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„Impact of cinnamon aroma compounds on mechanisms regulating satiety and lipid metabolism“

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1. Introduction

1.1. Overweight and obesity: a worldwide challenge

Overweight and obesity as the consequence of an imbalanced energy intake and expenditure is recognized as a major socioeconomic health challenge at present. As published by the World Health Organization (WHO), globally, obesity has nearly tripled since 1975[1, 2] and its prevalence constitutes a considerable challenge for public health systems worldwide, increasing the risk for non-communicable diseases such as cardiovascular disorders, diabetes mellitus type II or specific cancers[1, 3-9]. Additionally, concern has been expressed regarding a potentially subsequently increased susceptibility to obesity in offspring following maternal obesity and suboptimal dietary conditions[10-14]. In the management of obesity prevention and its treatment, lifestyle changes, such as restricting the caloric intake and increasing physical activity, are considered as key effective strategies. However, commitment to behavioral modifications is often low and accompanied by rising dependence on synthetic drugs, which are commonly known for adverse side effects[15-19]. Hence, there is great interest in discovering novel and safer anti-obesity agents, specifically focusing on naturally occurring plant-derived substances. As possible targets for weight reduction, for example, (i) decreasing energy intake through enhanced satiation and satiety, (ii) modulating the intestinal nutrient uptake or (iii) regulating endogenous lipid reservoirs via inhibition of adipogenesis, have been suggested[20-25].

1.2. Regulation of hunger and satiety

Regulation of food intake and maintenance of the body's homeostatic energy balance is controlled by the intricate mechanistic network of the hunger/satiety system. Here, the gut-brain axis plays an essential role, allowing nutritional, mechanical or hormonal signals from the gut as well as signals of palatability and social and environmental stimuli to be integrated in the brain[26-29]. Whereas homeostatic signaling promotes food intake in response to depleted energy stores, hedonic influences can result in food consumption devoid of the need for calories[30].

Satiation, which is defined as the process that terminates the food intake, and satiety, which describes the feeling of fullness, that continues after an eating episode and potentially inhibits its resumption until the hunger returns, are essential parts of the body's complex appetite control system that regulates energy intake[31, 32]. In general, satiation and satiety are modulated by short-term episodic signals, which are produced by episodes of eating, and by long-term tonic signals, which depend on the body's energy and nutrient status[33-36]. The gastrointestinal tract generates post-ingestive stimuli in the form of gastric distension and the release of hormones, which are then integrated in the brain to induce satiation[37, 38]. Moreover, apart from gastrointestinal hormonal episodic signals, satiety is also influenced by fluctuating levels of hormones like insulin

or adipostatic leptin, providing information about fat reservoirs in the body, thereby mediating tonic satiety signaling[32, 39-43].

1.2.1. Central control of food intake

In the central regulation of the hunger/satiety system, the hypothalamus, the brainstem and the cortico-limbic brain areas are considered key structures[44-47]. In response to peripheral stimuli acting in the brain via afferent nerve endings or through receptors directly, corresponding hypothalamic neurons synthesize a variety of neuropeptides and/ or neurotransmitters involved in the promotion or suppression of food consumption[36, 48]. These so-called anorexigenic and orexigenic pathways emerge from the hypothalamic arcuate nucleus (ARC), which mainly steers the energy intake and satiety[49]. There, two counter-operating populations of neurons are located: the proopiomelanocortin (POMC) and cocaine-and amphetamine-related transcript (CART) expressing neurons, which induce anorexigenic pathways, and the neuropeptide Y (NPY) and "Agouti-related" peptide (AgRP) expressing neurons, which exert appetite-stimulating effects [49-56]. These neurons have also been termed "first order" neurons, which subsequently project to "second order" neurons, localized inside and outside of hypothalamic regions[50]. The hypophagic impact of POMC/CART neurons is mediated by the secretion of the α- melanocyte stimulating hormone (α-MSH), which is generated from the precursor POMC, and a subsequent stimulation of melanocortin receptors 3 and 4 (MC3R and MC4R)[50, 57-63]. In addition, POMC in the ARC is co-localized with the CART peptide, a further inhibitor of food intake, whose specific receptor(s), however, to date have not been characterized[53, 64, 65]. POMC neurons have been linked to second-order neurons in the lateral hypothalamus (LH), the paraventricular hypothalamic nucleus (PVN), the dorsomedial hypothalamus (DMH) and the ventromedial hypothalamus (VMH)[66, 67]. After processing the input from first-order neurons, they project to various extrahypothalamic neural curcuits, thereby conveying the neuronal information into behavioral and metabolic responses that modulate energy intake and expenditure[68].

Neuropeptide Y (NPY)/AgRP neurons, representing the population of orexigenic neurons in the ARC, have also been shown to have vast projections within the hypothalamic region[68-71]. They express the neuropeptide NPY, which mediates its orexigenic pathways at least via the activation of Y1 and Y5 receptors[72, 73], and the AgPR, which, as a selective antagonist, binds to melanocortin receptor subtypes[74]. Moreover, NPY release has been demonstrated to inhibit POMC neurons via the stimulation of Y1 and possibly Y2 receptors expressed in the anorexigenic neurons[75, 76]. The lateral (LHA) regions of the hypothalamus comprise further neuronal populations, demonstrated to release the melanin concentrating hormone (MCH) and orexin[77, 78]. NPY/AgRP neurons as well the melanocortin system have been suggested to interact with the orexin- and MCH expressing neurons, which have also been indicated to play a role in promoting the food intake[79, 80].

Furthermore, NPY/AgRP as well as POMC/CART neurons express leptin receptors, presenting direct targets for leptin signaling[81-83]. On the one hand, POMC/CART neurons have been found to be activated by leptin, thus inhibiting food intake[82, 84, 85]. On the other hand, stimulation of leptin receptors also inhibits NPY/AgRP expressing neurons and as a consequence their orexigenic signaling[71, 86]. In contrast, ghrelin is likely to further feeding behavior via ghrelin receptor-mediated activation of NPY/AgRP neurons and was also shown to indirectly suppress POMC neurons[87-89]. Additionally, studies suggest, that the activity of POMC neurons is also partially modulated by insulin signaling as well and that glucose sensing by POMC neurons might be involved in regulation of the glucose homeostasis[90, 91].

1.2.2. Peripheral signals of satiety

Apart from its role in the digestive system, the gastrointestinal tract functions in the regulation of energy balance, especially in the short-term control of energy consumption. Several gastrointestinal peptides and hormones have been identified, that act in digestive processes as endocrines, which are released directly into the bloodstream, as paracrines or as neurocrines[92].

Cholecystokinine (CCK) constitutes the first anorexigenic hormone to be identified in the gut[93, 94]. It is primarily secreted by duodenal and jejunal I-cells into the intestinal lamina propria following nutritional cues in the gut, especially after fat and protein intake[95-97]. CCK mediates its satiating effect via activation of CCK receptors at afferent fibers of the vagus nerve, transferring and integrating the anorectic signals to the brain[98]. Of the two receptor subtypes that have been identified for CCK, the CCK-A receptor has been found to mediate satiating effects, whereas the CCK-B receptor, could not be linked to satiety [92, 99, 100].

Anorexigenic peptide tyrosine-tyrosine (PYY) is released into circulation from enteroendocrine L-cell as PYY1-36, which is subsequently metabolized to its active form, PYY3-36[101-103]. PYY3-36 exerts its satiating effects directly via binding to hypothalamic Y2 receptors of NPY/AGRP neurons, thereby inactivating them, as well as indirectly via vagal Y2 receptor activation[102, 104-107]. PYY levels, which are low under fasting conditions, increase proportional to the caloric load after food intake and maintain elevated for several hours[108]. Moreover, PYY has also been reported to inhibit gastric emptying[109].

Like CCK and PYY, glucagon-like peptide 1 (GLP-1) represents another satiating hormone which is also synthesized by intestinal L-cells[110-112]. After post-prandial secretion into the circulation, GLP-17-36 amide and GLP-17-37 as main bioactive forms bind to GLP-1 receptors, which have been identified in the gastrointestinal tract, pancreatic cells and hypothalamic brain regions[113-118]. GLP-1 is quickly inactivated by dipeptidyl peptidase-4, exhibiting a very short half-life of approximately 1.5 – 5 min in the plasma[119-121]. Additionally, GLP-1 is considered an incretin

hormone, which stimulates insulin secretion in a glucose-regulated manner, and is associated with decreased gastric emptying[122-124].

The “hunger hormone” ghrelin, which is mainly released from cells in the region of the gastric fundus, is the only known peripheral orexigenic hormone[125, 126]. Ghrelin is known as a growth hormone secretagogue and has been associated with increased gastric motility as well as with the inhibition of lipolysis and stimulation of fatty oxidation[127-129]. It has been demonstrated, that central and peripheral administration of ghrelin stimulated food intake and body weight gain and plasma levels of ghrelin increase during fasting[130-132]. Moreover, plasma ghrelin levels in humans have been shown to exhibit a circadian rhythm, fluctuating diurnally with preprandial increases, postprandial decreases throughout the day and maximum peaks at night[133]. Ghrelin conveys its appetite–stimulating effects via the growth hormone secretagogue receptor (GHSR-1a), which is expressed on vagal affarents and in the ARC[87, 125, 134].

Additional gut hormones, that have been linked to the control of food intake, include oxyntomodulin (OXM) or the pancreatic polypeptide (PP). OXM, which is secreted from intestinal L-cells, exerts moderate anorexigenic actions predominantly via the GLP-1 receptor[135, 136]. PP is released post-prandially from pancreatic PP cells and to a smaller extent also from intestinal endocrine cells and has been hypothesized to exert its anorexigenic effects by binding to the Y receptor family[137-142].

1.3. Serotonin

The monoamine neurotransmitter serotonin or 5-hydroxytryptamine (5-HT) plays a vital role in a multitude of physiological functions and behaviors, including sleep, mood, regulation of temperature, hormone release, cognition or the control of appetite and is synthesized in the central nervous system as well as in peripheral tissues. In the gastrointestinal tract, where more than 90 % of the endogenous serotonin production is believed to occur, the majority of serotonin is synthesized, stored and released by enterochromaffin cells (EC), constituting the most abundant enteroendocrine cell type, and only a smaller part by enteric neurons[142-160]. EC cells as nutrient and mechanical sensors respond to luminal stimuli by serotonin release[149, 161].

Synthesis and metabolization of serotonin comprises, firstly, the hydroxylation of its precursor amino acid L-tryptophan, which, in contrast to serotonin can pass the blood-brain-barrier. This rate limiting step is mediated by tryptophan hydroxylase 1 (TPH1), which is mainly expressed in peripheral tissues, or TPH2, which is believed to be localized almost exclusively in the brain, yielding 5-hydroxytryptophan (5-HTP)[143, 147, 159, 162, 163]. Secondly, 5-HTP is converted into serotonin via a decarboxylation step catalyzed by the aromatic amino acid decarboxylase (AAAD)[143, 147, 164]. Thirdly, after synthesis, serotonin is loaded into vesicles by means of the vesicular monoamine transporter (VMAT), where it is stored until secretion [143, 144, 147, 165]. As

shown and reviewed repeatedly, serotonin in the gut is secreted basally into the lamina propria of the mucosal layer, which is innervated by serotonin receptor expressing intrinsic and extrinsic afferent nerve fibers[166-168]. Thereby, multiple physiological and pathophysiological reactions are modulated, including gastrointestinal contractions or nausea[167, 169, 170]. More recently, serotonin releasing granula have also been detected in the apical membrane of intestinal cells, suggesting a release into the lumen as well [171, 172]. Finally, the biological activity of serotonin is terminated by its uptake through the serotonin reuptake transporter, which is widely expressed in gut, and its subsequent metabolization through mitochondrial membrane enzyme monoamine oxidase (MAO-A) and aldehyde dehydrogenase into 5-hydroxyindole acetic acid (5-HIAA)[143, 167, 173-175].

A large number of serotonin receptor subtypes have been detected in the serotonergic system, which are co-expressed in multiple cell types in the brain and peripheral tissues and reflect the complexity and wide range of regulatory actions that are associated with serotonin[143, 176]. To date, 14 serotonin receptors have been identified, which are grouped into seven families. Serotonin receptors 5-HT_1, 5-HT_2, 5-HT_4, 5-HT_5, 5-HT_6 and 5-HT_7 constitute G protein coupled receptors, whereas 5-HT_3 is the only ligand gated ion channel linked to serotonin[176].

1.3.1. Serotonin in the regulation of food intake and satiety

Among countless other functions, serotonin signaling also plays an important part in the control of satiety. Broadly speaking, elevated serotonin availability in the brain and activation of specific serotonin receptors reduce food intake, whereas serotonin depletion or lack of certain receptor subtypes induce food consumption[49, 177-182]. Over the last decades, several serotonin receptor subtypes, such as receptors 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2c}, 5-HT_6 and possibly also 5-HT_3, could be linked to modulatory functions in the consumption of food[154, 155, 178, 183-186]. Activation of receptor subtypes 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2c}, for instance, was reported to decrease food intake and enhance the onset of satiety[178, 187]. But also, inhibition of auto-receptor 5-HT_{1A} signaling as well as inactivation of 5-HT_6 receptor, in contrast, was found to reduce food intake[154, 188]. In the hypothalamus, serotonin has been demonstrated to stimulate the melanocortin pathways, mediating its anorexigenic effect possibly via activation of 5-HT_{2c} receptors, which are expressed by POMC/CART neurons[189-191]. Thus, an elevated secretion of anorexigenic α-MSH has been assumed[49].

Although more than 90 % of the body's serotonin is generated in the gastrointestinal tract, relatively little is known concerning the potential impact of peripheral serotonin in the regulation of food intake[173]. Even though serotonin is not able to pass the blood-brain-barrier, evidence from animal studies, demonstrating reduced meal sizes or an earlier onset of satiety after peripherally administered serotonin, suggests that peripheral serotonin is also somehow involved in the control of food intake[192-194]. As other peripheral satiety signals, serotonin has also been shown to stimulate

vagal afferent fibers innervating the gastrointestinal tract[195]. Specifically, the stimulation of serotonin release from EC cells in the stomach induced c-*fos* expression in specific brain regions such as the nucleus of the solitary tract or the PVN. Truncal vagotomy, in contrast, blocked c-*fos* expression, indicating the participation of vagal afferent pathways[196]. Moreover, activation of 5-HT_3 receptors, which are expressed in peripheral vagal afferent nerve endings, by serotonin released in response to luminal stimulation, might be involved in the signal transduction as well[196]. In humans, it was found, that inhibition of 5-HT_3 receptors by antagonist ondansetron significantly elevated the amount of a liquid meal at maximum satiation, further supporting the role of serotonin in the regulation of food intake[197]. Regarding its effect as satiety signal, also complex interactions between serotonin and other satiety factors have been implicated. It has been shown, for instance, that intestinal lipids and carbohydrates suppress food intake via simultaneous 5-HT_3 and CCK_1 receptor activation[198]. Synergistic effects of CCK and serotonin have also been demonstrated in the *nucleus paraventricularis*, pointing to a priming effect of serotonin in the hypothalamic satiation system before reacting to elevated levels of CCK[199]. A further satiety hormone, that has been associated with serotonin, is leptin. However, data is still inconsistent concerning their interaction in the control of food intake[200, 201].

1.4. Lipids

Dietary fat, providing an average of 9 kcal/g and therefore considered an important energy source in our food, is an essential part of a healthy nutrition. Stored as triglycerides in our adipose tissue, lipids serve as an important energy reservoir during times of a negative energy balance. Moreover, fat intake is crucial for supplying the body with essential fatty acids, which humans are not able to synthesize by themselves, and assist the uptake of fat soluble vitamins[202, 203]. In the Western diet, triglycerides account for up to 90-95 % of dietary lipids, comprising a large amount of saturated fatty acids, complemented by phospholipids and cholesteryl esters which make up for the remaining ~5-10 % [202, 204]. Nutritional recommendations to uphold a healthy and balanced diet encompass a daily fat intake of 20-35 % of the total caloric intake, which, in Western diets, however, is often exceeded by far[203].

1.4.1. Lipid digestion and absorption

Components of our food are merged together in the stomach with lingual and gastric lipases, which along with peristaltic emulsification assist the gastric phase of the lipid digestion. The gastric chyme, containing the lipid emulsion of small lipid droplets, is then transported into the duodenum, where bile and pancreatic enzymes are added, which are essential for facilitating the subsequent hydrolysis of lipids. After their breakdown in the intestinal lumen,

monoacylglycerides and free fatty acids are translocated through the apical membranes of the enterocytes, constituting the absorptive and predominant intestinal epithelial cell type[202, 204, 205].

On a cellular and molecular level, however, the absorption of free fatty acids into the enterocytes is not entirely understood yet. Diffusion as well as a protein-mediated transport of fatty acids have been suggested to exist side by side, the latter, though, is now believed to be the predominant intestinal uptake mechanism[206-209] (Figure 1). Short-chain fatty acids may travel through biological membranes by simple passive diffusion, but also long-chain fatty acids in their un-ionized form have been shown to diffuse across biological membranes via a "flip-flop" mechanism, where they flip between the membrane bilayers, while staying bound to the membrane[202, 206, 210]. In recent years, however, the identification of membrane associated fatty acid binding proteins, led to the understanding, that fatty acids are translocated through cell membranes mainly via a protein-mediated pathway. In fact, these fatty acid transport proteins have been suggested not only to enable, but also to assist in the regulation of the fatty acid absorption, demonstrable through their ability to cross between intracellular storage compartments and cell membranes[207, 211, 212]. Fatty acid transporters, that have been suggested to be involved in the intestines, comprise the cluster determinant 36 or - as it is also referred - fatty acid translocase (CD36/FAT)[213, 214], plasma membrane-associated fatty acid-binding proteins ($FABP_{pm}$)[215, 216] or the fatty acid transport proteins (FATPs)[208].

CD36, which is highly expressed on the apical brush border along the human digestive tract, possibly together with $FABP_{pm}$ has been suggested to act as an acceptor for luminal fatty acids and to actively transport them across the apical cell surface[214, 217-219]. It comprises a big extracellular and two transmembrane domains terminating in a short cytoplasmic tail, respectively[220, 221]. Fatty acids are believed to bind within a hydrophobic pocket found at the surface, where the carboxylic group of the fatty acid is positioned nearby lysine 164, and subsequently guided through a tunnel spanning the entire protein[220-222]. Located at the inner side of the membrane, cytoplasmic FABP ($FABP_c$), which is abundantly available in the enterocytic cytoplasm, presents a docking site for fatty acids, after which fatty acids are activated to acyl coenzyme A (CoA) esters, making them available for subsequent formation of complex lipids[202, 207, 223-226]. In the intestinal lipid absorption, however, the contribution of CD36 is thought to be marginal as the fatty acid uptake via fatty acid translocase is saturable and luminal free fatty acid concentrations exceed its capacity[227, 228].

