, and both incubations ran for 12h and 24h in the experiments described in this manuscript.

3.2 Basal Cytokine release

The SCFA used in this experiment did not affect the non-stimulated (basal) cytokines level (data not shown). Besides, the concentrations of butyrate (0.1mM) and propionate (0.3mM) used in ELISA assays and butyrate (5mM) and propionate (10mM) used in flow cytometry assays were not toxic for HUVEC (**supplementary Figure 2**).

3.3 The optimal concentrations of SCFA and stimulation time duration

The optimal concentrations of the three SCFA for inhibition of IL-6 and IL-8 release by LPS or TNFα were: 0.1mM for butyrate, 0.3mM for propionate and 10mM for acetate (data not shown). For acetate a pre-incubation time of 1h, 16h and 24h was most effective, whilst a 24h pre-incubation time with 1h, 16h and 24h stimulation times were chosen for butyrate and propionate. Under these experimental conditions, the most pronounced anti-inflammatory effects of SCFA were found (data not shown).

3.3.1 The effects of acetate on IL-6 and IL-8 release

Pre-incubation in acetate for 16h or 24h, but not for 1h, significantly attenuated IL-6 after LPS stimulation (**Figure 1A-C**) and TNFα stimulation

(**Figure 1D-F**). In the LPS-stimulated groups, IL-8 was significantly inhibited by acetate after 1h and 16h pre-incubation (Figures 2A, B), but not after 24h pre-incubation (**Figure 2C**). IL-8 production was inhibited after all pre-incubation times after TNFα-stimulation (**Figure 2D-F**).

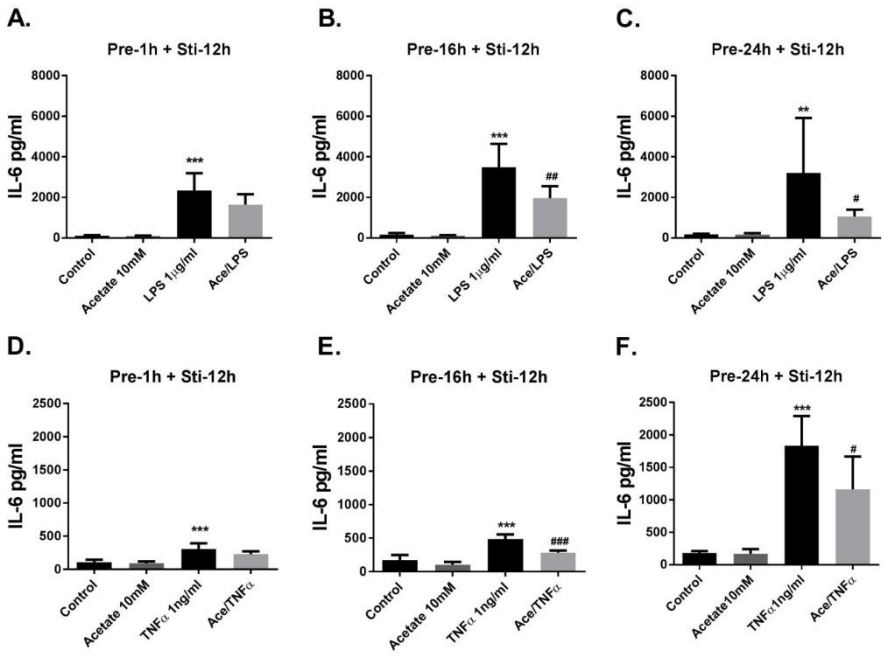

Figure 1. Acetate inhibited IL-6 release by HUVEC. Data show that pre-treatment of HUVEC with acetate (10mM) for 1h, 16h and 24h reduced IL-6 stimulated by LPS 1ug/mL (A-B) or TNFα 1ng/mL (D-F) for 12h. N=4, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with LPS or TNFα group.

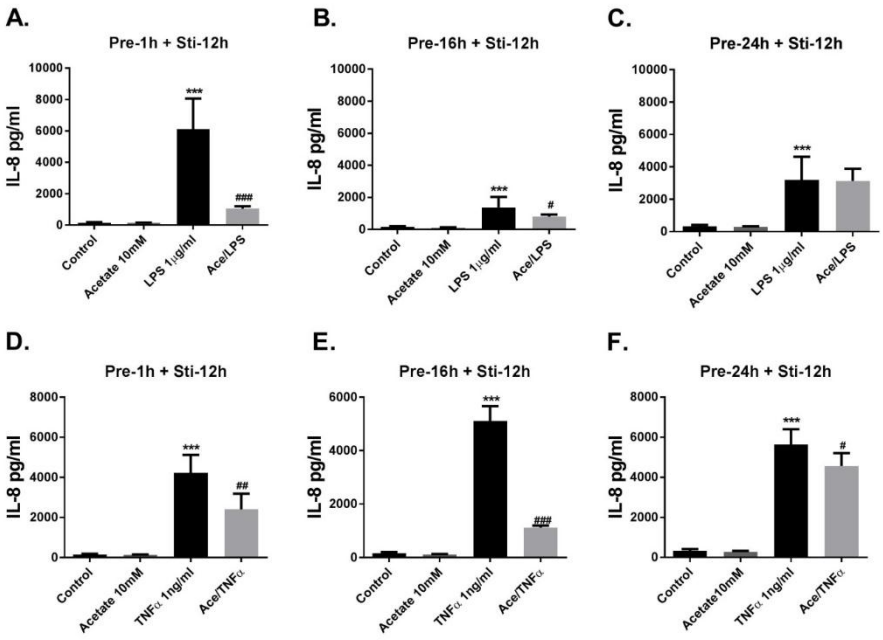

Figure 2. Acetate inhibited IL-8 release by HUVEC. Data show that pre-treatment of HUVEVC with acetate (10mM) for 1h, 16h and 24h reduced IL-8 production stimulated by LPS 1ug/mL (A-B) or TNFα 1ng/mL (D-F) for 12h. N=4, $^{***}p < 0.001$ compared with control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with LPS or TNFα group.

3.3.2 The effect of butyrate on IL-6 and IL-8 concentrations

Butyrate (24h pre-incubation) significantly attenuated IL-6 which reached significance in 24h LPS stimulation group (**Figure 3C**), and butyrate also decreased IL-6 production in 6h and 12h LPS stimulation groups (**Figure 3A, B**). With TNFα stimulation for 6h and 24h, IL-6 concentration was significantly decreased (**Figure 3D, F**) and a decrease was observed after 12h TNFα stimulation (**Figure 3E**).

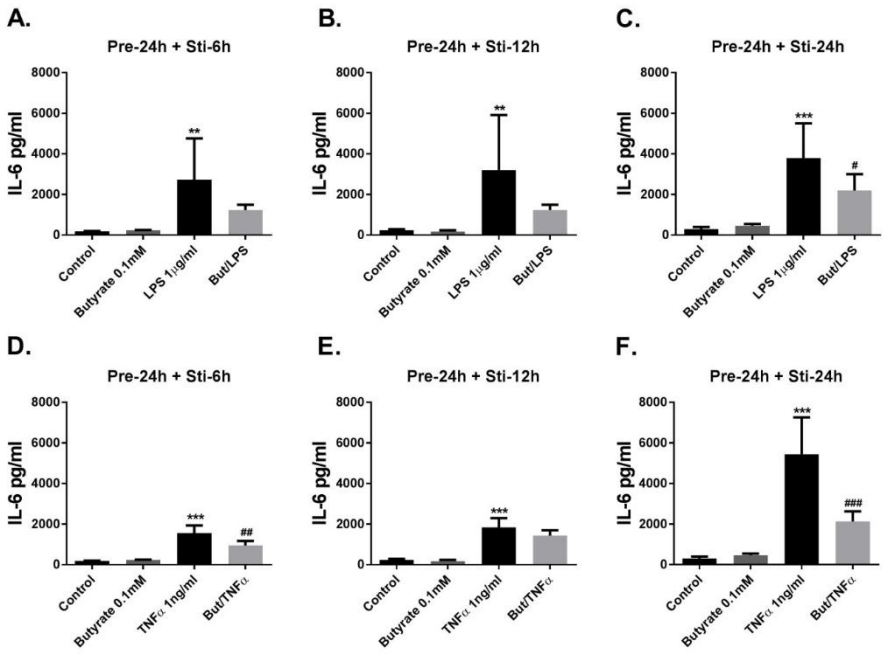

Figure 3. Effects of butyrate on IL-6 release. (A-F) show the effects of 0.1mM butyrate on IL-6 release with 24h pre-incubation and 6h, 12h or 24h stimulation. (A-C) Butyrate combined with LPS; (D-E) butyrate combined with TNFα. N=4, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with LPS or TNFα group.

IL-8 (24h pre-incubation) was significantly decreased by butyrate after 24h LPS-stimulation (**Figure 4C**). In contrast, IL-8 was significantly increased after 6h and 12h LPS stimulation compared to controls (**Figure 4A, B**). In the TNFα stimulated groups, butyrate increased IL-8 after 6h TNFα stimulation (*Figure 4D*), and decreased IL-8 after 24h TNFα stimulation (**Figure 4F**). No effect on IL-8 levels was observed after 12h TNFα stimulation (**Figure 4E**).

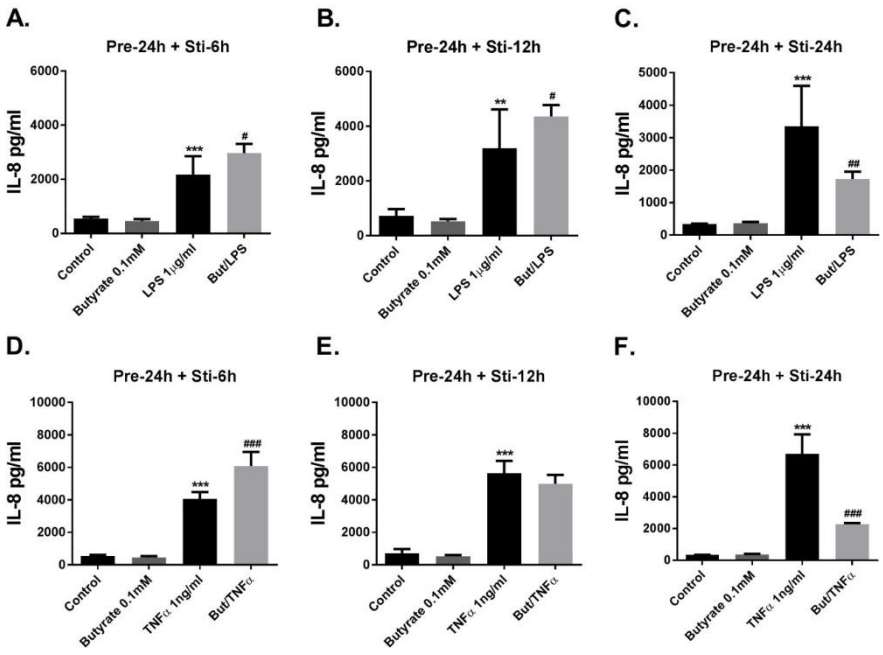

Figure 4. Effects of butyrate on IL-8 release. (A-F) show that 0.1mM butyrate inhibited IL-8 release after 24h pre-incubation and 6h, 12h or 24h stimulation. (A-C) butyrate combined with LPS; (D-F) butyrate combined with TNFα. N=4, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with LPS or TNFα group.

3.3.3 The effects of propionate on IL-6 and IL-8 release

Propionate (24h pre-incubation) significantly attenuated IL-6 production after LPS or TNFα stimulation (**Figure 5**). Propionate significantly inhibited IL-8 production after 24h LPS stimulation but not after 6h and 12 h (**Figure 6A-C**). However, propionate reduced IL-8 at all time-points after TNFα stimulation (**Figure 6D-F**).

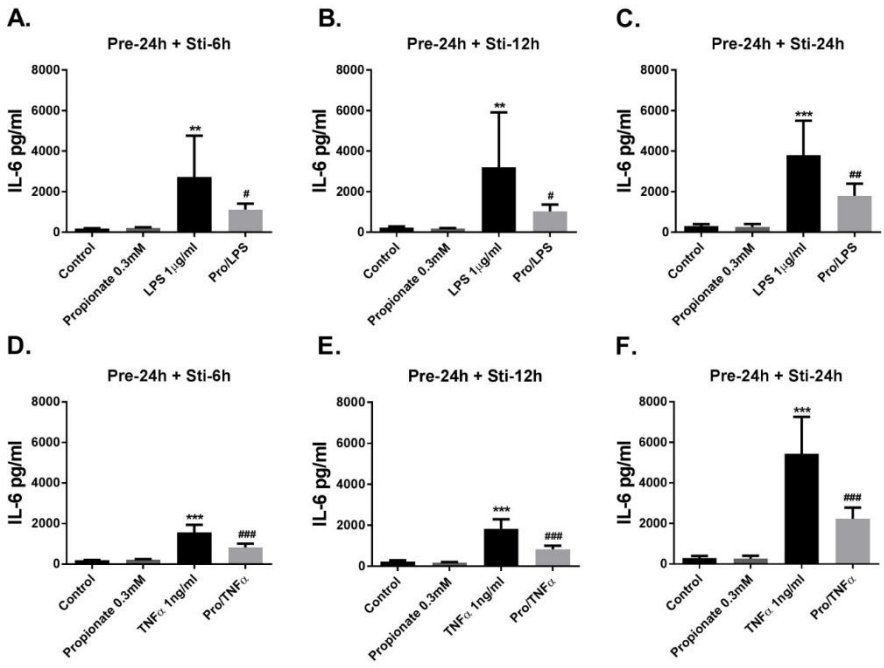

Figure 5. Effects of propionate on IL-6 release. (A-F) show that 0.3mM propionate inhibited IL-6 release after 24h pre-incubation and 6h, 12h or 24h stimulation. (A-C) propionate combined with LPS; (D-F) propionate combined with TNFα. N=4, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with LPS or TNFα group.

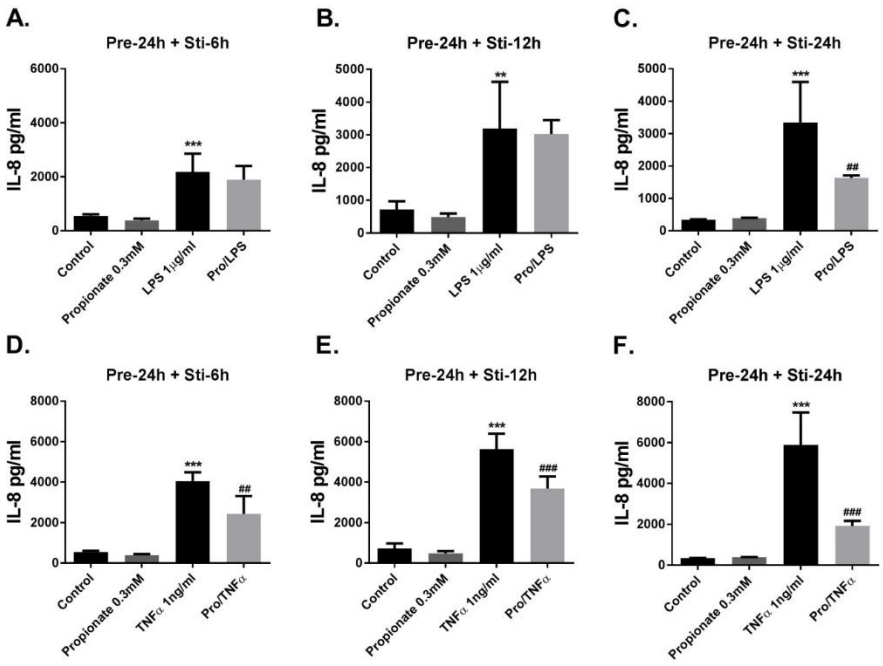

Figure 6. Effects of propionate on IL-8 release. (A-C) propionate combined with LPS; (D-E) propionate combined with TNFα. N=4, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.000$ compared with LPS or TNFα group.

3.4 Adhesion molecules

The effects of SCFA on LPS- and TNFα-induced expression of ICAM-1 and VCAM-1 were examined since these adhesion molecules are essential for adhesion and diapedesis of inflammatory cells. In a pilot study, butyrate (0.1mM) and propionate (0.3mM) showed no effects on ICAM-1 and VCAM-1 expression (data not shown), therefore, butyrate (5mM) and propionate (10mM) were used to investigate the effects on adhesion molecules expression.

3.4.1 LPS- and TNFα-induced increase expression of ICAM-1 and VCAM-1

On unstimulated HUVEC, ICAM-1 was expressed more than VCAM-1. Expression of both adhesion molecules were enhanced by LPS and TNFα stimulation (**supplementary Figure 3A, B**).

ICAM-1 expression was significantly increased after 8h of LPS or TNFα stimulation, and lasted for 24h (**supplementary Figure 3A**). VCAM-1 expression was significantly increased after 8h and 24h LPS and 8h of TNFα stimulation (**supplementary Figure 3B**). Therefore 8h stimulation time was used in the following experiments.

3.4.2 The effects of SCFA on ICAM-1 and VCAM-1 expression

HUVEC were pre-treated with acetate (10mM) for 24h, followed by 8h LPS or TNFα stimulation. Acetate did not affect ICAM-1 and VCAM-1 expression (**Figure 7**). Compared to the control group, the expression of ICAM-1 and VCAM-1 was increased after stimulation with LPS or TNFα. Butyrate profoundly inhibited VCAM-1 expression but not ICAM-1 (**Figure 8A-D**). Propionate did not affect the LPS- or TNFα-induced ICAM-1 expression (**Figure 9A, B**) and LPS-induced VCAM-1 expression (**Figure 9C**), but did reduced the TNFα-induced VCAM-1 expression (**Figure 9D**).

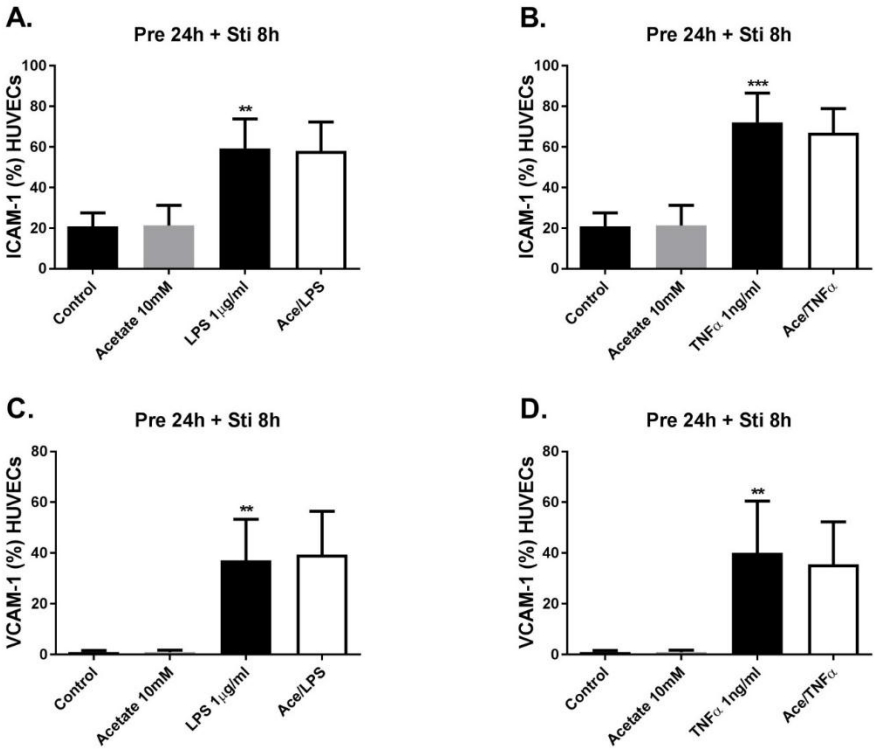

Figure 7. The effects of acetate on LPS- and TNFα-induced ICAM-1 and VCAM-1 expression. Acetate showed no effects on LPS- and TNFα- induced ICAM-1 (A and B) and VCAM-1 (C and D) expression. N=3 $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group.

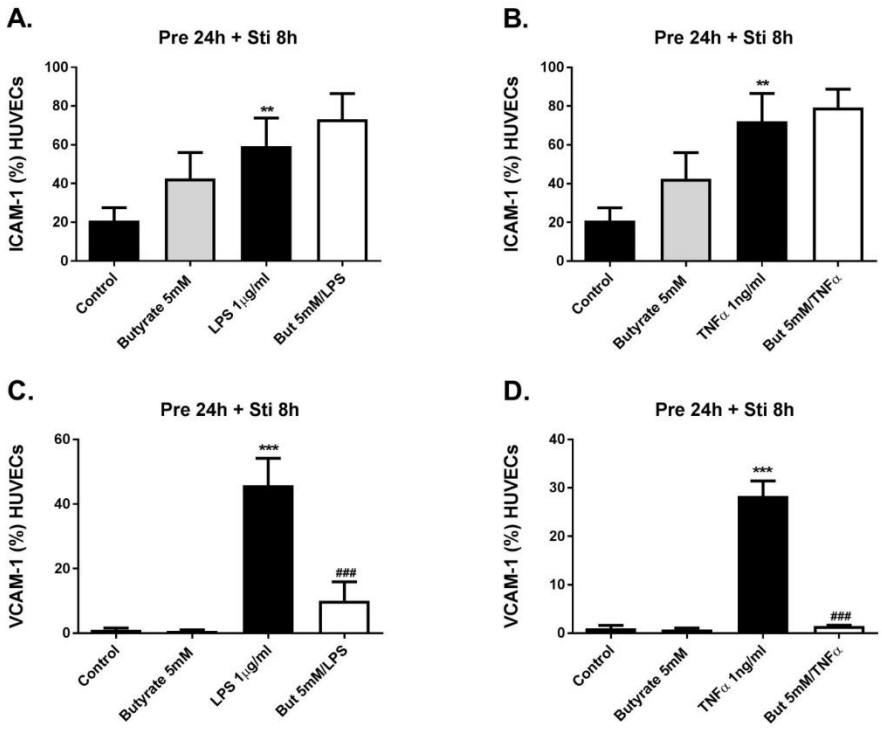

Figure 8. The effects of butyrate on LPS- and TNFα-induced ICAM-1 and VCAM-1 expression. (A and B) the effects of butyrate on LPS or TNFα induced ICAM-1 expression; (C and D) the effects of butyrate on LPS or TNFα induced VCAM-1 expression. N=3, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group. $^{###}p < 0.001$ compared with LPS or TNFα group.

3.5 Adhesion of mononuclear cells to endothelial cells

To investigate the functional role of propionate and butyrate on mononuclear cells adhesion to vascular endothelium, we conducted an adhesion assay by co-culturing mononuclear cells and HUVEC. The adhesion of mononuclear cells to HUVEC was significantly increased by TNFα (1ng/ml) which was significantly inhibited by butyrate. A similar tendency was observed for propionate, although it was less effective than butyrate (**Figure 10**).

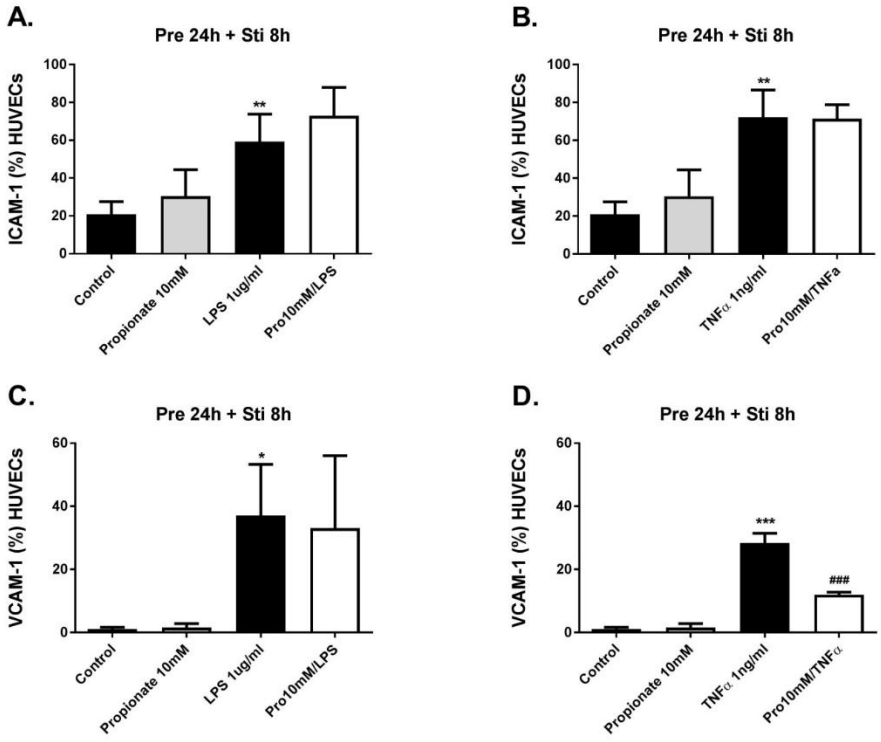

Figure 9. The effects of propionate on LPS- and TNFα induced VCAM-1 expression. (A and B) the effects of propionate on LPS or TNFα induced ICAM-1 expression; (C and D) the effects of propionate on LPS or TNFα induced VCAM-1 expression. N=3, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group. $^{\#\#\#}p < 0.001$ compared with LPS or TNFα group.

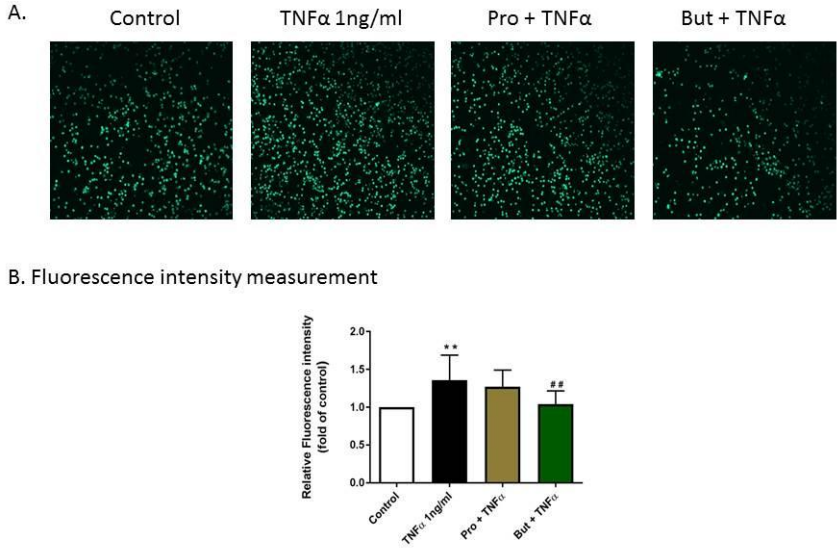

Figure 10. The functional role of butyrate and propionate on mononuclear cell adhesion to HUVEC monolayers. (A) adhesive mononuclear cells were visualized by Yokogawa CV7000S imager; (B) the mean data of relative fluorescence intensity of adhesive mononuclear cells presented as the fold of control. n=8. $^{**}p < 0.01$ compared with control group; $^{\#\#}p < 0.01$compared with TNFα group.

4. Discussion

Endothelium is important in the development of inflammatory diseases including atherosclerosis [26] and SCFA show potential roles in regulation of inflammation [27], however, the evidence on the effects of SCFA on endothelial activation are controversial and incomplete. Hence, in the present study, we used three different SCFA (acetate, butyrate and propionate), at various concentrations and incubation times to determine their effects on LPS or TNFα-induced endothelial activation including cytokines production and adhesion molecules expression. It is the first time to uncover the effects of SCFA on IL-6 and IL-8 production on LPS or TNFα-stimulated HUVEC and to investigate the effects of SCFA on ICAM-1 and

VCAM-1 expression under this experimental set-up. In the current study, SCFA demonstrated a variety of effects on cytokine (IL-6 and IL-8) release depending on the condition chosen. Moreover, butyrate and propionate, but not acetate, inhibited VCAM-1 expression without changing ICAM-1 expression.

Previous studies demonstrated that treatment of butyrate reduced IL-6 production in oxLDL treated endothelial cell line (EA.hy926) [28]. Thus, we expected that SCFA might inhibit cytokines production in LPS or TNFα-induced primary endothelial cells. Interestingly, we found SCFA reduced, increased or had no effects on the levels of IL-6 and IL-8 which depended on concentrations of SCFA used and pre-incubation/stimulation time applied. For example, pre-incubation with butyrate (0.1mM) for 1h increased IL-6 production, however, pre-incubation for 24h decreased IL-6 production. Or pre-treatment of acetate (10mM) for 12h decreased IL-6 production, while 100mM acetate increased IL-6 level under the same experiment set-up (data not shown). Similar effects of SCFA were found in peripheral blood monocytes and neutrophils: both acetate and butyrate, but not propionate, induced IL-8, IL-6 and IL-1β cytokine release when these cells were exposed to high levels (20mM) while lower levels (≤ 2mM) did not result in a significant increase in cytokine production [29]. These findings indicate that SCFA can have a biphasic effect on inflammation and different SCFA can have different effects. However, in the present study, we only focused on the anti-inflammatory effects of SCFA and tried to find regularity in the effects of each SCFA on IL-6 and IL-8 production. With 12h LPS- or TNFα-stimulation, acetate (10mM) inhibited or showed a tendency to decrease IL-6 and IL-8 production regardless of pre-incubation time. While for butyrate (0.1mM) and propionate (0.3mM), a pre-incubation period of 24h was needed to decrease IL-6 and IL-8. Butyrate and propionate showed similar effects on cytokine production but effects of acetate were different. The discrepancies between the SCFA might be due to different potencies on receptors or different duration of signaling pathways. For example, acetate can activate the G-protein coupled receptor 41/43 (GPR41/43, also known as FFA3/2 receptor),

while butyrate and propionate can both activate FFA2/3 receptors and inhibit HDACs [30]. Besides, their potencies on activation of FFA receptors and inhibition of HDACs are different. The potency orders of SCFA for FFA2 receptor is acetate ~ propionate > butyrate, whereas for the FFA3 receptor this is propionate ~ butyrate > acetate [30, 31]. In addition, butyrate is found to be the most potent HDAC inhibitor, propionate is less potent and acetate does not have HDAC inhibitory property [32]. These properties might explain the differences in effects between acetate and butyrate/propionate.

Furthermore, adhesion molecules-promoted adhesive interactions between leukocytes and endothelial cells and transmigration into the site of injury [33] are crucial steps in inflammation, immunity and atherosclerosis. On resting endothelial cells, ICAM-1 is usually expressed at low levels, while VCAM-1 is either absent or present in very low levels [34]. ICAM-1 and VCAM-1 expression was up-regulated at 8h after stimulation by either LPS or TNFα. However, the effects of SCFA on ICAM-1 and VCAM-1 expression on endothelial cells reported in previous studies are conflicting. Some found that butyrate increased adhesion molecules expression on HUVEC [35] but others observed inhibitory effects on expression of both ICAM-1 and VCAM-1 [21]. In this study, acetate had no effects on ICAM-1 and VCAM-1 expression. Propionate only inhibited TNFα-induced VCAM-1 expression, while butyrate selectively inhibited LPS and TNFα-induced VCAM-1 expression but not ICAM-1 expression which is in agreement with effects of butyrate on TNFα- or IL1- induced adhesion molecule expression on human vascular endothelial cells [21, 36]. The discrepancy between acetate and butyrate/propionate might be due to their inhibitory properties on HDAC: acetate does not inhibit HDAC activity and butyrate is more potent than propionate [37]. Moreover, the effect of butyrate on VCAM-1 expression was more potent than propionate, which was similar as their inhibitory effects on HDAC activity [30].Therefore, it can be speculated that the inhibition of VCAM-1 expression by butyrate and propionate might be regulated by HDACs. There was also discrepancy among the effects of butyrate/propionate on ICAM-1 and VCAM-1expression, which were regulated by different gene-

specific signal transduction routes [21]. Previous studies showed that TSA, an HDAC inhibitor, had no effect on TNF-α induced p65/p50 binding to a consensus NF-κB binding probe that plays an important role in ICAM-1 expression [38]. However, TSA could significantly reduce VCAM-1 expression as TSA might inhibit transcription factors, such as endothelial interferon-regulatory factor 1 and GATA instead of NF-κB, that are also involved in regulating VCAM-1 expression in activated HUVEC [38]. In our study, butyrate and propionate selectively inhibited VCAM-1 expression and VCAM-1 is more important in facilitating monocyte adherence [36]. Therefore, decreased VCAM-1 expression by butyrate/propionate might break the vicious cycle between cytokines production and immune cell adhesion and inhibit chronic inflammatory responses. In order to investigate the functional effects of butyrate and propionate on cell adhesion, human mononuclear cells were co-cultured with HUVEC monolayer. Mononuclear cells adhesion to endothelial cells was significantly inhibited by butyrate. Propionate was less effective than butyrate which might be the consequence of less pronounced suppression of VCAM-1 expression. By collecting these data, we have demonstrated a role for VCAM-1 in cell adhesion and that possible damage induced by mononuclear cells in cardiovascular diseases can be reduced by SCFA.

In conclusion, endothelial activation, characterized by excessive cytokines (IL-6 and IL-8) production and adhesion molecules (ICAM-1 and VCAM-1) expression, is a crucial factor in the development of atherosclerosis and SCFA regulated endothelial activation in different ways. We found that the pre-incubation time and concentration used are important factors for each SCFA to show inhibitory effects on IL-6 and IL-8 production. Moreover, butyrate and propionate but not acetate inhibited VCAM-1 expression without affecting ICAM-1 expression. The different mechanisms including activation of GPR41/43 and inhibition of HDACs by which SCFA influence endothelial function might be one way to explain these results and elucidating the inhibitory pathways induced by SCFA might open a new era in the treatment of (chronic) inflammatory (cardiovascular) diseases.

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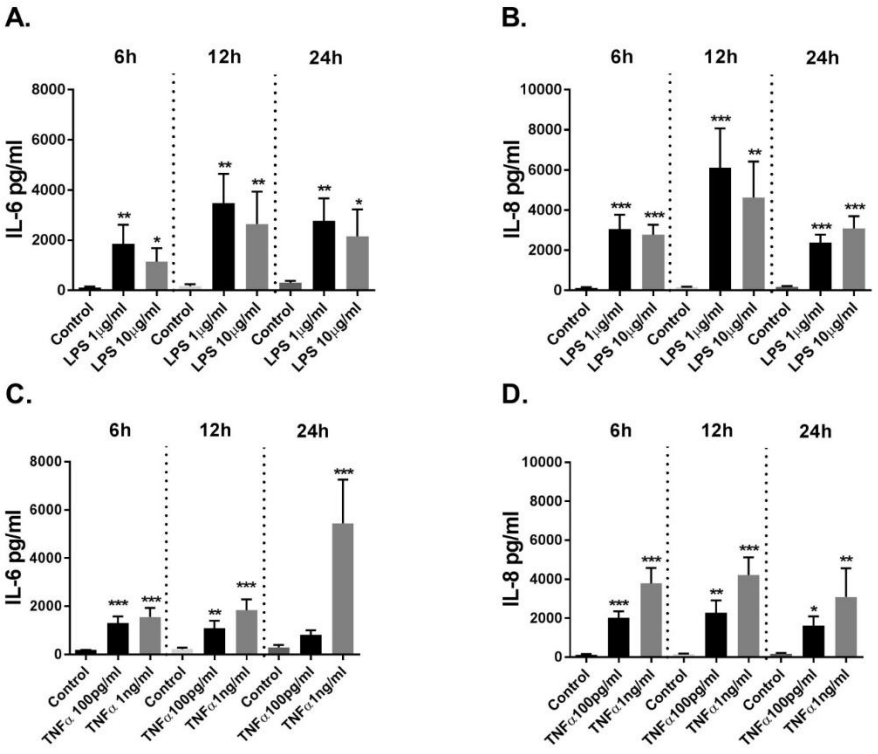
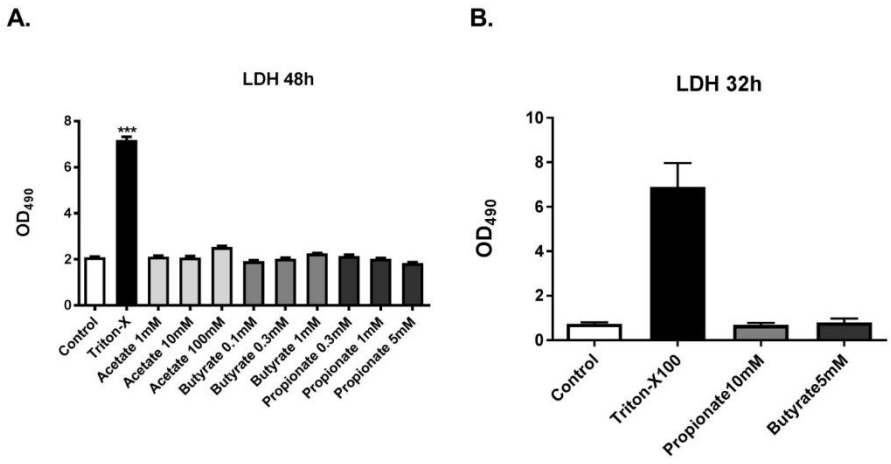
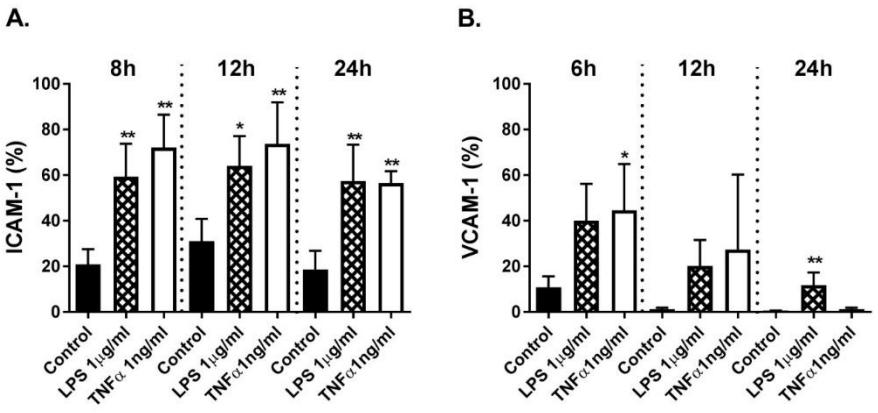
Supplementary data



Supplementary Figure S1. IL-6 and IL-8 release by HUVEC stimulated with different concentrations of LPS or TNFα at different time points. (A and B) show IL-6 and IL-8 production with LPS stimulation respectively, and (C and D) show IL-6 and IL-8 production with TNFα stimulation respectively. N=4, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group.



Supplementary Figure S2. The effects of different concentrations of acetate, butyrate and propionate on HUVEC. After 48h incubation, cells toxicity was determined by using LDH assay (A). Besides, high concentrations of butyrate 5mM and propionate 10mM were incubated for 32h and cell toxicity was tested by LDH (B). Cells treated with only medium were control groups and cells exposed to 1% Triton-X were used as positive control of cytotoxicity. N=5-6, $^{***}p < 0.001$ compared with control group.



Supplementary Figure S3. LPS and TNFα induced ICAM-1 and VCAM-1 expression. With LPS or TNFα stimulation for 8h, 12h or 24h, (A) ICAM-1 was significantly increased; (B) VCAM-1 expression was upregulated. Data was presented as percentage of positive population. N=3, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control group.

Chapter 5

The anti-inflammatory effects of short chain fatty acids on lipopolysaccharide- or tumor necrosis factor α-stimulated endothelial cells via activation of GPR41/43 and inhibition of HDACs

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ABSTRACT

Background and aim

Previously, we found that short chain fatty acids (SCFA) inhibit LPS or TNFα-induced endothelial inflammatory responses and excessive vascular cell adhesion molecule-1 (VCAM-1) expression, two important steps in the development of atherosclerosis. However, the mechanisms involved are still unclear. We hypothesized that the effects of SCFA are associated with activation of G-protein coupled receptor 41/43 (GPR41/43) and/or inhibition of histone deacetylases (HDACs).

Methods

The expression and location of GPR41/43 and HDAC3 in human umbilical vein endothelial cells (HUVEC) were confirmed. HUVEC were pre-incubated with acetate, butyrate or propionate alone or in combination with GLPG0974 (GLPG, antagonist of GPR43) or β-hydroxybutyrate (SHB, antagonist of GPR41) and then exposed to LPS or TNFα. Interleukin (IL)-6 and IL-8 levels and VCAM-1 expression were measured. HDAC activity was measured after treatment with butyrate, propionate and trichostatin A (TSA, HDAC inhibitor). The peripheral blood mononuclear cell (PBMC) adhesive level was also determined after TSA treatment.

Results

GPR41/43 were expressed on the membrane of HUVEC and HDAC3 was located in cytoplasm and nucleus. The GLPG and/or SHB treatments restored the inhibitory effects of acetate on IL-6 and IL-8 production and the inhibitory effects of butyrate or propionate on IL-6 production, but not on IL-8. In contrast, GLPG and/or SHB treatments did not affected the inhibitory effects of butyrate or propionate on TNFα-induced VCAM-1 expression. TSA showed similar effects on IL-8 production and VCAM-1 expression as butyrate and propionate. In addition, TSA significantly inhibited the adhesion of PBMC to an endothelial monolayer.

Conclusion

Activation of GPR41/43 mediates the effects of acetate on IL-6 and IL-8 production and the effects of butyrate and propionate on IL-6 production. Furthermore, inhibition of HDACs mediates the effects of butyrate and propionate on IL-8 production, VCAM-1 expression and PBMC adhesion to an endothelial monolayer. These data indicated the beneficial roles of SCFA in preventing vascular inflammation and relevant diseases by activation of GPR41/43 and inhibition of HDACs.

1. Introduction

Atherosclerosis, which is associated with chronic vascular inflammation, is an inflammatory disease with its most common pathological processes leading to cardiovascular disease [1]. The earliest event in atherosclerosis is increased monocyte adhesion to endothelial cells, which is primarily regulated by vascular inflammatory factors including cytokines such as interleukin (IL)-6, chemokines such as IL-8 and monocyte chemoattractant protein-1, and endothelial adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) [2]. Since increases in these factors also characterise endothelial activation [3], regulating inflammatory reactions in the vascular endothelium is a potential target for therapeutic intervention in the treatment of atherosclerosis [4].

Less endothelial activation and less low-grade inflammation have been associated, in clinical studies, with high fibre consumption [5, 6], which might be due to the production of short chain fatty acids (SCFA). SCFA, predominant acetate, propionate and butyrate, are the main anaerobic bacterial metabolites fermentation of dietary fibres in the colon which not only regulate proliferation, differentiation and gene expression in the colon but also have physiological relevance for the host outside of the gastro-intestinal tract [7]. SCFA have recently emerged as important signaling molecules regulating a variety of responses in the cardiovascular system [8]. Furthermore, as we have recently shown, SCFA play beneficial roles in decreasing endothelial activation leading to diminished cytokines production and expression of adhesion molecules [9]. SCFA may regulate endothelial function either by inhibiting histone deacetylases (HDACs) and/or activating G-protein coupled receptors (GPRs): GPR41 (also known as free fatty acid receptor3, FFAR3) and GPR43 (or FFAR2) and/or inhibition of histone deacetylases (HDACs) [10]. However, the roles played in endothelial activation by GPRs and HDACs are not clearly understood.

GPR41 and GPR43 are differentially expressed on the membrane of cells [7, 11, 12]. GPR41 is mainly expressed in blood vessel on endothelial cells, and

GPR43 is mainly expressed on immune cells [13]. GPR41 and GPR43 can both be activated by SCFA, but their specificities and potencies for ligands are different. The order of potency of the GPR41 is propionate ≈ butyrate >acetate and for GPR43 is propionate ≥ acetate ≈ butyrate [14], indicating that butyrate is more active on GPR41, propionate is the most potent agonist for both GPR41 and GPR43, and acetate is more selective for GPR43 [15]. Increasing evidence indicates the anti-inflammatory effects of GPR43 activation on immune cells. For example, deficiency in GPR43 increases the production of inflammatory mediators and immune cell recruitment and results in an increased inflammatory response in models of colitis, arthritis and asthma [16]. Their roles in endothelial activation remain unknown [13].

HDACs, a group of deacetylating enzymes, remove acetyl groups from both histones and non-histone proteins complexes that regulate gene expression [17]. HDACs are involved in atherosclerosis associated inflammatory processes [18]. HDAC inhibitors have shown potent anti-inflammatory activity in inflammatory diseases [19-21]. For example, HDAC3, by regulating NF-κB activity, is involved in the expression of inflammatory genes and monocyte recruitment to sites of inflammation [22]. A potent HDAC inhibitor, suberoylanilide hydroxamic acid, exhibits anti-inflammatory properties by attenuating the LPS-induced expression of NF-κB-regulated cytokines [23, 24]. Inhibition of HDACs is also a molecular mode of action of SCFA, and butyrate and propionate but not acetate can act as HDAC inhibitors [25]. It remains unclear whether HDACs are involved in the anti-inflammatory properties of SCFA in the process of endothelial activation.

We therefore investigated the roles of GPR41/43 and HDACs in the anti-inflammatory effects of SCFA on LPS- and TNFα-induced endothelial activation. This could offer new therapeutic ways to prevent the development of atherosclerosis.

2. Materials and Methods

2.1 Reagents and materials

Sodium butyrate, propionate, trichostatin A (TSA, a selective and reversible hydroxamate inhibitor of class I and II HDACs) [26], sodium β-hydroxybutyrate (SHB, antagonist for GPR41 receptor) [27] and LPS (*Escherichia coli* 0111:B4) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Sodium acetate was bought from Merck Millipore. A cytotoxicity detection kit (lactate dehydrogenase, LDH) was obtained from Roche. Human IL-6, IL-8 ELISA (enzyme-linked immunosorbent assay) kits and calcein-AM were purchased from Invitrogen. Human recombinant TNFα, anti-human CD106 (VCAM-1) PE and viability fixable dyes were bought from eBioscience. GLPG0974 (GLPG, antagonist of GPR43 receptor) [28] was obtained from Tocris Bioscience. Primary anti-GPR41 antibody, anti-GPR43 antibody and an HDAC activity assay kit were purchased from Abcam. EGM-2 Bulletkit was purchased from Lonza (Switzerland).

2.2 Cell culture

HUVEC from umbilical vein were provided by Mrs. J.H. van Kats-Renaud (University Medical Center, Utrecht). HUVEC were isolated and cultured by adapting the method of Jaffe *et al.* [29] HUVEC were cultured in EGM-2 (Lonza) containing 2% fetal bovine serum (FBS) and VEGF for rapid proliferation in a humidified incubator at 37°C in 5% CO_2 and medium was changed every 2-3 days. Cells of passage 2-7 were used. Informed consent was obtained from all subjects and was provided in accordance with the Declaration of Helsinki. Approval was obtained from the Medical Ethics Committee of the University Medical Center Utrecht (Utrecht, The Netherlands).

2.3 PBMC isolation

Human PBMC from healthy donors were isolated from buffy coats (Sanquin, Amsterdam, the Netherlands). Cells were purified using Ficoll-Paque Plus gradient centrifugation [30]. Briefly, PBMC were isolated by centrifugation

according to the manufacturer's instructions. PBMC above the polyester gel were collected, washed with PBS containing 2% FBS by centrifuging at 1000g for 13min, re-suspended cell pellet at a concentration of $2x10^6$cells/ml in RPMI1640 medium without phenol red containing 10% FBS and 1% penicillin-streptomycin. The viability of PBMC was determined by trypan blue staining and cell number was counted. Informed consent was obtained from all subjects and was provided in accordance with the Declaration of Helsinki.

2.4 Cell cytotoxicity (LDH) test

Based on the results published in the recent manuscript [9], we chose different exposure periods for each SCFA in the present study. HUVEC were treated with acetate (10mM), TSA (1µM), SHB (5mM) and GLPG (0.1µM) alone or combination for 28h. Treatment with propionate (0.3mM) and butyrate (0.1mM), alone or combination with antagonists or TSA lasted for 48h. Treatment with propionate (10mM) and butyrate (5mM), alone or combination with antagonists or TSA lasted for 32h. After treatments, supernatants were transferred into a new 96-well plate and 100ul reaction solution of LDH was added into each well. The plate was incubated in the dark at room temperature for 30 min and was then measured at 490 nm excitation and 650 nm emission by microplate reader. Positive control was 1% Triton-X treated cells, which led to 100% cell death.

2.5 Immunocytochemistry

HUVEC ($5x10^3$ cells) were seeded to 96-well plate and incubated at 37℃, 5% CO_2 incubator for two days. The cells then were processed for immunocytochemistry as previously described [31]. Briefly, the HUVEC were washed twice by cold PBS. For intracellularly located protein (HDAC3), cells were treated with permeabilization solution (0.25% Triton-X) for 10 min and then washed with cold PBS. HUVEC were then incubated with blocking buffer for 1h and washed with cold PBS. Cells were subsequently stained with primary antibodies, rabbit-anti-human GPR41 (1:300), rabbit-anti-human GRP43 (1:300) and rabbit-anti-human HDAC3 antibodies (1:800), overnight at 4 degrees. HUVEC were then incubated with goat anti-rabbit Alexa Fluor

488 or 568 secondary antibodies (1:400) for 1 h and washed with cold PBS three to five times. Negative controls were simply stained with secondary antibodies.

2.6 ELISA for pro-inflammatory cytokines detection

Confluent HUVEC were treated with acetate (10mM) alone or combination with GLPG (0.1μM) or SHB (5mM) for 16h and were then exposed to LPS (1μg/mL) or TNFα (1ng/mL) for 12 h. Propionate (0.3mM) and butyrate (0.1mM) alone or combination with GLPG or SHB were treated for 24 h, then stimulated with LPS or TNFα for 24 h. Supernatants were collected and stored at -20℃. The levels of inflammatory cytokines IL-6 and IL-8 were assayed by ELISA according to the manufacturer's instructions using standard curve. The optical densities of the samples were detected using a microplate reader at a wavelength of 450 nm.

2.7 HDAC activity assay

After treatment with propionate (0.3mM/10mM) and butyrate (0.1mM/5mM) for 3, 6, 12, or 48 h, cells lysates were collected and stored in -80℃ until measurement. HDAC activity was detected with fluorometric HDAC activity assay kit according to manufacturer's protocol. As a positive control, the effects of TSA (0.01μM, 0.1μM and 1μM) on HDAC activity were also measured after 48 h treatment. Fluorescence was monitored every 1min over 60 min at 37℃ using a Fluostar reader at excitation wavelength of 355 nm and emission wavelength of 460 nm.

2.8 Flow cytometry

Confluent HUVEC were incubated with propionate (10mM) and butyrate (5mM) alone or combination with GLPG, SHB or TSA for 24h. HUVEC were then stimulated with TNFα (1ng/mL) for 8h. After treatment, HUVEC were stained with human VCAM-1 PE-conjugated antibody and cell viability dye according to the manufacturer's protocol and then detached from the culture plates with 0.05% trypsin-EDTA. The stained HUVEC were measured by flow cytometer FACSCanto II and data was analysed by Flowlogic version7.

2.9 Endothelial cell monolayer adhesion experiment

HUVEC were seeded in 96-well plates until they were confluent. PBMC, isolated from different healthy donors, were washed with warm PBS three times, and PBMC ($2x10^6$/mL) labelled with 1µM calcein-AM for 30min according to the manufacturer's instruction. HUVEC were pre-incubated with TSA (1µM) for 24h, followed by 8h of TNFα stimulation. After stimulation, the medium was exchanged for fresh medium and co-cultured with labelled PBMC ($2x10^5$cells/well) for 30min. The un-adhesive PBMC were then washed away and HUVEC with adhesive PBMC were fixed with 4% paraformaldehyde. The fluorescence image of labelled PBMC was captured by Yokogawa CV7000S imager and relative fluorescence intensity was measured by fluoroskan Ascent™ FL with excitation wavelength 492nm and emission wavelength 518 nm.

2.10 Statistical analysis

All data are expressed as mean ± standard deviation (SD). Group comparisons were performed using the one-way ANOVA analysis of variance of the experiments. The method of least-significant difference (LSD) was used as a post hoc test for multiple comparisons, to determine significant difference between specific treatment groups. In all cases, *P-value* < 0.05 was considered statistically significant.

3. Results

3.1 No compounds used showed cytotoxicity

HUVEC were treated with different concentrations of SHB, GLPG and TSA to test cytotoxicity (**Supplementary Figure S1 A-C**). Non-toxic concentrations of SHB (5mM), GLPG (0.1µM) and TSA (1µM) were used in the following experiments.

HUVEC were treated for 28h or 48h with acetate (10mM), propionate (0.3mM) and butyrate (0.1mM) in combination with SHB (5mM), GLPG (0.1µM) or TSA (1µM) (**Supplementary Figure S1D-F**), while propionate

(10mM) and butyrate (5mM) combinations were treated for 32h (**Supplementary Figure S1G, H**). Medium was collected for the measurement of LDH release, an indicator of cytotoxicity. All the treatments with acetate, propionate or butyrate in combination with GLPG, TSA and SHB were nontoxic.