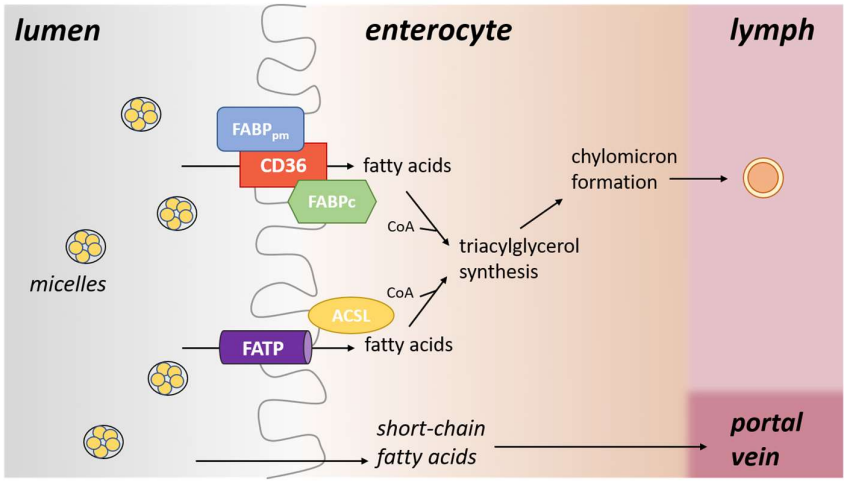

Figure 1. Regulation of intestinal fatty acid uptake (adapted from Wang et al. (2013)[202])

Members of the fatty acid transport proteins (FATPs), a family of transmembrane proteins consisting of six isoforms, are known to exert acyl-CoA synthetase activity and to have an extracellular fatty acid binding site as well as an ATP docking site, suggesting a role in both fatty acid transport as well as activation[229-231]. Current evidence indicates, that FATPs solely or together with isoforms of the specific long chain acyl CoA synthetase (Acsl) are needed for fatty acid uptake in a process referred to as vectorial acylation, a mechanism that is linked to esterification with coenzyme A[232-236]. In the small intestine, especially FATP2 and FATP4 have been found to be expressed[208, 237].

1.4.2. Inhibition of intestinal lipid absorption as a strategy for weight loss and maintenance

Apart from dietary restrictions, targeting the macronutrient absorption is regarded a potential strategy to achieve weight loss. Long-term intake of high-caloric foods such as high-fat diets are associated with the development of overweight and obesity as well as various co-morbidities[238-240]. Strategies to reduce excessive lipid absorption from the gastrointestinal tract include, for example, the application of low or non-caloric fat substitutes which can be incorporated in the daily diet[25]. Health benefits of fat substitutes, though, are still under debate due to various side effects such as flatulence, bowel cramps or loose stools as well as the frequent failure to achieve weight loss[241-243]. Apart from fat substitution, also focusing on lipid digestion per se has been considered as an effective approach in body weight reduction and maintenance in the therapy of obesity. Dietary intake of orlistat, a statin which belongs to the group of lipase inhibitors, has been shown to decrease body weight by >5 % in more than 50 % of treated subjects at a dose of 120 mg three times daily by inhibiting fat absorption by approximately 30 %[17, 244, 245]. In 2007,

the FDA has been approved orlistat as the anti-obesity drug Xenical[17, 244-246]. Adverse effects related to a long-term orlistat intake, however, also include gastrointestinal problems and decreased the intake of fat-soluble vitamins[18, 19, 244, 247-249]. Moreover, since its application is not automatically accompanied by dietary changes, weight gain after orlistat treatment is likely and might not present a long-term strategy to lose and maintain a healthy body weight. Therefore, in recent years, the focus of anti-obesity research has also shifted to fatty acid transporters, which function as regulatory gateways in the intestinal fatty acid uptake[22]. Located at the apical enterocytic membranes, their antagonism may constitute a potential preventive and therapeutic target to reduce excessive lipid absorption without gastrointestinal disturbances related to undigested lipids. Thus far, emphasis was placed especially on identifying compounds that block FATP2, which has been reported to be expressed in the human intestines and which has shown promising results in mice[22, 250, 251].

1.5. Adipose tissue and adipogenesis

Apart from thermal insulation and mechanical cushioning, adipose tissue constitutes the main site for storing excess calories, thereby functioning as an energy reservoir in times of higher need or fasting. As a highly complex endocrine organ, it also plays an important role in synthesizing and releasing hormones, which participate in the regulation of various metabolic pathways, including those of appetite and body weight control, immunity or lipid and glucose metabolism[252-257]. Adipose tissue in mammals can be characterized as white or brown, the latter being nearly absent in human adults. In contrast to white adipocytes, which primarily present a vital buffering system for balancing lipid homeostasis, brown adipocytes facilitate thermogenesis by generating heat by oxidation of fatty acids within the adipocyte[252, 253, 257].

The differentiation process of pre-adipocytes to mature adipocytes, which is referred to as adipogenesis, can occur throughout the entire life to adapt in cases of higher lipid storage requirements[252, 258, 259]. Adipocytes differentiate from mesenchymal precursor cells. Their differentiation is controlled by a complex regulatory system of stimulatory and inhibitory agents, inducing the development of an adipocyte specific phenotype via a signaling cascade of adipogenic transcription factors. These factors modulate the adipogenic process on a transcriptional level through elaborate interactions in a temporal network[260] (Figure 2).

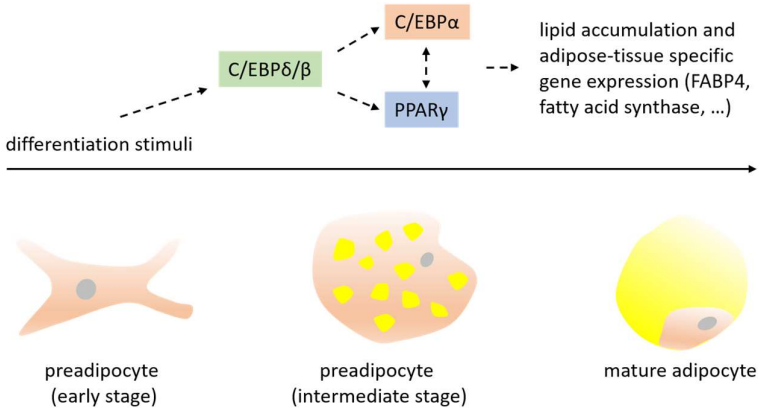

Figure 2. Schematic overview of the adipocyte differentiation process
Integration of adipogenic signals via transcription factors regulates the process of adipogenesis (adapted from Christodoulides[261] (2009))

At confluence, pre-adipocytes in culture, such as the murine 3T3-L1 cells, switch from cell proliferation to differentiation by undergoing cell cycle arrest at the G1/S phase of the cell cycle. This transition is accompanied by the expression of early indicators of differentiation such as lipoprotein lipase as well as the decrease of delta-like 1 homolog (DLK-1), a negative regulator of adipogenesis[262-267]. After reaching confluence, the supplementary addition of external stimuli is required to trigger a synchronous entry of the pre-adipocytes in culture into the adipogenic differentiation program. This "adipogenic cocktail" most commonly contains the cAMP elevating methylisobutylxanthine, the glucocorticoid analogue dexamethasone and insulin, which stimulates the insulin receptor (IR) as well as the insulin-like growth factor receptor (IGF-R)[262, 263, 268-272]. The phosphodiesterase inhibitor methylisobutylxanthine increases intracellular cAMP-levels, subsequently inducing protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac)[262, 273, 274]. PKA in turn has been proposed to be involved in mediating Rho and Rho kinase activity inhibition, as well as to potentially directly or indirectly regulate other adipogenic transcription factors[262, 274, 275]. The synthetic glucocorticoid dexamethasone has been suggested to induce its adipogenic modes of action via activation of the glucocorticoid receptor[276-278], whereas insulin is thought to induce early phases of the adipogenic program primarily via activation of the IGF-R, subsequently inducing ERK1/2 as well as a phosphoinositide 3-kinase (PI3K) signaling pathways[271, 275, 279-282]. Downstream, the signaling cascades also lead to the expression of cAMP response element – binding protein (CREB) and sterol regulatory element – binding protein 1 (SREBP-1)[274, 275, 283].

Within 1 h after adding the hormone cocktail to 3T3-L1 cells, the temporary expression of proto-oncogenes c-fos, c-jun, c-myc, which are believed to exert mitogenic properties, takes place over a period of 30 minutes to six hours. Moreover, a first wave of transcription factors, including

C/EBPβ and C/EBP δ, which play a role in directing the differentiation process, are expressed [263, 277, 284-286]. Pre-adipocytes simultaneously reenter the cell cycle to undergo a post-confluent mitosis and clonal expansion, where transcription factors gain access to adipogenic specific regulatory response elements[263, 286-290]. Approximately 48 hours after induction of adipogenesis, a final growth arrest (G_D) preludes the terminal differentiation[263, 291]. In a second wave, transcription factors peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα) levels are expressed shortly before and during G_D, mutually stimulating each other's expression[263, 284, 292-297]. C/EBPα and PPARγ, especially, are viewed as essential modulators in the adipogenic process, playing an important role in the committed development into the mature adipocyte phenotype[263, 265, 292-295, 298]. Three to four days post-induction, the formation of small lipid droplets starts, which in the mature adipocyte melts into on large lipid droplet [262, 263]. After the adipogenic differentiation process is terminated, lipids can be taken up and accumulate within the cells, causing adipocyte to expand in size (hypertrophy) [262, 263].

1.5.1. Regulating the adipogenic process as a strategy for weight loss and weight maintenance

A prolonged over-nutrition in the form of energy-dense diets and simultaneous decreased energy expenditure promotes hyperplasia and/or hypertrophy of adipocytes and eventually leads to a pathophysiological overgrowth of the adipose tissue[299]. This augmented lipid accumulation is considered a substantial risk factor for developing co-morbidities associated with obesity such as diabetes mellitus type 2, metabolic syndrome or cardiovascular diseases[238-240, 300, 301]. Although lifestyle adjustments as main therapeutic strategy in obesity treatment are, in general, very effective, their outcomes, nevertheless, are often poor due to the challenges of committing to behavioural adapations[302-306]. Therefore, obesity management is often assisted by the use of conventional drugs, which, however, are accompanied by numerous side effects[15, 19, 20, 247, 248]. Hence, great interest has evolved in identifying novel and more risk-free anti-obesity agents, especially focusing on naturally occurring plant-derived products[24, 307, 308]. Especially, products derived from herbs or plants, which are consumed in daily life, are commonly deemed more socially acceptable and might also often be relatively safer than synthetic drugs[24]. Moreover, bioactive dietary constituents as nutraceuticals might be consumed as part of the daily diet[308, 309]. Anti-obesity effects of bioactive compounds on metabolic and molecular levels, that might assist in reducing adipose tissue mass and, thereby help to maintain a healthy body weight, include the stimulation of catabolic pathways and induction of apoptosis in adipocytes, as well as the stimulation of browning white adipose tissue[310-313]. Moreover, targeting the adipogenic process to prevent the expansion of adipose tissue by inhibiting the differentiation of pre-adipocyte into mature adipocytes might be a promising new approach to assist in obesity prevention and treatment[23, 314]. Adipogenesis, which is a highly intricate multistep process, might be targeted by

anti-obesity compounds at very different stages and signaling pathways. To date, no anti-obesity drugs aiming at adipocyte hyperplasia specifically have been approved. However, many promising studies *in vitro* and *in vivo* have already reported the anti-adipogenic effects of bioactive plant derived compounds[23, 314].

1.6. Cinnamon and its constituents as anti-obesity agents

Growing research regarding the pharmacological activities of flavor and volatile compounds in plant and food products indicates, that, beyond their overall taste and flavor properties, aroma compounds might also provide the potential to exert various health benefits, including, among many others, anti-obesity effects[23]. Therefore, interest in terms of their use as health-promoting ingredients or as natural agents supplementing the daily diet is rising. Cinnamon bark of Cinnamomum species, for example, a widely known spice in traditional cuisines, has been used as herbal remedy for centuries[315]. For instance, its neuropathic use in managing health issues related to diabetes mellitus has led to great interest in identifying its bioactive constituents as well as potential further beneficial uses as natural agent[316]. Cinnamaldehyde, one of the major compounds in the cinnamon bark oil, has been associated with anti-oxidant and anti-inflammatory activity[317], has been shown to reduce blood glucose and plasma lipid levels[23, 318] as well as to increase insulin sensitivity[23, 319]. Additionally, mechanistic studies *in vitro* also revealed its anti-adipogenic potential[23].

1.6.1. Impact of cinnamon and its constituents on mechanisms regulating satiety and food intake

As mentioned before, cinnamon and its constituents have been associated repeatedly with favorable health effects, including decreased fasting and postprandial blood glucose levels [23, 320-322], ameliorated insulin sensitivity [23, 323], serum lipid profiles[320, 322] as well as body weight and body composition[320, 324]. Especially a long-term administration of cinnamon was found to be effective in the maintenance of body weight in humans as demonstrated by an increase in lean mass and a decrease in body fat in pre-diabetic humans with the metabolic syndrome after supplementing the diet with a water-soluble cinnamon extract (500 mg/d) for 12 weeks[320]. Similarly, it could be shown that daily consumption of 3 g cinnamon by study participants with metabolic syndrome resulted in a higher reduction in body weight (difference between means: 3 kg), percentage body fat (difference between means: 3 %) and waist circumference (difference between means: 4.8 cm) compared to the consumption of placebo vehicles[324]. But even though beneficial properties of cinnamon or numerous cinnamon-derived substances on food intake or long-term body weight maintenance have been suggested, their potential satiety-enhancing impact is still under debate, as, up to now, scientific evidence concerning a short-term effect of

cinnamon or its constituents on food intake and satiety is insufficient and contradictory [318, 321, 324-328]. In an animal model, e.g., a bolus dose of 250 mg cinnamaldehyde (CAL) per kg body weight (bw) was shown to decrease the cumulative food intake and to delay gastric emptying rates[318]. Additionally, chronic cinnamaldehyde supplementation (10 mg/kg bw) of a high fat diet dose-dependently reduced fasting-induced hyperphagia and increased anorectic gene expression in the hypothalamus in mice[329]. Besides upregulating mRNA expression of neuropeptides associated with satiety, cinnamaldehyde was also demonstrated to alter the release of satiety-related gut hormones, such as PYY or serotonin[156, 327]. However, despite this strong evidence regarding an impact of cinnamon constituents on food intake and maintenance of body weight in animals, there are conflicting findings in humans. Whereas, e.g., delayed gastric emptying in human subjects was reported after intake of a standardized meal supplemented with 6 g cinnamon, indicating prolonged post-meal satiety through enhanced gastric distension, satiety scores, which were assessed at different time points before and after the meal, did not change[321]. Furthermore, cinnamon supplementation in a lower amount of 3 g to a high-fat meal also showed no effect on gastric emptying parameters or rating of appetite sensations, analyzed by means of visual analogue scale (VAS) questionnaires before and after consumption of the test meal. Additionally, compared to the placebo control, cinnamon treatment showed no impact in energy intake or the quantity of food consumed[330]. These findings point to more pronounced effects regarding short-term effects on energy intake and parameters associated with post-meal satiety after administration of higher cinnamon doses or just the supplementation of individual cinnamon constituents. Since consumption of *Cinnamomum cassia* powder in higher quantities on a regular basis, also increases the risk to exceed the daily intake limit of carcinogenic and hepatotoxic coumarin, which has been set at 0.1 mg x kg^{-1} bw per day, administering bioactive cinnamon constituents in its pure form might be the better and safer option[331]. Overall, research is still warranted, especially regarding the intake of individual cinnamon constituents in humans.

1.6.2. Impact of cinnamon and its constituents on intestinal nutrient uptake

In the last decades, cinnamon and its constituents have gained interest especially in terms of their potential beneficial health effects under diabetic and obese conditions due to their hypoglycemic and lipid-modulating properties. *In vitro*, cinnamon extract in a concentration of 30 µg/ml was found to stimulate glucose uptake and translocation of GLUT4 to cell membranes in mature 3T3-L1 adipocytes[332]. *In vivo*, long-term as well as acute supplementation of cinnamon in form of powder or extracts has also been suggested to reduce fasting and postprandial blood glucose levels which might be linked to delayed gastric emptying and an increase in insulin sensitivity[320, 321, 323, 330]. Additionally, improved lipid profiles and body weight modulating effects were reported after cinnamon administration to metabolically challenged individuals[324]. Apart from cinnamon-derived phenolic compounds, cinnamaldehyde (CAL), in particular, is one of cinnamons major

constituents that has been repeatedly associated with anti-diabetic as well as anti-obesity activities. On a cellular level, the GLUT4 gene expression was upregulated in murine C2C12 muscle cells after treatment with 10-50 µM CAL[333] as well as the Glut4 protein level in the tissue of diabetic rats after an oral administration of 40 mg/kg bw CAL[334]. Moreover, in L929 fibroblast cells CAL was revealed to exert dual effects on the glucose uptake activity of GLUT1, which was found to be stimulated under basal conditions, but to be inhibited when glucose transport was activated upon glucose deprivation[335]. Similarly, also structurally related cinnamon constituent and potential *in vivo* CAL metabolite cinnamic acid (CAC) in a concentration of 25 µM elevated glucose uptake in differentiated 3T3-L1 adipocytes[336]. Furthermore, CAC was also revealed to improve glucose uptake in insulin-resistant FL83B hepatocytes[337]. In obese animal models, chronic consumption of 0.2 % CAL in a high fat chow was shown to ameliorate plasma glucose levels (8.4 ± 0.3 mmol/L after high fat chow vs. 7.5 ±0.3 mmol/L after CAL-supplemented high fat chow) and to decrease body fat mass gain (5.2 ± 0.3 g after chronic high fat diet vs. 4.5 ± 0.3 g after CAL-supplemented high fat chow)[318]. Moreover, compared to a high fat chow control group, a 4-week CAL (40 mg/kg) containing high fat diet has been demonstrated to decrease plasma levels of triglycerides by 39 %, free fatty acids by 31 % and weight gains by 67.3 % in mice[23]. Compared to cinnamaldehyde, structurally related cinnamic acid in even lower doses of 5 and 10 mg/kg was also reported to decrease acute blood glucose concentrations (with maximum decrease of approximately 28% after 10 mg/kg cinnamic acid supplementation) and improve glucose tolerance in diabetic rats[338]. Altogether, *in vitro* and *in vivo* data indicate an impact of cinnamon and several constituents on mechanisms and pathways associated with the uptake of glucose and its metabolism. But whereas results from *in vivo* studies also point to hypolipidemic effects of cinnamon constituents, such as CAL, through reduced intestinal lipid uptake, relatively little is known concerning their impact on intestinal uptake of other macronutrients. However, since (i) a potential to lower plasma free fatty acids and triglycerides was demonstrated for various cinnamon constituents, and (ii) lipid profiles can be changed through dietary alterations like a decreased fat intake, in particular, studies concerning the intestinal absorption of fatty acids in the gastrointestinal tract as the first point of contact are warranted.

1.6.3. Impact of cinnamon and its constituents on adipogenesis

Apart from a plasma lipid modulating impact, cinnamaldehyde has been demonstrated repeatedly to exert potent anti-adipogenic properties in cell culture and animal studies, leading to a growing interest concerning its potential application as a natural agent in the prevention or therapy of overweight and obesity. On the one hand, these anti-adipogenic effects were demonstrated in form of reduced lipid accumulation in 3T3-L1 cell models after a 10 - 40 µM CAL treatment[23]. On the other hand, decreased body weight gains and reduction of murine epididymal fat cells in number and size, following a one month CAL (40 mg/kg) administration to mice as compared to

a high-fat diet control group support the CAL-mediated regulation of the lipid metabolism *in vivo* as well[23]. Ongoing research indicates, that cinnamaldehyde might not be the only bioactive cinnamon derived-compound associated with an anti-adipogenic activity. Other structurally related cinnamon constituents, such as cinnamyl alcohol (C-ALC) or CAC, have also been shown to reduce triglyceride accumulation in lipid cell models[311, 339]. However, mechanisms of action underlying their modulating impact on adipogenesis are not completely understood yet. It was found, that (i) the inhibiting effect on fat cell augmentation after CAL treatment is linked to a downregulation of transcription factors which play an essential role in regulating the adipogenic process. PPARγ as well as C/EBPα mRNA levels were downregulated in 3T3-L1 cells after a 10-40 μM CAL treatment during differentiation, accompanied by a specific inhibition of PPARγ transcriptional activity, pointing also to a direct antagonistic activity of cinnamaldehyde[23]. Additionally, (ii) a regulating effect of cinnamaldehyde on lipid biosynthesis and lipolysis in mature adipocytes has been suggested, indicated by an upregulation of hormone sensitive lipase expression levels and a downregulation of perilipin and glycerol-3-phosphate dehydrogenase expression levels [329]. Furthermore, (iii) metabolic reprogramming and thermogenic stimulation via stimulation of protein kinase A signaling, phosphorylation of the hormone sensitive lipase and elevated expression of genes associated with thermogenesis have been discussed as potential cinnamaldehyde-mediated modes of action[340]. Finally, cinnamaldehyde has also been demonstrated to (iv) activate AMP-activated protein kinase and subsequent acetyl-CoA carboxylase phosphorylation[23], thereby affecting constituting key enzymes in the catabolic and anabolic processes, which maintain metabolic lipid homeostasis. However, it is still under debate, whether AMPK signalling also functions in the adipogenic process[23]. In conclusion, despite consistent evidence concerning anti-adipogenic properties of cinnamaldehyde and various structural analogues in cell culture experiments and animal studies, on a mechanistic level, complete signaling cascades are still lacking, especially concerning the pathway through which cinnamon constituents as potential exogenous inhibitory agents modulate the downstream cascade of adipogenic transcription factors or lipid biosynthesis and lipolysis in mature adipocytes.

1.7. The role of transient receptor potential channel A1 (TRPA1) in adipogenesis and peripheral serotonin release

The transient receptor potential Ankyrin 1 channel (TRPA1) or wasabi receptor, as it is also known, is a non-selective, ligand-activated ion channel, that has been shown to be localized in sensory neurons[341-344]. There, as cation entryway, it plays an important role in nociception, evoking pain and neuronal inflammation by operating as sensor molecules for chemical, thermal and mechanical stimuli[345-350]. By now, however, also various non-neuronal cells, including, among others, endothelial cells, smooth muscle cells or enterochromaffin (EC) cells of the gut

have been found to express TRPA1 channels[156, 351, 352]. Analyzing the architecture of the human TRPA1 channel revealed its structure as a homotetramer assembled through "domain swap" interactions, with each TRPA1 subunit being composed of six transmembrane helices. Within the intracellular N- and C-terminal domains, constituting the majority of the channel's mass, several prominent elements have been characterized, including the C-terminal coiled-coil or the large N-terminal ankyrin repeat domain (ARD), giving the TRPA1 channel its name[353]. The ARD is further linked to the S1 helix through the pre-S1 region, which is of notable importance due to its cysteine and lysine residues, which play a critical role in the channel's activation by electrophilic agonists[353, 354]. Moreover, a TRP-like domain, which is also distinctive of other TRP channels, has been identified in hTRP1, functioning in the allosteric regulation by interacting with pore-forming domains[353]. TRPA1 channel agonists comprise a range of structurally different noxious and pungent chemical irritants including environmental toxins, such as acrolein, plant derived substances, such as allyl isothiocyanate, cinnamaldehyde, eugenol or gingerol as well as proalgesic agents, such as bradykinin [345, 355-361]. Multiple TRPA1 activators, e.g. cinnamaldehyde and other unsaturated carbonyl-containing substances, are considered potent electrophilic compounds, which can activate the channels via covalent modification of the lysine and cysteine residues of the N-terminal TRPA1 domains[354, 360]. Here, a direct addition of residue thiol groups to the carbonyl carbon of the α,β-unsaturated carbonyl agonist has been proposed to be favored over conjugate reactions[362]. For the dietary agonist cinnamaldehyde, an EC_{50} value of 6.8 µM has been determined for hTRPA1, whereas as structural derivatives such as cinnamyl alcohol or cinnamic acid, have been reported to have much lower up to insignificant TRPA1 potential[355, 362, 363]. Moreover, phospholipase C has been suggested to be an involved signaling factor for TRPA1 activation[355]. The pore opening of TRP channels can induce the influx of ions into the cell, thereby altering cytoplasmic concentrations of cations such as Ca^{2+} [348, 349]. Thus, also the activation of the Ankyrin 1 channel might be associated with the potential to modulate a wide range of intracellular Ca^{2+}-signaling processes.

Over the past few decades, a TRPA1-dependency has already been discovered in numerous cinnamaldehyde-mediated physiological responses, including anti-inflammatory[364] and vasodilatory effects[365] or the release of digestive hormones such as ghrelin[318] and PYY[327]. Moreover, experimental data suggest an involvement of TRPA1 in peripheral serotonin release, as serotonin was shown to be secreted TRPA1-dependently by enterochromaffin cells[156, 366]. Additionally, a TRPA1 agonists-mediated delay in gastric emptying involving serotonergic pathways was assessed *in vivo*[367]. Since the intake of the TRPA1 agonist cinnamaldehyde has also been demonstrated to reduce visceral fat tissue and weight gains as well as to exhibit hypolipidemic effects by lowering the plasma concentrations of triglycerides and free fatty acids in animal models, the potential mechanistic involvement of TRPA1 and Ca^{2+}-signaling in pathways of the adipogenic process and the lipid metabolism has also become a subject of

growing research[23, 329, 368, 369]. This hypothesis is further supported by studies reporting the impact of increased intracellular Ca^{2+}-concentrations via stimulation of Ca^{2+}-influx as well as suppression of endoplasmic reticulum Ca^{2+}-ATPase on the different stages of the adipogenic process as well as in the lipid metabolism of adipocytes[368, 369]. However, possible modes of action, including the potential involvement of TRPA1 regarding the impact of cinnamaldehyde and possibly also its cinnamon-derived structural analogues on molecular pathways of the lipid metabolism and the adipogenesis are yet to be examined in more detail.

2. Objectives

The increasing prevalence of overweight and obesity as a consequence of continued immoderate caloric intake and insufficient physical activity poses major challenges for global health care systems. Dietary management is considered an essential part of lifestyle adjustments in treating obesity and maintaining a healthy body weight. Therefore, discovering new anti-obesity agents, especially in the form of naturally occurring plant-derived substances which potentially target macronutrient absorption or overall food consumption via modification of anorexigenic and orexigenic signals, is of great interest. Food intake and satiety are steered by an intricate system of hormonal, neuronal and metabolic signals originating in peripheral tissues, subsequently interacting with central signals, and thereby regulating hypothalamic orexigenic and anorexigenic neurons. Signals arising from the gastrointestinal tract comprise nutrient uptake as well as satiety-related peptides and hormones, which unfold their effects via vagal afferents. Moreover, serotonin signaling in the brain plays a vital part in the control of food intake and satiety. However, the majority of endogenous serotonin derives from the gut and evidence from animal studies suggest, that peripheral serotonin is also involved in the control of food intake via activation of vagal afferent pathways. A large number of health-promoting effects have been associated with naturally derived aroma compounds found in herbs and spices, including, among many others, cinnamon. Beneficial health effects that have been linked to cinnamon and its constituents include ameliorated blood glucose levels, plasma lipid profiles and insulin sensitivity, as well as an anti-adipogenic and a body weight-modulating impact, pointing to its potential application under diabetic and obese conditions. Especially cinnamaldehyde, constituting a major cinnamon-derived bioactive aroma compound, has been studied in terms of its hypoglycemic, hypolipidemic and anti-adipogenic potential. Its administration in animal models has been repeatedly reported to decrease food intake, gastric emptying rates and body weight gain as well as to improve plasma glucose and lipid concentrations. *In vitro*, also potent anti-adipogenic properties have been identified. Additionally, a few animal studies also hint at an effect of cinnamaldehyde on mechanisms regulating satiety, including an impact on the release of satiety-related hormones, such as PYY and serotonin, or the upregulation of hypothalamic expression of anorectic genes. In contrast to long-term outcomes of cinnamon or cinnamon compound administration on the maintenance of body weight, however, data concerning their short-term impact of dietary consumption and satiety is limited and conflicting, especially in humans.

Collectively, these data provide strong evidence that a dietary supplementation with cinnamon, cinnamaldehyde and potentially also other bioactive cinnamon-derived substances might be a promising complementary strategy on top of traditional therapeutic measures in treating obesity or just to help maintain a healthy body weight by modulating food intake, plasma nutrient levels or body composition. However, cinnamon intake is self-limited due to its distinctive and potent aroma characteristics and the fact, that a continued intake of *Cinnamomum cassia* powder in

higher quantities increases the risk of exceeding the recommended limit of dietary coumarin intake. On the other hand, consumption of cinnamaldehyde or potentially other constituents in cinnamon powder in naturally occurring concentrations might be too low for their anti-diabetic and anti-obesity effects. Hence, supplementing bioactive cinnamon constituents in their pure form might be the more effective and safer option. Cinnamaldehyde as a potential anti-obesity agent might also be somewhat restricted in its application due to its specific odor, spicy flavor descriptors and its nociceptive properties. As a further potentially bioactive cinnamon constituent, the less spicy cinnamyl isobutyrate (CIB), which is described as a moderately fruity and sweet aroma compound, was investigated more closely in the present thesis regarding its potential short-term impact on food intake and satiety.

As part of this work, firstly (i) a satiating effect of CIB *in vivo* was proposed, which was studied by measuring *ad libitum* energy intake, subjective hunger perceptions, postprandial blood glucose levels as well as plasma levels of selected orexigenic and anorexigenic peptides and hormones, such as ghrelin, PYY_{3-36}, GLP-1 or serotonin, following an oral glucose tolerance test (OGTT) with or without CIB supplementation in a short-term human cross-over intervention trial.

Although there is strong evidence suggesting effects of cinnamon constituents, such as cinnamaldehyde, on energy intake, nutrient absorption, on adipogenesis as well as on plasma levels of free fatty acid and triglycerides, a direct impact on intestinal lipid absorption has not been investigated so far. Following the short-term intervention study, this work aimed to examine possible modes of action underlying this reported impact on the lipid metabolism *in vitro*. One the one hand, (ii) using differentiated Caco-2 cells as a model for the intestinal barrier as first point of contact, the potential impact and mechanistic pathways of selected cinnamon compounds and structural analogues CIB, CAL, CAC and C-ALC on fatty acid uptake were studied. Since CAL has also been identified as a potent anti-adipogenic agent in adipose tissue, inhibiting the differentiation of pre-adipocytes into mature adipocytes and decreasing lipid accumulation in mature adipocytes in the 3T3-L1 cell model and in animal models, on the other hand, (iii) an anti-adipogenic effect of the structural analogue CIB was hypothesized in the present thesis as well. For this purpose, long-term lipid accumulation during the adipogenic process of 3T3-L1 cells, fatty acid uptake in mature 3T3-L1 adipocytes as well as underlying mechanisms of action were investigated after treatment with CIB or CAL in dietary relevant concentrations. Due to the known TRPA1 agonistic activity of CAL, its potential action via TRPA1 was hypothesized and mechanistically studied *in vitro* as well.

3 Results

3.1 Cinnamyl Isobutyrate Decreases Plasma Glucose Levels and Total Energy Intake from a Standardized Breakfast: A Randomized, Crossover Intervention

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In this randomized, short-term crossover intervention study, the impact of cinnamyl isobutyrate, administered at a bolus of 0.45 mg in a 75 g glucose-water solution, on *ad libitum* energy intake from a standardized breakfast, postprandial plasma glucose levels and subjective hunger perceptions was investigated. Additionally, plasma levels of ghrelin, glucagon-like-peptide1 (GLP-1), PYY_{3-36}, and serotonin were measured as markers of hunger and satiety.

In this publication, the PhD candidate participated in scientific discussion regarding the research topic, contributed in performing statistical data analysis and wrote the manuscript draft.

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Cinnamyl Isobutyrate Decreases Plasma Glucose Levels and Total Energy Intake from a Standardized Breakfast: A Randomized, Crossover Intervention

*Christina M. Hochkogler, Julia K. Hoi, Barbara Lieder, Nicole Müller, Joachim Hans, Sabine Widder, Jakob P. Ley, and Veronika Somoza**

Scope: Cinnamon is associated with anti-obesity effects, regulating food intake, improving plasma glucose levels and lipid profiles in vivo. In the present study, the impact of cinnamyl isobutyrate (CIB), one constituent of cinnamon, on ad libitum food intake from a standardized breakfast and outcome measures of hormonal regulation of appetite were investigated.
Methods and results: In this randomized, short-term crossover intervention study, a 75 g per 300 mL glucose solution solely (control) or supplemented with 0.45 mg CIB was administered to 26 healthy volunteers. Prior to and 2 h after receiving control or CIB treatment, subjective hunger perceptions were rated using a visual analog scale. Food intake from a standardized breakfast was assessed 2 h after treatments. Plasma peptide YY_{3-36}, glucagon-like-peptide1, ghrelin, and serotonin as well as plasma glucose and insulin were measured in blood samples drawn at fasting and 15, 30, 60, 90, and 120 min after treatment. CIB administration decreased total energy intake and delta area under curve plasma glucose by 4.64 ± 3.51% and 49.3 ± 18.5% compared to control treatment, respectively.
Conclusions: CIB, administered at a 0.45 mg bolus in 75 g glucose–water solution, decreased ad libitum energy intake from a standardized breakfast and postprandial plasma glucose levels.

1. Introduction

Combating the rising prevalence of overweight and obesity, associated with an increased risk of developing various comorbidities, has become a worldwide challenge.[1–3] Therefore, medical treatment of obesity may become inevitable, taking into account, however, that combined treatment strategies are regarded more effective than drug application solely. Reducing food intake via modulation of anorexigenic and orexigenic signals as well as enhancing energy expenditure are strategies to combat overweight and maintain a healthy body weight.[4] Hence, there is considerable interest in identifying anti-obesity agents, focusing on bioactive dietary compounds that target food intake and promote feelings of satiety.

A variety of beneficial health effects have been attributed to naturally occurring aroma compounds in herbs and spices, such as cinnamon.[5] Obtained from the bark of *Cinnamomum* species, it has been used for centuries for treating ailments such as respiratory and digestive disorders.[6] More recently, cinnamon and its constituents have gained attention for potential application under diabetic and obesity conditions. Chronic and acute cinnamon supplementation have been reported to decrease fasting and postprandial plasma glucose levels and to delay gastric emptying in healthy and diabetic subjects.[7,8] Apart from reducing gastric emptying, increased insulin sensitivity has been proposed to explain the hypoglycemic effect of cinnamon.[9–11] Regarding the active principles of cinnamon responsible for its hypoglycemic effects, plant secondary metabolites of phenolic compounds, including catechins, epicatechins, or proanthocyanidins, have been associated with beneficial effects in insulin signaling as well as antioxidative properties.[12–14]

Moreover, some studies hint at an impact of cinnamon and constituents on mechanisms regulating satiety in humans.[8,15–17] Despite evidence suggesting beneficial long-term effects of cinnamon on maintenance of body weight in humans, to our knowledge, scientific data regarding its short-term impact on food intake and satiety is scarce and inconsistent.[15,16] A search of

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compounds linked to antidiabetic and anti-obesity properties of cinnamon led to the identification of cinnamaldehyde, constituting 80–90% of the essential oil of cinnamon bark. Cinnamaldehyde administration has been associated repeatedly with body weight and lipid-modulating effects in animal models, including reduced cumulative food intake and gastric emptying rates,[18,19] as well as decreased weight gain, plasma triglycerides, and free fatty acid.[20] In addition, ameliorated blood glucose levels and glucose tolerance in mice have been reported for cinnamaldehyde.[18]

In addition to reduced fasting-induced hyperphagia and upregulated mRNA expression of hypothalamic neuropeptides associated with satiety after chronic cinnamaldehyde supplementation (10 mg kg^{-1} body weight) in mice,[21] cinnamaldehyde was also found to modulate the secretion of satiety-related gut hormones, including peptide YY (PYY) or serotonin.[22,23]

Taken together, evidence suggests that consumption of cinnamon, cinnamaldehyde, and possibly other bioactive cinnamon constituents might be a promising approach in helping to maintain a healthy body weight by affecting food intake, blood glucose levels, and body composition through a satiety-enhancing impact.[17] However, consumption of cinnamon is self-limiting due to strong and unique aroma values. Moreover, dietary intake of cinnamaldehyde and additional possibly bioactive constituents might be insufficient for antidiabetic and anti-obesity effects.[24,25] Frequent consumption of cinnamon in larger amounts, especially *Cassia cinnamon*, might increase the risk of exceeding daily intake limit of coumarin (0.1 mg kg^{-1} body weight per day), which has been linked to hepatotoxicity and carcinogenic effects.[26] Consequently, selecting individual bioactive cinnamon compounds might be a safer application option. Cinnamaldehyde, although repeatedly demonstrated to affect glucose and lipid metabolism in animal models, might also be limited in its consumption due to characteristic cinnamon odor and spicy flavor characteristics.[24] Thermosensitive transient receptor potential cation channel A1 (TRPA1) channels are activated by cinnamaldehyde, thereby evoking nociceptive responses and painful sensations.[27] Cinnamaldehyde has also been reported to cause skin irritation and allergic contact dermatitis in humans.[28,29]

To identify further potentially bioactive and less spicy cinnamon-derived constituents, the aroma compound cinnamyl isobutyrate (CIB), naturally occurring in the essential oil of cinnamon bark, was chosen for the present study. Here, we propose a satiating effect of CIB. Despite structural similarities with cinnamaldehyde, CIB is mainly known for its moderate fruity and sweet flavor descriptors.[30] It has been categorized as "Generally Recognized As Safe" (GRAS) by Flavor Extract Manufacturers Association (FEMA),[31] and added to the EU list of flavoring substances (EFSA; Regulation EU 872/2012).

As our main hypotheses, we studied whether a bolus dose of 0.45 mg CIB has an impact on the ad libitum energy intake from a standardized breakfast (primary outcome measure), subjective hunger perceptions, and on postprandial blood glucose levels (secondary outcome measures) in moderately overweight volunteers following an oral glucose tolerance test (OGTT) with or without CIB supplementation. Additionally, ghrelin, glucagon-like-peptide1 (GLP-1), PYY_{3-36}, and serotonin concentrations were measured in the plasma as orexigenic and anorexigenic markers. The crossover study design in this short-term intervention trial was conducted based on previous works which reported an impact of other aroma active food constituents on food intake and hormonal regulation of satiety.[32,33]

2. Experimental Section

2.1. Study Population

This human intervention study was carried out with 26 metabolically healthy, moderately overweight (BMI between 25 and 32 kg m^{-2}) male human subjects aged 21 to 43 years. Recruitment criteria for volunteers additionally required no tobacco consumption, alcohol, or drug abuse. Written consent was obtained from all participants after detailed instructions regarding the intervention. The Ethics Committee of the University of Vienna authorized the study design (registration no. 00163) which followed the guidelines of the Declaration of Helsinki. To determine eligibility in terms of study participation, subjects were medically screened beforehand. Determination of a hemogram as well as analysis of liver enzymes (aspartate aminotransferase, alanine aminotransferase alkaline phosphatase, and γ-glutamyl transpeptidase), blood lipids (triglycerides, total, LDL, and HDL cholesterol), creatinine, glomerular filtration rate, thyroid-stimulating hormone, and blood glucose levels (after 12 h fasting as well as 1 and 2 h after an OGTT) were performed by a medical laboratory (Blutlabor Dr. Greiner, 1220 Wien, Austria). A urine test was carried out to exclude glucosuria. Body weight and height were recorded using a digital scale (Seca Bella 840, Germany) to an accuracy of 100 g and a stadiometer with a precision of 0.1% (Seca 213, Germany), respectively.