3.2 GPR41 and GPR43 was expressed on the HUVEC membrane

GPR41 and GPR43 are the two targets of SCFA which have been indicated to modulate the inflammation and immune responses in non-endothelial cells [32, 33]. To ascertain the expression of GPR41 and GPR43 on the HUVEC membrane, HUVEC were stained with GPR41 and GPR43 antibodies and checked with a Yokogawa CV7000S imager. GPR41 (**Supplementary Figure S2C**) and GPR43 (**Supplementary Figure S2D**) were expressed on HUVEC. Cells stained only with the secondary antibodies were regarded as backgrounds (**Supplementary Figure S2A, B**).

3.3 HDAC3 was located in the cytoplasm and nucleus

HDACs activity can be inhibited by SCFA, especially butyrate and propionate, and regulate gene expression mediating the effects of SCFA [10, 33]. HDACs, including HDAC3, a class I HDAC, are highly expressed in endothelial cells [34]. We confirmed that HDAC3 was expressed in HUVEC, and that HDAC3 was highly expressed in the cytoplasm and nucleus (**Supplementary Figure S2E**).

3.4 Acetate inhibited pro-inflammatory cytokines (IL-6 and IL-8) production in LPS- or TNFα-stimulated HUVEC via activation of GPR41 and GPR43

To determine whether GPR41/43 are involved in the decrease of IL-6 and IL-8 production by acetate, we compared the effects of acetate alone or in combination with antagonists of GPR41/43. Optimum concentrations and incubation times for SCFA and LPS/TNFα were determined previously [9]. Acetate treatment alone showed no significant effects on IL-6 and IL-8 production compared to the control group (data not shown).

3.4.1 Acetate-inhibited IL-6 production was associated with activation of GPR41/43

LPS (1μg/ml for 12h) significantly enhanced the IL-6 production of HUVEC (**Figure 1A**). Similar results were observed for TNFα (1ng/ml, **Figure 1B**). IL-6 production was significantly reduced by pre-incubation with acetate (10mM for 16h) and was completely restored by the GPR41 or GPR43 antagonist (SHB or GLPG) alone or in combination (**Figure 1A, B**).

Furthermore, in the acetate and GPR41/43 antagonists combination group, IL-6 production showed no significant difference between acetate combined with GPR41 or GPR43 antagonist alone (**Figure 1A**). Similar effects of acetate on TNFα-induced IL-6 production were observed, however treatment with acetate in combination with GPR41 antagonist (SHB) partially increased IL-6 production (**Figure 1B**). These data indicate that both GPR41 and GPR43 are involved in the inhibitory effects of acetate on IL-6 production.

3.4.2 Acetate-inhibited IL-8 production was associated with activation of GPR41/43

After pre-incubation with acetate for 16h, the IL-8 release was significantly decreased in LPS stimulated group (12h) (**Figure 1C**). IL-8 levels were partially restored by GLPG (GPR43 antagonist) or SHB (GPR41 antagonist), but still lower than the LPS stimulation group (**Figure 1C**). However, IL-8 production was completely restored in the GLPG/SHB-treated group (**Figure 1C**). In addition, acetate in combination with both GPR41 and GPR43 antagonist showed additional effects on IL-8 production compared with each antagonist alone (**Figure 1C**). Results suggest that the effects of acetate on IL-8 production are mediated by both by GPR41 and GPR43. In the TNFα-treated groups, IL-8 production was significantly reduced by acetate, an effect that was significantly inhibited by treatment with GPR43 antagonist and partially inhibited by GPR41 antagonist (**Figure 1D**). However, this decrease in TNFα-induced IL-8 production was not completely restored by GPR41/43 antagonist treatment, which led to levels still significantly lower than those

of the LPS-stimulated group. These results together indicate that both GPR41 and GPR43 are involved in the effects of acetate on TNFα-induced IL-8 production.

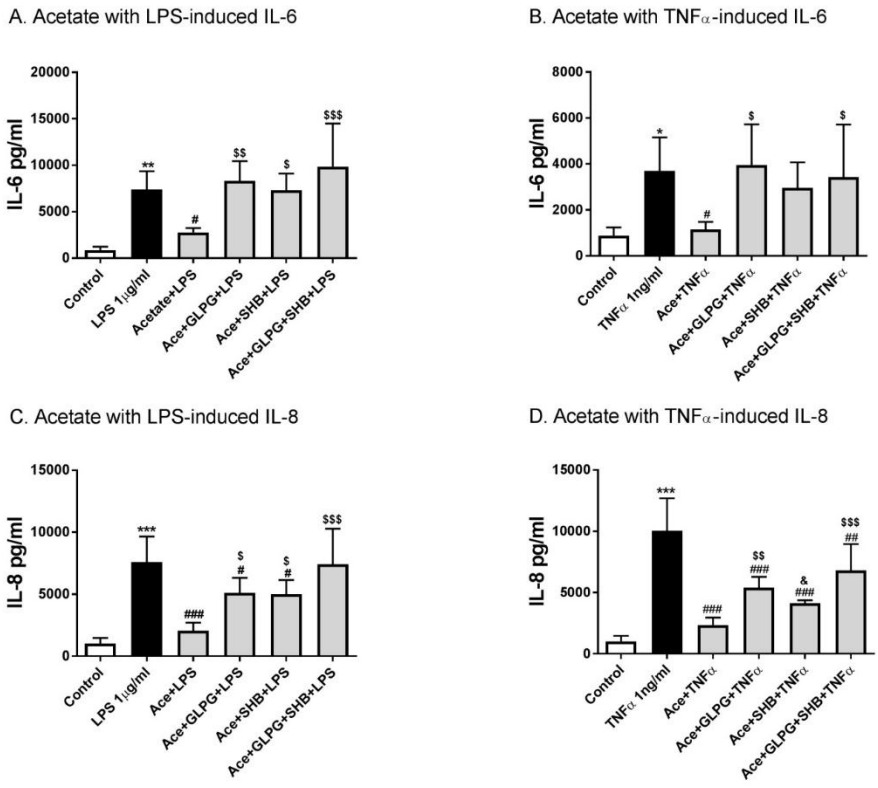

Figure 1. The effects of acetate alone or in combination with GLPG and/or SHB on IL-6 and IL-8 production by LPS- or TNFα-stimulated HUVEC. (A) and (B) show the effects of pre-treatment with acetate alone or combined with GLPG and/or SHB for 16h on IL-6 production stimulated by LPS or TNFα for 12h. (C) and (D) show the IL-8 production level after pre-incubating acetate or combination with GLPG and/or SHB for 16h and 12h LPS or TNFα stimulation. N=4, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with LPS or TNFα group; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ compared with acetate alone group; $^{\&}p < 0.05$ compared with acetate combination with GLPG/SHB (AGS) group.

3.5 Butyrate and propionate inhibited IL-6 production mainly via activation of GPR41/43

Butyrate or propionate treatment alone showed no significant effects on IL-6 production compared to the control group (data not shown). IL-6 production was significantly decreased by pre-treatment with butyrate for 24h in the LPS-stimulated (12h) group. The effect of butyrate treatment alone was reversed when combined with GLPG or SHB (**Figure 2A**). Similar effects of butyrate in combination with GLPG were found in TNFα-activated groups (**Figure 2B**). However, the effect of butyrate on IL-6 production was only partially reversed by SHB treatment but not significantly different from the TNFα stimulated group (**Figure 2B**). IL-6 production was also inhibited by propionate treatment (**Figure 2C, D**). Again, this was significantly reversed when combined with GLPG or SHB (**Figure 2C, D**).

3.6 HDACs are involved in butyrate and propionate-reduced IL-8 production in LPS or TNFα-stimulated HUVEC

Butyrate or propionate treatment alone showed no significant effects on IL-8 production compared to the control group (data not shown). In both the 24h LPS and TNFα-activated groups, IL-8 production was significantly decreased by 24h pre-treatment with butyrate. These effects were not altered by GLPG or SHB (**Figure 3A, B**). Similar effects on IL-8 production were shown by treatment with propionate production (**Figure 3C, D**). Therefore, we hypothesized that HDACs might be involved in the regulation of IL-8.

For this reason, we measured the effects of butyrate and propionate on HDAC activity with different concentrations (butyrate: 0.1mM and 5mM; propionate: 0.3mM and 10mM) within 48h. Results indicated that butyrate (0.1mM) and propionate (0.3mM) inhibited HDAC activity after 12h treatment. Butyrate (5mM) significantly inhibited HDAC activity after 6h treatment, while the inhibitory effect of propionate (10mM) on HDAC activity reached significance after 48h treatment (**Figure 4A, B**). TSA, as a positive control, showed a concentration-dependent inhibitory effect on HDAC

activity (**Supplementary Figure S3**). Moreover, inhibitory effects on IL-8 production were also shown by the HDAC inhibitor TSA (**Figure 4C**). These data indicate that HDACs might be involved in the effects of butyrate and propionate on IL-8 production.

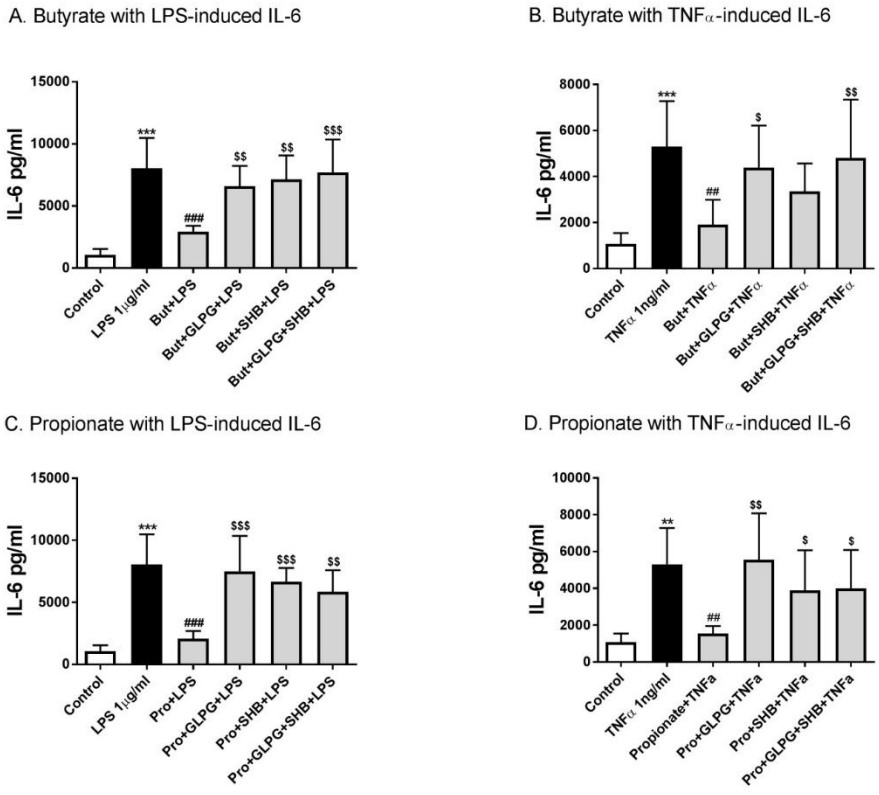

Figure 2. Butyrate and propionate inhibited IL-6 in LPS and TNFα-stimulated HUVEC. Pre-treatment with butyrate (0.1mM) and propionate (0.3mM) for 24h significantly reduced IL-6 levels in 12h LPS- or TNFα-stimulated groups. (A) and (B) show the effects of butyrate; (C) and (D) show the effects of propionate; N=4, $^{**}p < 0.01$,$^{***}p < 0.001$ compared with control group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with LPS or TNFα group; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ compared with butyrate or propionate alone group.

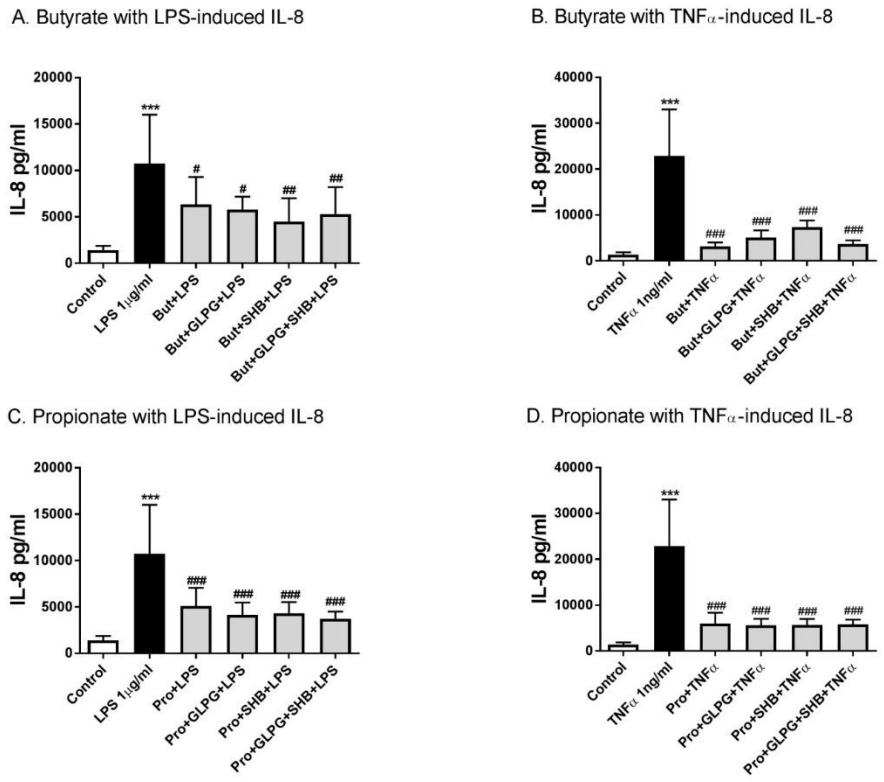

Figure 3. Butyrate and propionate inhibited IL-8 production in LPS and TNFα-stimulated HUVEC. With 24h pre-treatment, butyrate and propionate reduced IL-8 production in 24h LPS or TNFα stimulation. (A) and (B) are the data of butyrate effects on LPS- and TNFα-induced IL-8 production respectively; (C) and (D) are the data of propionate treatment. N=4, $^{***}p < 0.001$ compared with control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with LPS or TNFα group.

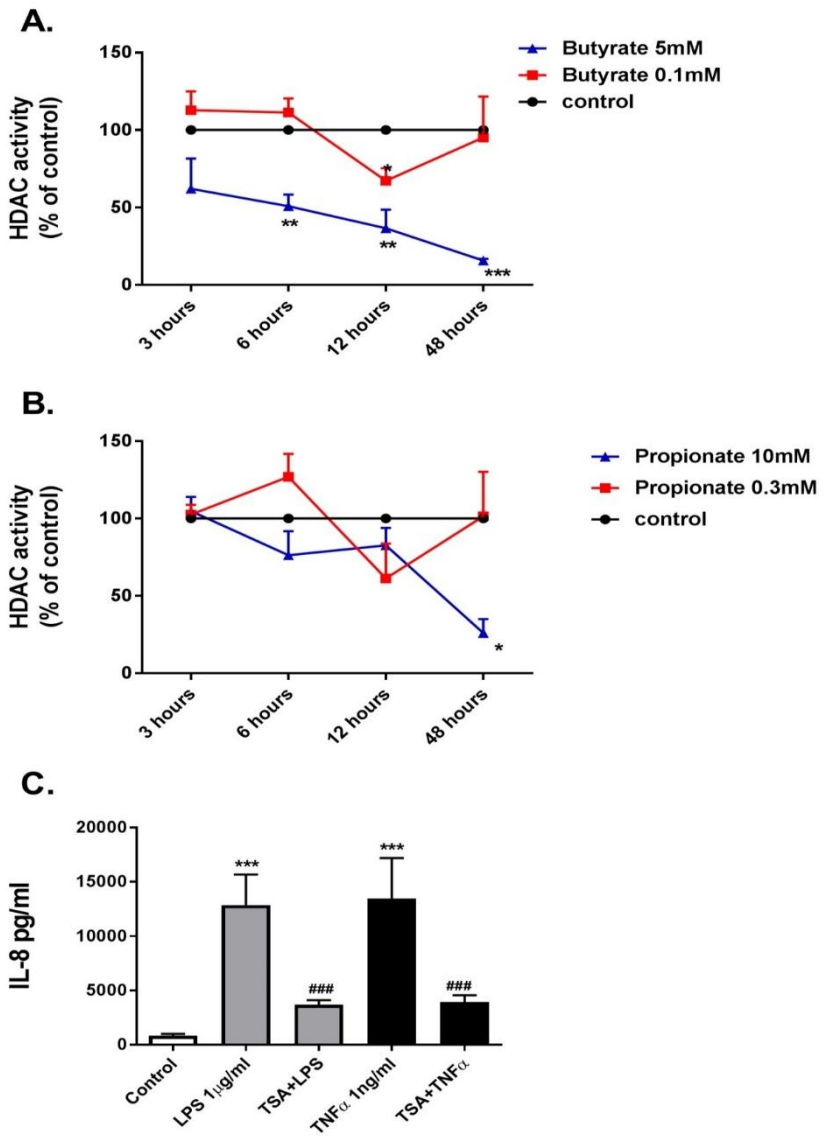

Figure 4. Butyrate, propionate and TSA inhibited HDACs activity in HUVEC. (A) and (B), HDACs activity were inhibited in HUVEC by treatment of butyrate and propionate. The results were normalized using the control as 100%. (C), TSA inhibited IL-8 production in HUVEC. N=3-4 for each group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$compared with control group; $^{\#\#\#}p < 0.001$ compared with LPS or TNFα group.

3.7 Butyrate and propionate suppression of VCAM-1 in TNFα-stimulated HUVEC were associated with inhibition of HDACs but not with activation of GPR41/43

Based on previous experiments [9], propionate (10mM), butyrate (5mM) and TSA (1μM), either alone or combination with antagonists, were used to pretreat confluent HUVEC for 24h and then exposed to TNFα (1ng/mL) for 8h. TNFα increased the VCAM-1 expression (**Figure 5**). Butyrate, propionate and TSA all inhibited TNFα-induced VCAM-1 expression, and the effect was not reversed by GLPG and/or SHB treatment (**Figure 5A, B**). These results indicate that, unlike GPR41 and GPR43, HDACs were involved in the inhibitory effects of butyrate and propionate on TNFα-induced VCAM-1 expression.

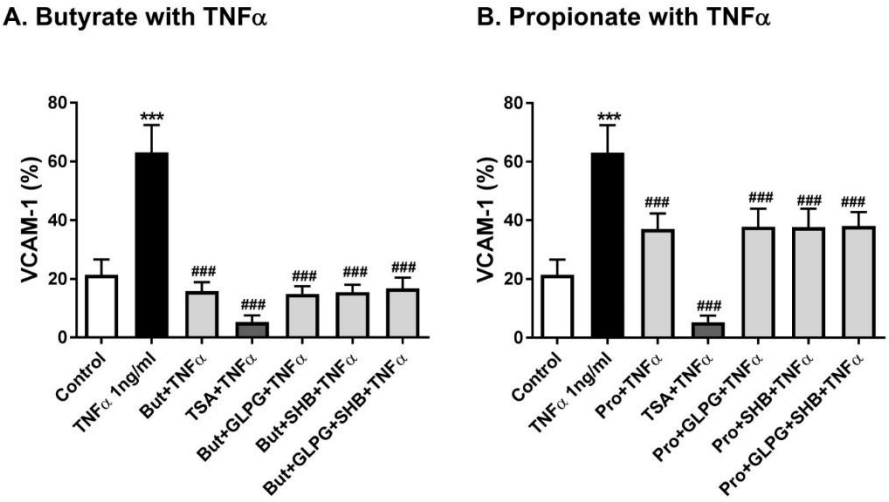

Figure 5. Butyrate and propionate inhibited TNFα-induced VCAM-1 expression. HUVEC were pre-treated with propionate (10mM), butyrate (5mM), GLPG (0.1μM), SHB (5mM), TSA (1μM) or combinations and then exposed to TNFα (1ng/mL). (A) shows the effects of butyrate or its combination with GLPG and/or SHB on VCAM-1 expression; (B) shows the effects of propionate or its combination with GLPG and/or SHB on VCAM-1 expression. N=5. $^{***}p < 0.001$ compared with control group; $^{###}p < 0.001$ compared with TNFα group.

3.8 TSA inhibited adhesion of PBMC to endothelial cells.

To investigate the functional role of TSA on PBMC adhesion to vascular endothelium, we conducted an adhesion assay by co-culturing PBMC and HUVEC. The adhesion of PBMC to HUVEC was significantly increased by TNFα (1ng/ml). In a previous study, we showed that butyrate and propionate inhibited the adhesion of activated mononuclear cells to stimulated HUVEC [9]. In the current study we also observed a significant inhibition by TSA (**Figure 6**).

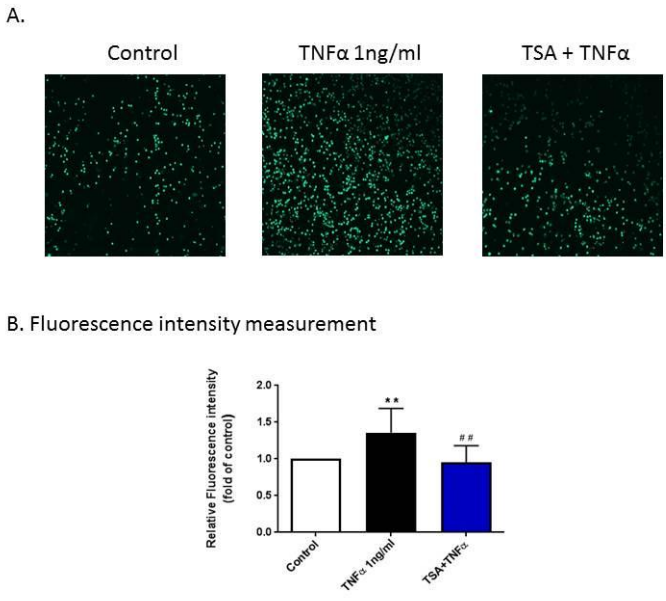

Figure 6. The functional role of TSA on PBMC adhesion to HUVEC monolayer. Until HUVEC were confluent, cells were pre-treated with TSA (1μM) for 24h, followed by 8h TNFα stimulation, then co-cultured with calcein-AM labelled PBMC for 30min. (A) shows adhesive PBMC visualized by Yokogawa CV7000S imager; (B) shows the mean data of relative fluorescence intensity of adhesive PBMC presented as the fold of control. N=8. $^{**}p < 0.01$ compared with control group; $^{\#\#}p < 0.01$ compared with TNFα group.

4. Discussion

Excessive pro-inflammatory cytokines production and adhesive molecule expression are two important steps in the development of atherosclerosis [2]. We have previously demonstrated that SCFA pre-treatment could significantly inhibit IL-6 and IL-8 production as well as VCAM-1 expression on activated endothelial cells [9]. However, the mechanisms involved in their anti-inflammatory effects remained unclear and an understanding of their mechanisms of action could be important for new therapeutic options for atherosclerosis and for related lifestyle adjustments. We confirmed the membrane expression of GPR41/43 and the intracellular location of HDAC3 on HUVEC, which indicate that the anti-inflammatory effects of SCFA on activated endothelial cells might be mediated by activation of GPR41/43 and/or inhibition of HDACs. Furthermore, we found that GPR41/43 are involved in cytokine production but not in adhesion molecule expression and that HDACs are involved in the butyrate and propionate-induced decrease in IL-8 production and VCAM-1 expression.

Inflammatory mediators, such as IL-6 and IL-8, are crucial to the pathophysiology of atherosclerosis [35, 36]. IL-6 is an important upstream inflammatory cytokine in propagating the downstream inflammatory response for atherosclerosis [37]. IL-8 is a prominent chemokine that triggers the adhesion, transmigration and retention of monocytes and macrophages to the atherosclerotic sites [38]. Therefore, decreasing IL-6 and IL-8 should be an important step in preventing the development of atherosclerosis. In the present study, acetate reduced IL-6 and IL-8 production in LPS- or TNFα-activated HUVEC, which were prevented by co-treatment with GPR41 or GPR43 antagonist. Furthermore, the effects of GPR43 antagonist seem more profound than those of the GPR41 antagonist, which corresponds with the activation potency order of acetate on GPR41 and GPR43 [14]. Butyrate and propionate have effects on IL-6 production similar to those of acetate, but IL-8 production by stimulated HUVEC was unchanged by GPR41 or GPR43 antagonist treatments, suggesting that the inhibitory effects of butyrate and

propionate on IL-8 production were not mediated by activation of GPR41/43. IL-8 is encoded on the CXCL8 gene, which is normally repressed due to histone deacetylation [39], thus theoretically, inhibition of histone deacetylase results in hyper-acetylation of histones and, increases IL-8 production. However, in our study, butyrate and propionate inhibited IL-8 production and HDAC activity. Furthermore, TSA, as a potent HDACs inhibitor, also reduced IL-8 production. These findings are in agreement with previous studies that show a reduction in IL-8 gene expression by butyrate but accompanied with induced histone H4 hyper-acetylation in its inducible promoter [40, 41]. Taken together, these results indicate that HDACs might be involved in the effects of butyrate and propionate on the IL-8 production.

In addition to the cytokines and chemokines, monocyte migration and adhesion to the inflammation site on endothelial cells is another essential cellular event for initiation of inflammatory processes associated with atherosclerosis [42]. Cell adhesion is a multi-step process including rolling, firm adhesion and transmigration the endothelial monolayer and is regulated by a combination of endothelial cell surface adhesion molecules including VCAM-1, ICAM-1 and selectins [4, 43]. Furthermore, deletion of VCAM-1 expression has been shown to suppress atherosclerotic lesions in hyper-lipidemic mouse models [43]. This shows the importance of VCAM-1 in cell adhesion and the development of atherosclerosis. VCAM-1, also known as CD106, is extensively expressed on activated endothelial cells and mediates both rolling-type adhesion and firm adhesion steps during monocyte adhesion and transmigration [43, 44]. Therefore, decreasing VCAM-1 expression might be beneficial in inhibiting inflammation and the development of atherosclerosis. In a recent study, we showed that VCAM-1 expression is inhibited by butyrate and propionate but not by acetate [9].

VCAM-1 expression in TNFα stimulated endothelial cells has been shown to be regulated by the acetylation status and TSA increased intracellular acetylation leading to significantly suppress TNFα-induced VCAM-1 expression in *in vitro* and *in vivo* experiments [4, 45]. Furthermore, siRNA

knockdown of HDAC3 in HUVEC reduced VCAM-1 expression and hence suppressed monocyte adhesion [46]. Such data demonstrates the involvement of HDACs, especially HDAC3, in regulating VCAM-1 expression. We found that acetate, which has no HDAC inhibitory activity [47], showed no effect on TNFα-induced VCAM-1 expression [9]. Moreover, activation of GPR41/GPR43 did not mediate the suppression of VCAM-1 expression by butyrate and propionate. Finally, TSA, a potent HDAC inhibitor, significantly inhibited VCAM-1 expression, and this effect was more potent than butyrate and propionate. Furthermore, the PBMC adhesion level was significantly inhibited by TSA which showed effects similar to those of butyrate and propionate [9]. Based on our data, we conclude that the effects of butyrate and propionate on VCAM-1 expression were probably mediated by inhibition of HDACs thereby inhibiting the subsequent immune cell adhesion and preventing the development of atherosclerosis.