2.2. Study Design

Following the medical screening, participants of this open, randomized, controlled, crossover study were randomly allocated and required to attend two separate study days after an overnight fast. At the first visit, the control group received a glucose solution (75 g glucose + 300 μL ethanol) devoid of additives, whereas the intervention group received a glucose solution with CIB supplementation (75 g glucose + 300 μL ethanol + 0.45 mg CIB). Study groups were switched at the second visit after 1 week to ensure that each subject participates once in the control and once in the intervention group. CIB was not sensorically detectable in the glucose solution at the applied dose of 0.45 mg. A visual analog scale (VAS) was applied to determine subjective perceptions of hunger, which describes the food intake depending on energy depletion and nutritional status in contrast to appetite, characterizing the desire to eat.[34] It was completed by study volunteers before and 2 h after receiving the glucose solution. To assess their ad libitum energy intake, a standardized breakfast providing an average of 12.1 MJ with a total of 335 g carbohydrates, 126 g fats, and 80 g proteins was served 2 h after the OGTT. The meal comprised four rolls, four slices of dark bread, four slices of ham and cheese, 100 g honey and strawberry jam, 80 g butter, 180 g berry yogurt, 50 g coffee cream, 20 g sugar as well as 200 mL of coffee or tea and water as desired. The quantity consumed was determined by weighing the remaining food, in order to calculate the ad libitum energy and macronutrient intake

using the German Food Code and nutrient database "Bundeslebens mittelschlüssel," according to Hochkogler et al.[32] Apart from assessing the potential impact on satiation, which determines the size of a meal, participants were asked to document their diet using provided forms by estimating their food intake on both study days for 24 h post-intervention to evaluate an influence on satiety, describing the inter-meal period following the end of an eating episode.[35] Analysis of the diet records was achieved by means of the software program nut.s (dato Denkwerkzeuge, nut.s science, v1.29.34; Austria) as described by Hochkogler et al.[36]

2.3. Blood Sample Collection

Venous blood samples were taken at six time points over the course of 2 h using a venous catheter. The first blood sample was drawn at fasting, further drawings followed at 15, 30, 60, 90, and 120 min after consumption of the glucose solution. EDTA-coated tubes (Sarstedt, Germany) were used for sample collection in preparation for ghrelin, GLP-1, $PYY_{3–36}$, and serotonin measurement, whereas heparin- or fluoride-coated tubes (both Sarstedt, Germany) were used to determine plasma insulin and glucose concentrations, respectively. Plasma was obtained by centrifugation of blood collection tubes for 15 min at 1800 × *g* at 4 °C.

Determination of $PYY_{3–36}$ concentration in the plasma required addition of the serine protease inhibitor AEBSF (4-[2-aminoethyl benzene] sulfonyl fluoride; Merck Millipore, Darmstadt, Germany) as well as a dipeptidyl peptidase protease inhibitor (DPP IV; Merck Millipore, Darmstadt, Germany) to the whole blood sample before centrifugation. For ghrelin analysis, AEBSF was added to the blood samples as well, which was promptly followed by centrifugation and plasma acidification with hydrochloric acid (0.05 M, final concentration). Aliquots of all samples were stored at −80 °C until further analysis.

2.4. Glucose, Insulin, $PYY_{3–36}$, GLP-1, Ghrelin, and Serotonin Assays

Determination of glucose concentrations in plasma was carried out by means of a colorimetric assay kit (Cayman Europe, Tallinn, Estonia) with an intra-assay variation of 4.6–8.1% and an inter-assay variation of 1.7–11.3%, whereas an enzyme-linked immunosorbent assay (ELISA) kit (DRG Instruments GmbH IASON, Graz, Austria) with an intra-assay variation of 1.8–2.6%, an inter-assay variation of 2.9–6% was used to measure insulin concentrations in the plasma. ELISA assays, purchased from DLD Diagnostika (Hamburg, Germany) and Merck Millipore (Darmstadt, Germany), were also performed to assess plasma serotonin levels (intra-assay variation: 4.7–6%; sensitivity: 5 ng mL^{-1}) as well as total GLP-1 (intra-assay variation: 1–2%; inter-assay variation: 10–12%), $PYY_{3–36}$ (intra-assay variation: 7–15%; inter-assay variation: 6–11%), and ghrelin (intra-assay variation: 1.11–1.91%; inter-assay variation: 5.18–7.74%) concentrations. All assays were implemented according to specifications given by the manufacturer's protocols.

2.5. Statistical Analysis

All statistical analyses were carried out using SigmaPlot 13.0 (Systat Software GmbH). Normality of data was determined by Shapiro–Wilk test and if not indicated otherwise, normally distributed data are shown as mean ± standard error of mean. For determination of a sample size of 44, a power analysis was performed using G*Power 3, based on energy intake as main outcome measure from a pilot study with crossover design. This pilot test ($n = 4$) was conducted with male volunteers, who received an OGTT with or without 0.45 mg CIB supplementation on two separate study days. Taking into account the percentage change of energy intake (−12.3 ± 28.3%), an effect size of 0.44 was calculated (paired sample *t*-test). From a total of 50 screened subjects, 34 were eligible to participate in the study. After further dropouts in the course of the study, 26 volunteers completed the intervention.

To assess statistical differences between control and CIB groups for mean delta values at t_x–t_0 for glucose and insulin, a two-way repeated measures ANOVA for time and treatment followed by a Student–Newman–Keuls post hoc test was performed. Delta area under curve ($^{\Delta}$AUC) was determined for glucose, insulin as well as GLP-1, $PYY_{3–36}$, and ghrelin plasma concentrations over time. To test differences between treatment groups, a paired Student's *t*-test (one or two tailed) was applied.

Moreover, a paired Student's *t*-test (one tailed) was carried out to examine a statistical decrease in ad libitum energy intake, macronutrient intake, and hunger perceptions after CIB treatment compared to control treatment. For statistical analysis of energy and macronutrient intake assessed by food records, a paired Student's *t*-test was performed as well.

3. Results

3.1. Total ad libitum Energy and Macronutrient Intake from Breakfast and Perceptions of Hunger

To determine the ad libitum energy intake, participants received a standardized breakfast 2 h after administration of a glucose solution with or without supplementation of 0.45 mg CIB. In the control group, a mean total energy intake of 5.81 ± 0.28 MJ was demonstrated compared to 5.37 ± 0.19 MJ in the intervention group, revealing a difference (−4.64 ± 3.51%; $p = 0.03$) in food intake (**Figure 1**A). Administration of CIB also showed a reduction in fat intake by 8.63 ± 3.53% as well as in protein intake by 7.91 ± 2.65% in comparison to the control treatment (Figure 1B). However, no changes in carbohydrate intake between treatments were detected.

A continuous VAS, 100 mm in length, was used by participants to rate their subjective hunger perceptions before and 2 h after administering glucose solution with or without CIB supplementation. As presented in **Figure 2**, there was no significant difference in the feeling of hunger.

3.2. Energy and Macronutrient Intake from a 1 Day Food Record

Assessment of the dietary intake of study participants was achieved by keeping a 1 day estimated food record. CIB and

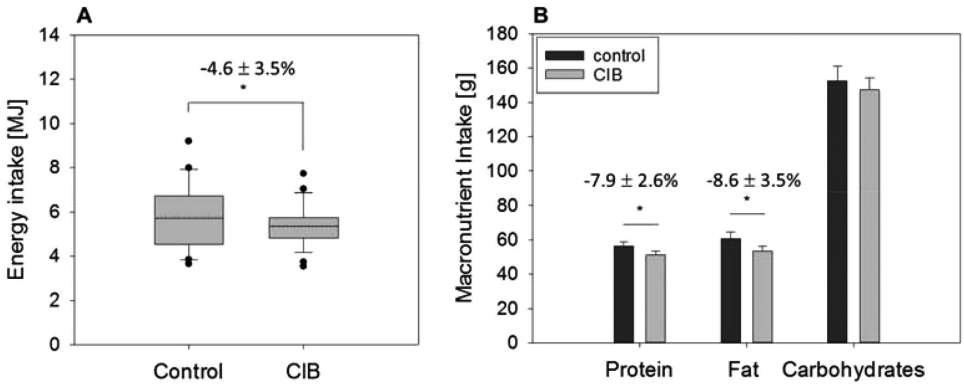

Figure 1. A) Total energy and B) macronutrient intake from a standardized breakfast 2 h after an OGTT administered solely (control) or with 0.45 mg cinnamyl isobutyrate (CIB) in 26 male healthy subjects. Statistical analysis ($p \leq 0.05$) was conducted by a paired Student's *t*-test (one tailed). Means are presented as dotted lines.

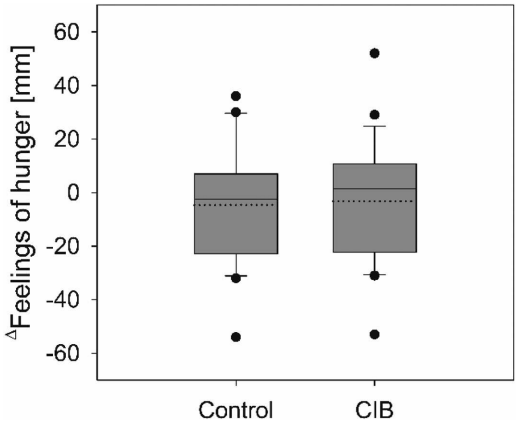

Figure 2. Mean Δ values of self-reported hunger perceptions assessed by a 100 mm visual analog scale before and 2 h after an OGTT with or without (control) 0.45 mg cinnamyl isobutyrate (CIB) supplementation ($n = 26$). Statistical difference ($p \leq 0.05$) was determined by a paired Student's *t*-test (one tailed). Mean is presented as dotted line.

control group did not differ in energy or macronutrient intake on day 1 (**Table 1**), apart from a decrease in saturated fatty acids (60.9 ± 4.27 control vs 53.5 ± 3.22 CIB, $p = 0.05$).

3.3. Plasma Concentrations of Glucose and Insulin

Ninety minutes after administering a 75 g glucose solution supplemented with CIB, reduced delta glucose levels of 0.01 ± 0.31 mmol L^{-1} were calculated compared to 0.59 ± 0.24 mmol L^{-1} in the control group ($p = 0.02$; **Figure 3**A). No difference between treatment groups (control vs CIB) could be shown at any other time point. Moreover, $^{\Delta}$AUC values (2765 ± 371.3 control, 1988 ± 434.4 CIB, $p = 0.03$) demonstrated a decrease in plasma glucose concentrations after CIB intervention by 49.3 ± 22.8% compared to control treatment (Figure 3B). No significant differences, however, were shown for plasma insulin levels between treatment groups at any time point (AUC: 7663 ± 711.9 control vs 7000 ± 641.7; Figure 3C,D).

Table 1. Energy (MJ day^{-1}), macronutrient (g day^{-1}), and fatty acid (g day^{-1}) intake after administration of 75 g glucose without (control) and with supplementation of 0.45 mg cinnamyl isobutyrate determined by an estimated food record over a period of 24 h post-intervention. Values are presented as mean ± SEM. For statistical analysis, a one-sample Student's *t*-test (one tailed) was performed ($p \leq 0.05$).

	Control	Cinnamyl isobutyrate	*p*
Energy	12.2 ± 0.65	11.9 ± 0.64	0.34
Protein	128 ± 10.6	121 ± 8.57	0.23
Carbohydrates	311 ± 23.3	294 ± 21.9	0.21
Fat	122 ± 7.81	111 ± 7.33	0.12
SFA	60.9 ± 4.27	53.5 ± 3.22	0.05
MUFA	38.9 ± 2.79	36.9 ± 2.99	0.27
PUFA	16.3 ± 1.68	15.2 ± 1.56	0.29

3.4. Plasma Ghrelin, PYY_{3-36}, and GLP-1 Levels

Ghrelin, PYY_{3-36}, and GLP-1 levels were assessed as short-term markers of hunger and satiety before and at five time points after the OGTT (t_{15}, t_{30}, t_{60}, t_{90}, t_{120}). As presented in **Figure 4**A,B, $^{\Delta}$AUC values for PYY_{3-36} and GLP-1 levels in the plasma did not differ between CIB and control treatment. Administration of CIB also did not affect plasma concentrations of ghrelin, as no difference could be demonstrated for ghrelin concentrations between the two treatment groups (Figure 4C).

3.5. Plasma Serotonin Concentrations

Results of serotonin concentrations determined in the plasma after CIB treatment in comparison to control treatment are shown in **Figure 5**A. Statistical analysis revealed no difference in $^{\Delta}$AUC values between treatment groups. Two hours after administration of CIB, calculated percentage changes of plasma serotonin levels from fasting to t_{120} showed a statistical trend (10.36 ± 10.06 control vs 49.36 ± 22.82 CIB; $p = 0.07$) to be higher than after receiving glucose solution solely (Figure 5B).

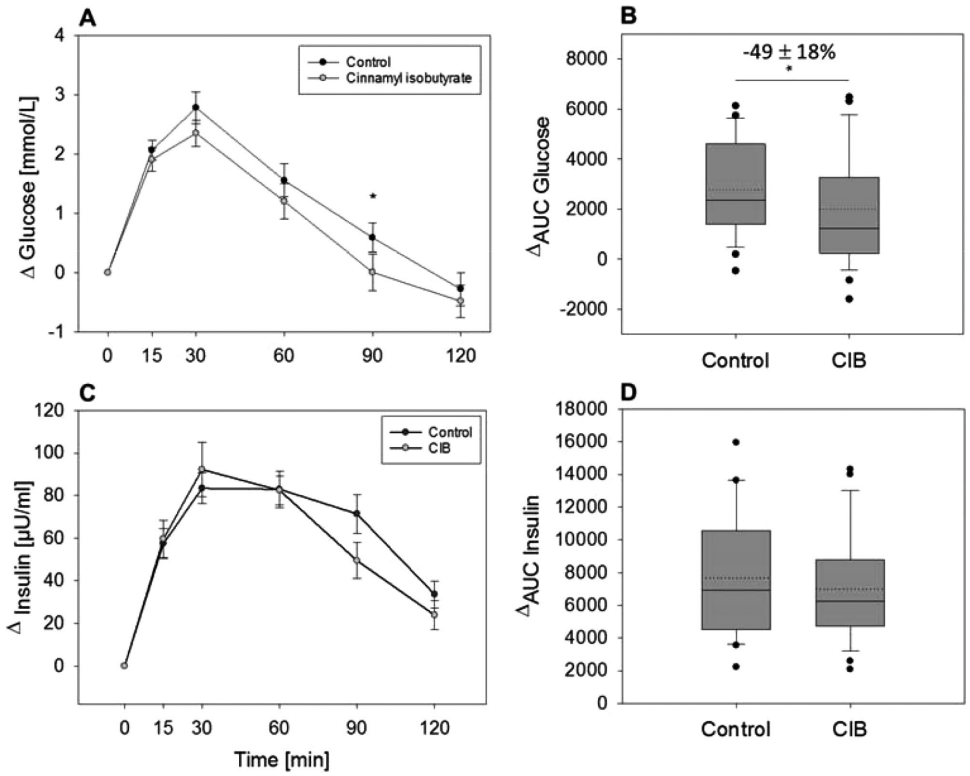

Figure 3. A) Mean Δ plasma glucose concentrations and B) AUC values as well as C) mean Δ plasma insulin concentration D) AUC values before and 15, 30, 60, 90, and 120 min after an OGTT with or without (control) 0.45 mg cinnamyl isobutyrate (CIB) supplementation ($n = 25–26$). Values are demonstrated as mean ± SEM. For statistical analysis, a two-way repeated measures ANOVA for time and treatment (A,C) was performed. Significant differences ($p < 0.05$) between control and intervention treatments for each time point are marked with "*". To assess the difference between AUC values (B,D), a paired Student's *t*-test (one or two tailed) was performed (*$p \leq 0.05$). Mean is presented as dotted line.

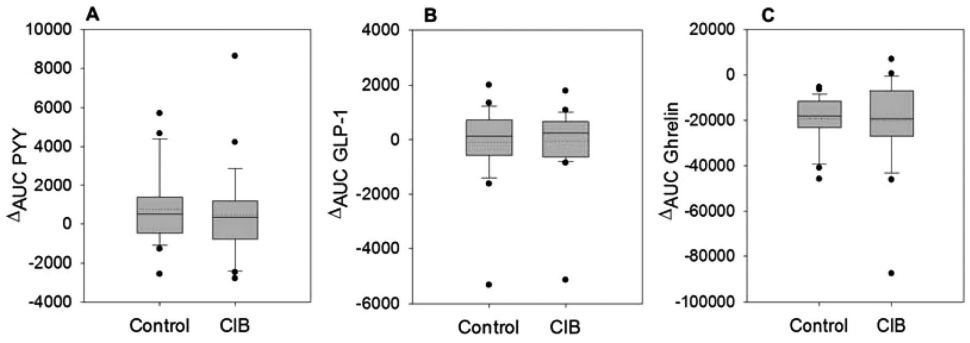

Figure 4. Mean ΔAUC values for A) $PYY_{3–36}$, B) GLP-1 and C) ghrelin concentrations from baseline levels 15, 30, 60, 90, and 120 min after administration of 75 g glucose without (control) and with supplementation of 0.45 mg cinnamyl isobutyrate (CIB) ($n = 24–26$). Values are shown as mean ± SEM. Statistical difference ($p \leq 0.05$) was determined by a paired Students *t*-test (one tailed). Mean is presented as a dotted line.

4. Discussion

In this crossover human intervention trial, a 0.45 mg bolus administration of CIB reduced ad libitum energy intake from a standardized breakfast and plasma glucose levels in moderately overweight men.

Cinnamon has previously been linked to beneficial health properties, including improved blood glucose levels,[13,15,37,38] insulin sensitivity[39,40] as well as serum lipid profiles,[7] and to maintenance of body weight and body composition.[15,16] In terms of hypoglycemic effects of cinnamon, high concentrations of antioxidative phenolic compounds like proanthocyanidins might contribute to its beneficial effects on postprandial glucose levels.[13,14] Reports of other active ingredients, with the exception of cinnamaldehyde, or molecular pathways responsible for the above-mentioned effects are relatively unknown and inconsistent. Anti-obesity and antidiabetic properties of cinnamon are often attributed to its major flavoring component, cinnamaldehyde, which has been shown to reduce short-term energy intake and cumulative body weight in animal models.[18,23]

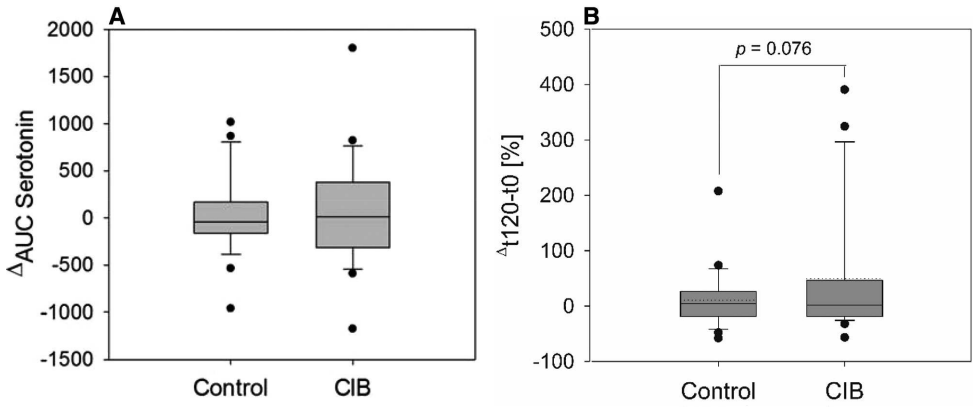

Figure 5. A) Mean $^{\Delta}$AUC values for serotonin concentrations from baseline levels 15, 30, 60, 90, and 120 min after administration of 75 g glucose without (control) and with supplementation of 0.45 mg cinnamyl isobutyrate (CIB) and B) percentage changes of plasma serotonin levels from fasting to t_{120} ($n = 26$). Values are shown as mean ± SEM. Statistical difference ($p \leq 0.05$) was determined by a paired Students *t*-test (one tailed). Mean is presented as a dotted line.