The present study offers novel insights into the anti-inflammatory mechanisms of SCFA in stimulated human primary endothelial cells and shows the different involvement of GPR41/43 and HDACs in the anti-inflammatory process of SCFA including inhibition of the pro-inflammatory cytokines production and down-regulation of adhesion molecule expression on HUVEC. We found that both activation of GPR41/43 and inhibition of HDACs are involved in decreasing pro-inflammatory cytokines production, IL-6 and IL-8, in LPS- and TNFα-activated HUVEC. Inhibition of HDACs, and not activation of GPR41/43, significantly attenuated VCAM-1 expression in TNFα-activated HUVEC and consequently inhibited PBMC adhesion to endothelial monolayer. Thus, modulating GPR41/43 and/or HDACs might be a promising therapeutic pathway for the treatment of vascular inflammation relevant diseases, including atherosclerosis. Moreover, these findings argue for altering lifestyles in the direction of increased dietary intake of fibers that promote the production of SCFA.

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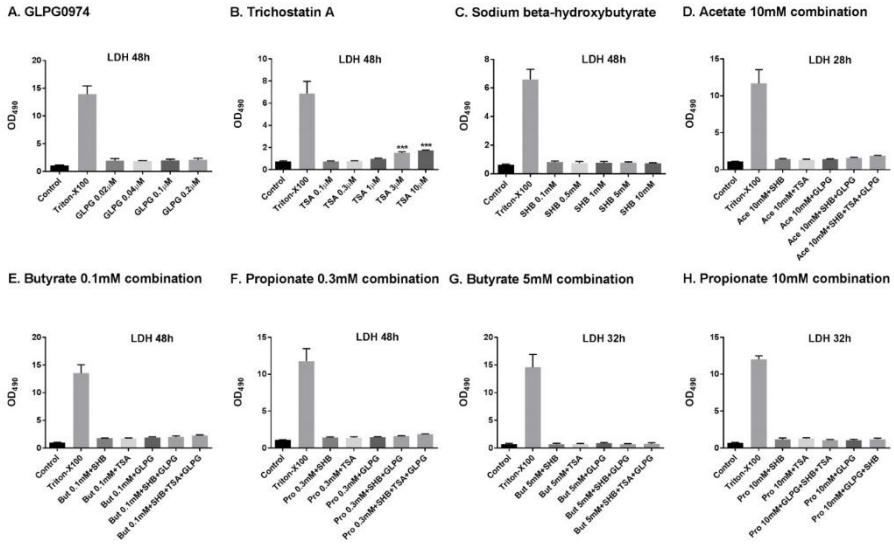
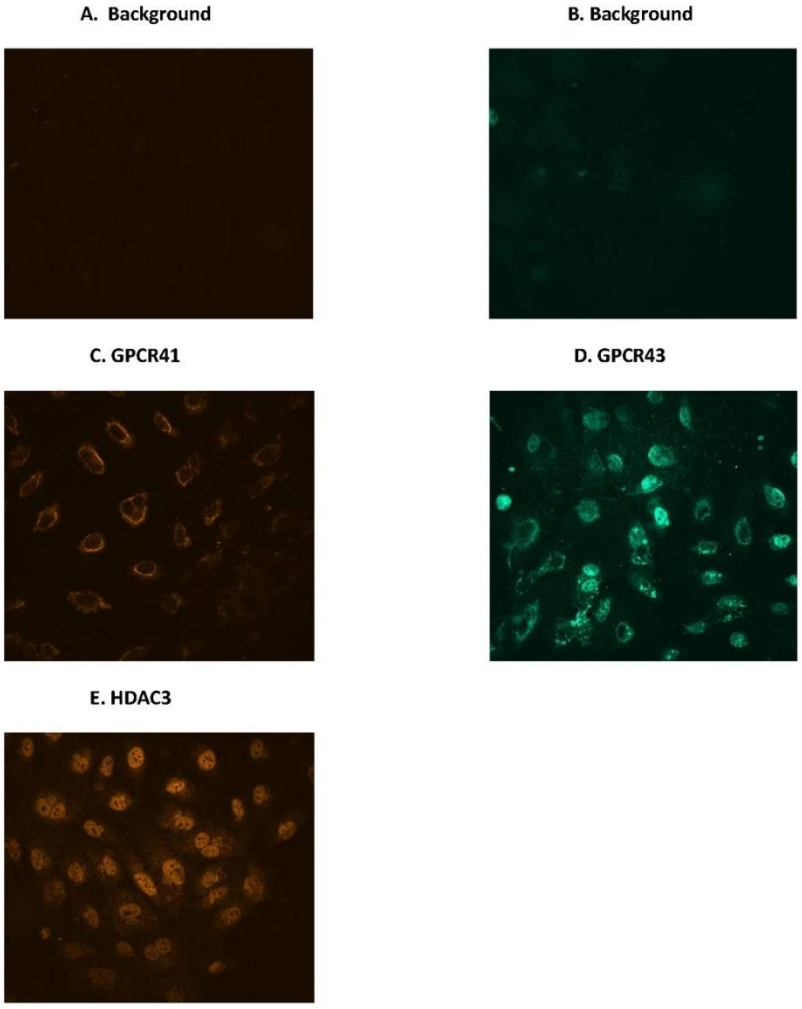
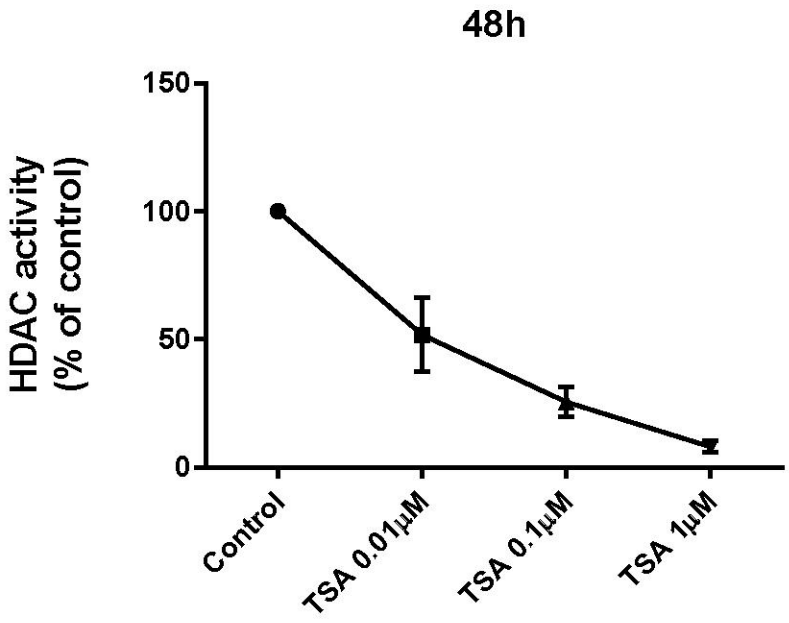
SUPPLEMENT



Supplementary Figure S1. LDH cell toxicity test. Toxic effects of (A) GLPG, (B) TSA, (C) SHB, (D) acetate (10mM) combination, (E) butyrate (0.1mM) combination, (F) propionate (0.3mM) combination, (G) butyrate (5mM) combination, and (H) propionate (10mM) combination. Cells treated only with medium were control groups and cells exposed to 1% Triton-X were used as a positive control of cytotoxicity. N=5-6, $^{***}p < 0.001$ compared with control group.



Supplementary Figure S2. GPR41 and GPR43 were expressed on HUVEC membrane. Only staining with secondary antibodies (A) and (B) was regarded as background staining. HUVEC were stained with (C) rabbit anti-human GPR41 and (D) GPR43 antibodies, then stained with goat anti-rabbit Alexa Fluor 488 (green) or 568 (orange) second antibodies. HDAC3 was located in cytoplasm and nucleus in HUVEC (E). HUVEC were fixed and permeabilized by 1% paraformaldehyde and 0.25% Triton-X. Fixed cells were stained with HDAC3 primary antibody and Alexa Fluor 568 (orange) secondary antibody.



Supplementary Figure S3. TSA inhibited HDAC activity in HUVEC. TSA concentration-dependently inhibited HDAC activity in HUVEC at 48h. The results were normalized using the control as 100%. N=4.

Chapter 6

Butyrate and propionate suppress TNFα-induced endothelial activation by regulating HDACs/IL-33/NF-κB and HDACs/MAPK signaling pathways

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Submitted for publication

ABSTRACT

Background and purpose

To investigate whether endogenous IL-33 mediates the effects of butyrate and propionate on TNFα-induced endothelial activation by regulating NF-κB and MAPK signaling pathways.

Experimental approach

The location of IL-33 was determined in primary human umbilical vein endothelial cells (HUVEC). IL-33 concentration was measured in supernatants and cell lysates every 3h during 24h TNFα stimulation. HUVEC were then pre-incubated with butyrate, propionate or trichostatin A (TSA) for 24h followed by 24h TNFα stimulation and subsequent endogenous IL-33 concentration measurements. Furthermore, the effects of butyrate, propionate and TSA on TNFα-induced IL-8, vascular cell adhesion molecule-1 (VCAM-1), NF-κB and MAPK signaling pathways in normal HUVEC and IL-33 siRNA (siIL-33) transfected HUVEC were compared.

Key results

Endogenous IL-33 was highly expressed in perinuclear of HUVEC which was significantly reduced by TNFα stimulation. This reduction was prevented by pre-incubation with butyrate or propionate. Low concentrations of butyrate (0.1mM) and propionate (0.3mM) inhibited both IL-8 production and activation of NF-κB, which were restored in siIL-33 transfected HUVEC. In contrast, the effects of butyrate (5mM) and propionate (10mM) on VCAM-1 expression and activation of MAPK signaling pathways were not affected by siIL-33 transfection. TSA showed similar effects to butyrate and propionate on IL-33 expression, IL-8 production, VCAM-1 expression, and activation of NF-κB and MAPK signaling pathways.

Conclusions and implications

The inhibitory effects of butyrate and propionate on TNFα-induced IL-8 production were mediated by the HDACs/IL-33/NF-κB pathway, while the effects on VCAM-1 expression might be associated with the HDACs/MAPK signaling pathway, independent of IL-33.

1. Introduction

Atherosclerosis is a chronic inflammatory disease of the vascular system and causes a significant increase in morbidity and mortality of cardiovascular diseases, which is initiated by endothelial activation [1, 2]. The molecular signs of cytokine-induced endothelial activation include up-regulated expression of cellular adhesion molecules, cytokines and chemokines [3, 4]. Therefore, understanding the precise mechanisms driving endothelial activation may elucidate the pathogenesis of atherosclerosis. The risk of developing atherosclerosis is reduced by consuming a diet high in fibre, possibly due to the production of short chain fatty acids (SCFA) [5]. Furthermore, we previously found the SCFA butyrate and propionate could improve endothelial cell function by reducing the TNFα-induced production of chemokines (IL-8), vascular cell adhesion molecule-1 (VCAM-1) expression and peripheral blood mononuclear cell (PBMC) adhesion to endothelial cells [6] via inhibition of histone deacetylases (HDACs) [7]. However, the resultant changes downstream following HDAC inhibition are unknown. Studies demonstrated that HDACs, especially HDAC3, regulated IL-33 expression in PBMC [8, 9] and epithelial cells [10]. Trichostatin A (TSA), a HDAC inhibitor, reduced intracellular IL-33 levels, without increasing IL-33 in the serum in lipopolysaccharide stimulated-PBMC [8, 9] and lung epithelial cells [10]. However, whether butyrate and propionate improve endothelial activation via regulation of HDAC/IL-33 is unknown.

IL-33, a novel member of the IL-1 family of cytokines, is constitutively expressed in the nuclei of endothelial cells from both large and small vessels during homeostasis and released by damaged endothelial cells [11]. IL-33 is thus regarded as an 'alarmin' signal that alerts immune cells of tissue damage [11]. Immuno-modulatory properties of IL-33 have been studied previously [12] and its role in the modulation of inflammatory pathologies of the respiratory system, gastrointestinal track and other inflammatory diseases such as atherosclersis have also been reported [13, 14]. Given its prominent involvement in health and disease, a good understanding of IL-33 biology and

the mode of action is crucial. IL-33 can be a dual function protein, acting both extracellularly as an IL-1 family cytokine and intracellularly as a nuclear factor regulating gene transcription [15]. As a cytokine, IL-33 binds to ST2 (interleukin-1 receptor-like 1) and induces the activation of endothelial cells towards an inflammatory phenotype by upregulating expression of inflammatory proteins, including adhesion molecules (VCAM-1), chemokines and cytokines (IL-6 and IL-8) [14, 16, 17]. Contrary to its pro-inflammatory effect, extracellular IL-33 also exerts protective effects in the cardiovascular system. For example, IL-33 treatment of $ApoE^{-/-}$ mice alleviates atherosclerosis [18].

Despite these significant findings related to the role of extracellular IL-33, the nuclear function of IL-33 currently remains unclear. Intracellular IL-33 can modulate inflammatory responses by regulating gene expression in two ways: firstly, IL-33 directly localizes to the nuclei and associates with the histones H2A-H2B and chromatin with its chromatin-binding motif being located in its N-terminal region, indicating a critical role for nuclear localization and chromatin associaton; secondly, nuclear IL-33 interacts with NF-κB [19]. The NF-κB family consists of five members: p65 (or RelA), RelB, c-Rel, p50/p105 and p52/p100, and is involved in the regulation of a variety of physiologic processes [20]. The TNFα-induced activaton of NF-κB contributes to the activation of endothelial cells by up-regulation of pro-inflammatory cytokines and adhesion molecules in endothelial cells [21]. However, both pro- and anti-inflammatory effects of nuclear IL-33 were reported by either enhancing or reducing NF-κB activity [17, 22]. Although IL-33 appears to act as a multifunctional protein in the nucleus, no study has been performed to demonstrate whether endogenous IL-33 is involved in the effects of butyrate and propionate on the regulation of TNFα-induced endothelial activation via modulating activation of NF-κB. Besides, TNFα also induces activation of the MAPK signaling patwhays, including ERK1/2, JNK and p38MAPK, which are also involved in regulation the expression of adhesion molecules [23-25]. Normally, activation of JNK and p38MAPK signaling pathways are essential for VCAM-1 expression in endothelial cells [26-29].

SCFA can modulate the MAPK signaling pathways by inhibiting HDAC activity in immune cells [30] but the modulating mechanisms of intracellur pathways in endothelial cells are currently unkown. In this study we investigated which intracellular pathways were involved in the HDAC regulated anti-inflammatory responses of butyrate and propionate in TNFα-induced endothelail activation. It was found that the HDACs/IL-33/NF-κB signaling cascade was involved in the effects of butyrate and propionate on IL-8 production and HDACs/MAPK signaling pathways were involved in their effects on VCAM-1 expression. These findings offer a better understanding of the anti-inflammatory effects of butyrate and propionate on TNFα-induced endothelial activation.

2. Methods

2.1 Materials

Sodium butyrate, propionate, TSA and protease inhibitor cocktail were purchased from Sigma-Aldrich. Human recombinant TNFα was bought from eBioscience. The human IL-8 enzyme-linked immunosorbent assay (ELISA) kit, Lipofectamine 2000 (lipo-2000) and BLOCK-iT Alexa Fluor Red Fluorescent control were purchased from Invitrogen. NF-κB p65 (pS536) ELISA kit was bought from Cell signaling technology. IL-33 ELISA kit was purchased from U-CyTech biosciences. IL-33 monoclonal antibody and Sliencer GAPDH siRNA (human) were bought from Thermo Fisher Scientific. Silencer pre-designed on-Targetplus SMARTpool siRNA IL-33 and siGENOME Non-Targeting siRNA pool (Silencer negative control) were bought from Dharmacon. The following monoclonal antibodies: anti-phospho p38 (phospho T180 + Y182) antibody, anti-JNK1+JNK2 (phospho T183 + Y185) antibody, anti-ERK1/2 (phospho Thr202/Tyr204) antibody, anti-GAPDH antibody, rabbit anti-mouse IgG H&L (HRP) conjugated antibody and goat anti-rabbit IgG H&L (HRP) conjugated antibody were purchased from Cell signaling technology and Abcam.

2.2 HUVEC culture

HUVEC were isolated from umbilical vein by adapting the method of Jaffe *et al.*[31] and were kindly provided by J.H. van Kats-Renaud (University Medical Center, Utrecht). HUVEC were cultured in EGM-2 (Lonza) containing 2% fetal bovine serum and VEGF for rapid proliferation. HUVEC cultures were maintained in a humidified incubator at 37°C and 5% CO_2, and medium was changed every two-three days. Passages two to seven of HUVEC were used. Informed consent was obtained from all subjects and was provided in accordance with the Declaration of Helsinki. Approval was obtained from the medical ethics committee of the University Medical Center Utrecht (Utrecht, The Netherlands).

2.3 Small interfering RNA (siRNA) and cytotoxicity test

siRNA transfection was performed as described previously.[14] HUVEC were seeded on 96-well plates and 6 well-plates at 1.25 x 10^4 and 2 x 10^5 cells/well, respectively, until cells reached 60%-80% confluence. First, a siRNA transfection efficiency test in HUVEC was performed. HUVEC were incubated with a mixture of fluorescence labelled siRNA (Block-iT) in 96-well plates, at a final concentration of 50nM, and different amounts of lipo-2000 according to the manufacturer's instructions. After 6h transfection, HUVEC were washed with PBS and cultured in regular growth medium. Fluorescence images were collected. Secondly, HUVEC in 6-well plates were transfected with IL-33 siRNA along with lipo-2000, or with silencer negative control siRNA, which has no significant sequence similarity to human gene sequences combined with lipo-2000. After 6h transfection, medium was replaced by regular growth medium. The protein expression level of intracellular IL-33 was measured at 48h, 72h and 96h after start of transfection by western blot. Lastly, a WST-1 test in 96-well plates was performed to measure cell proliferation and viability after 48h siIL-33 transfection.

2.4 Immunocytochemistry

HUVEC were seeded in 96-well plates and incubated at 37°C and 5% CO_2 for two days. Cells were treated with or without TNFα for 24h and then processed for immunocytochemical analysis as previously described.[32] Briefly, cells were first treated with permeabilization solution (0.25% triton-X) for 10min and then washed with cold PBS. Cells were then incubated with blocking buffer for 1h and then washed with cold PBS. Next, cells were stained with IL-33 primary antibodies (1:300) for 1h at room temperature and with goat anti-rabbit Alexa Fluor 568 second antibody (1:400) for 1h. The nuclei of cells were stained by exposure to DAPI at a concentration of 300nM for 5 min. The negative controls were stained only with the second antibody. The images of stained cells were collected by a Yokogawa CV7000S imager.

2.5 IL-33 ELISA assay

Cells were treated with TNFα for 24h and supernatants and cell lysates were collected every 3h to determine the appropriate stimulation time. Proteins in supernatants were concentrated and re-suspended in ELISA assay buffer with protease inhibitor. Cells lysates were collected by splitting cells with cell lysis buffer containing protease inhibitor. IL-33 concentration was measured in supernatants and cell lysates by ELISA according to the manufacturer's instructions by using a standard curve. The optical densities of samples were detected using a microplate reader at a wavelength of 450nm. Cells were then pre-treated with butyrate (0.1mM and 5mM), propionate (0.3mM and 10mM) or TSA (0.5μM) for 24h, then trerated with TNFα for 24h. The endogenous IL-33 concentration was assayed by ELISA.

2.6 IL-8 production in siIL-33 transfected HUVEC

siIL-33 transfected HUVEC were pre-incubated with butyrate (0.1mM), propionate (0.3mM) or TSA (0.5μM) for 24h, followed by TNFα stimulation for 24h. The supernatants were collected and stored at -20°C for later analysis. The IL-8 concentration in the supernatant was measured by ELISA according to the manufacturer's instructions.

2.7 VCAM-1 expression in siIL-33 transfected HUVEC by Flow cytometry

After transfection with IL-33 siRNA for 48h, HUVEC were pre-incubated with butyrate (5mM), propionate (10mM) or TSA (0.5μM) for 24h and followed with 8h TNFα stimulation. Cells were then stained with human VCAM-1 PE-conjugated antibody and cell viability dye according to the manufacturer's protocol and then detached from the culture plates with 0.05% trypsin-EDTA. VCAM-1 expression on siIL-33 transfected HUVEC was measured by a flow cytometer (FACSCanto II), and data was analysed by Flowlogic, version7.

2.8 Activation of NF-κB p65 in normal and siIL-33 transfected HUVEC

HUVEC were first treated with TNFα for 4h and cell lysates were collected at 0min, 5min, 10min, 30min, 60min, 120min, 180min and 240min to determine the optimal stimulation time. Normal and siIL-33 transfected HUVEC were then pre-incubated with butyrate (0.1mM and 5mM), propionate (0.3mM and 10mM) or TSA (0.5μM) for 24h, followed by 10min TNFα stimulation. Cell lysates were collected in cell lysis buffer containing protease inhibitors, and samples stored at -80°C. The phosphorylation level of p65 (S536) in the cell lysate was measured by ELISA according to the manufacturer's instructions.

2.9 Activation of MAPK signaling pathway

Again, HUVEC were treated with TNFα for 2h and cell lysates were collected at 0min, 2min, 5min, 10min, 20min, 30min, 60min and 120min to determine the best stimulation time. Normal and siIL-33 transfected HUVEC were then pre-incubated with butyrate (0.1mM and 5mM), propionate (0.3mM and 10mM) or TSA (0. 5μM) for 24h, followed by 30min TNFα stimulation. Cells were lysed in RIPA buffer containing protease and phosphatase inhibitor. Proteins were separated on 15% polyacrylamide gel and transferred to a methanol-activated PVDF membrane. The membrane was blocked for 1h in 5% milk and then incubated with primary antibodies at 4°C overnight. Secondary antibody incubation was performed at room temperature for 1h. Primary antibodies used were: anti-phospho p38 (phospho T180 + Y182;

1:1000) antibody, anti- JNK1+JNK2 (phospho T183 + Y185; 1:1000) antibody, anti-ERK1/2 (phospho Thr202/Tyr204; 1:1000) antibody and anti-GAPDH antibody (1:1000). Secondary antibodies used were goat anti-rabbit IgG H&L (HRP) conjugated antibody (1:10,000) and rabbit anti-mouse IgG H&L (HRP) conjugated antibody (1: 10,000).

2.10 Statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology [33].All data are expressed as mean ± SD. Group comparisons were performed with one-way ANOVA using a post hoc Bonferroni correction. SPSS was used for all statistical analyses. In all cases, *P-value* < 0.05 was considered statistical significant.

3. Results

3.1 IL-33 was expressed in the cytoplasm and perinuclear of HUVEC

Under non-stimulated conditions, IL-33 was expressed in the cytoplasm and perinuclear. IL-33 expression was high near the nuclear membrane (**Figure 1A**) and was decreased after TNFα stimulation (**Figure 1A**). HUVEC were treated with TNFα and supernatants and cell lysates were harvested every 3h for a period of 24h. IL-33 concentrations in cell lysates were significantly reduced after 6h TNFα stimulation and lasted for 24h. In contrast, although the proteins in the supernatant were concentrated 10 times, IL-33 was not detectable in the supernatant (**Figure 1B**), which indicated that TNFα treatment did not result in extracellular release of IL-33, nor did induce apoptosis.

3.2 Butyrate and propionate prevented the TNFα-induced decrease in intracellular IL-33 level

The TNFα-induced decrease in intracellular IL-33 concentration after 24h stimulation was prevented when the HUVEC were pre-incubated for 24h with

butyrate (0.1mM or 5mM) and propionate (0.3mM or 10mM) (**Figure 2**). TSA (0.5µM), the HDAC inhibitor, showed similar effect (**Figure 2**).

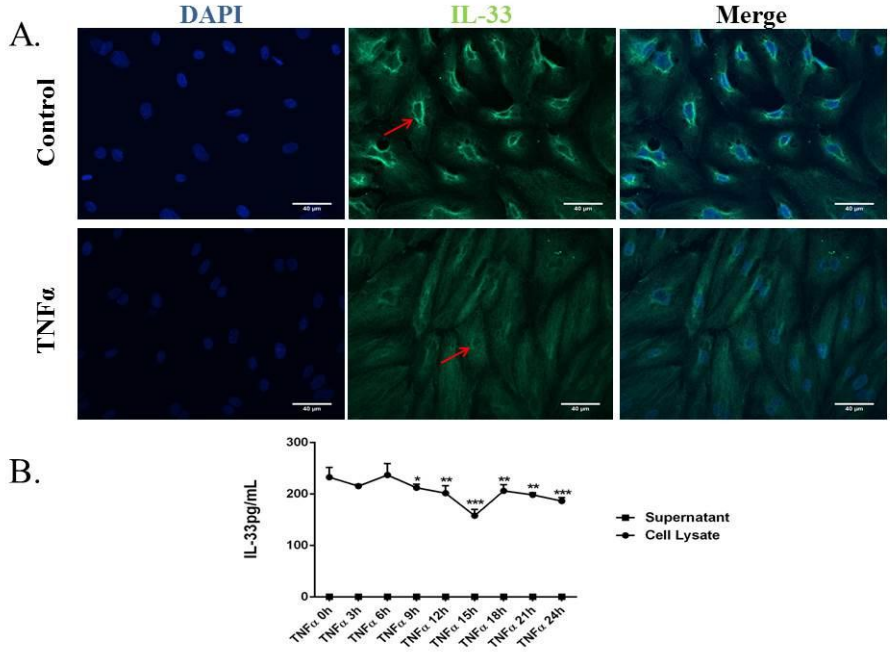

Figure 1 The effects of TNFα on IL-33 expression in HUVEC. (A) Representative images of DAPI and IL-33 duplicate fluorescence staining showing intracellular IL-33 expression and location in normal and TNFα (1ng/mL)-treated HUVEC. Cell nucleus was visualized by a blue signal and IL-33 was visualized by a green signal. Red arrows indicate high expression of IL-33 near the nuclear membrane in normal cells which was decresed in TNFα stimulated HUVEC.Scale bar is 40µm. (B) ELISA analysis of the effect of TNFα stimulation on expression of intra- and extracellular IL-33 during 24h. N=4, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group.