Besides a delay in gastric emptying rates, which has been associated with reduced hunger and increased satiety,[19] regulation of satiety- and appetite-related hormones might play a role in the cinnamaldehyde-induced effect on energy intake.[18,22,23] Overall, literature evidence suggests more pronounced effects after administration of individual constituents like cinnamaldehyde compared to cinnamon powder or extract. However, knowledge of anti-obesity or antidiabetic activities of minor cinnamon-derived compounds is sparse. In addition, usage of cinnamon and its major ingredient cinnamaldehyde is also restricted due to its strong spicy flavor and pungency, and potential hazardous impact from chronic consumption of higher amounts.[24,26] Thus, in the present study, CIB, exhibiting structural similarities to cinnamaldehyde, whereas presenting a less spicy, no pungent, and only a weak cinnamon-specific taste and odor, was examined for its impact on short-term energy intake. Here, bolus administration of CIB reduced short-term energy intake from a standardized breakfast compared to control intervention by 4.64 ± 3.51%. As indicated by our results, this small, but significant decrease in energy intake can be attributed to a decline in fat and protein intake by 8.63 ± 3.53% and 7.91 ± 2.65%, respectively, whereas consumption of carbohydrates from the standardized meal was not affected. Despite administering CIB as a bolus in a low amount of 0.45 mg compared to effective short-term applications of cinnamaldehyde in animal studies, ranging from 10–250 mg kg^{-1} body weight,[18,23] a reduction in food intake was seen in this study. However, the high number of dropouts leading to reduced sample size may have resulted in an inadequately powered study. This is a restricting factor for interpreting our outcomes which have to be verified in larger intervention studies to come. Whether the effect size might increase dose dependently as well, as it has been shown for cinnamaldehyde,[18,22] also needs to be investigated in future studies.

According to our main objective, we hypothesized that a decrease in short-term energy intake is determined by decreased hunger perception. In contrast to this hypothesis, the slight reduction in food intake after CIB intervention was not accompanied by equally reduced hunger perceptions, as differences between treatment groups assessed by VAS were not detectable. It is not clear why CIB intake did not decrease hunger perceptions in the present study. Hlebowicz et al.[8] demonstrated that adding 6 g cinnamon to a 300 g rice pudding delayed gastric emptying, suggesting extended post-meal satiety due to increased gastric distension, but also did not affect satiety scores, estimated at several time points before and after the start of the meal. In addition, bolus administration of CIB did not affect cumulative food intake over a period of 24 h post-intervention, as demonstrated by analyzing total energy intake by an estimated food record for 1 day.

Short-term antidiabetic effects of cinnamon and its main constituent cinnamaldehyde have been demonstrated repeatedly,[8,18] suggesting increased glucose utilization and insulin activity in animal models after long-term cinnamon administration.[39] In this study, the impact of 0.45 mg CIB on plasma glucose concentrations was analyzed over a time course of 2 h after performing an OGTT with or without CIB supplementation. Administration of CIB reduced $^{\Delta}$AUC levels compared to control treatment. Examination of the time course of glucose levels revealed a significant reduction 90 min after the OGTT compared to the control treatment, indicating an impact of CIB on glucose metabolism. In contrast to decreased glucose levels, insulin responses showed no changes at any time point between control and CIB treatment. These results are in accordance with another study demonstrating decreased postprandial glucose levels after bolus administration of 6 g cinnamon.[8] Likewise, chronic oral administration of cinnamaldehyde was also reported to decrease blood glucose levels in diabetic rats (20 mg kg^{-1}),[41] and in obese mice (250 mg kg^{-1}).[18] In addition, Solomon et al.[40] hypothesized delayed effects of cinnamon supplementation on insulin response and sensitivity, which was detected 2 weeks after daily cinnamon supplementation (6 × 500 mg), as opposed to a more immediate impact on postprandial glucose response. In contrast to high doses of cinnamon and cinnamaldehyde applied in previous studies, bolus administration of the comparatively small amount of 0.45 mg CIB significantly decreased postprandial plasma glucose levels in the present study. However, whether a long-term administration enhances the demonstrated hypoglycemic effects or alters insulin responses need to be addressed in future studies.

In order to provide further insights into a potential appetite-modulating impact of CIB, additional outcome measures associated with sensations of hunger and satiety were investigated. Short-acting satiety signals in the gastrointestinal tract are generated primarily by gastric distension and the secretion of enteroendocrine peptides as mediators of intestinal satiety signaling.[42] Anorexigenic peptides released in response to food intake include GLP-1 and PYY, which are produced mainly by intestinal L-cells.[42] In addition, ghrelin levels, which are known for appetite-inducing effects, were assessed in the present study. However, contrary to cinnamon and cinnamaldehyde, whose administration resulted in modulating effects on the secretion of anorexigenic hormones,[23,38] no differences in GLP-1, PYY_{3-36}, or total ghrelin levels were found in this study.

Having been shown to exert inhibitory effects on appetite, to generate feelings of satiety, and leading to suppression of food intake,[43,44] serotonin is also considered a marker of satiety. Although peripheral serotonin is not able to cross the blood–brain barrier, it has been shown repeatedly in vivo that peripheral administration also resulted in reduced food intake and accelerated satiety.[32,45–47] Whereas cinnamaldehyde has been shown to enhance serotonin release from enterochromaffin cells in vitro via TRPA1 stimulation,[22] plasma levels of serotonin did not change after a CIB bolus in the present study. However, 2 h after receiving the glucose solution supplemented with CIB, a trend for a serotonin increase ($10.3 \pm 10.0\%$ control vs $49.3 \pm 22.8\%$ CIB, $p = 0.076$) compared to control treatment was demonstrated. Future studies need to elucidate whether a more pronounced decrease in food intake at higher or frequent CIB supplementation in studies with larger sample sizes show a regulation on hormonal level. In addition, an involvement of TRPA1 has been discussed to be involved in cinnamaldehyde-induced hormone release in the gastrointestinal tract.[22,23,48] A TRPA1-independent mode of action of cinnamaldehyde and potentially other cinnamon-derived constituents concerning its impact on food intake cannot be excluded, but needs to be addressed in future studies as well.

Postprandial secretion of selected anorexigenic and orexigenic hormones as well as subjective hunger perceptions were not affected by CIB, although energy intake was reduced. Apart from energy expenditure, meal initiation and meal size not only depend on the nutritional status but also other signals, including palatability traits affecting taste and smell, influence our food intake.[35,49] Hunger perceptions, consequently, must be distinguished from appetite, which, by contrast, describes the desire to eat and is stimulated by availability of food and pleasure of eating.[34] Thus, based on the results of the present study, we hypothesize an effect of CIB on energy intake and appetite, but cannot suggest a long-term satiating impact of CIB, which is in accordance with other studies related to a satiating impact of cinnamon conducted in humans.[8,50]

In conclusion, our study results point to a short-term satiating effect of 0.45 mg CIB, corresponding to 1.5 ppm, an amount, which is used in nonalcoholic beverages, on energy intake. However, taking into consideration the small sample size as a limitation of this study, our results warrant larger intervention trials. These future studies will also need to elucidate dose-dependent long-term effects of CIB on energy intake and outcome measures of satiety to verify its potential activity as an anti-obesity agent that might help to reduce food intake, and to maintain a healthy body weight and body composition.

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Conflict of interest

The authors declare no conflict of interest.

Keywords

blood glucose, cinnamyl isobutyrate, energy intake, satiety, serotonin

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3.2 Identification of Cinnamaldehyde as Most Effective Fatty Acid Uptake Reducing Cinnamon-Derived Compound in Differentiated Caco-2 Cells Compared to Its Structural Analogues Cinnamyl Alcohol, Cinnamic Acid, and Cinnamyl Isobutyrate

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In this publication, the impact of cinnamon derived aroma compound cinnamaldehyde and its structurally related constituents cinnamyl alcohol, cinnamic acid, and cinnamyl isobutyrate on TRPA1-dependent fatty acid uptake and serotonin release in differentiated Caco-2 cells was analyzed.

The PhD candidate participated in the experimental design and analyzed fatty acid uptake and serotonin release as well as neurotransmitter uptake after stimulation with structurally related cinnamon constituents cinnamaldehyde, cinnamyl isobutyrate, cinnamic acid and cinnaml alcohol in the presence and absence of TRPA1 inhibitor AP-18. Moreover, the PhD candidate determined gene expression levels of selected serotonin receptors and fatty acid transporter by performing microarray analysis and qPCR. Additionally, trans-well uptake studies were performed to investigate the intestinal cellular uptake of the cinnamon test compounds. Moreover, data analysis and preparation of the publication draft was carried out by the PhD candidate.

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Identification of Cinnamaldehyde as Most Effective Fatty Acid Uptake Reducing Cinnamon-Derived Compound in Differentiated Caco-2 Cells Compared to Its Structural Analogues Cinnamyl Alcohol, Cinnamic Acid, and Cinnamyl Isobutyrate

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Supporting Information

ABSTRACT: Naturally occurring cinnamon compounds such as cinnamaldehyde (CAL) and structurally related constituents have been associated with antiobesity activities, although studies regarding the impact on intestinal fatty acid uptake are scarce. Here, we demonstrate the effects of CAL and structural analogues cinnamyl alcohol (CALC), cinnamic acid (CAC), and cinnamyl isobutyrate on mechanisms regulating intestinal fatty acid uptake in differentiated Caco-2 cells. CAL, CALC, and CAC (3000 μM) were found to decrease fatty acid uptake by 58.0 ± 8.83, 19.4 ± 8.98, and 21.9 ± 6.55%, respectively. While CAL and CALC at a concentration of 300 μM increased serotonin release 14.9 ± 3.00- and 2.72 ± 0.69-fold, respectively, serotonin alone showed no effect on fatty acid uptake. However, CAL revealed transient receptor potential channel A1-dependency in the decrease of fatty acid uptake, as well as in CAL-induced serotonin release. Overall, CAL was identified as the most potent of the cinnamon constituents tested.

KEYWORDS: *intestinal fatty acid uptake, serotonin, TRPA1, Caco-2, cinnamaldehyde*

INTRODUCTION

The rising prevalence of obesity and associated comorbidities have become a major challenge for our health system worldwide and are considered major risk factors for developing diseases such as metabolic syndrome, diabetes mellitus type 2, or cardiovascular events.[1] Hence, there is considerable interest in identifying strategies to counteract or prevent this development. Apart from physical exercise, dietary approaches are considered a vital strategy to achieve body weight loss and maintain a healthy body weight, especially taking into consideration plant-based compounds that might act as antiobesity agents by targeting total energy intake.[2] Cinnamon, gained from the inner bark of *Cinnamomum* spp., has been a popular natural flavoring agent in kitchens for thousands of years.[3] Because of numerous benefits for human health, it has also been used in traditional and modern medicines.[4] Many studies have reported a variety of potential therapeutic and preventive effects of cinnamon, including antimicrobial,[5] antioxidant,[6] or anti-inflammatory[7] properties. Additionally, several studies point to the therapeutic efficiency of cinnamon and constituents under diabetic conditions.[8−10] Apart from improving glycemic indices, supplementation of cinnamon bark powder (1 g/day) was also reported to positively affect lipid profiles of patients with type II diabetes after long-term application.[10] In this study, all assessed anthropometric outcomes including body mass index (BMI), body fat, and visceral fat decreased after the 3 month cinnamon administration, further pointing to an impact on the lipid metabolism.[10] Interestingly, most pronounced effects of the cinnamon supplementation were detected in participants with higher baseline BMI, indicating a better response in overweight patients.[10] A recent meta-analysis assessing randomized controlled trials with up to 750 participants further supports the lipid lowering effects of cinnamon regarding the reduction of total plasma cholesterol and triglycerides.[11] Results from animal studies also suggest that cinnamaldehyde (CAL) as the major constituent might be one of the cinnamon bioactive compounds contributing to lipid profile ameliorating effects: administration of 40 mg of CAL per kg body weight decreased the levels of plasma triglycerides, nonesterified fatty acid, and cholesterol in obese mice on a high-fat diet.[12,13] However, an impact of CAL on fatty acid uptake in the gut directly has not been reported yet. Further indication for an antiobesity activity of CAL was demonstrated by its ability to inhibit adipogenesis in vitro when applied in concentrations of 10−40 μM and to reduce body weight and visceral fat depots in diabetic subjects after receiving a dose of 40 mg/kg body weight for 1 month.[12] Its mode of action is reflected not only by regulating the expression of adipogenic transcription factors and enzymes

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involved in the maintenance of lipid homeostasis[12] but also by activating thermogenic and metabolic actions, which was recently shown in mouse and human primary subcutaneous adipocytes.[14] Besides CAL, the structurally related compounds cinnamyl alcohol (CALC) and cinnamic acid (CAC), naturally occurring in cinnamon plants as well, are being discussed as potential candidates to beneficially modulate lipid homeostasis. CAC, considered a major metabolite of CAL, was found to improve body weight gain and lipid profiles in the blood by inhibiting lipid digestive enzymes in diet-induced obese rats after long-term treatment with 30 mg/kg[15] and, similar to CAL, to inhibit adipogenesis via C/EBPα and PPARγ pathways, stimulate lipid catabolism, and induce white fat browning in concentrations of 50−200 μM.[16] In addition, CALC has also been suggested to be a potential agent in prevention or treatment of obesity, suppressing adipocyte differentiation in vitro as well.[17]

Apart from modulating food intake via inhibition of macronutrient uptake, reduced energy intake due to increased satiety or an impact on satiety-related hormones might be another target of antiobesity compounds. In this context, cinnamon and cinnamon-derived aroma compounds have been linked to effects on mechanisms regulating satiety after short- or long-term application.[9,18,19] Again, CAL has been proposed as a potential candidate for causing several satiety-related in vivo effects associated with cinnamon supplementation. In mice, a reduction of cumulative food intake as well as a delay in gastric emptying was found after a CAL gavage of 250 mg/kg.[19] Finally, CAL was also reported to mediate the release of satiety-related gastrointestinal (GI) hormones, including PYY after a 0.1−10 mg/kg body weight administration to mice[20] or serotonin after administration of a concentration of 500 μM to mice.[21] In these pathways, the transient receptor potential cation channel A1 (TRPA1), a nonselective cation channel expressed in neuronal and non-neuronal cells such as enterochromaffin (EC) cells in the GI tract, has been suggested to act as a sensor molecule.[21] CAL, a known potent TRPA1 agonist, has, therefore, been shown to play a role in TRPA1-dependent hormone secretion.[19−21] Besides CAL, it cannot be excluded that other cinnamon constituents have an effect on food intake. Cinnamyl isobutyrate (CIB), for instance, also occurring naturally in the essential oil of cinnamon bark in very low concentrations, decreased total energy intake from a standardized breakfast in moderately overweight men when administered in bolus amounts of 0.45 mg.[22]

Altogether, evidence suggests an impact of cinnamon and several of its constituents on mechanisms and pathways associated with lipid homeostasis. Although most of the aforementioned cinnamon compounds have been studied in more detail concerning long-term effects on the lipid metabolism after their absorption in the gut, not much is known regarding their short-term impact on intestinal lipid uptake. Because (1) a reduction of plasma lipids could be demonstrated repeatedly and (2) lipid concentrations in the plasma are modifiable through dietary changes such as reduced fat intake, we hypothesized an impact of CAL and selected structural analogues on fatty acid uptake in enterocytes as the first point of contact. As an in vitro model for the intestinal barrier, differentiated Caco-2 cells were chosen. Moreover, the stimulating effect of CAL on serotonin release from enriched rat small intestine EC cell fractions as well as from rat and human pancreatic endocrine cell lines has been reported.[21,23] Mechanistically, short-chain fatty acid uptake in the gut has also been associated with serotonergic pathways by increasing serotonin synthesis in EC cells and its excretion into the lumen.[24,25] Serotonin as an important signaling agent throughout the gut is involved in various GI processes.[26] Therefore, we also hypothesized a mediating effect of CAL on serotonin release in differentiated Caco-2 cells as an enterocyte model, possibly via involvement of TRPA1, and a potential impact on fatty acid uptake. Hydrolysis and metabolization of cinnamon derivatives have been reported to occur quickly in vivo.[27] Therefore, we collectively aimed to investigate the effect of several structurally related cinnamon compounds or potential metabolites of each other in order to allow a comparison of their effectiveness in one system. To investigate the potential bioavailability and intestinal cellular uptake of the cinnamon constituents tested, an in vitro transport study using a differentiated Caco-2 cell monolayer as the model for the intestinal barrier was carried out in a first step. Cinnamon derivatives chosen comprised CIB representing a cinnamic ester, CALC, CAL, and CAC. All of them are flavoring substances (EFSA, Regulation EU 872/2012) that have been categorized as "Generally Recognized As Safe" (GRAS) by FEMA[27] and were applied in concentrations roughly following but not exceeding maximum average use levels.

MATERIALS AND METHODS

Chemicals. All chemicals and reagents were purchased from Sigma-Aldrich (Vienna, Austria) unless stated otherwise. All cinnamon compounds were of >97% purity. The human colon carcinoma cell line Caco-2 was purchased from ATCC.

Cell Culture. Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2% L-glutamine, and 1% penicillin/streptomycin at 37 °C and 5% CO_2 at humidified atmosphere. Cells were passaged at 80−90% confluence and used until passage 25. Upon differentiation for 21 days, Caco-2 cells develop enterocyte-specific characteristics and can be used as a model for the intestinal brush border membrane.[28] For cell culture experiments, cells were seeded in the desired plate formats, and the medium was changed every second until the third day. Cells were used for experiments on day 21 ± 1.[29]

Stock solutions of the test compounds CAL, CAC, CALC, CIB, thapsigargin (TG), and oligomycin were prepared in ethanol, whereas 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), dorsomorphin (compound c), calcimycin (A23187), 9-β-D-arabinofuranosyladenine (ara-A), 4-(4-chlorophenyl)-3-methylbut-3-en-2-oxime (AP-18), and sulfo-*N*-succinimidyl oleate (SSO) were prepared in dimethyl sulfoxide (DMSO). Final concentrations of ethanol and DMSO never exceeded 0.2% on the cells.

Cell Viability. To exclude negative effects of all test compounds on metabolic activity, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were carried out using a 96-well format.[28] For this purpose, cells were starved in a medium devoid of FBS as well as FBS- and glucose-free medium before adding the test compounds alone or combinations thereof diluted in Hanks' balanced salt solution supplemented with 20 mM HEPES (HBSS/HEPES) for 120 min and 3 h, respectively. After treatment with the test compounds, the medium was substituted by the MTT working solution containing a final concentration of 0.83 mg/mL of MTT diluted in phosphate-buffered saline (PBS). After an incubation time of 15−20 min, the MTT working solution was removed and replaced by DMSO, added to solve purple formazan. Determination of absorbance was carried out at 550 nm with 690 nm as a reference wavelength using a multiwell plate reader (Tecan infinite M200, Tecan Austria). To calculate changes in metabolic activity, absorbance measurements were assessed relative to untreated control cells.

Trans-Well Studies and Identification of Test Compounds by Gas Chromatography−Mass Spectrometry. After a 21 day

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Table 1. Sequence of Primers Used in qRT-PCR Experiments

target	forward primer	reverse primer
HTR1A	TCATCGTGGCTCTTGTTCTG	CGGGGTTAAGCAGAGAGTTG
HTR1B	CTGGTGTGGGTCTTCTCCAT	AGAGGATGTGGTCGGTGTTC
HTR2A	GTTGCTTACTCGCCGATGATA	TGCCAAGATCACTTACACACAAA
HTR2C	GCGGTGCATTCATTCCTTGTGCA	TGAAGCGTCCACCATCGGAGGT
HTR3C	TTCCGGTCTCACTGCCTATATC	AAGGTGAAGGTACAGTTCTGTTG
CD36	TGTAACCCAGGACGCTGAGG	GAAGGTTCGAAGATGGCACC
GAPDH	AGGTCGGAGTCAACGGATTTG	GGGGTCATTGATGGCAACAATA
HPRT	CCTGGCGTCGTGATTAGTGA	CGAGCAAGACGTTCAGTCCT

differentiation in trans-well inserts of a six-well plate format, the medium from basolateral and apical compartments was aspirated, and Caco-2 cells were washed with PBS. Afterward, CAL, CIB, CALC or CAC were added into the apical chamber in a final concentration of 3 mM (solved in HBSS/HEPES with 0.1% ethanol) in DMEM without serum, glucose, L-glutamine, and phenol red for 180 min at 37 °C. Incubation medium without test compounds was added to the basolateral chamber. To reduce the loss of the compound because of evaporation, plates were sealed with the foil, and to ensure integrity of the cell monolayer, the transepithelial electrical resistance (TEER) was measured after 30, 90, and 180 min. After treatment of the cells, cultivation media of apical and basolateral chambers were collected and stored at −80 °C for further sample preparation. For quantitation, an internal standard Benz-$^{13}C_6$-aldehyde was added to the samples obtained from the uptake assay, before they were extracted with 2 × 500 μL of methyl *tert*-butyl ether and filtered through a nylon filter (0.2 μM, Phenomenex, Aschaffenburg, Germany). In the case of CAC detection, caffeic acid was used as an internal standard, and after the solvent was evaporated with a stream of nitrogen, N,O-bis-(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane and pyridine were added to the extraction sample and heated at 60 °C for 1 h as described by Isodorov and Vinogorova (2003).[30] Samples were analyzed using a Shimadzu GCMS-QP 2010 Ultra (Shimadzu, Vienna, Austria) equipped with a capillary column (ZB-Wax Zebron capillary column, 30 m × 0.25 mm, 0.25 μm film thickness). Helium was used as a carrier gas at a constant flow rate of 1.02 mL/min. A sample volume of 1 μL was injected in splitless mode at 40 °C column oven temperature, and a temperature rise to 280 °C was programmed at a rate of 9 °C/min and held for 5 min. Limits of detection were determined to be 0.04 μg/mL for CAL, 0.05 μg/mL for CALC, 0.05 μg/mL for CIB, and 0.10 μg/mL for CAC.

Fatty Acid Uptake. To assess the uptake of free fatty acids in differentiated Caco-2 cells, the QBT fatty acid uptake kit (Molecular Devices Germany GmBH, Germany) was used following manufacturers' instructions. The assay is based on the trafficking of fluorescently labeled fatty acid analogue BODIPY-C12 into the cells, resulting in intracellular fluorescence signals accompanied by quenching of extracellular fluorescence at the same time. For implementation of the assay, Caco-2 cells were differentiated in 96-well plates and starved for 1 h in a serum-free medium. Afterward, cells were treated with the cinnamon test compounds (0.3−3000 μM) diluted in HBSS/HEPES for 30 min at 37 °C. For mechanistic experiments, cells were cotreated with AMP-activated protein kinase (AMPK) activators AICAR (1 mM) and oligomycin (1 μM), CD36 inhibitor SSO (10 and 100 μM), calcium ionophore A23187 (10 μM), or sarco-/endoplasmic reticulum calcium ATPase (SERCA) inhibitor TG (5 μM) diluted in HBSS/HEPES for 30 min at 37 °C. For further coincubation experiments, AMPK inhibitors compound c (10 μM) and ara-A (1 mM), the calcium/calmodulin-dependent protein kinase kinase (CaMKKβ) inhibitor KN-93 (10 μM), and the TRPA1 inhibitor AP-18 (5 μM) were added 20 min prior to the test compounds. The concentrations of AICAR,[31,32] oligomycin,[33] compound c,[34] and ara-A[35,36] were based upon their use as activators and inhibitors in cardiomyocytes and oocytes after negative effects on cell viability were excluded. Subsequently, the loading dye, containing BODIPY-C12 and 0.2% essentially fatty acid-free BSA diluted in HBSS/HEPES, was added. Fluorescence was assessed for 60 min at 515 nm emission and 485 nm excitation every 20 s using a Tecan plate reader (Tecan infinite M200, Tecan Austria). Area under the curves (AUCs) from corresponding signal/time plots were calculated using SigmaPlot and determined relative to untreated control cells (100%).

Custom-Made DNA Microarrays. The effects of CAL, CIB, CALC, and CAC on the genomic level in Caco-2 cells were examined applying customized cDNA microarrays as described before.[37,38] Whole genome DNA microarrays, including 4−15 60mer oligonucleotide probes of genes chiefly associated with the metabolic cascade of satiety, were synthesized on glass slides implementing the method of maskless array synthesis.[39,40] Briefly, Caco-2 cells were differentiated in a six-well format and incubated with 300 μM CAL, CIB, CALC, and CAC for 3 h. RNA was isolated applying the RNeasy Mini Kit (Qiagen, Hilden, Germany), and labeling was accomplished during transcription of the corresponding cDNA using Cy-3-labeled random nonamer primers (Tebu Bio, Offenbach, Germany) as described by Ouellet et al.[41] Hybridization of labeled cDNA with the microarrays was carried out for 24 h at 42 °C, subsequently scanning the arrays using an Axon GenePix 4100A microarray scanner (Molecular Devices, Sunnyvale, CA, USA). Scanned images were then evaluated by the NimbleScan 2.1 software (NimbleGen, Madison, USA), performing a robust multichip analysis for normalization. To identify genes that show the highest degree of regulation after treatment with the cinnamon test compounds, fold changes were calculated using Microsoft Excel 2017. Subsequently, regulated genes were analyzed using pathway analysis software DAVID (DAVID; http://david.abcc.ncifcrf.gov) after extracting fold changes below 0.5 or above 2 to identify and select genes showing potential regulation by the test compounds for further validation by quantitative real-time PCR (qRT-PCR).

Quantitative Real-Time PCR. After differentiation in a six-well plate format, Caco-2 cells were treated with 300 μM CAL, CIB, CALC, or CAC for 3 h. After washing the cells with PBS, RNA was isolated using a RNA isolation kit (MasterPure Complete DNA & RNA Purification Kit, Biozym), followed by reverse transcription with the high capacity cDNA Kit (Life Technology, Carlsbad, CA, USA). The qRT-PCR measurements were performed in triplicates using SYBR Green MasterMix (Life Technology, Carlsbad, CA, USA) on a StepOnePlus device before being analyzed by LinRegPCR v.2012.2. Gene expression data were normalized to *HPRT*[42] and *GAPDH*[29] reference genes. *HTR1A*,[38] *HTR1B*,[38] *HTR2A*,[38] and *CD36*[42] primer sequences were applied as published by Rohm et al. (2013)[38] and Riedel et al. (2014).[42] Sequences of reverse and forward primers are shown in Table 1.

Serotonin Release. Caco-2 cells were seeded in a 12-well plate format and differentiated for 3 weeks. In a first step, cells were washed with PBS, before they were treated with Krebs Ringer HEPES buffer as a control and 300 μM CAL, CIB, CALC, and CAC diluted in Krebs Ringer HEPES buffer (pH 7.4). To investigate the involvement of TRPA1, the inhibitor AP-18 (30 μM) was added 20 min prior to the test compounds. After a 5 min incubation period, the supernatant was removed for the analysis of serotonin concentration using a serotonin-sensitive ELISA kit (DLD Diagnostika, Hamburg, Germany), which was applied according to manufacturers' protocol.

Neurotransmitter Uptake. To investigate the effect of the cinnamon test compounds on serotonin uptake into Caco-2 cells, the

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Neurotransmitter Transporter Uptake Assay Kit (Molecular Devices Germany GmBH, Germany) was applied. The assays determine the transport of a fluorescent analogue of biogenic amine neurotransmitters using a quencher technology, allowing only determination of intracellular fluorescence intensity. For experiments, Caco-2 cells were cultivated in 96-well plates. Cultivation medium was removed in the first step, before a 10 min pretreatment with the test compounds diluted in HBSS/HEPES buffer was carried out. For coincubation experiments with AP-18 and test compounds, the inhibitor (5 μM) was added 20 min prior to CAL or CALC. After pretreatment with test compounds, the loading dye was transferred onto the cells. Fluorescence was monitored in real time for 60 min at 520 nm emission and 440 nm excitation using a Tecan plate reader (Tecan infinite M200, Tecan Austria). AUCs were assessed using SigmaPlot and calculated relative to control cells treated with HBSS/HEPES alone (100%).

Transepithelial Electrical Resistance. To assess TEER, trans-well inserts (Sarstedt, 0.4 μm pore size) in a six-well plate format were used to differentiate Caco-2 cells. After a 1 h starvation in serum-free DMEM, CAL, CIB, CALC, and CAC dissolved in HBSS/HEPES (0.1% ethanol) were added to the upper compartment in a final concentration of 3 mM. TEER values were measured applying the EVOM resistance meter (World Precision Instruments, Germany) and the EndOhm chamber (World Precision Instruments, Germany) after treatment periods of 30, 90, and 180 min at 37 °C. The corresponding unit area resistance ($\Omega \cdot cm^2$) was determined by multiplication with the surface area of the trans-well insert.

Statistical Analysis. In vitro data are displayed as means ± SD, unless stated otherwise, as well as fold changes (T/C) from at least three biological and two technological replicates. To exclude outliers from statistical analysis, the Nalimov outlier test was applied. To test significant differences between test compounds versus untreated control cells, one-way analysis of variance (ANOVA) followed by the Holm−Sidak post hoc test, Kruskal−Wallis one-way ANOVA on Ranks followed by Dunn's method as well as Man−Whitney Rank Sum Test was applied, whereas a two-way ANOVA was carried out to test differences between different treatments. Statistical analysis was performed using SigmaPlot 13.0.

RESULTS

Cell Viability. MTT assays were applied to exclude negative effects on cell viability after the treatment of differentiated Caco-2 cells with the test compounds CAL, CIB, CALC, and CAC in concentrations up to 3 mM for 3 h, as well as after treatment with combinations of 300 μM CAL, CALC, or CAC with or without AP-18 (5 μM) for 90 or 110 min. Cell viability was also determined after a 90 or 110 min treatment of differentiated Caco-2 cells with AICAR (1 mM), oligomycin (1 μM) as well as compound c (10 μM) and ara-A (1 mM) alone and after cotreatment with 300 μM CAL. Additionally, the CaMKKβ inhibitor KN-93 (10 μM) as well as calcium ionophore A23187 (10 μM) and TG (5 μM) were tested for 90 or 110 min. No significant differences ($p < 0.05$) were detected between treatments and untreated control cells (data not shown).

Cellular Uptake of CAL, CIB, CALC, and CAC. To investigate the intestinal bioavailability of the test compounds CAL, CIB, CALC, and CAC as a requirement and indicator for potential effects on intracellular pathways, their transport through a confluent Caco-2 cell monolayer was analyzed. The TEER was measured to ensure integrity of the monolayer. Untreated, differentiated Caco-2 cells showed a mean TEER of 420 $\Omega \cdot cm^2$, which is comparable to those of other studies.[28] After a 180 min apical treatment with 3 mM of CAL, 295 ± 56.7 μM CAL was recovered apically, whereas 7.53 ± 3.67 was recovered in the basolateral incubation medium (Supporting Information, Table S1). In the apical incubation medium, also CALC, hydrocinnamaldehyde, and 3-phenylpropanol were detected, pointing to degradation and metabolization of CAL. No CIB was detected in the incubation medium after a mean apical and basolateral recovery of 180 ± 23.2 and 5.19 ± 4.34 μM, respectively. CAC was detectable in traces but not quantifiable after 180 min of exposure.

Effects of CAL, CIB, CALC, and CAC on Fatty Acid Uptake in Differentiated Caco-2 Cells. In order to investigate the impact of CAL, CIB, CALC, and CAC on fatty acid uptake, Caco-2 cells were pretreated with each test compound in a concentration range of 0.3−3000 μM. CAL in concentrations of 30−3000 μM reduced fatty acid uptake in Caco-2 cells dose-dependently by up to 58.0 ± 8.83% ($p < 0.001$) compared to nontreated controls (Figure 1). CAC and CALC in a concentration of 3000 μM also decreased fatty acid uptake by 21.9 ± 6.55 and 19.4 ± 8.98% ($p < 0.001$), respectively. In contrast, no effect was seen for CIB.

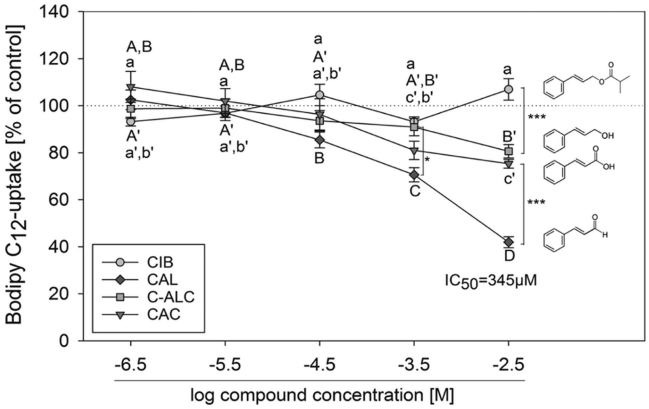

Figure 1. Fatty acid uptake after a 30 min pretreatment with CAL, CIB, CALC, and CAC in concentrations of 0.3−3000 μM. Values are displayed as mean ± SEM in percent compared to the control of 100 ± 1.34%; (buffer with 0.1% ethanol, displayed as the dotted line). n = 3−8 (tr = 2−3). The IC_{50} value for CAL was calculated using one-site competition curve fitting in SigmaPlot 13.0. Significant differences between control and treatments are tested with a one-way ANOVA, followed by the Holm−Sidak post hoc test, and significant differences between treatments are tested with a two-way ANOVA. Statistical differences within treatments are marked with different letters (ctrl = A, a, A′, a′), and differences between treatments are marked with *$p < 0.05$ and ***$p < 0.001$.

Effects of CAL, CIB, CALC, and CAC on Gene Expression in Caco-2 Cells. The impact of the selected cinnamon-derived compounds CAL, CIB, CALC, and CAC in a concentration of 300 μM on gene expression in Caco-2 cells was screened applying customized DNA microarrays after a 3 h treatment.[38,40] Scatterplots (Supporting Information, Figure 1) illustrating the effects of 300 μM cinnamon compounds showed changes in gene regulation following exposure of the cells to each of the compounds compared to the untreated control cells. Altogether, 805 and 1095 genes with fold changes below 0.5 or above 2 were determined after treatment with 300 μM CAL and CIB, respectively, revealing more pronounced effects compared to CAC and CALC. To determine changes in individual gene expression, an enrichment analysis of gene expression data was performed by applying the functional annotation clustering software DAVID using genes with fold changes < 0.5/>2 as thresholds (Supporting Information,

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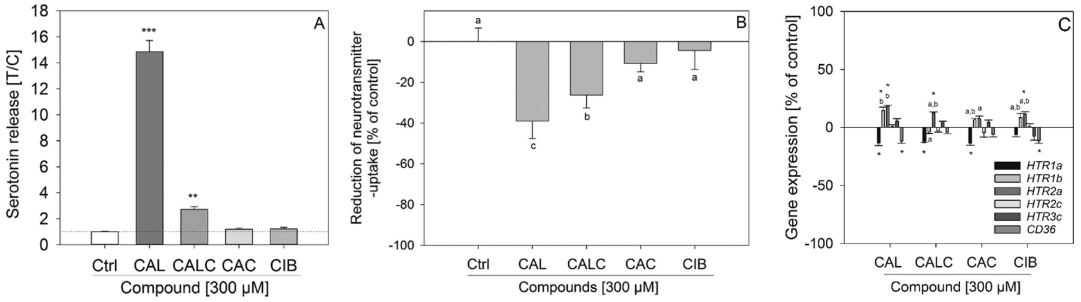

Figure 2. (A) Serotonin release after 5 min stimulation with 300 μM CAL, CIB, CALC, and CAC. Values are presented as mean ± SEM in % compared to control (buffer with 0.1% ethanol, set to 100%); n = 4−8 (tr = 1−2). Significant differences between treated and untreated control cells are tested with a Kruskal−Wallis one-way ANOVA on ranks, followed by Dunn's method. Significant differences between control and treatments are marked with ***p < 0.001 and **p < 0.01. (B) Neurotransmitter reuptake after a 10 min stimulation with 300 μM CAL, CALC, CIB, and CAC. Values are shown as mean ± SD in percent compared to control (buffer with 0.1% ethanol, set to 0%); n = 5 (tr = 2−3). Significant differences between different treatments are tested with a one-way ANOVA, followed by the Holm−Sidak post hoc test and marked with different letters (ctrl = a). (C) Gene expression levels for *HTR1A*, *HTR1B*, *HTR2A*, *HTR2C*, *HTR3C*, and *CD36* after treatment with 300 μM CAL, CIB, CALC, and CAC. Data are shown as mean ± SEM in % compared to the controls of 0.00 ± 1.06−3.73% (buffer with 0.1% ethanol, displayed as the dotted line); n = 4−5. Significant differences versus controls are tested with a one-way ANOVA, followed by the Holm−Sidak post hoc test and marked with *. Significant differences between treatments are tested with a Kruskal−Wallis one-way ANOVA on ranks, followed by Dunn's method and marked with different letters.

Table S2). Clusters with the highest enrichment scores (CAL: 7.14; CALC: 5.56; CAC: 9.45; CIB: 7.04) indicated an involvement of genes linked to serotonin receptor activity as well as the fatty acid transporter CD36. Gene expression of selected serotonin receptors as well as of fatty acid translocase CD36 was validated by the more sensitive qRT-PCR method. As shown in Figure 2C, a regulation on the gene expression level was demonstrated for several serotonin receptors. CAL in a concentration of 300 μM was shown to downregulate the gene expression of *HTR1A* and to upregulate that of *HTR2A* and *HTR1B* after a 3 h treatment. CIB treatment also resulted in an upregulation of *HTR2A*. CALC showed a downregulation of *HTR1A* and an upregulation of *HTR2A*, whereas CAC only showed an effect on *HTR1A*. Moreover, *CD36* levels were downregulated after 3 h treatment of Caco-2 cells with CIB and CAL.

Effects of CIB, CAL, CALC, and CAC on Serotonin Release and Uptake in Differentiated Caco-2 Cells. Because a regulation on gene expression was demonstrated for several serotonin receptors after a 3 h incubation with 300 μM CIB, CAL, CALC, or CAC, their potential impact on serotonin release was tested. Caco-2 cells were stimulated with the selected cinnamon compound for 5 min in a concentration of 300 μM, respectively. As presented in Figure 2A, CAL treatment enhanced the serotonin release 14.9 ± 3.00-fold (p < 0.001) compared to nontreated controls. CALC treatment in the same concentration also resulted in a 2.72 ± 0.69-fold increase (P < 0.01) of serotonin levels in the extracellular supernatant compared to controls, whereas neither CIB nor CAC affected serotonin release in differentiated Caco-2 cells.

To effectively substantiate increased extracellular serotonin concentrations in the cellular supernatant, the impact of CAL, CALC, CIB, and CAC on serotonin uptake by the cells was examined as well. To study this influence on serotonin reuptake transporter (SERT) activity, cells were pretreated with the cinnamon compounds in a concentration of 300 μM each. As displayed in Figure 2B, CAL and CALC decreased serotonin uptake by the cells by 39.0 ± 8.6% (p < 0.001) and 26.3 ± 6.3% (p < 0.001), respectively. No impact of CIB and CAC, also found in the measurement of serotonin release, was substantiated in this assay as well.

Impact of Serotonin on Fatty Acid Uptake in Differentiated Caco-2 Cells. Because CAL (300 μM) and CALC (300 μM) were shown to stimulate serotonin release in Caco-2 cells, their potential impact of 5-hydroxytryptamine on fatty acid uptake was examined in a first approach. However, a 30 min pretreatment with 5-hydroxytryptamine in concentrations of 0.001−100 μM did not result in an altered fatty acid uptake (data not shown).