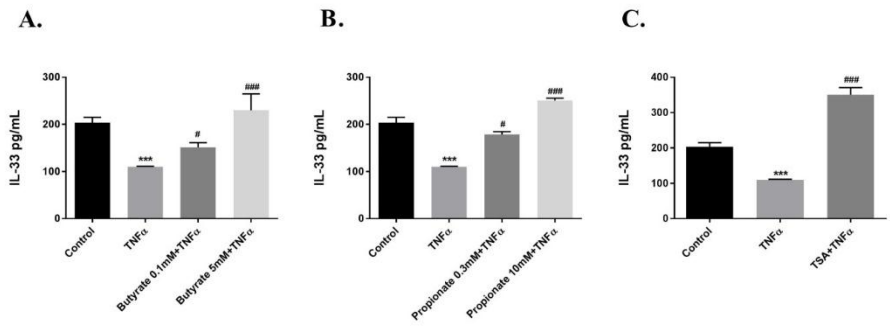

Figure 2 The effects of butyrate, propionate and TSA on expression of endogenous IL-33 in HUVEC. TNFα treatment significantly decreased intracellular IL-33 level, which was prevented by butyrate, propionate and TSA treatments. N=4, $^{***}p < 0.001$ compared with control group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with TNFα group.

3.3 siIL-33 transfection

IL-33 expression was silenced in HUVEC by siRNA transfection. The weight ratio of 1:3 between siRNA and lipo-2000 produced a higher transfection efficiency than a weight ratio of 1:1.5 (**Figure 3A**). The expression level of IL-33 protein was then measured by western blot after transfection with IL-33 siRNA for 48h, 72h and 96h. IL-33 expression was efficiently suppressed by siIL-33 transfection compared with the negative control at all three-time points (**Figure 3B**). All experiments that were performed with siIL-33transfected HUVEC started from 48h after transfection. We therefore, performed a WST-1 test to measure cell proliferation and viability at 48h after siIL-33 transfection, and found no effect of siIL-33 transfection on cell proliferation and viability (**Figure 3C**).

3.4 The inhibition of the TNFα-induced IL-8 production by butyrate and propionate is IL-33 dependent

The IL-8 production induced by TNFα-induced HUVEC was suppressed by butyrate and propionate (**Figure 4A, B**). These inhibitory effects of butyrate and propionate on IL-8 production were significantly prevented in siIL-33 transfected HUVEC (**Figure 4A, B**). Similar results were obtained by pre-incubation with the HDAC inhibitor, TSA (**Figure 4C**).

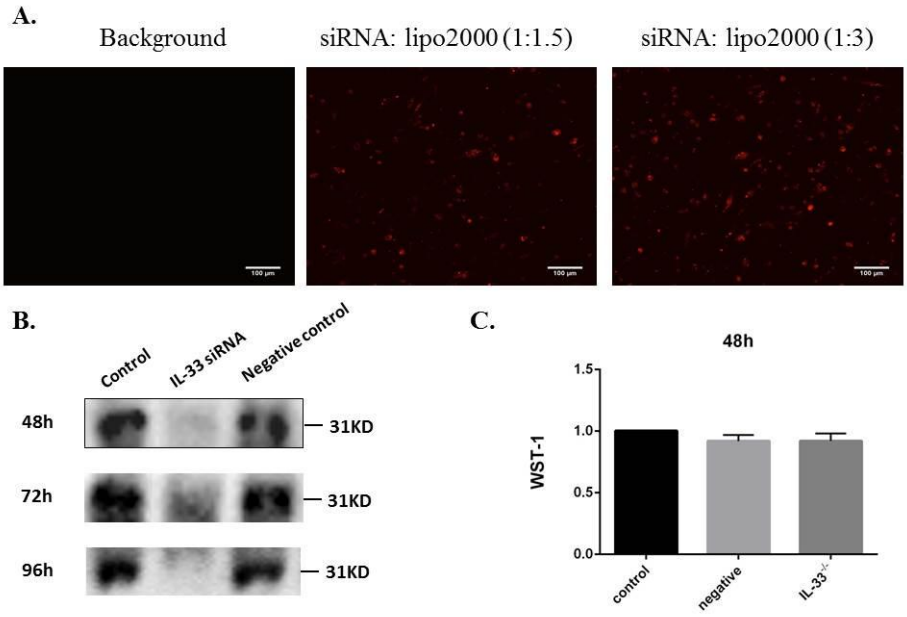

Figure 3 siIL-33 transfection in HUVEC. (A) Transfection efficiency test. HUVEC were transfected with different ratios of Block-iT and lipo-2000 for 6h. Incubation with only fluorescence labelled Block-iT without transfection reagent was regarded as background. Scale bar is 100µm. (B) Western blot analysis of confluent primary HUVEC lysates was performed using antibodies against IL-33 and GAPDH. Similar amounts of GAPDH were observed in the presence or absence of siRNA to IL-33 (control). IL-33 expression was significantly silenced by siRNA transfection after 48h, 72h and 96h. In silencer negative control, IL-33 expression was not affected. (C) After HUVEC being transfected by IL-33 siRNA for 48h, WST-1 test was performed to indicate cell viability and showed no influence on cell viability. N=6.

3.5 The inhibition of the TNFα-induced VCAM-1 expression by butyrate and propionate is IL-33 independent

The VCAM-1 expression induced on TNFα-stimulated HUVEC was suppressed by butyrate and propionate (**Figure 4D**). In contrast to the IL-8 production, the inhibitory effects of butyrate and propionate on VCAM-1 expression were not different in siIL-33 transfected HUVEC (**Figure 4D**). TSA pre-incubation also blocked VCAM-1 expression in siIL-33 transfected HUVEC (**Figure 4D**).

3.6 The butyrate and propionate-inhibited NF-κB activation is IL-33 dependent

Phosphorylation level of p65, the subunit of NF-κB, was induced by TNFα in a time-dependent manner and reached a maximum after 10min stimulation (**Figure 5A**). Pre-incubation of normal HUVEC with a low concentration of butyrate (0.1mM) and propionate (0.3mM) inhibited TNFα (10min)-induced phosphorylation of p65, which was restored in siIL-33 transfected HUVEC (**Figure 5B, C**). However, high concentrations of butyrate (5mM) and propionate (10mM) showed no effect on TNFα-induced (p)p65 levels (**Figure 5B, C**). Furthermore, TSA treatment also showed inhibitory effects on phosphorylation of p65 in normal cells which was reversed in siIL-33 transfected HUVEC (**Figure 5D**).

3.7 Butyrate and propionate modulated the MAPK signaling pathway independently of IL-33

To investigate whether the MAPK signaling pathways are also affected by butyrate and propionate treatment and whether IL-33 modulates activation of the MAPK signaling pathways, phosphorylation levels of MAPK family proteins, including ERK1/2, JNK and p38MAPK, in normal and siIL-33 transfected HUVEC were investigated by western blot. Phosphorylation of JNK and p38MAPK, but not ERK1/2, was activated by TNFα stimulation in a time-dependent manner (**Figure 6A**), with phosphorylation levels of JNK and p38MAPK peaking around 20-30min (**Figure 6A**).

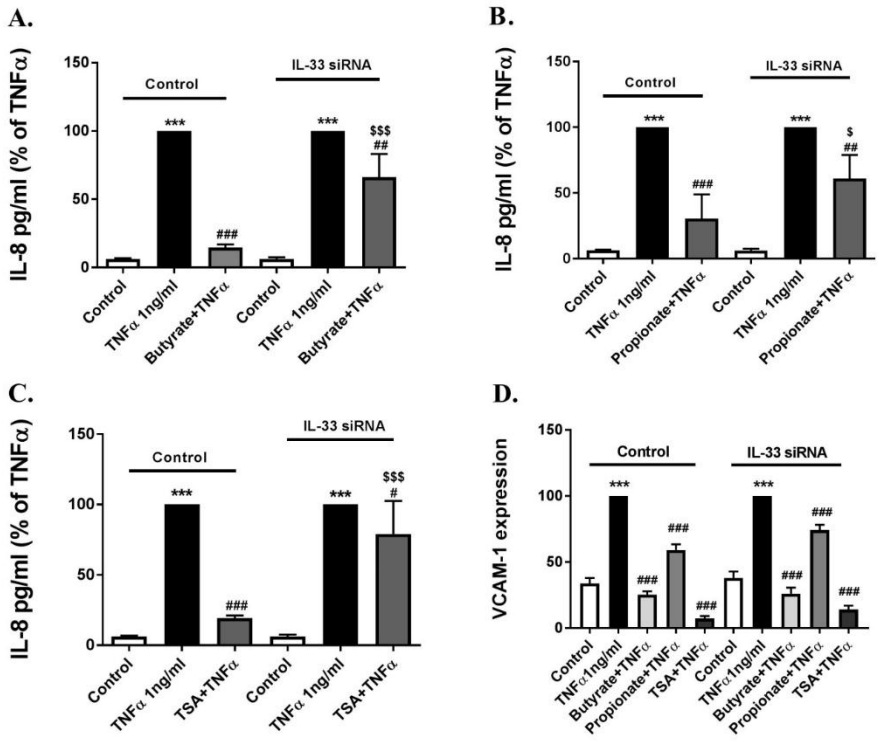

Figure 4 The effects of butyrate and propionate on IL-8 production and VCAM-1 expression in TNFα-stimulated normal and siIL-33 transfected HUVEC. (A) Butyrate treatment significantly reduced TNFα-induced IL-8 production in normal HUVEC which was significantly restored in siIL-33 transfected HUVEC. Similar effects were found in propionate (B) and TSA (C) treated cells. (D) In siIL-33 transfected HUVEC, TNFα-increased VCAM-1 expression was inhibited by butyrate, propionate and TSA treatments, which were similar in normal HUVEC. N=4, $^{***}p < 0.001$ compared with control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with TNFα group; $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ compared with the effects of butyrate, propionate or TSA treatment in normal HUVEC.

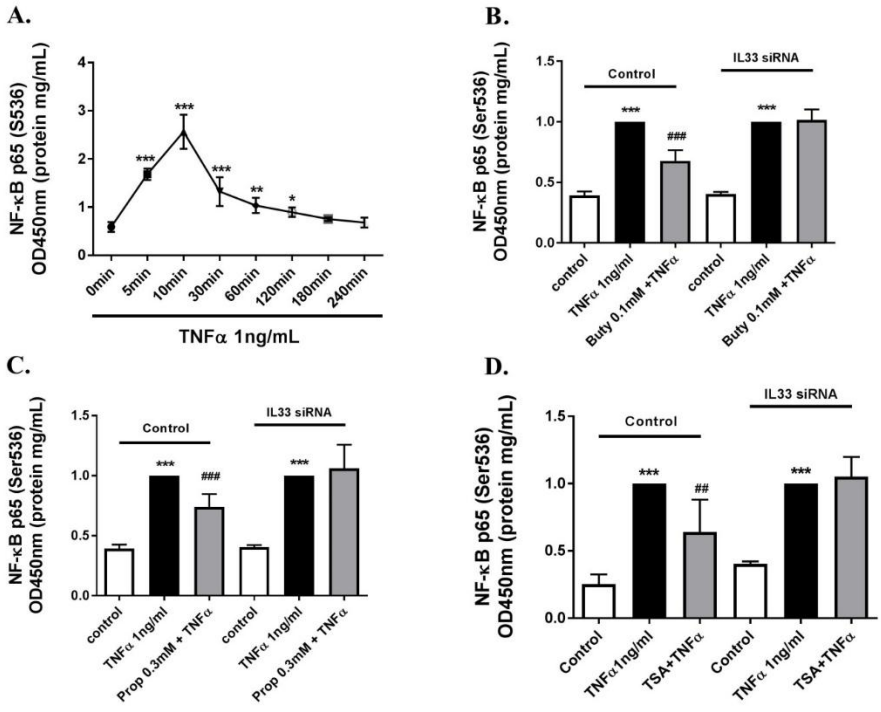

Figure 5 Butyrate and propionate inhibited activation of NF-κB was IL-33 dependent. (A) TNFα induced phosphorylation of p65 in a time-dependent manner during 240min. N=4. (B) Butyrate (0.1mM) treatment inhibited TNFα-induced (p)p65 level which was restored in IL-33 siRNA transfected HUVEC. Similar effects were obtained in propionate treated groups (C). (D) TSA also inhibited p65 activation and its effect was restored in siIL-33 transfected HUVEC. N=4 $^{*}p < 0.05$, $^{*}p < 0.01$, $^{***}p < 0.001$ compared with control group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with TNFα group.

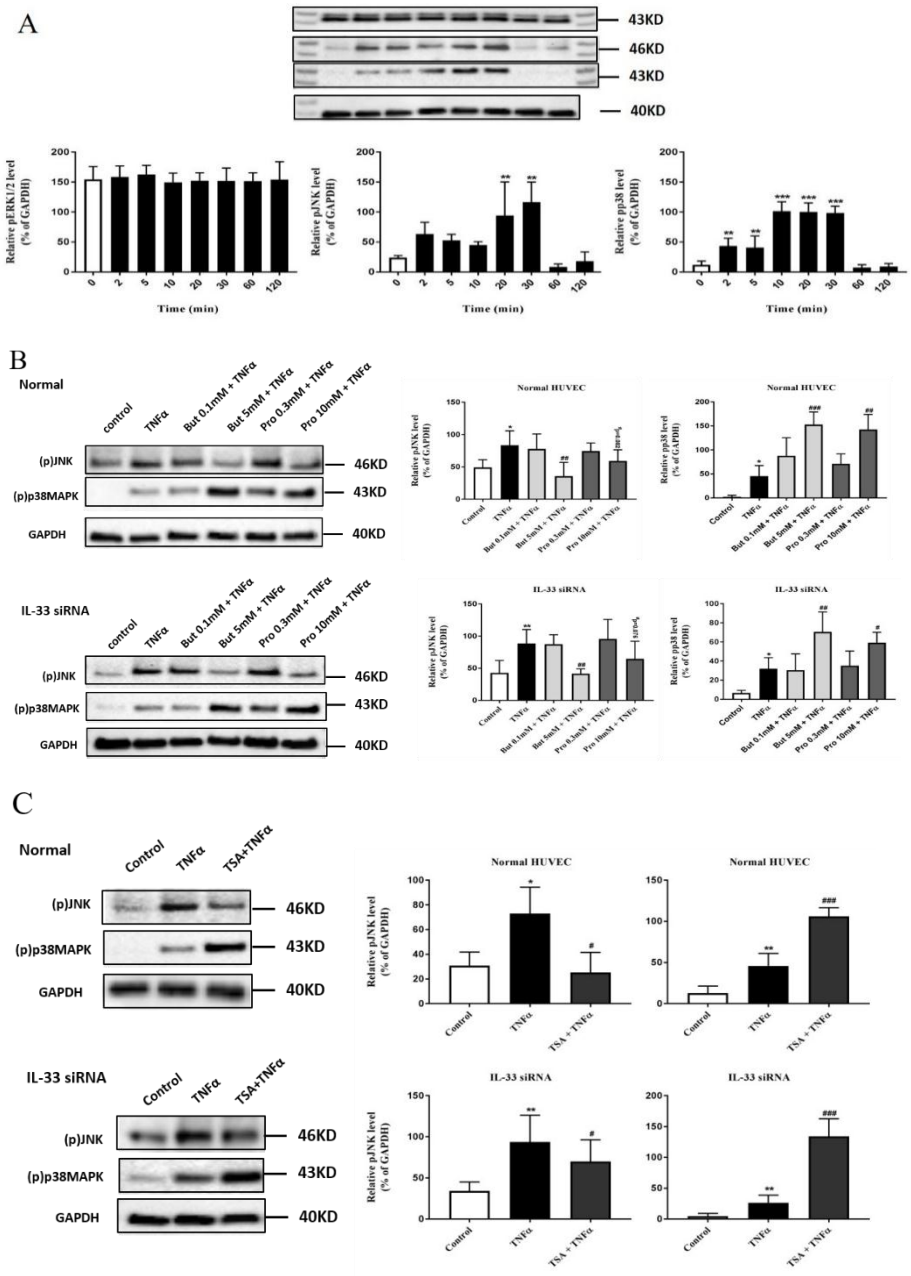

Figure 6 The effects of butyrate and propionate on TNFα-induced MAPK signaling pathways activation in normal and siIL-33 transfected HUVEC. (A) Western blot analysis demonstrated time-dependent activation of JNK and p38MAPK, but not ERK1/2, by TNFα in HUVEC; (B) Butyrate (5mM) and propionate (10mM) inhibited activation of JNK, but facilitated activation of p38MAPK in normal HUVEC, which

were not affected by IL-33 transfection. (C) TSA showed similar effects with butyrate and propionate on activation of JNK and p38MAPK. N=3. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with TNFα group.

The effects of butyrate (0.1mM and 5mM) and propionate (0.3mM and 10mM) on protein levels of (p)JNK and (p)p38MAPK induced by 30min TNFα stimulation were then investigated. In normal HUVEC, low concentrations of butyrate (0.1mM) and propionate (0.3mM) had no significant effects on phosphorylation of JNK and p38MAPK (**Figure 6B**). However, high concentrations of butyrate (5mM) and propionate (10mM) inhibited increased levels of (p)JNK increased protein level of (p)p38MAPK (**Figure 6B**). These effects were not changed in siIL-33 transfected HUVEC (**Figure 6B**). TSA showed similar effects as high concentrations of butyrate and propionate on the phosphorylation of JNK and p38MAPK, both in normal and siIL-33 transfected HUVEC (**Figure 6C**).

4. Discussion

Accumulating evidence indicates that endothelial activation is an early marker for atherosclerosis [34] which is characterized by increased production of cytokines and chemokines and expression of adhesion molecules [3, 4]. In a previous study, we showed that TNFα-induced endothelial activation was diminished by SCFA, especially butyrate and propionate, via inhibition of HDAC activity. HDACs regulate the expression of nuclear IL-33 [10] which is involved in inflammatory response in immune cells by regulating NF-κB and MAPK signaling pathways.[22] However, the molecular and downstream mechanisms underlying HDAC inhibition by butyrate and propionate are completely unknown in endothelial cells. Therefore, we investigated whether nuclear IL-33 mediates the beneficial effects of butyrate and propionate on endothelial activation by regulation of NF-κB or MAPK signaling pathway. In present study, we demonstrated several novel findings:

First, we found that IL-33 was located in the cytoplasm and highly centralized near the nucleus. Nuclear IL-33 was significantly decreased by TNFα stimulation and restored by butyrate and propionate. Moreover, IL-33 was not detectable in the supernatant with or without TNFα treatment. Second, the inhibitory effects of butyrate and propionate on IL-8 production were IL-33 dependent, while their effects on VCAM-1 expression were not. Third, low concentrations of butyrate (0.1mM) and propionate (0.3mM), which affected IL-8 production, inhibited phosphorylation of p65(S536) in an IL-33-dependent way. Fourth, high concentrations of butyrate (5mM) and propionate (10mM) which affected TNFα-induced VCAM expression, inhibited activation of the JNK pathway and facilitated activation of p38MAPK pathway in an IL-33-independent way. Finally, TSA (an HDAC inhibitor) showed similar effects as butyrate and propionate on IL-8 production, VCAM-1 expression, and activation of NF-κB and MAPK signaling pathways in normal and siIL-33 transfected HUVEC. Understanding the underlying mechanisms of action by butyrate and propionate on endothelial activation offers a novel therapeutic strategy in the treatment of atherosclerosis.

IL-33, belonging to the IL-1 family, can function both as a ligand for ST2 [35] and as a nuclear factor [17] to modulate inflammatory responses. IL-33 is abundantly expressed in the nuclei of endothelial cells during homeostasis [11] and can be induced [36] or suppressed [9] by different kinds of stimulation. So far, IL-33 expression at the mRNA and protein level in endothelial cells has been shown *in vivo* [11]. In contrast, the expression of IL-33 in primary human endothelial cells (HUVEC) has not been reported. In the present study, we found IL-33 consistitutively expressed in the cytoplasm and nucleus of HUVEC. Moreover, IL-33 was highly concentrated near the nucleus, which was reduced by TNFα treatment. As IL-33 was not detected in the supernatant of normal or TNFα stimulated HUVEC, we can infer it was behaving as a nuclear factor in this study. The TNFα inhibited IL-33 expression was prevented by butyrate and propionate treatment which might be associated with their inhibitory effects on HDAC activity. HDACs, especially

HDAC3, are involved in the regulation of endogenous IL-33 levels in PBMC, and inhibition of HDACs by TSA reduced IL-33 in LPS-stimulated PBMC [8]. In our previous study, HDAC3 was highly expressed in HUVEC and butyrate or propionate inhibited HDAC activity [7]. In order to further support the hypothesis that butyrate- and propionate-modulated endogenous IL-33 expression was mediated by inhibition of HDAC activity, the effects of butyrate/propionate and TSA, a potent HDAC inhibitor, on IL-33 expression were compared. Interestingly, TSA increased intracellular IL-33 expression and had similar effects as butyrate and propionate. All these data suggested IL-33 was regulated by HDACs and acted as nuclear factor rather than as a cytokine under this experiment set-up and might be involved in the effects of butyrate and propionate on regulation of TNFα-induced endothelial activation.

IL-8 is a prominent chemokine released by endothelial cells which triggers the recruitment of immune cells to the atherosclerotic sites and mediates inflammatory responses [37]. VCAM-1 expression can be induced by TNFα and mediates monocyte adhesion and transmigration [6] (Figure 8). NF-κB regulates the TNFα-induced inflammatory response in endothelial cells, including the production of pro-inflammatory cytokines such as IL-8 and expression of adhesion molecules such as VCAM-1 [38, 39]. Therefore, inhibition of NF-κB can dampen TNFα-induced endothelial activation. IL-33 functions as a nuclear factor with pro- and anti-inflammatory properties by regulating the activation of NF-κB [19]. Nuclear IL-33 can function as a direct transcriptional activator of NF-κB that up-regulates the basal expression of NF-κB p65 showing pro-inflammatory property [17]. Nuclear IL-33 can also interact with the p65 and reduce p65 binding to its cognate DNA and dampen NF-κB-stimulated gene transcription, leading to anti-inflammatory responses [22]. In the present study, we found the reduced phosphorylation of p65 and IL-8 production by butyrate (0.1mM) and propionate (0.3mM) was IL-33 dependent. p65 is the specific subunit of NF-κB that can bind to the IL-8 promoter to regulate IL-8 expression [40]. Therefore, we can predict that nuclear IL-33 was involved in the inhibitory effects of butyrate and

propionate on IL-8 production by interacting with p65 and reducing p65 binding to IL-8 promoter, explaining the molecular mechanism behind the anti-inflammatory properties of the SCFA. In contrast, butyrate (5mM) and propionate (10mM) showed no effects on activation of NF-κB p65 and their inhibitory effects on VCAM-1 expression were not changed in siIL-33 transfected HUVEC. This indicates that the effect of butyrate and propionate on VCAM-1 expression was IL-33 and NF-κB independent, thus mediated by another mechanism. In previous studies, activation of JNK [29, 41] and p38MAPK signaling pathways [26, 28] was involved in modulation of VCAM-1 expression. In this study, JNK and p38MAPK pathways, but not ERK1/2, were activated by TNFα, and subsequently regulated by butyrate and propionate. This indicates the involvement of JNK and p38MAPK signaling pathways in the effects of butyrate and propionate on VCAM-1 expression. Interestingly, we found that butyrate (5mM) and propionate (10mM) inhibited the activation of JNK but facilitated the activation of p38MAPK pathway, independently of IL-33. Despite the opposite effects on activation of JNK and p38MAPK, butyrate and propionate inhibited VCAM-1 expression in TNFα-induced endothelial cells, and might be the result of integration between JNK and p38MAPK signaling pathways. p38MAPK can negatively regulate JNK activity by inhibiting the activity of MLK3, upstream of JNK [42]. Activation of p38MAPK induced by butyrate and propionate might result in the inhibition of JNK. Moreover, TSA showed similar effects with butyrate and propionate on VCAM-1 expression and activation/inhibition of JNK and p38MAPK. Therefore, the inhibitory effects of butyrate or propionate on VCAM-1 expression might be mediated by HDACs/JNK or p38MAPK signaling cascade.

5. Conclusions

In conclusion, this is the first study demonstrating the involvement of nuclear IL-33 in the anti-inflammatory effects of butyrate and propionate on TNFα-activated endothelial cells and IL-33 as a nuclear factor showed anti-inflammatory properties. Furthermore, the effects of butyrate and

propionate on IL-8 production were mediated by the HDAC/IL-33/NF-κB pathway, while their effects on VCAM-1 expression were associated with the IL-33 independent HDAC/MAPK signaling pathways (*Figure 7*). These findings provide a better understanding of the beneficial effects of butyrate and propionate on TNFα-induced endothelial activation, and represent a novel protective pathway in the prevention of atherosclerosis and opportunities for therapeutic intervention.

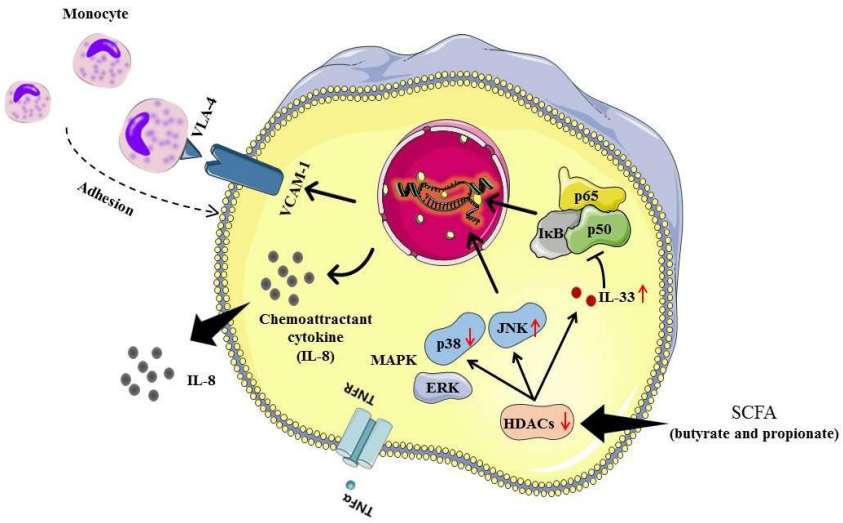

Figure 7 Schematic overview of the anti-inflammatory effects of SCFA on TNFα-induced endothelial activation. SCFA, especially butyrate and propionate, decreased TNFα-induced endothelial activation by inhibiting HDAC activity and two following signaling pathways. By inhibiting HDACs, butyrate and propionate increased intracellular IL-33 production, which inhibited activation of NF-κB and consequent IL-8 production and secretion. Furthermore, by inhibiting HDACs, butyrate and propionate regulated MAPK signaling pathways including JNK and p38MAPK but not ERK1/2 which inhibited TNFα-induced VCAM-1 expression and the following adhesion of monocytes to endothelial monolayer.

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Chapter 7

The protective and restorative effects of short chain fatty acids on the barrier function of human bronchial airway epithelium

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ABSTRACT

Background

Barrier dysfunction of the airway epithelium contributes to the development of allergies, airway hyper-responsiveness and respiratory diseases. Short-chain fatty acids (SCFA) can enhance and restore the barrier function of the intestinal epithelium. The purpose of this study was to investigate whether SCFA are able to enhance the barrier function of bronchial epithelium by regulating tight junction protein expression and leaky potassium channels. Furthermore, the roles of MAPK signaling pathways in the SCFA induced effects were explored.