Impact of Cinnamon Compounds on TEER. To identify the potential effect of the selected cinnamon compounds on the paracellular barrier function, the TEER was determined at different time points over the course of 3 h. As depicted in Figure 3, no changes in the TEER values could be shown for

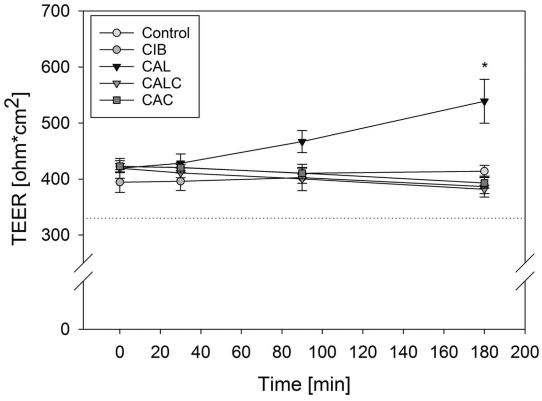

Figure 3. Evaluation of the TEER of differentiated Caco-2 cells after treatment with 3000 μM CAL, CIB, CALC, and CAC for 30, 90, and 180 min or nontreated control cells. Values are presented as mean ± SEM. n = 3−5 (tr = 1−2). The significant difference between control and treatment is analyzed with a Mann−Whitney Rank Sum Test and marked with *p < 0.01.

CIB, CAC, or CALC, indicating an intact membrane integrity. Despite a steady increase, a 90 min treatment with CAL (3 mM) did not affect TEER values (CAL: 467 ± 19.6 Ω·cm^2 vs control: 410 ± 9.12 Ω·cm^2). After a 3 h CAL treatment (3 mM), an impact on the paracellular barrier function could be

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determined via increased TEER values (CAL: 539 ± 39.1 Ω·cm^2 vs control: 414 ± 10.8 Ω·cm^2; p = 0.02).

Involvement of CD36/FAT, CaMKKβ, and AMPK Regulation in Lipid Absorption in Differentiated Caco-2 Cells. To investigate the regulating effect on the FAT/CD36 transport protein in the CAL-, CAC- or CALC-induced effect on fatty acid uptake, mechanistic experiments considering downstream signaling were carried out. A 30 min pretreatment of differentiated Caco-2 cells with the AMPK activators AICAR (1 mM) and oligomycin (1 μM), as well as AMPK inhibitors compound c (10 μM) and ara-A (1 mM) to test the potential impact on the fatty acid uptake via regulation of AMPK did not alter fatty acid uptake compared to untreated control cells (Supporting Information, Figure 2). Additionally, cotreatment of activators and inhibitors of AMPK with CAL (300 μM) did not change the fatty acid uptake compared to the decrease of CAL solely (Supporting Information, Figure 2). To further examine the involvement of the Ca^{2+}-dependent CaMKKβ acting upstream of AMPK, cells were incubated with the CaMKKβ inhibitor KN-93 (10 μM) as well as with the calcium ionophore A23187 (10 μM) and TG (5 μM), an inhibitor of the SERCA. As shown in Figure 4, a 30 min pretreatment with this calcium ionophore (10 μM) decreased the fatty acid uptake in Caco-2 cells by 12.8 ± 7.48% compared to the control (p = 0.04). Applying the CD36 inhibitor SSO did not reduce fatty acid uptake (data not shown).

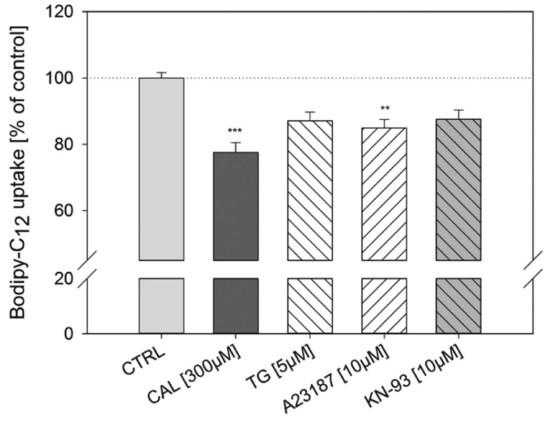

Figure 4. Fatty acid uptake after a 30 min pretreatment with CAL (300 μM), TG (5 μM), calcium ionophore A23187 (10 μM), and KN-93 (10 μM). Values are presented as mean ± SEM in % compared to control (buffer with 0.1% ethanol, set to 100%). n = 5−6 (tr = 2−3). Significant differences between treatments are tested with a one-way ANOVA, followed by the Holm−Sidak post hoc test. Differences between control and treatments are marked with **$p \leq$ 0.01 and ***$p \leq$ 0.001.

Involvement of TRPA1 in Decreased Fatty Acid Uptake in Differentiated Caco-2 Cells. To investigate the TRPA1 involvement in the CAL- or CALC-mediated effect on the fatty acid uptake, Caco-2 cells were incubated with the TRPA1 inhibitor AP-18 (5 μM) for 20 min, prior to the addition of the test compounds. As shown in Figure 5, inhibition of TRPA1 channels lowered the effect of the TRPA1 agonist CAL (300 μM) on fatty acid uptake by 9.97 ± 9.57% (p = 0.045). The decrease in fatty acid uptake after 3 mM CALC treatment by 19.7 ± 6.25% was not changed by TRPA1 inhibition. Pretreatment with antagonist AP-18 also did not affect the CAC-induced decreased fatty acid uptake (data not shown).

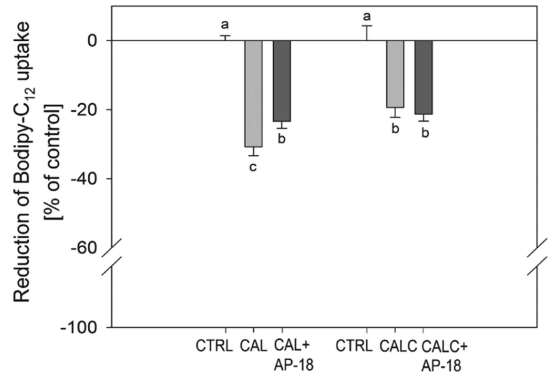

Figure 5. Reduction of fatty acid uptake after a 30 min pretreatment with 300 μM CAL and 3 mM CALC alone and after cotreatment with the TRPA1 inhibitor AP-18 (5 μM). AP-18 was added 20 min prior to the test compounds. Values are displayed as mean ± SEM in percent compared to control (set to 0%). n = 6 (tr = 1−3). Significant differences between treatments are tested with a one-way ANOVA, followed by the Holm−Sidak post hoc test and marked with different letters (ctrl = a).

Involvement of TRPA1 in CAL- and CALC-Induced Effect on Serotonin Release and Serotonin Uptake in Differentiated Caco-2 Cells. Examining a potential TRPA1 involvement in the CAL- and CALC-mediated increase in serotonin release, differentiated Caco-2 cells were pretreated with the TRPA1 inhibitor AP-18 for 20 min before test substances were added. As displayed in Table 2A, inhibition of TRPA1 decreased CAL-induced serotonin release by up to 30.7 ± 12.1% (p < 0.001) compared to the effect of CAL alone. Pretreating the cells with AP-18, however, did not lower the effect of CALC on serotonin release (CALC: 100 ± 9.00-% vs. CALC + AP-18: 86.3 ± 12.8-%) (Table 2A).

Table 2. (A) Serotonin Release after a 5 min Stimulation with 300 μM CAL or CALC Alone (Set to 100%) and after Cotreatment with the TRPA1 Inhibitor AP-18 (30 μM)[a]; (B) Neurotransmitter Uptake after a 10 min Stimulation with 300 μM CAL and CALC Alone (Set to 100%) and after Cotreatment with the TRPA1 Inhibitor AP-18 (5 μM)[b]

(A)	CAL (%)	CALC (%)
compound (300 μM)	100 ± 12.7^a	100 ± 9.00^a
compound + AP-18 (30 μM)	69.3 ± 12.1^b	86.3 ± 12.8^a
(B)	CAL (%)	CALC (%)
compound (300 μM)	100 ± 0.57^a	100 ± 5.01^a
compound + AP-18 (5 μM)	109 ± 2.77^b	103 ± 13.8^a

[a]AP-18 was added 20 min prior to test compounds. Values are presented as mean ± SEM in % compared to CAL/CALC (set to 100%); n = 4−5 (tr = 2). Significant differences between treatments are tested with a one-way ANOVA, followed by the Holm−Sidak post hoc test and marked with different letters (CAL or CALC alone = a). [b]AP-18 was added 20 min prior to the test compounds. Values are shown as mean ± SD in percent compared to control (set to 100%); n = 3−4 (tr = 1−3). Significant differences between treatments are tested with a one-way ANOVA, followed by the Holm−Sidak post hoc test and marked with *p < 0.05.

The TRPA1-dependent effect of cinnamon compounds on serotonin reuptake in Caco-2 cells was tested as well by adding the AP-18 inhibitor 20 min prior to the test compounds. As

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displayed in Table 2B, TRPA1 inhibition decreased the CAL-induced effect on neurotransmitter uptake into the cells by 9.30 ± 2.77% ($p = 0.04$), whereas no TRPA1 involvement was demonstrated for CALC.

DISCUSSION

Cinnamon and its constituents, first and foremost CAL, have been studied extensively not only in the context of glucose metabolism[8,9,19] but also for their antiobesity activities and their impact on food intake and mechanisms regulating satiety.[9,10,19] In vivo evidence suggests ameliorating effects on parameters associated with lipid homeostasis, including decreased accumulation of visceral fat and triglyceride and fatty acid plasma levels after administration of cinnamon (1 g/day) or CAL (10−40 mg/kg body weight).[11,12,19,43] These reported hypolipidemic effects of CAL not only point to anti-adipogenetic effects[12] but also indicate an impact on dietary lipid absorption. In the present work, the influence of selected cinnamon-derived aroma compounds on intestinal fatty acid uptake was investigated in differentiated Caco-2 cells as an in vitro enterocyte model constituting the first point of contact of dietary lipid absorption in the gut. The cinnamon compounds tested included CAL, the major constituent of cinnamon bark oil, as well as its structural analogues or potential metabolites formed in vivo, CALC, CAC, and the cinnamic ester CIB. CIB was recently demonstrated to have an effect on total energy intake in a short-term human intervention study.[22]

To investigate the intestinal cellular uptake of the cinnamon constituents tested, an in vitro transport study using a differentiated confluent Caco-2 cell monolayer as the model for the intestinal barrier was carried out in a first step. In general, recovery in apical and basolateral compartments was low, probably because of the high volatility of the test compounds. Nevertheless, CAL and CALC were detected on the basolateral side, indicating an uptake and transport through the cells. Traces of CALC, hydrocinnamaldehyde, and 3-phenylpropanol were detected apically as metabolites or degradation products of CAL, and CAL was also detected as the potential metabolite of CALC. As reviewed by Adams et al. (2004),[27] for cinnamyl derivatives, a rapid absorption and metabolization have been shown, as evidenced by their excretion from the body within 24 h.[27] In vivo oxidation of primary alcohols to aldehydes and, subsequently, carboxylic acids has been reported to be catalyzed by alcohol and aldehyde dehydrogenases.[27] While Caco-2 cells were shown to express aldehyde dehydrogenase isoenzymes, no alcohol dehydrogenase activity was found,[44] pointing to a non-enzymatic oxidation of CALC to CAL in the incubation medium of the here presented experiments. Moreover, CIB was not detected at all, suggesting evaporation or hydrolysis and formation of metabolites such as CALC, which was determined on the apical side after a 3 h CIB treatment. In general, aromatic esters have been demonstrated to hydrolyze quickly into its respective components before, during, or after being absorbed in the gut.[27] CAC, which was used at 300 μM due to significantly increased membrane permeability in higher concentrations in the low glucose incubation medium, was detectable in traces in the apical but not in the basolateral compartment, possibly due to lower concentrations, losses in sample preparation, or the formation of metabolites not detectable by gas chromatography−mass spectrometry. Collectively, data from trans-well uptake studies revealed the highest recovery and uptake for the test compound CAL.

Analyzing the impact of the test compounds on fatty acid uptake in Caco-2 cells demonstrated CAL to dose-dependently and most effectively decrease fatty acid uptake. CAC and CALC in higher concentrations also reduced fatty acid uptake, whereas no reduction on fatty acid uptake could be determined for the cinnamyl ester. Taking into account that quantitative analysis of commercial cinnamon powder by Friedman et al. (2000)[45] revealed a mean concentration of 8.2 ± 0.5 mg CAL per gram, an amount of about 50 mg of CAL, corresponding to about 400 μM, could theoretically reach the intestines following an intake of 6 g of cinnamon powder, an amount that has been administered in human intervention studies before.[9] To the best of our knowledge, we could show for the first time an impact of CAL, CAC, and CALC on fatty acid uptake in an enterocyte cell model, revealing CAL as the most promising metabolite. An influence of CAL on lipid metabolism, however, has already been reported. The antiobesity effects of CAL were shown in vitro and in vivo by inhibiting adipogenesis in adipocytes and reducing visceral fat depots, respectively.[46] In 3T3-L1 preadipocytes, a 4 day cell treatment with CAL (10−40 μM) decreased lipid accumulation accompanied by the downregulated expression of peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT/enhancer-binding proteins (C/EBPs), essential transcription factors in downstream activation of adipocyte related genes.[12] Administration of CAL (40 mg/kg bw) to mice on a high-fat diet resulted in lowered body weight gains, plasma triglyceride, and free fatty acid levels compared to a control group, suggesting a modulated lipolytic activity as well.[12] Recently, CAC was also suggested as a further antiobesity agent, decreasing C/EBPα and PPARγ expression in 3T3-L1 cells, stimulating lipid catabolism, and inducing white fat browning.[16] However, as far as we could ascertain, mechanisms of action regarding the inhibitory effect of CAL, CALC and CAC on lipid absorption have not been investigated so far.

To gain first insights into potential mechanistic pathways that might be involved in the modes of action underlying the observed decrease in fatty acid uptake, customized DNA microarrays were applied to screen CAL, CIB, CALC, and CAC concerning a potential impact on gene expression in differentiated Caco-2 cells. The impact on fatty acid uptake was screened in a concentration range of 0.3−3000 μM. For mechanistic studies, a higher concentration of 300 μM was chosen because only CAL showed a significant effect at a lower concentration of 30 μM. Scatterplots displaying treatment versus control showed changes in gene expression for all compounds but revealed more regulated genes after a 300 μM CAL and CIB incubation. A pathway analysis applying the database for annotation, visualization, and integrated discovery (DAVID) for regulated probes was performed. Interestingly, the cluster with the highest enrichment score after 3 h CAL incubation identified by functional annotation clustering did not primarily reveal an impact on pathways associated with lipid metabolism but indicated a distinctive impact of CAL on serotonin receptor activity and signaling pathways. A closer examination of the highest scoring clusters of the other cinnamon compounds also suggested a regulative but less prominent effect on genes associated with serotonin receptor activity. To validate this impact, the gene expression of selected serotonin receptors, that have been previously associated with mechanisms regulating satiety,[47−51] was analyzed by qRT-PCR after a 3 h compound treatment at a concentration of 300 μM. These results confirmed a regulation on the gene expression

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level for several serotonin receptors. Therefore, we hypothesized an impact of the test compounds on serotonin release, which in turn might influence lipid absorption. Indeed, stimulation with 300 μM CAL strongly increased serotonin release in differentiated Caco-2 cells, and CALC in the same concentration also raised serotonin levels in the cellular supernatant. CAC and CIB, in contrast, did not affect serotonin release at all. These results demonstrated that CAL, which has been reported to release serotonin from enriched rat small intestine EC cell fractions as well as from rat and human pancreatic endocrine cell lines,[21,23] also induced 5-HT release from differentiated Caco-2 cells as a human enterocyte in vitro model.

In this context, uptake of serotonin into the cell, which is dependent on the SERT, also plays a pivotal role.[52] To assess if a CAL or CALC stimulation of Caco-2 cells effectively results in enhanced extracellular serotonin concentrations and not only in short-term serotonin release, their impact on the uptake of a neurotransmitter imitating fluorophore was tested. The results on neurotransmitter uptake after pretreatment with 300 μM CAL or CALC are in agreement with their elevating effects detected on serotonin release. Both compounds decreased the neurotransmitter uptake, suggesting not only a stimulating effect on serotonin release but also an inhibiting effect on SERT activity. Expression levels of SERT in cell membranes are dependent on SERT trafficking processes between the cell interior and surface.[52] Besides selective SERT inhibitors, decreasing serotonin transport into the cell by directly binding to SERT, compound interactions with intracellular proteins and kinase/phosphate systems might be involved in the regulation of SERT activity as well.[52] However, serotonin itself also plays a role in modulating the surface expression of SERT, influencing its own signaling transduction.[52] Moreover, involvement of serotonin autoreceptors, such as *5-HT$_{1B}$*, in the regulation of SERT activity has been discussed.[53] Whether CAL or CALC directly inhibits or reduces SERT activity or decreases serotonin uptake via interacting with regulatory mechanisms of SERT trafficking cannot be explained at this point. It can also not be excluded that the elevated extracellular serotonin levels additionally affect SERT activity as described above.

Serotonin, which is secreted by EC cells throughout the gut, plays an important role in multiple physiological and pathophysiological GI mechanisms via activation of the enteric intrinsic and extrinsic nervous system.[26] Primarily, this hormone is thought to be released basally into the lamina propria, where serotonin receptors of vagal afferents are located, although apical serotonin excretion has been demonstrated as well.[54,55] Peripherally circulating serotonin mostly originates from the gut, constituting the body's major serotonin source, and is determined frequently as reflection of its mucosal availability.[26] In response to mechanical, chemical, or nutritive stimuli in the lumen, serotonin functions in peristaltic activity, secretory processes, as well as in modulating pain perception and nausea.[26] Serotonin signaling is also an established key aspect in regulating food intake and causing satiety signals in the hypothalamus.[56] Peripheral serotonin, however, not being able to pass the blood−brain barrier, has been suggested to exert its satiating effects in the brain via vagal afferent fibers.[57,58] In the case of cinnamon, modulating effects on parameters linked to satiety have already been reported in humans, reflected by increased GLP-1 plasma levels after a 3 g and delayed gastric emptying rates after 6 g cinnamon administration, although an effect on subjective feelings of satiety was not detectable.[9,59] One of the cinnamon-derived compounds indicative for the regulating effect on satiety-related hormones and energy intake might be CAL, as evidenced by increased plasma PYY levels in mice after receiving a dose of 10 mg CAL per kg body weight by gavage,[20] reduced food intake in animal studies after the gavage of 250 mg/kg body weight,[19] as well as enhanced expression of anorectic hypothalamic genes accompanied by decreased HFD-evoked hyperphagia in mice after daily administration of 10 mg/kg body weight for 14 weeks.[46] Altogether, our data demonstrated increased extracellular levels of serotonin after Caco-2 cell stimulation with CAL and CALC. Additionally, a regulation on the gene expression level could be demonstrated for several serotonin receptors associated with mechanisms regulating satiety, including the serotonin auto-receptor 5-HT$_{1A}$[47] as well as 5-HT$_{1B}$,[48] 5-HT$_{2A}$,[49] after CAL and CALC treatment. These findings further emphasized that Caco-2 cells, which do not represent an enteroendocrine cell line, might be used as a potential cell model to assess serotonin release in the intestinal epithelium as previously proposed by Lieder et al. (2017).[29]

To elucidate cellular mechanisms that might have an impact of the test compounds on fatty acid uptake, the potential involvement of several mechanistic pathways has been explored. Serotonergic pathways in the gut have been linked to the presence of short-chain fatty acids by increasing synthesis and luminal serotonin concentrations.[24,25] The reverse effect of serotonin as a key regulator on fatty acid uptake has not been shown so far. Because CAL and CALC were shown to stimulate serotonin release in Caco-2 cells, the potential impact of serotonin on fatty acid uptake was hypothesized but could not be substantiated in a first approach. In an attempt to explain the reduction in fatty acid uptake via alterations in the membrane integrity of Caco-2 cells, the TEER of treated and untreated cell monolayers was monitored. As the permeability of the epithelial membrane is dependent on tight junction barrier function, which at a large part determines paracellular substrate transport,[60] the TEER was measured as a parameter for tight junction permeability. TEER values steadily increased only after the CAL treatment, demonstrating significant effects of CAL on the membrane integrity after 3 h. These results point to an effect of CAL on epithelial barrier function, possibly via the modulating effect on tight junction proteins as it was shown before in intestinal porcine epithelial cells.[61] However, our measurements did not demonstrate a significant TEER increase over the 90 min treatment length, suggesting that an impact of CAL on paracellular transport routes might not be the main mode of action in decreasing the fatty acid uptake but cannot be excluded entirely as an additional contributing factor.