Methods

First, the effects of SCFA on the development of normal lung epithelial barrier function were studied. Then, the restorative effects of SCFA (acetate, propionate and butyrate) on damaged epithelial barrier function induced by IL-4 and IL-13, house dust mite extract (HDM) or spadin (Trek1 inhibitor) were investigated. The epithelial barrier function of 16HBE was evaluated by measuring by the transepithelial electrical resistance (TEER). In addition, the effects of SCFA on IL-4-induced MAPK (ERK1/2 and JNK) signaling pathways and the expression of zonula occluden-1 (ZO-1) were measured by western blot assays.

Results

Propionate and butyrate enhance the barrier function of developing bronchial epithelium in a concentration-dependent manner. Furthermore, incubation with propionate or butyrate results in complete recovery of the barrier function after exposure of bronchial epithelium to IL-4, IL-13, HDM or spadin. Acetate induces partial recovery of the epithelium after exposure to HDM or spadin but held no additive effect on the recovery of the barrier properties after exposure to the IL-4 or IL-13. Furthermore, IL-4 decreased ZO-1 protein expression and induced phosphorylation of ERK1/2 and JNK.

Reduced ZO-1 expression and activated ERK1/2 signaling pathway were restored by SCFA treatment.

Conclusions

SCFA, especially butyrate and propionate, showed prophylactic and restorative effects on airway epithelial barrier function by increasing tight junction protein (ZO-1) expression which is associated with inhibition of ERK1/2 signaling pathway. Trek1 regulated lung epithelium barrier function and this might be an additional pathway by which SCFA exert their effect. SCFA may open a new avenue for the treatment of respiratory diseases.

1. Introduction

The airway epithelium is an important mucosal barrier involved in the protection of the host against inhaled pathogens, allergens and other noxious substances. The barrier is constituted by the formation of complexes which consist of adherens junctions, tight junctions (TJs) and desmosomes [1-4]. The complexes form intercellular contacts between the epithelial cells, thereby limiting the passage of liquids and macromolecules across the epithelium via paracellular diffusion [5, 6]. TJs, the most apical intercellular junctions of epithelial cells, are formed by occludin, claudins, junctional adhesion molecules (JAMs) and zonula occludens-1 (ZO-1). Under physiological conditions, the tight junction proteins cover the subapical regions of the epithelial cells and preserve the integrity of the epithelium [5, 7]. However, reduced airway epithelial cell barrier integrity is increasingly associated with allergic inflammation and asthma [2] and the mechanisms involved remain poorly understood. The permeability of the airway epithelium can significantly increase when the epithelial cells are exposed to pro-inflammatory cytokines, pathogens or allergens. For example, IL-4, IL-13 and components of house dust mite can cause barrier dysfunction [2, 8, 9]. Analysis of primary airway epithelial cell obtained from asthmatic subjects revealed diminished epithelial barrier function that was associated with decreased ZO-1 expression [10]. ZO-1 is critical to TJs formation and organization by linking the transmembrane protein occludin to other cytoplasmic components of TJs complex and to the actin cytoskeleton [11]. Mitogen-activated protein kinases (MAPK), which includes extracellular signal-regulated protein kinases 1/2 (ERK1/2), c-Jun N-terminal kinases (JNK) and p38MAPK, are Ser/Thr protein kinases that respond to extracellular stimuli and regulate various cellular activities. The expression of ZO-1 can be modulated by MAPK signaling pathways leading to the altered permeability and most studies demonstrated the relationship between ERK1/2 and ZO-1 [12].

Recently, it was demonstrated that Trek1, a leaky potassium channel, is involved in the maintenance of membrane potential and regulates the barrier function of epithelial tissues [13-15]. Trek1 is expressed in airway endothelium and epithelium, as well as in intestinal epithelium [14]. Exposure of nasal and intestinal epithelium to pro-inflammatory cytokines, like IL-4, IL-5 and IL-13, results in a reduced expression of Trek1 via upregulation of histone deacetylase 1(HDAC1) and modulation of the MAPK signaling pathways [14, 15]. However, it is not known whether such effects also occur in activated airway epithelium, which also expresses the potassium channel.

The SCFA, mainly acetate, propionate and butyrate, are metabolites of the bacterial fermentation of indigestible carbohydrates and proteins in the colon [16-18]. SCFA can be absorbed by the colonocytes, after which they are used as energy source for the cellular metabolism. Fatty acids that manage to escape the metabolism of the colonocytes can diffuse through the basolateral membrane and reach hepatic portal vein. The fatty acids possess potent anti-inflammatory effects by inhibition of class I and IIa HDACs and activation of free fatty acid 2 and 3 (FFA2/3) receptors and therefore can potentially be used for modulation of inflammatory disorders [19, 20]. Interestingly, it was reported that sodium butyrate can recover the Trek1 expression in intestinal epithelium after exposure to pro-inflammatory cytokines [14].

The potency of SCFA to cause above-described effects combined with their capability to reach the systemic circulation, justifies research into the effects of these fatty acids in tissues remote from the site of production and may open an area of therapeutic options. Therefore, the aim of the present *in vitro* study was to determine whether SCFA can modulate the development of barrier function of bronchial airway epithelium and whether SCFA can be used for the treatment of existing damage to the barrier caused by exposure to type 2 cytokines IL-4 and IL-13, house dust mite extract (HDM) or spadin (a synthetic Trek1 inhibitor). Furthermore, we investigated whether SCFA

restore IL-4-induced barrier dysfunction by regulating tight junction protein (ZO-1) expression via modulation of MAPK (ERK1/2 and JNK) signaling pathways.

2. Methods

2.1 Reagents

Minimal Essential Medium (MEM) and Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) with glutamax were purchased from Gibco (Thermo Fisher Scientific, Noord-Brabant, The Netherlands). Fetal bovine serum (FBS) was purchased from Bodinco (Noord-Holland, The Netherlands). The bovine collagen type I solution was obtained from Advanced BioMatrix (CellSystems Biotechnologie Vertrieb, Troisdorf, Germany). The sodium salts of propionate and butyrate, as well as fibronectin from human plasma and penicillin-streptomycin (pen-strep) solutions were purchased from Sigma (Breda, Noord-Brabant, The Netherlands). Sodium acetate was obtained from Merck Millipore (Amsterdam-Zuidoost, Noord-Holland, The Netherlands). The synthetic Trek-1 potassium channel blocker Spadin and recombinant human IL-13 were purchased from R&D systems (Abingdon, Oxfordshire, UK). Recombinant human IL-4 was purchased from ProSpec-Tany TechnoGene (Ness-Ziona, Israel). The house dust mite extract was obtained from GREER (Lenoir, NC, USA). Trichostatin A (TSA), a potent class I and II HDAC inhibitor, was purchased from Selleckchem (Huissen, Gelderland, The Netherlands). The following monoclonal antibodies: anti-JNK1+JNK2 (phospho T183 + Y185) antibody, anti-ERK1/2 (phospho Thr202/Tyr204) antibody, anti-GAPDH antibody, rabbit anti-mouse IgG H&L (HRP) conjugated antibody and goat anti-rabbit IgG H&L (HRP) conjugated antibody were purchased from Cell signaling technology and Abcam.

2.2 Cells and culture conditions

The experiments were performed using the SV40-transformed and immortalized human bronchial airway epithelial cell 16HBE14o- (16HBE), which were kindly provided by the University of California, San Francisco, CA,

USA [21, 22]. The cells were cultivated in MEM supplemented with 10% (V/V) FBS in combination with 1% pen-strep. The cells were grown in plastic flasks coated with a coating solution consisting of fibronectin and collagen. The coating solution was prepared by diluting concentrated fibronectin derived from human plasma and bovine collagen type I solutions to a concentration of 30 µg/mL in DMEM/F12 with glutamax. The resulting solution was sterilized by leading it through a 0.2 µm filter. Prior to cell seeding, the plastic flasks and 96 well plates were coated overnight in an incubator at a temperature of 37 °C. After seeding, the cells were incubated at 37 °C and an atmosphere consisting of 5% CO_2.

2.3 Transepithelial electrical resistance (TEER)

The 16HBE cells were seeded at a density of 10^5 cells per well on permeable transwell inserts with a polyester membrane with a diameter of 12 mm and a pore size of 0.4 µm (cat. no. 3460, Corning). Before seeding, the apical side of the insert was coated with a 30 µg/mL bovine collagen type I solution in 70% ethanol. The solution was filtered, after which the insert was coated by applying 70 µL to the apical side of the transwell. Then, the ethanol was allowed to evaporate overnight in a laminar flow hood under exposure to UV light to preserve sterility. The 16HBE cells were grown under liquid-covered culture conditions wherein the apical and basolateral fluid volumes were set at 250 and 900 µL, respectively. The epithelial barrier function of 16HBE was evaluated by measuring the transepithelial electrical resistance (TEER) using an EVOM volt-ohm-meter and a STX2 “chopstick” electrode (World Precision Instruments). The TEER values measured were corrected for the background resistance of an empty insert containing only medium and calculated as $\Omega \cdot cm^2$.

In the first series of experiments, the effects of SCFA on the development of the barrier function of 16HBE were investigated. Previous study has shown that 16HBE cells show a sharp increase in TEER starting from day 3 after seeding, with a maximum value that is reached between the 5th and 8th day [23]. Then, a decrease in TEER follows, which from day 11 fluctuates around

approximately 400 Ω·cm^2. After seeding, the culture medium on both the apical and basolateral side was replaced daily with fresh culture medium. TEER values were determined daily prior to the replacement of the medium for 15 days, wherein the first measurement took place 24 h after seeding. Starting from day 4, the cells were given 900 µL medium or medium containing 5 or 10 mM acetate, 0.5 or 1 mM propionate, 0.5 or 1 mM butyrate administered to the basolateral side of the insert. Statistical analysis was carried out on TEER values between the different treatments on day 5, 10 and 15.

In the following series of experiments the restorative effects of SCFA after barrier disruption were investigated. Again, 16HBE cells were cultured on inserts for 14 days with daily replacements of culture medium. Subsequently, the cells were stimulated for 24 h by replacing the basolateral medium with 900 µL of a 50 ng/mL solution of IL-4 or IL-13 in medium or a 5 µM spadin solution in medium. Stimulation with house dust mite was accomplished by replacing the apical medium by a solution containing 200 µg/mL of HDM in medium and allowing incubation for 48 h. After the pre-incubation period, the basolateral medium was replaced with solutions of 10 mM acetate, 0.5 mM propionate, 1 mM butyrate or 0.5 µM TSA in medium. The cells were then incubated for 24 h. TEER measurements were performed prior to replacement of the medium at the time of pre-incubation with a stimulus, incubation with SCFA or TSA, and 24 h after addition of last mentioned substances.

All experiments were repeated two times with cells originating from different passages to ascertain reproducibility. The solutions of SCFA, TSA and stimuli were sterilized after preparation by passing them through a 0.2 µm filter. All solutions were warmed to 37 °C before they were added to the inserts.

2.4 Cytotoxicity

To determine the integrity of the cell membrane after exposure to SCFA, TSA, spadin or HDM for 72 h and/or 11 days, the release of the cytosolic enzyme lactate dehydrogenase (LDH) was determined using the cytotoxicity

detection^{Plus} kit from Roche Diagnostics (Almere, Flevoland, The Netherlands). To determine the cytotoxic effects of 72 h of exposure to above-mentioned substances, a 96-well plate was coated overnight using above-described collagen and fibronectin coating solution. Subsequently, the 16HBE cells were seeded and grown until visual confluence was reached based on microscopic inspection. Next, the cells were exposed to the various concentrations of SCFA, TSA, spadin or HDM applied in the experiments for 72 h. For the determination of the cytotoxic effects of daily exposure to these substances for 11 days, the supernatant of the basolateral compartment of the inserts used in the first series of experiments (effects of SCFA on development of barrier function) was used. Maximum release of LDH was achieved by lysing the cells with a 1% Triton X-100 solution in culture medium for 10 min at 37 ℃. Thereafter, 100 µL of the supernatant was transferred four times to a 96-well plate and 100 µL of the reaction mix of the cytotoxicity kit was added to each well. The plate was then incubated for 30 min at room temperature. Subsequently, absorbance was determined at 492 nm using a microplate reader (iMark microplate reader, Bio-Rad laboratories, Veenendaal, Utrecht, The Netherlands) and the absorbance values of the four replicates were averaged.

2.5 Western blot

Confluent 16HBE were exposed to medium with or without IL-4 and HDM and then treated with SCFA. After treatment, cells were washed with cold PBS and lysed on ice in RIPA buffer with Protease and Phosphatase Inhibitor Cocktails, followed by scraping. Proteins were resolved on SDSPAGE 4–15% and transferred to nitrocellulose membranes. Membranes were incubated in blotting solution (5% nonfat dry milk in TBS/0.1% Tween 20) at room temperature for 1h prior to overnight incubation with primary antibodies. After overnight incubation at 4°C, the blots were washed in TBS/0.1% Tween-20 followed by incubation with secondary antibodies. The blots were exposed to ECL and images were acquired. Primary antibodies used were: anti- JNK1+JNK2 (phospho T183 + Y185; 1:1000) antibody, anti-ERK1/2

(phospho Thr202/Tyr204; 1:1000) antibody and anti-GAPDH antibody (1:1000) and rabbit anti-ZO-1 (1:1000). Secondary antibodies used were goat anti-rabbit IgG H&L (HRP) conjugated antibody (1:10,000) and rabbit anti-mouse IgG H&L (HRP) conjugated antibody (1: 10,000).

2.6 Statistical analysis

All data are expressed as means $\pm$ standard error from mean (SEM). The results of the LDH assays and the TEER data of the first series of experiments were analyzed using a one-way factorial analysis of variance (ANOVA), followed by a Dunnett's multiple comparison test. The results of the experiments investigating the restorative effects of SCFA on the barrier function have been analyzed with a two-way ANOVA, also followed by a Dunnett's multiple comparison test. *P* values < 0.05 were considered as significant. The statistical analysis was performed using GraphPad Prism 6.14 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1 Assessment of cell damage

Incubation of the concentrations of SCFA, TSA, spadin and HDM applied to epithelial cells for 72 h show no indication of cytotoxicity. Except for the positive control (1% Triton X-100 solution in culture medium) no significant differences in cytotoxicity appear to exist between applied treatments and the control. Similarly, no significant differences in cytotoxicity were found between the applied concentrations of SCFA and the control after daily administration of the fatty acids for 11 days (data not shown).

3.2 Effects of SCFA on development of the epithelial barrier function of 16 HBE

The effects of repeated daily administration of SCFA on the development of the barrier function of 16HBE were investigated. **Figure. 1** illustrates the development of TEER as function of time, in which **Figure. 1A** (acetate), **1B** (propionate) and **1C** (butyrate) show the effects of repeated administration

for 11 days (starting on day 4 and ending on day 15). The development pattern of the barrier function shown in the graphs of **Figure. 1** corresponds with the pattern previously described in literature. The TEER value in controls was 534±6 Ω·cm^2 (**Figure. 1D**).

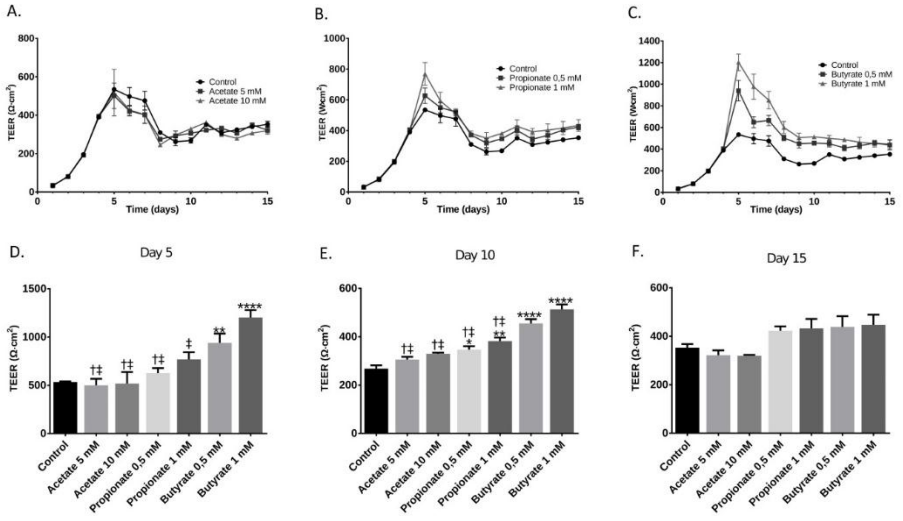

Figure 1. The effects of SCFA on the development of the barrier function of 16HBE grown on transwells expressed as a function of time. The medium on both sides of the transwells was changed daily with prewarmed, fresh medium. Barrier function was determined daily prior to replacement of the medium using TEER measurements and were corrected for the background resistance due to the membrane and medium, and membrane surface. Starting from day 4 after seeding, the medium in the basolateral compartment of the transwells was replaced daily by medium containing different concentrations of SCFA. **A**, **B** and **C** show the effects of SCFA on the barrier function, when treatment is continued until day 15. The TEER values shown in **D**, **E** and **F** correspond to the measurements on day 5, 10 and 15. All data are expressed as mean±SEM of n=3 experiments. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ and ****: $p < 0.0001$ when compared to the control group. † and ‡ indicate $p < 0.05$ when compared to the 0.5 mM and 1 mM butyrate treatment groups, respectively.

Butyrate concentration-dependently increased the TEER values: 940±95 Ω·cm^2 and 1203±77 Ω·cm^2 corresponding with 0.5 and 1 mM butyrate, respectively. Similar results were obtained with propionate. Acetate gave no

significant differences in TEER values. The TEER values of 0.5 mM propionate and both concentrations of acetate are significantly lower when compared to both concentrations of butyrate. The earlier observed significant increases in TEER (268±13 Ω·cm^2) still exists on day 10, when the cells are incubated with butyrate or propionate and were also concentration-dependent for butyrate (**Figure. 1E**). There were no significant differences between the incubations with acetate and the control. On day 15 (**Figure. 1F**), the observed effects on TEER appear to have worn.

3.3 Restorative effects of SCFA on affected airway epithelium barriers

The restorative effects of SCFA on damaged airway epithelium after exposure to three inflammatory stimuli (IL-4, IL-13 and HDM) and the Trek1 inhibitor spadin were investigated (**Figure. 2-4**).

IL-4

Pre-incubation with IL-4 (50 ng/mL) for 24 h, resulted in a significant reduction in TEER when compared to the control (413±2 and 517±2 Ω·cm^2, respectively). Replacement of the basolateral medium with fresh culture medium without IL-4 resulted in a partial recovery of the TEER value during the next 24 h. Nevertheless, the TEER value remains significantly lower when compared to the control group (473±5 and 524±3 Ω·cm^2, respectively). Similar results were observed after incubation of acetate (10 mM) following IL-4 exposure (**Figure. 2A**), which resulted in a significantly lower TEER value relative to the control group (470±4 and 524±3 Ω·cm^2, respectively). In contrast, propionate (0.5mM) (**Figure. 2C**) or butyrate (1 mM) (**Figure. 2E**) incubation without prior IL-4 stimulation, resulted in significantly higher TEER values when compared to the control and the IL-4 stimulated group (588±7, 703 ± 4, 524 ± 3 and 473 ± 5 Ω·cm^2, respectively). Interestingly, both propionate and butyrate incubation following IL-4 exposure reversed the decrease in TEER values induced by IL-4, into an increase up to the levels of propionate and butyrate alone and far above control and the IL-4 stimulated group at 48 h (588±1, 693±11, 524±3 and 473±5 Ω·cm^2, respectively).

IL-13

Pre-incubation with IL-13 (50 ng/mL) for 24 hs, induced a significant reduction in TEER when compared to control (420±2 and 517±2 Ω·cm^2, respectively). Replacement of the basolateral medium with fresh culture medium without IL-13 caused a partial recovery of the TEER value during the next 24 h, although remaining lower compared to the control group (**Figure. 2B**; 497±2 and 524±2 Ω·cm^2, respectively). As observed with IL-4, acetate incubation following IL-13 exposure did not affect the increase in TEER values due to the replacement with fresh medium (without IL-13). Both groups, IL-13 and IL-13/acetate remain below the control group. Again as observed for IL-4, propionate (**Figure. 2D**) and butyrate (**Figure. 2F**) reversed the decrease in TEER values induced by IL-13, into an increase up to the levels of propionate and butyrate alone and far above control and the IL-13 stimulated group at 48 h (593±2, 708±2, 524±3 and 497±2 Ω·cm^2, respectively).

HDM

Pre-incubation with HDM for 48 h resulted in a significant reduction in TEER when compared to the control group (371 ±2 and 489 ± 4 Ω·cm^2, respectively). Replacement of the apical compartment with fresh medium without HDM for 24 h resulted in an even further reduction of the barrier function, which remains significantly lower than the control (320±9 and 524 ± 3 Ω·cm^2, respectively). Incubation with acetate following HDM stimulation significantly increased TEER values when compared to HDM treatment group (320±9 Ω·cm^2) although levels remained significant lower compared to the control groups (474 ± 3, 477 ± 6 and 524 ± 3 Ω·cm^2, respectively) (**Figure. 3A**). Both propionate (**Figure. 3B**) and butyrate (**Figure. 3C**) alone increase the TEER values above control values. Interestingly, Incubation with propionate (**Figure. 3B**) or butyrate (**Figure. 3C**) following HDM exposure resulted in significantly higher TEER values when compared to the control group and HDM stimulated group (558±8, 702±8, 524±3 and 320±9 Ω·cm^2, respectively) indicating that the sustained decline in TEER induced by HDM was reversed by propionate and butyrate to TEER values above control.

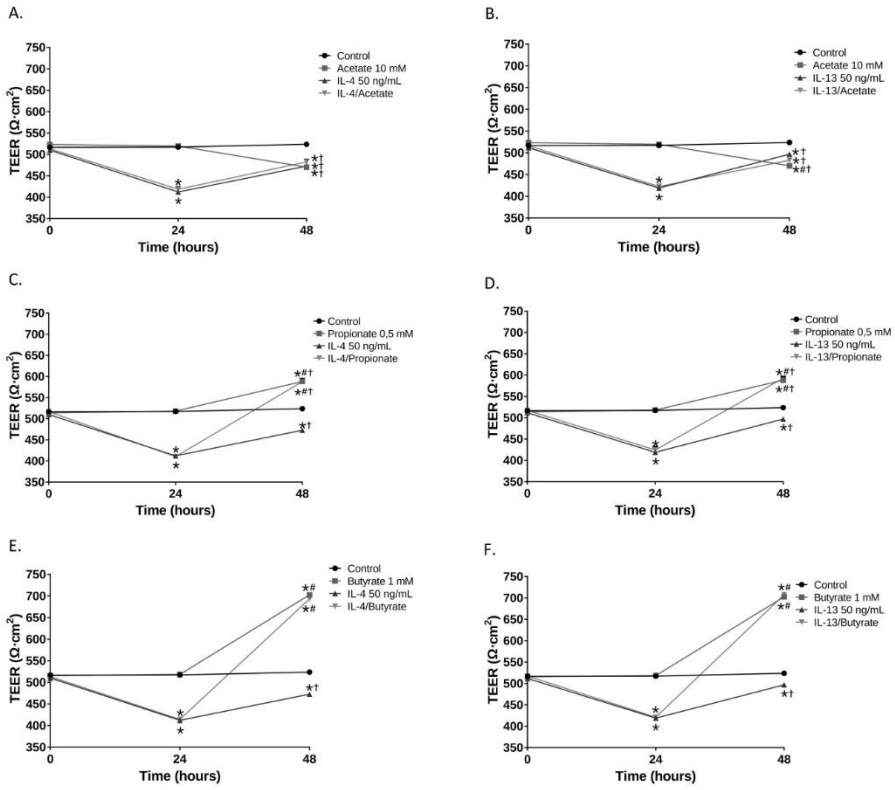

Figure 2. The effects of SCFA on the recovery of the barrier function of airway epithelium after stimulation with IL-4 or IL-13. 16HBE cells were grown on transwells for 14 days with daily refreshment of the culture medium. The cells were then stimulated at the basolateral side of the membrane with 50 ng/mL IL-4 or IL-13 for 24 h. After stimulation, the basolateral medium was replaced with medium alone or medium containing 10 mM acetate, 0.5 mM propionate or 1 mM butyrate. The barrier function was determined via TEER measurements prior to stimulation, prior to addition of SCFA and 24 h after replacement of the basolateral medium. The values measured were corrected for background resistance and for membrane surface. A-F show the effects of 10 mM acetate, 0.5 mM propionate and 1 mM butyrate on the restoration of the barrier after stimulation with IL-4 or IL-13, respectively. All data are expressed as mean±SEM of n=3 experiments. *: $p < 0.0001$ when compared to control, #: $p < 0.0001$ when compared to IL-4 or IL-13 and $: $p < 0.0001$ when compared to the treatment group that only received the corresponding SCFA.

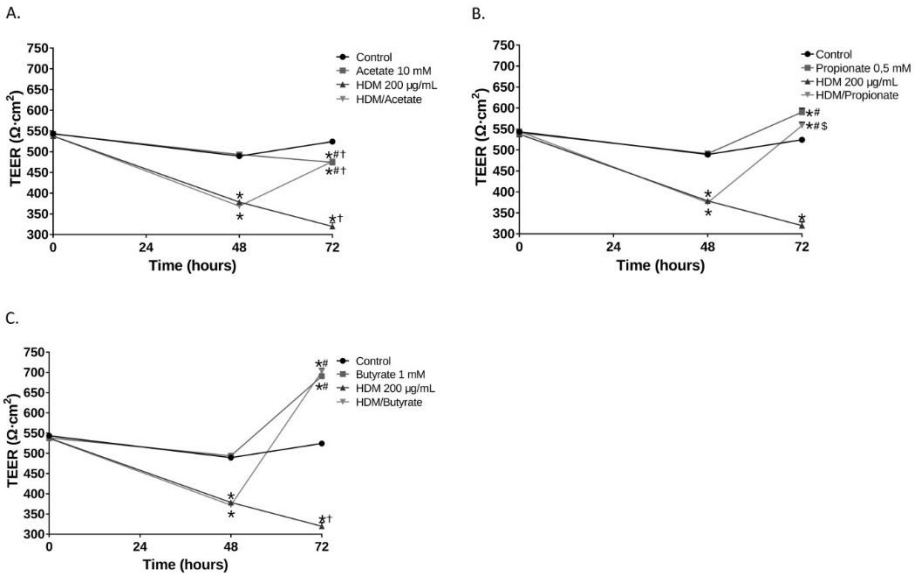

Figure 3. The effects of SCFA on the recovery of the barrier function of airway epithelium after stimulation with house dust mite extract (HDM). 16HBE cells were grown on transwells for 14 days with daily refreshment of the culture medium. The cells were then stimulated at the apical side of the membrane with 200 µg/mL HDM for 48 h. After the stimulation the basolateral medium was replaced with medium alone or medium containing 10 mM acetate, 0.5 mM propionate, 1 mM butyrate or 0.5 µM TSA. The barrier function was determined by means of TEER measurements prior to stimulation, prior to addition of SCFA or TSA, and 24 h after replacement of the basolateral medium. The values measured were corrected for the background resistance and for the surface of the membrane. **A-C** show the effects of 10 mM acetate, 0.5 mM propionate and 1 mM butyrate on the restoration of the barrier, respectively. All data are expressed as mean±SEM of n=3 experiments. *: $p < 0.0001$ when compared to control, #: $p < 0.0001$ when compared to IL-4 or IL-13 and $: $p < 0.0001$ when compared to the treatment group that only received the corresponding SCFA.

Spadin

Pre-incubation with spadin for 24 h leads to significant reductions in TEER (between 86 and 99 $\Omega \cdot cm^2$) when compared to the control (424 ± 2 versus 517 ± 2 $\Omega \cdot cm^2$, respectively). Replacement of the basolateral medium with

fresh medium without spadin did not affect the TEER values which remain

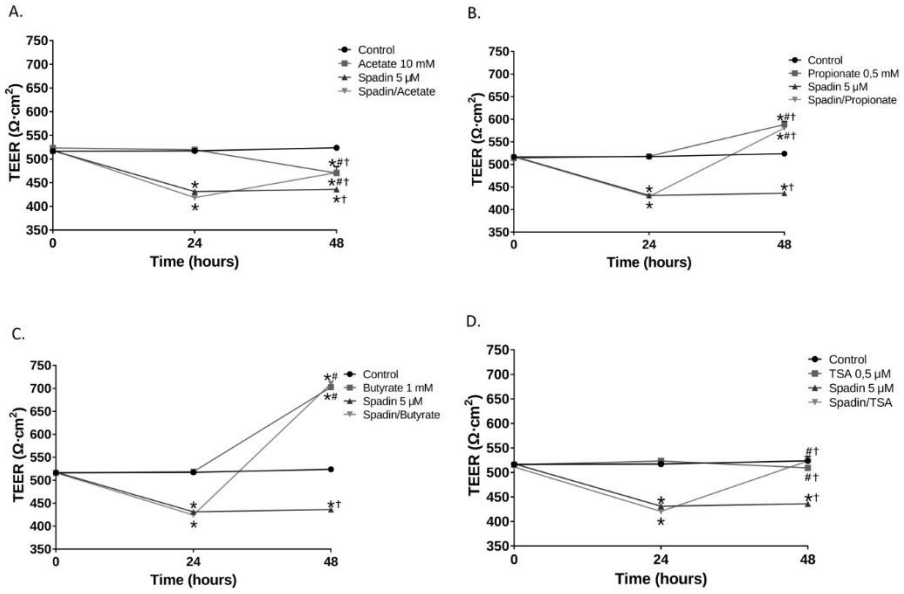

Figure 4. The effects of SCFA on the recovery of the barrier function of airway epithelium after incubation with spadin, a potent Trek-1 potassium channel inhibitor. 16HBE cells were grown on transwells for 14 days with daily refreshment of the culture medium. The cells were then incubated at the apical side of the membrane with 5 µM spadin for 24 h. After the stimulation the basolateral medium was replaced with medium alone or medium containing 10 mM acetate, 0.5 mM propionate, 1 mM butyrate or 0.5 µM TSA. The barrier function was determined by means of TEER measurements prior to incubation, prior to addition of SCFA or TSA, and 24 h after replacement of the basolateral medium. The values measured were corrected for the background resistance and for the surface of the membrane. A-D show the effects of 10 mM acetate, 0.5 mM propionate, 1 mM butyrate or 0.5 µM TSA on the restoration of the barrier, respectively. All data are expressed as mean±SEM of n=3 experiments. *: $p < 0.0001$ when compared to control, #: $p < 0.0001$ when compared to IL-4 or IL-13 and $: $p < 0.0001$ when compared to the treatment group that only received the corresponding SCFA.

significantly lower compared to the control (436 ± 3 and 524 ± 3 Ω·cm^2, respectively). Incubation with acetate (10 mM) for 24 h following spadin incubation (**Figure. 4A**) results in significantly higher TEER values

relative the spadin treatment group (471 ± 13, 436 ± 3 Ω·cm^2, respectively), although levels are lower when compared to the control group. Incubation with propionate (**Figure. 4B**) or butyrate (**Figure. 4C**) resulted in increased TEER values relative to the control. Incubation with spadin/propionate (**Figure. 4B**) or spadin/butyrate (**Figure. 4C**) resulted in significantly higher TEER values when compared to the control group and spadin exposed treatment group (581±8, 710±2, 524±4 and 436±4 Ω·cm^2, respectively). Incubation with TSA following spadin exposure (**Figure. 4D**) resulted in a significant higher TEER value when compared to the spadin treatment group (523±10 and 436±3 Ω·cm^2, respectively), although no significant differences in TEER could be demonstrated between the spadin/TSA treatment group and the control group.

3.4 SCFA inhibited IL-4-induced activation of ERK1/2 and JNK signaling pathways

Confluent 16HBE were solely stimulated with IL-4 (50ng/mL) for 4h. Cell lysates were collected at 0, 5, 10, 30, 60, 120, 180and 240min. We found that IL-4-induced phosphorylation of ERK1/2 reached at peak after 30min stimulation, while phosphorylation of JNK induced by IL-4 increased after 5min stimulation (**Figure. 5A**). Confluent 16HBE were then stimulated with IL-4 (50ng/mL) for 30min in the presence or absence of acetate (10mM), butyrate (1mM) or propionate (0.5mM). We found that SCFA inhibited activation of ERK1/2 but not JNK signaling pathways (**Figure. 5B**).

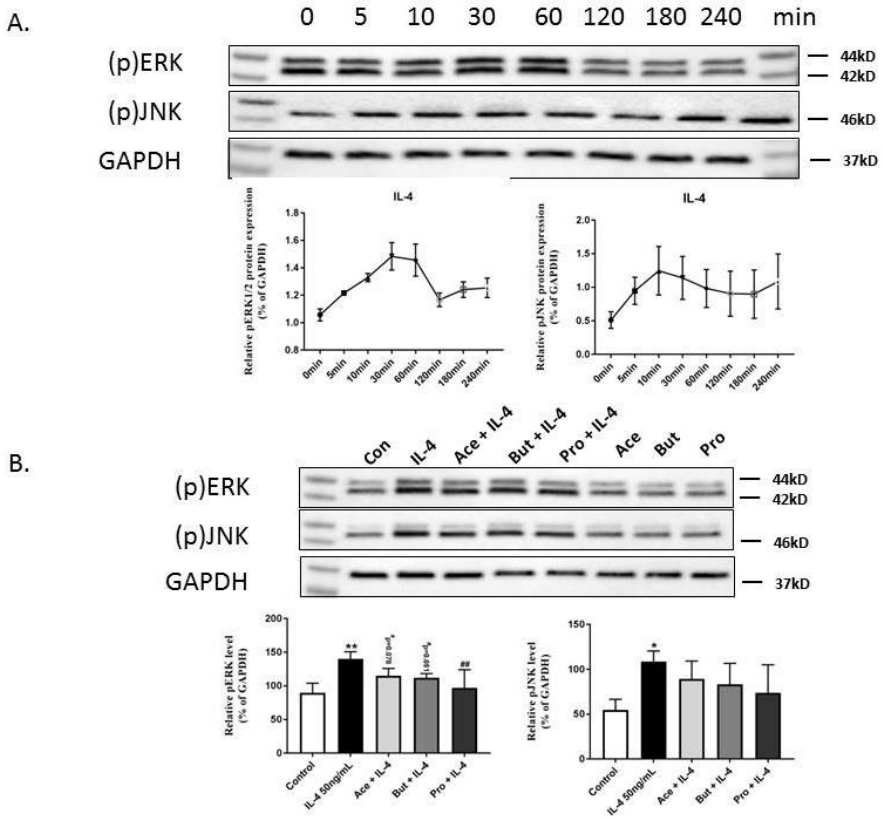

Figure 5. The effects of SCFA on IL-4-induced activation of MAPK signaling pathways in 16HBE. **A** shows IL-4 (50ng/mL) induced phosphorylation of ERK1/2 and JNK signaling pathways in 4h. **B** shows the inhibitory effects of 10 mM acetate, 0.5 mM propionate and 1 mM butyrate on IL-4-induced activation of ERK1/2 and JNK signaling pathways. GAPDH is shown as a control. N=3

3.5 SCFA increased ZO-1 expression in IL-4-stimulated 16HBE

To examine the effects of IL-4 on proteins associated with tight junctions, protein expression of ZO-1 was measured. Confluent 16HBE were stimulated with IL-4 (50ng/mL) for 6, 24 and 48h. We found that after stimulation with IL-4, ZO-1 expression was significantly decreased for up to 48h (**Figure. 6A**). Accordingly, the epithelial barrier function is also reduced. Confluent 16HBE were stimulated with IL-4 for 24h and cells were then treated with acetate

(10mM), butyrate (1mM) or propionate (0.5mM) for 24h. SCFA treatment restored ZO-1 expression in IL-4 stimulated epithelial cells (**Figure. 6B**).

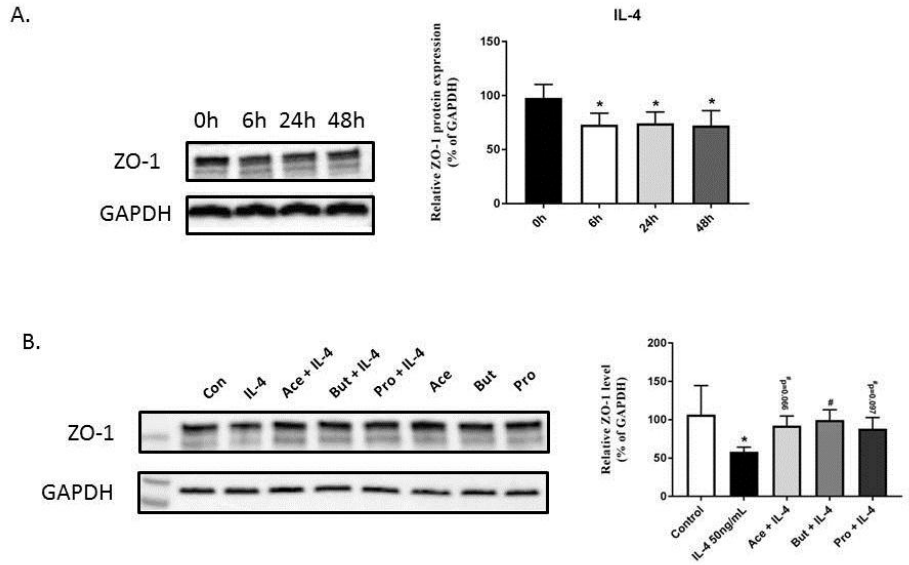

Figure 6. The effects of SCFA on IL-4-decreased ZO-1 expression in 16HBE. **A** shows that confluent 16HBE were treated with or without IL-4 (50ng/mL) for 6h, 24h and 48h and ZO-1 expression was decreased by IL-4 stimulation. **B** shows that treatment with 10 mM acetate, 0.5 mM propionate and 1 mM butyrate for 24h restored ZO-1 expression in 24h IL-4 stimulated 16HBE. GAPDH is shown as a control. N=3

4. Discussion

The current study explored the effects of SCFA on the barrier function of human bronchial airway epithelium. We found that propionate and butyrate enhanced the barrier function of the bronchial epithelium as demonstrated by increased TEER values. The most powerful effects of SCFA observed on day 5 of barrier development are thought to result from an extra sensitivity to modulation of the barrier function of the epithelium barrier as it is still in development. Furthermore, SCFA induced complete recovery of the breached airway epithelial barrier function caused by exposure of bronchial epithelium to IL-4, IL-13 and HDM.

The airway epithelium represents an important barrier that protects against the intrusion of the internal environment by inhaled harmful substances from the external environment [1-4]. An impaired barrier function of the epithelium underlies a multitude of inflammatory lung diseases and contributes to the onset of exacerbations of existing respiratory diseases, as the passage of harmful substances occurs more easily [10]. Patients with inflammatory respiratory diseases appear to be far more sensitive to exposure to allergens and viral infections. For example, bronchial epithelial cells from patients with asthma prove to be much more susceptible to barrier dysfunction caused by exposure to HDM [24].

Exposure to IL-4, IL-13 or HDM resulted in a deterioration of the barrier function of the epithelium. Epithelial barrier function is mediated by a complex of proteins, including ZO-1, which constitutes the tight junctions. Inflammatory cytokines are known to disrupt barrier function and apical junctional complexes in intestinal epithelial cells. In a previous study, IL-4 decreased ZO-1 expression in airway epithelial cells is associated with MAPK/ERK1/2 signaling pathway [11]. SCFA restored the barrier function of the damaged lung epithelium. The protective effects of SCFA on bronchial epithelial barrier function may be mediated by restoring tight junction protein (ZO-1) expression via inhibition MAPK (ERK1/2 and JNK) signaling pathways [12]. In the present study, we found that IL-4 stimulation significantly decreased expression of ZO-1 in lung epithelial cells, which was associated with decreased lung epithelial barrier function. The decreased ZO-1 expression was restored by SCFA treatment. It was reported that short-chain fatty acids (SCFA) can modulate activation of MAPK signaling pathways to regulate inflammatory responses in intestinal epithelial cells [25]. In agreement, we found IL-4 stimulation activated ERK1/2 and JNK pathways and SCFA treatment inhibited IL-4-induced activation of the ERK1/2 pathway. These data indicate the effects of SCFA on ZO-1 may be mediated by inhibiting ERK1/2 signaling pathway.

In addition, the protective effects of SCFA on bronchial epithelial barrier function may further be mediated via Trek1. Recent studies showed that Trek1 is involved in the regulation of the barrier function of the intestinal epithelium and expression of Trek1 is reduced by stimulation with pro-inflammatory cytokines such as IL-4 and IL-13 [14]. In our studies, inhibition of Trek1 by spadin also showed the HDAC regulated functional role of Trek1 in maintaining airway epithelial barrier integrity. Previous studies in intestinal and nasal epithelium demonstrated that Trek1 expression is downregulated by increased HDAC1 expression and activated MAPK pathways [14, 15]. The protective effects of the SCFA on barrier function of the bronchial epithelium may therefore be partly related to their inhibitory property on HDACs, but other mechanisms might be involved. We found SCFA also inhibited MAPK (ERK1/2) signaling pathway which might also be involved in regulating Trek1. However, further investigation is required to confirm the roles of MAPK in the regulation of Trek1 leading to an increased expression of Trek1.

5. Conclusions

A compromised epithelium is one of the symptoms of many immunological respiratory diseases, including asthma, and is often responsible for an increased sensitivity to inhaled substances and exacerbations of these airway disorders [10, 24]. SCFA increase the barrier function of developing bronchial epithelial cells. Moreover, SCFA contribute to preservation and recovery of barrier properties during the progression of barrier impairment which may be mediated by increasing the expression of ZO-1 and Trek1. The ability of SCFA to induce recovery makes it possible to treat both apical and basolateral-induced barrier damage. Therefore, SCFA, especially butyrate and propionate, may open a new avenue for the treatment of respiratory diseases with an epithelial component like T2-high asthma or COPD.

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Chapter 8

Summary and discussion

With the changes in lifestyle, diet, hygiene, microbiome, industrial technologies and infections, the morbidity of non-communicable diseases (NCDs), such as cardiovascular diseases and chronic respiratory diseases, has significantly increased, posing a great threat to health worldwide. Decreasing the incidence of NCDs associated mortality is a goal of the WHO global health task force, which aims at a 25% reduction before 2025. A better understanding of the underlying mechanisms contributing to the increase in NCDs will be beneficial for their prevention and treatment. This includes understanding the roles of endothelial and airway epithelial cells, which are very important in maintaining the homeostasis in cardiovascular and respiratory systems. Therefore, the main aim of this thesis was to better understand the role of short-chain fatty acids (SCFA) in stimuli-induced endothelial activation and abnormal lung epithelial function, as well as the mechanisms involved.

Cardiovascular system

Chapter 2 reviews what is known about SCFA including their synthesis, transportation, metabolism and excretion, and shows that some human cardiovascular disorders are caused by a loss of endothelial functions or by accumulation of triglycerides in the heart muscle due to imbalances in the metabolism of lipids and glucose [1, 2]. SCFA are able to restore the imbalances in lipid and glucose metabolism, and thus can contribute to the prevention and treatment of cardiovascular diseases [3-8]. However, for the development of therapies with SCFA in cardiovascular disorders, some aspects are still insufficiently mapped, making it unclear whether all preconditions for correct treatment can be met.

In chapter 3, we present an overview of the effects of SCFA on the cardiovascular system and summarize the effects of SCFA on LPS- or TNFα-induced immune and endothelial cell activation. The effects of SCFA on immune cell function and the mechanisms involved are quite well documented. In contrast, knowledge of the effect of SCFA on endothelial activation, an important step in the initiation and development of

atherosclerosis, is much more limited. Further, in the few studies that have been published, the effects of SCFA on adhesion molecules including ICAM-1 and VCAM-1 are inconsistent. For example, butyrate and propionate were able to inhibit the expression of both ICAM-1 and VCAM-1 [9, 10], only inhibited VCAM-1 expression [11] or augmented ICAM-1 expression [12]. Furthermore, there is no relevant information available regarding the effects of SCFA on the production of pro-inflammatory cytokines and the underlying mechanisms. This supports the main direction of our investigation in this thesis.

Chapter 4 focused on the effects of SCFA, mainly acetate, butyrate and propionate, on LPS or TNFα-induced HUVEC activation. The effects of different concentrations of each SCFA, different pre-incubation times of SCFA and different stimulation times of LPS or TNFα were interrogated for their effects on the modulation of pro-inflammatory cytokine (IL-6 and IL-8) production and adhesion molecule expression (ICAM-1 and VCAM-1), which are two important characteristics in endothelial activation. SCFA showed both pro-and anti-inflammatory properties in endothelial activation, depending on SCFA concentration, SCFA pre-incubation period and LPS or TNFα stimulation period. We selected conditions from these experiments to further investigate the mechanisms behind the anti-inflammatory effects of SCFA (**Figure 1**).

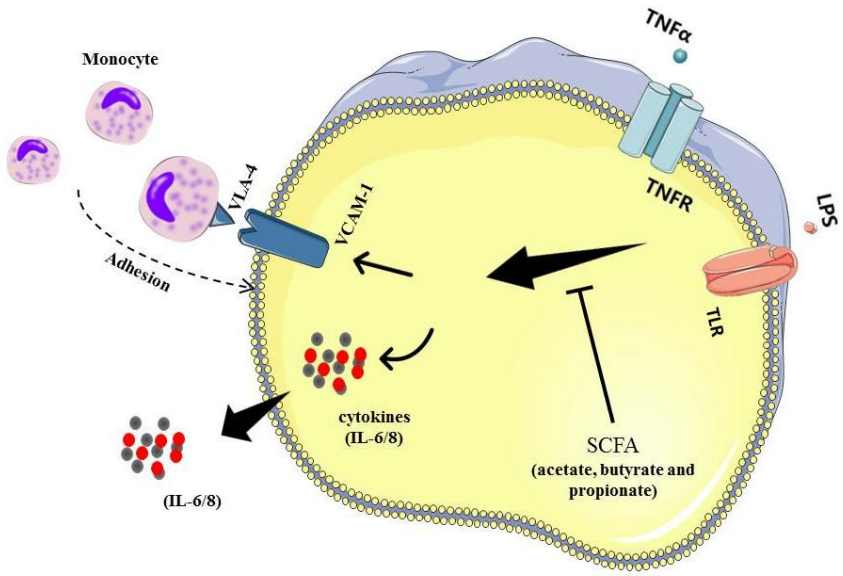

Figure 1. The effect of SCFA on LPS or TNFα-induced endothelial activation. LPS- or TNFα-induced endothelial activation was characterized by increased pro-inflammatory cytokines (IL-6 and IL-8) production, adhesion molecules (ICAM-1 and VCAM-1) expression and immune cells adhesion. Pre-treatment with SCFA, mainly acetate, propionate and butyrate, suppressed endothelial activation.

Possible explanations for these results are that: (1) SCFA, especially butyrate and propionate, are HDAC inhibitors inhibiting class I and II HDACs [13, 14]. Individual HDAC enzymes could have pro- and/or anti-inflammatory functions [15]. Alternatively, (2) the effects of SCFA may be mediated by activation of GPR41/43. Each SCFA has a different potency on the activation of GPR41 and GPR43 [16], which might explain the different effects of SCFA on cytokine production. For therapeutic purposes and restricted time-lines, we mainly focused on the anti-inflammatory properties of SCFA in LPS- or TNFα-induced endothelial activation.

In chapter 5, we chose the optimal inhibitory condition of the SCFA based on the results of chapter 4 and investigated the roles of both GPR41/43 and HDACs by using specific antagonists and an enzyme inhibitor. We

demonstrated that the effects of acetate were largely mediated by activation of GPR41/43, while the effects of butyrate or propionate were mediated by both activation of GPR41/43 and inhibition of HDACs (**Figure 2**).

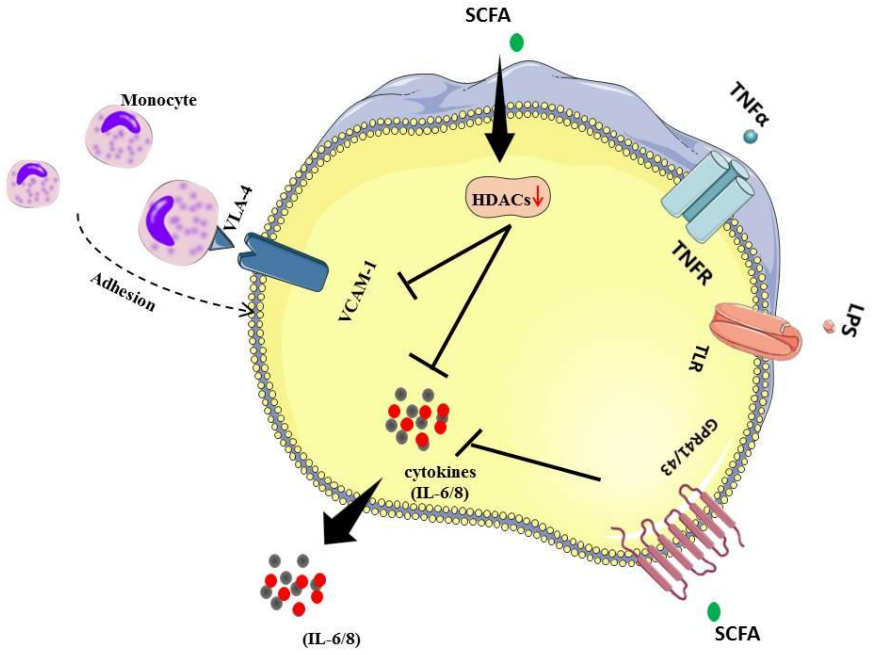

Figure 2. Activation of GPR41/43 and inhibition of HDACs involved in the effects of SCFA on endothelial activation. The effect of acetate on cytokines production was largely mediated by activation of GPR41/43, while the effects of butyrate or propionate on cytokines production, adhesion molecules expression and immune cell adhesion were mediated by both activation of GPR41/43 and inhibition of HDACs.

Previous studies have demonstrated that GPR41/43 activated by different SCFA will trigger different downstream signaling cascades, including $G_{\alpha i}$, $G_{\alpha q}$ and β-arrestins-2, and then regulate intracellular signaling pathways (e.g. NF-κB) and gene expression [17, 18]. The downstream cascades of inhibition of HDACs were unknown. It has been shown previously that inhibition of HDACs was shown to regulate inflammatory responses by modulating NF-κB and MAPK signaling pathways in non-endothelial cells. Using the data collected from chapter 5, we also concluded that inhibition of HDACs by butyrate or propionate was associated with their inhibitory effects on two important

factors in the initiation of immune cell adhesion to endothelium: IL-8 production and VCAM-1 expression. However, the links between inhibition of HDACs and NF-κB or MAPK signaling pathways in endothelial cells are still unknown. Studies have also demonstrated that HDACs, especially HDAC3, regulates IL-33 expression in monocytes[19, 20] and epithelial cells [21].

Furthermore, intracellular IL-33 can modulate inflammatory responses by regulating NF-κB [22].

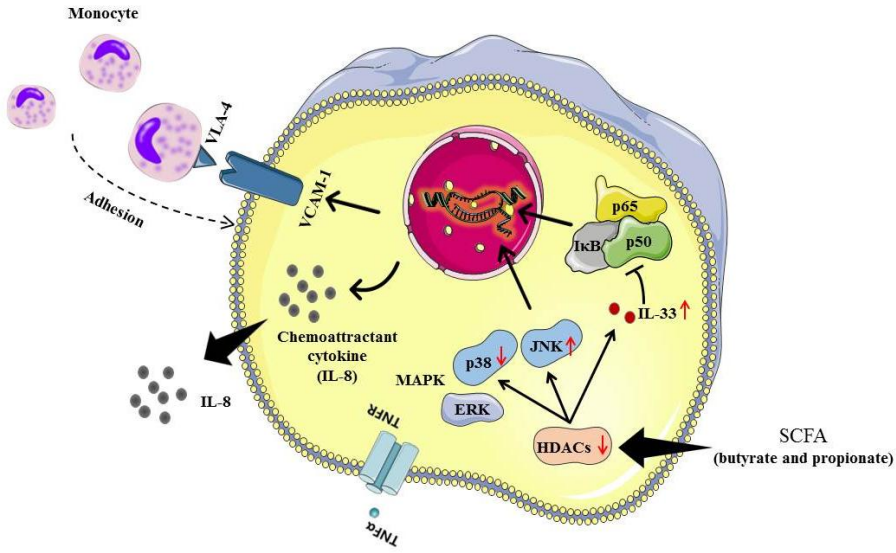

Figure 3. HDAC/IL-33/NF-κB and HDAC/MAPK signaling cascades involved in the effects of SCFA on endothelial activation. Propionate and butyrate affected HDAC activity, and then regulated both the IL-33/NF-κB signaling pathway associated with downregulation of IL-8 production and the MAPK signaling pathway (especially JNK and p38MAPK) involved in the suppression of VCAM-1 expression.

Given this, **in chapter 6**, we investigated whether endogenous IL-33 could link HDACs and NF-κB or MAPK signaling pathways and whether IL-33 was involved in the beneficial effects of butyrate and propionate on TNFα-induced endothelial activation. In these experiments, the effects of butyrate and propionate on TNFα-induced IL-8 production and VCAM-1 expression in normal cells were compared with IL-33 siRNA transfected HUVEC.

Interestingly, the effects of butyrate and propionate on TNFα-induced IL-8 production were mediated by regulating the HDAC/IL-33/NF-κB signaling cascade, while their effects on VCAM-1 expression were mediated by the HDAC/MAPK signaling pathway and were IL-33 independent (**Figure 3**). The findings described in chapters four through six provide a better understanding of the anti-inflammatory effects of SCFA on endothelial activation and show the potential of SCFA to suppress the development of inflammatory disorders, including atherosclerosis.

Respiratory system

The airway epithelium is an important mucosal barrier involved in the protection of the host against inhaled pathogens, allergens and other noxious substances. Airway epithelial barrier dysfunction is increasingly associated with allergic inflammation and asthma [23-26], moreover, study indicates SCFA can enhance and restore the barrier function of intestinal epithelium. However, its role in lung epithelium is unknown, even though many data indicate that SCFA can reach all organ systems via the circulation. Therefore, **in chapter 7**, we investigated whether SCFA are able to enhance the barrier function of bronchial epithelium by regulating tight junction protein expression (ZO-1) and leaky potassium channels (Trek1). In addition, we investigated whether the effects of SCFA (on IL-4-induced barrier dysfunction and decreased expression of ZO-1) were mediated by modulation of MAPK signaling pathways. We demonstrated that SCFA enhanced the barrier function development of normal airway epithelium in a concentration-dependent manner and recovered the diminished barrier function induced by the exposure of epithelial cells to IL-4, IL-13, house dust mite (HDM) and spadin. Furthermore, IL-4 decreased ZO-1 expression which is consistent with the fact that the epithelial barrier was also reduced. The decreased ZO-1 expression was restored by SCFA treatment. The effects of SCFA on epithelial barrier function and expression of tight junction protein might be associated with regulation of MAPK signaling pathways, especially ERK1/2. Besides, Trek1 was also involved in regulating lung epithelium barrier

function and this might be an additional pathway by which SCFA exert their effects. However, further investigation is required to confirm the relationship between Trek1 and HDAC and/or MAPK signaling pathways (**Figure 4**). These findings may open a new avenue for the treatment of respiratory diseases with an epithelial component, like Th2-high asthma or COPD.

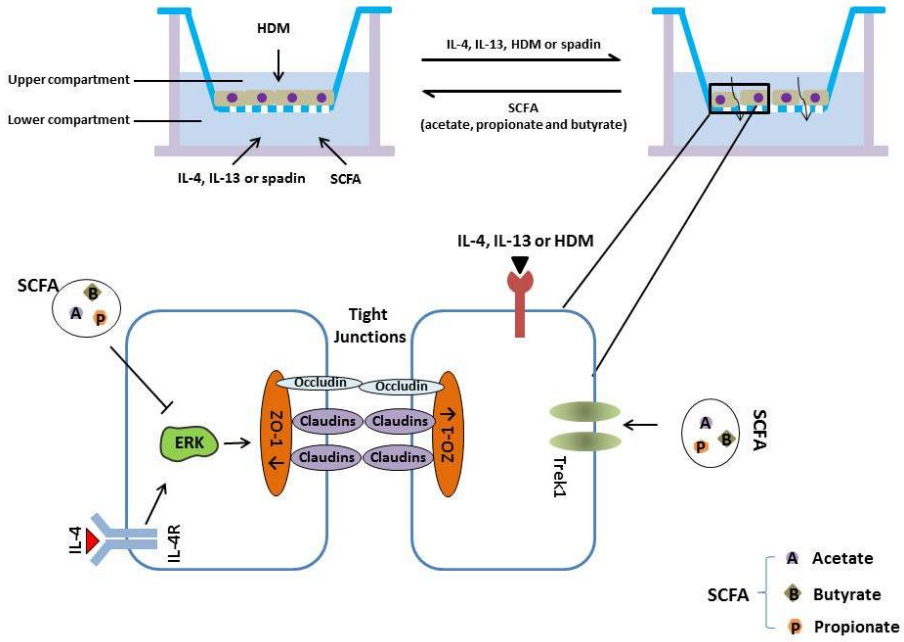

Figure 4. Schematic overview of the restorative effects of SCFA on lung epithelial barrier function. Damage to the confluent 16HBE was induced by IL-4, IL-13, and spadin (basolateral) or HDM extract (apical) and SCFA restored stimuli-induced airway epithelial barrier dysfunction. The restorative effects of SCFA on damaged epithelial barrier might be mediated by two signaling pathways: one way is associated with regulating Trek1; another way is by increasing the expression of tight junction protein (ZO-1) via inhibition of MAPK (ERK1/2) signaling pathways.

In conclusion, the findings in this thesis strongly highlight the roles SCFA plays in the prevention and treatment of inflammatory diseases in the cardiovascular and respiratory systems. Furthermore, we partly clarified the mechanisms involved in the effects of SCFA on endothelial activation and found their potential roles in developing and restoring the epithelial barrier. Since SCFA can be produced by the fermentation of dietary fibres, one

method to suppress the incidence of NCDs might be a balanced microbiome and consuming more dietary fibres. Moreover, the results of this thesis may open a new avenue for both protective as well as therapeutic pathways in cardiovascular and respiratory diseases and beyond.

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Nederlandse samenvatting

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De belangrijkste vormen van niet-overdraagbare ziekten (*non-communicable diseases, NCDs*) bij de mens zijn hart- en vaatziekten, chronische aandoeningen van de luchtwegen (chronische obstructieve longziekte [COPD] en astma), kanker en diabetes. Volgens de Wereldgezondheidsorganisatie (WHO) vormt de toenemende last van deze niet-overdraagbare ziekten momenteel de grootste bedreiging voor de wereldwijde gezondheid in zowel ontwikkelde als ontwikkelingslanden. De morbiditeit ten gevolge van niet-overdraagbare aandoeningen is in de laatste decennia aanzienlijk toegenomen door veranderingen in levensstijl, voeding, hygiëne, microbioom en infecties. Een beter begrip van de onderliggende mechanismen die bijdragen aan de toename van niet-overdraagbare ziekten is belangrijk voor preventie en een verbeterde behandeling. Het is duidelijk dat chronische en systemische ontstekingsprocessen een wezenlijke rol spelen bij het ontstaan en in stand houden van de verschillende niet-overdraagbare ziekten.

De geschatte jaarlijkse sterfte als gevolg van hart- en vaatziekten zal tegen het jaar 2030 wereldwijd de 25 miljoen benaderen. Eén mogelijke oorzaak van hart- en vaatziekten is een lokale infectie leidend tot verhoogde systemische ontsteking en verminderd functioneren van de endotheellaag in de bloedvaten. Endotheelcellen zijn betrokken bij het handhaven van homeostase en kunnen worden geactiveerd door endogene cytokinen (zoals TNFα) of bacteriële componenten (zoals endotoxinen, lipopolysacchariden [LPS]). Activering van endotheelcellen is een centraal onderdeel van het vasculaire ontstekingsproces bij de initiatie en ontwikkeling van atherosclerose. Hierbij spelen de verhoogde expressie van adhesiemoleculen (zoals ICAM-1 en VCAM-1) en een toename van de productie van pro-inflammatoire cytokinen (zoals IL-6) en chemokinen (zoals IL-8), leidend tot een verhoogde adhesie van immuuncellen aan de endotheellaag, belangrijke rollen.

Chronische aandoeningen van de luchtwegen, met name COPD, zijn ongeneeslijk en behandeling houdt alleen de symptomen onder controle. Het luchtwegepitheel is nauw betrokken bij de bescherming van de gastheer tegen ingeademde pathogenen, allergenen en andere schadelijke stoffen. Het luchtwegepitheel is echter meer dan alleen een structurele barrière. Epitheelcellen zijn ook een van de eerste cellen die reageren op geïnhaleerde antigenen en andere schadelijke stoffen door een verhoogde secretie van cytokines en daardoor het rekruteren van immuuncellen naar de luchtwegen.

Aangezien chronische ontsteking een veel voorkomend kenmerk is van alle niet-overdraagbare aandoeningen, kan het verminderen van het risico op ontstekingsreacties een goede benadering zijn met betrekking tot zowel preventie als behandeling. Modulatie van het microbioom is een veelbelovende optie om het immuunsysteem en daarmee ontstekingsprocessen te beïnvloeden. Deze modulatie kan plaatsvinden via verschillende routes waaronder toevoegingen aan het dieet van (1) probiotica, gedefinieerd als levensvatbare micro-organismen, in voldoende hoeveelheden aanwezig om de darm in een actieve toestand te bereiken en aldaar positieve gezondheidseffecten te induceren, (2) prebiotica, gedefinieerd als een selectief ingrediënt dat door het microbioom gefermenteerd kan worden die specifieke veranderingen, zowel in de samenstelling en/of activiteit in de gastro-intestinale microbioom veroorzaken die voordelen oplevert voor het welzijn en de gezondheid van de gastheer, (3) synbiotica, gedefinieerd als een synergetische combinatie van pro- en prebiotica en meer recentelijk, (4) postbiotica, de geproduceerde metabolieten of fragmenten afgegeven door het microbioom.

Vetzuren met een korte keten (*short-chain fatty acids,* SCFAs), voornamelijk acetaat, propionaat en butyraat, zijn fermentatieproducten van niet-verteerbare voedingsvezels geproduceerd door micro-organismen in de dikke darm en bezitten ontstekingsremmende en immuun-modulerende eigenschappen. Epidemiologische studies tonen aan dat een verhoogde consumptie van voedingsvezels de systemische ontsteking en het risico op

cardiovasculaire aandoeningen, immuunstoornissen en longziekten vermindert. Dit kan dus mogelijk verklaard worden door de productie van de SCFAs. Deze SCFAs kunnen hun werking op de cel uitoefenen via activatie van G-eiwit gekoppelde receptoren (*GPCRs*: GPR41 en GPR43, ook bekend als FFA3- en FFA2-receptoren) en/of via remming van histondeacetylases (HDACs).

De experimenten beschreven in dit proefschrift zijn uitgevoerd om meer kennis te verkrijgen over de effecten van SCFAs op het functioneren van luchtwegepitheel en van endotheelcellen van bloedvaten onder (chronische) ontstekingsomstandigheden.

Hoofdstuk 2 geeft een algemeen literatuuroverzicht van SCFAs, waaronder de synthese, transport, metabolisme en excretie en beschrijft de potentie van deze metabolieten in het voorkomen en behandelen van cardiovasculaire aandoeningen. SCFAs zijn in staat om de verstoringen in het lipide- en glucosemetabolisme te herstellen en kunnen daarmee bijdragen aan de preventie en behandeling van hart- en vaatziekten. **Hoofdstuk 3** richt zich op de rol van GPR41/43 en HDACs in de effecten van SCFAs op geactiveerde immuun- en endotheelcellen en de mogelijke mechanismen die hierbij zijn betrokken. De effecten van SCFAs op de werking van het immuunsysteem zijn redelijk goed gedocumenteerd. Daarentegen is de kennis over de effecten van SCFAs op activering van endotheelcellen, een belangrijke stap in de initiatie en ontwikkeling van atherosclerose, beperkt. Verder zijn de effecten van SCFAs op de expressie van de adhesiemoleculen ICAM-1 en VCAM-1 in de verschillende gepubliceerde studies inconsistent.

Activering van endotheelcellen kan geïnduceerd worden door TNFα of LPS en wordt gekenmerkt door een verhoogde productie van pro-inflammatoire cytokinen en verhoogde expressie van adhesiemoleculen. In **hoofdstuk 4** zijn drie factoren onderzocht die betrokken zijn bij de ontstekingsremmende eigenschappen van SCFAs (acetaat, propionaat en butyraat) op de endotheelcellen, namelijk (1) verschillende pre-incubatieperioden met SCFAs, (2) verschillende concentraties van SCFAs, en (3) verschillende

stimulatieperioden met ofwel TNFα of LPS. Uit de resultaten bleek dat SCFAs zowel pro-inflammatoire als ontstekingsremmende kenmerken induceerde bij de endotheelcellen en dat dit afhankelijk was van de gebruikte SCFA-concentratie, SCFA-pre-incubatieperiode en LPS- of TNFα-stimuleringsperiode. In **hoofdstuk 5** is onderzocht hoe GPR41/43 en HDACs betrokken zijn bij de door de SCFAs-geïnduceerde veranderingen in het functioneren van endotheelcellen. Daarbij zijn de meest optimale ontstekingsremmende condities van de SCFAs gebruikt op basis van de resultaten van hoofdstuk 4. Aangetoond is dat de effecten van acetaat grotendeels werden gemedieerd door activering van GPR41/43, terwijl de effecten van butyraat en propionaat werden veroorzaakt door zowel activering van GPR41/43 als remming van HDACs.

IL-33, een nieuw lid van de IL-1-familie van cytokines, wordt constitutief tot expressie gebracht in de kern van endotheelcellen en wordt gemoduleerd door HDACs. Nucleair IL-33 beïnvloedt ontstekingsreacties door de activering van NF-κB te reguleren. De rol van dit endogene IL-33 in de effecten van SCFAs is echter nog steeds onduidelijk. Daarom werd in **hoofdstuk 6** de rol van IL-33 in de effecten van butyraat en propionaat op door TNFα-geïnduceerde activering van endotheelcellen nader onderzocht. Het bleek dat de effecten van butyraat en propionaat op TNFα-geïnduceerde IL-8-productie werden gemedieerd door het reguleren van de HDAC / IL-33 / NF-κB-signaaltransductieroute, terwijl hun effecten op VCAM-1-expressie werden gemedieerd door de HDAC / MAPK-signaaltransductieroute.

De bevindingen beschreven in hoofdstuk vier tot en met zes geven een beter inzicht in de ontstekingsremmende effecten van SCFAs op de activering van endotheelcellen en tonen de potentie van SCFAs om de ontwikkeling van inflammatoire aandoeningen van het cardiovasculair systeem, waaronder atherosclerose, te onderdrukken.

Het luchtwegepitheel is een belangrijke mucosale barrière die betrokken is bij de bescherming van de gastheer tegen ingeademde pathogenen, allergenen en andere schadelijke stoffen. Het disfunctioneren van de

epitheelbarrière wordt steeds meer geassocieerd met allergische ontsteking en astma. Ander onderzoek wijst erop dat SCFAs de barrièrefunctie van epitheellaag in de darmen kan versterken en herstellen. De effecten van SCFAs op het longepitheel zijn echter onbekend, hoewel veel gegevens erop wijzen dat SCFAs via de bloedsomloop alle orgaansystemen kunnen bereiken. Daarom is in **hoofdstuk 7** onderzocht of SCFAs in staat zijn om de barrièrefunctie van bronchiaal luchtwegepitheel te moduleren via de zonula occludens (ZO-1; *tight junctions*) en kaliumkanalen (Trek1). Daarnaast is onderzocht of de effecten van de *SCFAs* op IL-4-geïnduceerde barrièredysfunctie en verminderde expressie van ZO-1 werden gemedieerd door MAPK-signaaltransductieroutes. Er kon worden aangetoond dat SFCAs de ontwikkeling van de barrièrefunctie van normaal luchtwegepitheel verhoogden en de verminderde barrièrefunctie geïnduceerd door blootstelling aan IL-4, IL-13 en huisstofmijt, herstelden. Deze effecten van de SCFAs waren geassocieerd met de regulatie van MAPK-signaaltransductieroutes. Bovendien was Trek1 ook betrokken bij het reguleren van de barrièrefunctie van het longepitheel en dit zou een extra route kunnen zijn waardoor SCFAs hun effecten uitoefenen. Er is echter nader onderzoek nodig om de relatie tussen Trek1 en HDACs en de MAPK-signaleringsroutes te bevestigen.

Hoofdstuk 8 vat de belangrijkste bevindingen beschreven in dit proefschrift samen en stelt een toekomstige onderzoeksrichting voor die nodig is voor een verder begrip van de effecten van de SCFAs op ontstekingsprocessen in het cardiovasculaire systeem en de barrièrefunctie van het epitheel in het ademhalingssysteem. Omdat de SCFAs worden geproduceerd door fermentatie van voedingsvezels, is het wellicht mogelijk om de incidentie van niet-overdraagbare ziekten te verminderen door veranderingen in het voedingspatroon. Door meer voedingsvezels te consumeren kan een gebalanceerd microbioom worden verwezenlijkt en zou dit tevens beschermende en therapeutische effecten kunnen hebben op cardiovasculaire aandoeningen en ziekten van de luchtwegen.

中文总结

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随着人类生活方式、饮食起居、环境卫生、工业进程和微生物感染的改变，非传染性疾病（non-communicable diseases, NCDs）包括心血管疾病和慢性呼吸道疾病的发病率一直在增加，这也在世界范围内对人类健康造成了巨大的威胁。世界卫生组织（world health organization, WHO）因此提出了全球卫生工作组的目标：在 2025 年之前，将与非传染性疾病相关的死亡率降低 25%。因此，全面理解非传染性疾病的发病机制将会有利于预防和治疗非传染性疾病。这其中具有正常功能的血管内皮细胞和呼吸道上皮细胞对维持心血管和呼吸系统的稳态是非常重要的。因此，该论文的主要目的就是研究，在刺激因素诱导中，短链脂肪酸（short chain fatty acids, SCFA）对血管内皮细胞和呼吸道上皮细胞功能异常的保护作用及机制。

心血管系统

在**第二章**中，我们叙述了一些关于短链脂肪酸（主要是乙酸盐，丙酸盐和丁酸盐）的基本信息，包括其合成，运输，代谢和排泄。同时指出心血管功能异常的主要原因可能是由于血管内皮细胞功能的丧失，或是由于脂质和糖代谢失衡引起的心肌内甘油三酯的累积。短链脂肪酸能通过修复脂质和葡萄糖代谢平衡来预防和治疗心血管疾病。但是，对于用短链脂肪酸治疗心血管疾病的研究仍不是很充分，因此还需要更进一步的研究。

在**第三章**中，我们概述了短链脂肪酸在心血管系统中的作用，重点描述了其对 LPS 或 TNFα 诱导的免疫及血管内皮细胞功能活化的影响和潜在机制。我们得出的结论是：短链脂肪酸对刺激诱导的免疫细胞功能障碍的作用及机制是较清楚的，但是有关短链脂肪酸在该功能障碍中的作用及机制研究是相当少的，而血管内皮功能障碍是引起动脉粥样硬化的重要原因。而且在有限的研究中，短链脂肪酸的作用也是不一致的。例如，在短链脂肪酸对粘附分子（包括 ICAM-1 和 VCAM-1）的作用中，有的研究表明丁酸盐（butyrate）和丙酸盐（propionate）可以同时抑制 ICAM-1 和 VCAM-1 的表达，有的研究表明其只能抑制 VCAM-1

的表达，亦或是增加 ICAM-1 的表达。此外，文献中也没有关于短链脂肪酸对刺激诱导的内皮细胞分泌的促炎细胞因子的调节作用及机制的研究。因此，这些问题将成为本论文的主要研究方向。

在**第四章**中，我们重点研究短链脂肪酸（主要是乙酸盐 acetate，丙酸盐 propionate 和丁酸盐 butyrate）对 LPS 或 TNFα 诱导的原代人脐静脉内皮细胞（human umbilical vein endothelial cells, HUVEC）活化的影响。在本章节中，我们研究了短链脂肪酸调节内皮细胞活化的浓度依赖性和时间依赖性，包括其对炎症因子（IL-6 和 IL-8）产生和粘附分子（ICAM-1 和 VCAM-1）表达的调节作用。我们发现，短链脂肪酸表现出促炎和抑炎两种特性，其对炎症因子的调节取决于短链脂肪酸的浓度，预孵育时间和刺激时间长短。此外，我们发现丙酸盐和丁酸盐能够抑制 VCAM-1 的表达及其介导的外周单核细胞的粘附，而对刺激诱导的 ICAM-1 表达无作用。另外乙酸盐对刺激诱导的粘附分子 ICAM-1 和 VCAM-1 都没有作用。我们通过选择短链脂肪酸抑制炎症的实验条件来为下一步做铺垫。但是，此时我们还不清楚其表现促炎和抑炎两种特性的原因以及抑炎的机制。

根据文献报道，对于出现这样结果可能的解释：第一，短链脂肪酸尤其是丁酸盐和丙酸盐能广泛地抑制组蛋白脱乙酰酶（histone deacetylases, HDACs）的活性，尤其是抑制 I 类和 II 类 HDACs。而每一类单独的 HDAC 都拥有促炎和抑炎两种活性。第二，短链脂肪酸可能是通过激活 G 蛋白偶联受体 41 和 43（G-coupled protein receptor 41/43, GPR41/43）来发挥作用。而乙酸盐，丙酸盐和丁酸盐对 GPR41/43 的激活具有不同的效力。出于治疗的目的和时间的限制，我们主要关注短链脂肪酸的抑炎作用。因此，在**第五章**中，我们使用 GPR41/43 的拮抗剂和 HDAC 的抑制剂来研究 GPR41/43 和 HDAC 在短链脂肪酸抑炎作用中的扮演的角色。我们发现，乙酸盐是通过激活 GPR41/43 发挥作用，而丙酸盐和丁酸盐是通过激活 GPR41/43 和抑制 HDAC 活性发挥作用。

先前的研究表明，不同的短链脂肪酸激活 GPR41/43 后将引发不同的下游信号级联反应，包括 Gαi，Gαq 和 β-arrestins-2 的激活，然后调节胞

内信号通路（例如 NF-κB）和基因的表达。但是，短链脂肪酸抑制 HDAC 后的下游通路还未知。而且，从我们第五章的研究结果也表明，短链脂肪酸抑制 IL-8 生成和 VCAM-1 表达与其 HDAC 活性抑制有关。研究表明，在非内皮细胞中，抑制 HDAC 活性能通过调节 NF-κB 和 MAPK 信号通路来调节炎症反应。然而，在血管内皮细胞中，短链脂肪酸通过抑制 HDAC 产生的抗炎活性是否与调节 NF-κB 和 MAPK 信号通路有关仍然未知。此外，研究表明，在非血管内皮细胞中，HDAC 可以调节 IL-33 的表达，而 IL-33 也通过调节 NF-κB 从而参与调节炎症反应。因此，我们在**第六章**中研究：内源性 IL-33 是否是连接 HDAC 和 NF-κB 或 MAPK 信号通路的媒介，以及 IL-33 是否参与介导短链脂肪酸在内皮细胞内的抗炎作用。我们在正常的以及 IL-33 转染的内皮细胞内，将短链脂肪酸对 TNFα 诱导的 IL-8 生成和 VCAM-1 表达的调节作用进行对比。实验结果表明：丙酸盐和丁酸盐对 IL-8 的调节作用是通过调节 HDAC/IL-33/NF-κB 信号通路实现的，而其对 VCAM-1 表达的调节作用是通过 HDAC/MAPK 信号通路实现的，且是 IL-33 非依赖性的。从第四章到第六章，我们一步一步的深入研究了短链脂肪酸在 LPS 或 TNFα 尤其是 TNFα 诱导的内皮细胞功能障碍中的作用以及机制。这些研究结果使我们对短链脂肪酸的抗炎作用有了更好的理解，也展示了其预防与治疗包括动脉粥样硬化在内的炎症疾病的潜力。

呼吸系统

呼吸道上皮细胞屏障是保护宿主免受吸入性病原体，过敏原和其他有毒物质的重要粘膜屏障。而呼吸道上皮细胞屏障功能障碍与过敏性炎症和哮喘有关，然而，引起呼吸道上皮细胞屏障功能障碍的机制仍不甚清楚。因此，在第七章中，我们研究短链脂肪酸是否能改善不同刺激诱导的人支气管上皮细胞功能损伤，以及其作用是否是通过调节紧密连接蛋白（ZO-1）和钾离子通道 Trek1 来达到的。此外，我们还研究了，短链脂肪酸对 IL-4 诱导的上皮细胞屏障功能损伤及 ZO-1 表达降低的抑制作用是否通过调节 MAPK 信号通路来达到的。我们发现：第一，短链脂肪酸浓度依赖性地促进支气管上皮细胞屏障功能的形成，而且短链脂肪酸也能修复由于 IL-4，IL-13，HDM 或 spadin 刺激导致的屏障

功能损伤;第二，IL-4 刺激能降低紧密连接蛋白 ZO-1 表达，这与其损伤上皮细胞屏障功能相一致，而短链脂肪酸能有效的上调 ZO-1 的表达。第三，短链脂肪酸对上皮细胞紧密连接蛋白表达的作用可能与其调节 MAPK 信号通路尤其是 ERK1/2 信号通路有关。此外，Trek1 也参与调节上皮细胞屏障功能，而且这可能是短链脂肪酸实施其作用的另外一条通路。但是，还需要进一步的研究去证明 Trek1 和 HDAC 或 MAPK 之间的联系。这些结果将会开启一条用于治疗呼吸系统疾病如过敏性哮喘或 COPD 的新的道路。

总结：

本文的研究结果突出了短链脂肪酸在预防和治疗心血管和呼吸系统炎性疾病中的作用及其机制。这些研究表明短链脂肪酸（膳食纤维的代谢产物）有益于 NCDs 的治疗和预防，这可能会引导人们维持一个更健康的饮食习惯，也为研究者们提供一个治疗心血管和呼吸系统疾病的新靶点。

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List of publications

List of publications

Levi B. Richards, **Meng Li**, Betty C.A.M van Esch, Johan Garssen, Gert Folkerts. The effects of short-chain fatty acids on the cardiovascular system. PharmaNutrition. 2016;4:68-111.

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Meng Li, Levi B. Richards, Gert Folkerts, Johan, Betty C.A.M van Esch. The protective and restorative effects of short chain fatty acids on the barrier function of human bronchial airway epithelium via regulation MAPK signaling pathways. (submitted for publication)

Curriculum vitae

The author of this thesis, Meng Li, was born on 5th Oct. 1988 in Bengbu, Anhui, China. In June 2011, she got her bachelor degree from GuangDong Medical University and majors in pharmacy. In September of the same year, she started her further study as a master student in pharmacology in China Pharmaceutical University. She was supervised by prof. Dezai Dai and prof. Feng Yu with research project on cardiovascular diseases. In 2014, she was offered a position as a PhD student in the department of Pharmacology in Utrecht University and funded by China Scholarship Council. During her last four years, she was supervised by prof. dr. Johan Garssen, prof. dr. Gert Folkerts, dr. Betty C.A.M. can Esch and dr. P.A.J. (Paul) Henricks. Her research aimed to expand the current knowledge regarding to the anti-inflammatory effects of short chain fatty acids on human endothelial and lung epithelial cells. She was trained in the Drug Innovation PhD program of the Graduate School of Life Sciences, Utrecht University.