Apart from passive diffusion, mechanisms on a cellular level involved in the transport of intraluminal fatty acids into the absorptive cells also include a protein-mediated transfer.[62] Gene reports in the clusters with the highest enrichment scores for CALC, CAC, and CIB as well as further clusters for CAL with lower enrichment scores identified by using functional annotation clustering for microarray analysis also revealed a regulation for gene expression of the fatty acid transporter CD36. Because the downregulation of *CD36* gene expression was shown after a 3 h treatment with 300 μM CAL and CIB by means of qRT-PCR, the potential involvement of CD36 in the decreased lipid uptake was investigated further. Because of lack

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of specific functioning CD36 activators, an activation via AMPK downstream signaling was approached because in isolated cardiomyocytes, an AMP-mediated increase in fatty acid uptake via CD36 was reported.[63] Treating cardiomyocytes simultaneously with activators of Ca^{2+}-signaling and AMPK phosphorylation further increased the AMPK-mediated substrate uptake.[63] Contrary effects have been reported for the adipose tissue, where the AMPK activating compound AICAR was found to reduce the uptake of fatty acids into the cells and to inhibit lipogenesis and fatty acid oxidation in adipocytes.[64] Moreover, AMPK was upregulated in 3T3-L1 cells after CAL treatment,[12] and CAC was found to activate AMPK signaling pathways[16] as well. Thus, we hypothesized the potential impact of the test compounds on CD36 via regulation of AMPK and CaMKKβ, which have been shown to act upstream of AMPK. However, an involvement of AMPK and CaMKKβ in the fatty acid uptake could not be confirmed. Interestingly, also the CD36 inhibitor SSO did not reduce fatty acid uptake, indicating that CD36 in general does not play a major role in the fatty acid uptake in differentiated Caco-2 cells and, therefore, appears not to be involved in the CAL-, CALC-, or CAC-mediated effect on fatty acid uptake. However, pretreatment with the calcium ionophore A23187 decreased fatty acid uptake, suggesting an involvement of Ca^{2+}-signaling. The potential effect of CAL, CALC, and CAC on other fatty transport proteins such as FATPs or FABP4 cannot be ruled out though and warrants further investigation. Finally, the CAL-induced effect on fatty acid uptake in differentiated Caco-2 cells also raised the question concerning the potential involvement of TRPA1 channels. As Ca^{2+} entrance routes, the ligand-activated cation channels might be involved in modulating multiple cellular processes.[21] CAL and other unsaturated carbonyl-containing compounds are known to covalently modify specific cysteine residues on the intracellular N-terminus of TRPA1. Thereby, an activation of the cation channel via direct addition reactions at the carbonyl carbon has been suggested to be preferred over conjugate additions at the β-carbon.[65] EC_{50} values of CAL for hTRPA1 have been reported to be 6.8 μM.[66] Compounds such as cinnamic or acrylic acid, only undergoing conjugate addition, were found to have an insignificant TRPA1 potential.[65] Moreover, calcium imaging studies using mTRPA1-expressing CHO cells further did not indicate a channel activation for CAC applied in a concentration of 1 mM.[67] When used at the same concentration of 1 mM, CALC, on the other hand, induced a calcium signal, exhibiting approximately one-third of the effect shown for 50 μM CAL, which might indicate a weak TRPA1 activation of CALC as well.[67] To investigate the potential TRPA1 dependency of the CAL-stimulated fatty acid uptake in the here presented study, coincubation experiments were conducted applying the competitive TRPA1 inhibitor AP-18. Our results revealed the CAL-induced effect to be attenuated by TRPA1 inhibition, which was neither the case for CAC nor for CALC, indicating that a TRPA1 activation might in fact play a minor role in the CAL-induced effect on fatty acid uptake in Caco-2 cells. The weak effect size, however, suggests that other pathways might be involved as well. Applying the calcium ionophore A23187 also suggested an involvement of Ca^{2+}-signaling in the CAL-mediated fatty acid uptake. However, whether calcium influx via TRPA1 also functions in the reduced fatty acid uptake needs to be investigated in further studies. Moreover, it can also not be excluded that CAL, CALC, and CAC directly affect other fatty acid transporters besides CD36 in Caco-2 cells.

Furthermore, the possibility of a TRPA1-mediated pathway involved in serotonin release was tested. Because only CAL and CALC stimulated serotonin release in Caco-2 cells and both compounds have been previously suggested to activate TRPA1,[67] we hypothesized a potential involvement of TRPA1 in serotonin secretion. Our results demonstrated that inhibition of TRPA1 decreased CAL-mediated serotonin release by about 31%. Because the effect of CAL was not completely inhibited, CAL might induce other pathways as well. Moreover, data also indicated a TRPA1-independent stimulatory effect of CALC on serotonin release. The potency of CAL to activate TRPA1 and the weak activation that has been shown for CALC could explain these findings. On the basis of these outcomes, a TRPA1 inhibition was also applied testing serotonin reuptake into the cells, showing that TRPA1 inhibition also attenuated the CAL-induced effect on neurotransmitter uptake and further substantiating a contributing impact of TRPA1 in the CAL-mediated effect on the serotonin system in Caco-2 cells. Collectively, these data suggest that TRPA1 activation, which is associated with a calcium influx, might play a role in the CAL-stimulated enhanced extracellular serotonin concentrations, although further analyses by means of, for example, Western blotting, are needed to verify the here presented results. It is acknowledged that Ca^{2+} is a required key factor in evoking exocytic pathways of chemical messengers stored in intracellular vesicles.[68] Enteroendocrine cell types in the gut respond by secretion of various hormones upon stimulation by numerous components from luminal contents. Also, it has been reported that serotonin release is regulated mainly by an extracellular Ca^{2+} influx through voltage-gated Ca^{2+}-channels.[68] Therefore, we hypothesize that a Ca^{2+}-dependent secretion of serotonin similar to other endocrine cells might be involved in the CAL-mediated pathway as well, and this occurs at least to some extent via Ca^{2+}-entry through TRPA1. To fully elucidate the mechanisms by which CAL induces fatty acid uptake in Caco-2 cells, its effects on internal calcium mobilization, which is linked to TRPA1 activation and serotonin release, need to be further investigated with respect to additional cellular pathways. Moreover, to perform an adequate correlation analysis and draw conclusive information, more concentrations and more substances need to be tested. There is a range of other structurally related compounds that might be of interest regarding a study on structure−activity relationships and are the topic of future projects.

In conclusion, analyzing the impact of several cinnamon-derived structurally related aroma compounds on fatty acid uptake and serotonin release in differentiated Caco-2 cells revealed their potential as bioactive dietary compounds to potentially target intestinal fatty acid uptake and the release of the anorexigenic hormone serotonin. Among the cinnamon constituents tested, CAL not only most potently reduced fatty acid uptake by intestinal Caco-2 cells but also showed most pronounced effects on serotonin release and TRPA1-dependent pathways.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b04274.

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Absolute and relative recovery (μM) of cinnamon compounds in apical and basolateral compartments; annotation cluster with the highest enrichment score found by DAVID using input data of microarray probes with fold changes below 0.5 or above 2 after treatment with 300 μM CAL, CIB, CALC, and CAC for 3 h; scatterplot of log 2 fluorescence intensity after 3 h incubation of Caco-2 cells with 300 μM CIB, CAL, CALC, and CAC (n=1); fatty acid uptake by Caco-2 cells after a 30 min pretreatment with CAL (300 μM), AICAR (1 mM), and oligomycin (1 μM); and undifferentiated and differentiated Caco-2 cells (PDF)

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Notes

The authors declare the following competing financial interest(s): The authors J. Hans and J.P. Ley are employees at Symrise AG, Holzminden, Germany.

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3.3 The TRPA1 agonist cinnamaldehyde decreases adipogenesis in 3T3-L1 cells more potently than the non-agonist structural analog cinnamyl isobutyrate

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In this manuscript, the anti-adipogenic and lipid-accumulation inhibiting effect of cinnamyl isobutyrate in 3T3-L1 adipocyte cells was investigated in comparison to TRPA1 agonist cinnamaldehyde. Moreover, a compound-mediated impact on key adipogenic transcription factors PPARγ, C/EBPα and C/EBPβ was analyzed.

The PhD candidate participated in the experimental design and analyzed fatty acid uptake and lipid accumulation after stimulation with structurally related cinnamon constituents cinnamaldehyde and cinnamyl isobutyrate, in the presence and absence of TRPA1 inhibitor AP-18. Moreover, the PhD candidate participated in determining gene expression and protein levels of selected key adipogenic transcription factors by performing qPCR and ELISAs. Moreover, data analysis and preparation of the publication draft was carried out by the PhD candidate.

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Article

TRPA1 Agonist Cinnamaldehyde Decreases Adipogenesis in 3T3-L1 Cells More Potently than the Non-agonist Structural Analog Cinnamyl Isobutyrate

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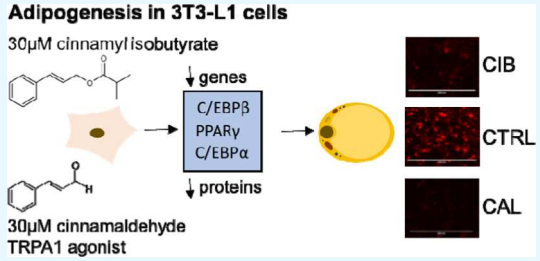
ABSTRACT: The cinnamon-derived bioactive aroma compound cinnamaldehyde (CAL) has been identified as a promising antiobesity agent, inhibiting adipogenesis and decreasing lipid accumulation in vitro as well as in animal models. Here, we investigated the antiadipogenic effect of cinnamyl isobutyrate (CIB), another cinnamon-derived aroma compound, in comparison to CAL in 3T3-L1 adipocyte cells. In a concentration of 30 μM, CIB reduced triglyceride (TG) and phospholipid (PL) accumulation in 3T3-L1 pre-adipocytes by 21.4 ± 2.56 and 20.7 ± 2.05%, respectively. CAL (30 μM), in comparison, decreased TG accumulation by 37.5 ± 1.81% and PL accumulation by 28.7 ± 1.83%, revealing the aldehyde to be the more potent antiadipogenic compound. The CIB- and CAL-mediated inhibition of lipid accumulation was accompanied by downregulation of essential adipogenic transcription factors PPARγ, C/EBPα, and C/EBPβ on gene and protein levels, pointing to a compound-modulated effect on adipogenic signaling cascades. Coincubation experiments applying the TRPA-1 inhibitor AP-18 demonstrated TRPA1 dependency of the CAL, but not the CIB-induced antiadipogenic effect.



1. INTRODUCTION

Health care systems worldwide struggle with the challenges associated with a rising prevalence of obesity and its comorbidities.[1] A sustained positive energy balance due to a caloric intake exceeding energy consumption ultimately leads to hyperplasia and/or hypertrophy of the adipose tissue.[2] This pathophysiological overgrowth of adipose tissue increases the risk of developing noncommunicable diseases, calling for effective countermeasures.[1] A potential approach to achieve an adipose tissue function that helps to maintain a healthy body weight and body composition is to target adipogenesis, the development of pre-adipocytes into mature adipocytes.[3,4] Recent studies proposed antiadipogenic effects of naturally occurring bioactive aroma compounds, for example, present in red pepper[5] or cinnamon spice.[6] The antiobesity properties of cinnamon have been mainly allocated to its most abundant constituent in the essential oil of cinnamon bark, cinnamaldehyde (CAL), which has been hypothesized as a potential agent in preventing or treating overweight and obesity.[4,7] It has been shown not only to exert anti-adipogenic effects in 3T3-L1 pre-adipocytes following a 4-day treatment with 10−40 μM CAL, but also to lower body weight gain, plasma lipids, and epididymal fat cell hypertrophy in mice after a 40 mg/kg CAL supplementation for 1 month compared to a high-fat diet control group.[4] Moreover, CAL, in a concentration of 30 μM, has been shown to reduce fatty acid uptake in Caco-2 cells, pointing to an antiobesity effect as well.[8] However, the molecular mechanisms regulating the CAL-mediated impact on adipocytes and lipid metabolism have not been entirely understood yet. Several possible modes of action, such as an impact on the adipogenic signaling cascade,[4] on enzymes associated with the lipid metabolism[9] as well as on thermogenesis have been described.[7] Apart from CAL, also other cinnamon-derived aroma compounds such as cinnamyl alcohol and cinnamic acid, which exhibit structural similarities with CAL and constitute potential metabolites, have been reported to inhibit adipocyte differentiation in concentrations of 40−200 μM, accompanied by downregulation of CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) pathways.[10,11] Moreover, for CAL, as a potent transient receptor potential channel A1 (TRPA1) agonist, a potential TRPA1 dependency in the CAL-induced effect on adipogenesis has been proposed, but not yet proven.[9,12] Activation of TRPA1, however, is also associated with nociceptive reactions and sensation of pain.[13] Considering its distinctive odor and spicy flavor qualities, the

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consumption of CAL is self-limited.[14] A less well-investigated compound regarding potential antiobesity effects that is present in the essential oil of cinnamon bark is cinnamyl isobutyrate (CIB). Unlike its structural relative CAL, no strong flavor and pungent effects, but sweet and fruity flavor characteristics and a moderate strength of spicyness have been described for CIB.[15]

Because antiadipogenic effects for CIB have not been reported yet, we hypothesized such a potential role of the cinnamyl ester CIB in 3T3-L1 cells as a structural analog of CAL. To target this hypothesis, we investigated the impact of CIB in comparison to CAL on the adipogenesis of the well-defined model for adipocytes, 3T3-L1 cells, during differentiation and maturation.

The adipogenic pathways of 3T3-L1 cells from the initiation of differentiation into mature adipocytes is well investigated and constitutes an intricate operational sequence, determined by the integration of stimulating or repressing signaling factors via a cascade of transcription factors, ultimately driving the downstream expression of adipocyte specific genes.[16] To that effect, complex interactions among various adipogenic transcription factors consecutively or synergistically play a decisive role in modulating the differentiation of adipocytes on a transcriptional level.[16−19] Especially the PPARγ and members of the C/EBP family are considered key modulators in adipogenesis and lipid storage.[18] However, many other transcription factors have been reported to have a regulatory effect in the different stages of the adipogenic network. Activation of the glucocorticoid receptor, cAMP response element—binding proteins (CREB) as well as ERK pathways are involved in the expression of C/EBPβ in the early stages of the adipogenic program.[20−22] C/EBPβ in turn induces the expression of C/EBPα and PPARγ, which at the same time further stimulate the mutual expression of each and, as later adipogenic markers, regulate final differentiation processes, leading to the development of the mature adipocyte phenotype.[16,23,24]

The main objectives of the present study were (I) to compare the impact of the structural analogs CIB and CAL on the differentiation process of 3T3-L1 pre-adipocytes into mature adipocytes and (II) to assess potential underlying mechanisms of action. For this purpose, long-term lipid accumulation during differentiation, the short-term fatty acid uptake in mature adipocytes, the regulation of selected key transcription factors and markers of adipogenesis, and a potential involvement of TRPA1 were examined following treatment with CIB and CAL. Both compounds are flavoring substances (EFSA, Regulation EU 872/2012) and were tested in concentrations roughly following average use levels and as previously applied by Hoi et al. (2018).[8]

2. RESULTS

2.1. Cell Viability. To rule out effects on cell viability after treatment with the test substances CIB, CAL, and AP-18 as well as combinations thereof, MTT assays were performed. No decrease in 3T3-L1 cell viability was determined after a 90 min treatment of fully matured adipocytes with CIB or CAL in concentrations of 0.3−300 μM compared to the untreated control cells. Additionally, no significant differences in cell viability were detected after treatment with 0.3 to 30 μM CIB or CAL with or without the addition of 2.5 μM AP-18 for 12 days compared to the control cells (data not shown). Higher concentrations of 300 μM tested for 12 days, however, significantly reduced cell viability.

2.2. Impact of CIB and CAL on Lipid Accumulation. To assess and compare the impact of CIB and CAL on lipid accumulation, which is considered a marker for the extent of adipogenesis,[16] 3T3-L1 cells were treated with the test compounds during their differentiation and maturation in concentrations of 0.3−30 μM. First, the staining of lipids was carried out using the widely applied lysochrome diazo dye oil red O, which is considered a standard method for assessing lipid accumulation. The results demonstrated a decrease in lipid accumulation by 32.0 ± 3.10 and 17.2 ± 3.71% compared to the untreated control after treatment with 30 μM CAL and CIB, respectively (Figure 1). Moreover, CAL showed a significantly stronger decrease in lipid accumulation compared to CIB. Second, staining was also performed using the lipophilic stain nile red, which allows a further distinction between neutral and polar lipids. CAL and CIB, applied in a concentration of 30 μM, reduced triglyceride accumulation by 37.5 ± 1.81 and 21.4 ± 2.56%, respectively, compared to the untreated solvent control (Figure 2A). Additionally, both compounds also decreased phospholipid accumulation compared to the control after 12-day treatment in the same concentration by 28.7 ± 1.83% in the case of CAL and 21.2 ± 1.95% in the case of CIB (Figure 2B). In both cases, the decrease of lipid accumulation was stronger after 30 μM CAL compared to 30 μM CIB treatment ($p \leq 0.05$). Moreover, calculations regarding the 30 μM CAL-mediated decrease in lipid accumulation applying a *t*-test revealed a stronger effect on the reduction of triglyceride compared to phospholipid accumulation ($p = 0.001$). In the case of 30 μM CIB treatment, there was no significant difference between the decrease in triglyceride and phospholipid accumulation.

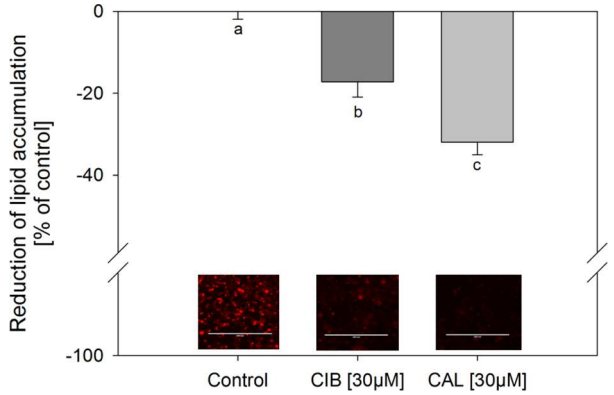

Figure 1. Reduction of lipid accumulation in % of control (0.1% ethanol; set to 0%) after addition of 0.3−30 μM CAL or CIB during differentiation and maturation of 3T3-L1 cells. Lipids in fully mature adipocytes were stained 12 d after initiation of differentiation with oil red O. Data are displayed as mean ± SEM. $N = 6$ (tr = 1−4). Significant differences are tested with one-way ANOVA followed by the Holm−Sidak post hoc test and marked with different letters (a = control).

2.3. Impact of CIB and CAL on the Fatty Acid Uptake. To test the effect of the cinnamon compounds on short-term fatty acid uptake, fully mature adipocytes were pretreated with 0.3−300 μM CIB or CAL. As depicted in Table 1, both compounds did not change BODIPY-C_{12} uptake by the cells compared to the solvent control.

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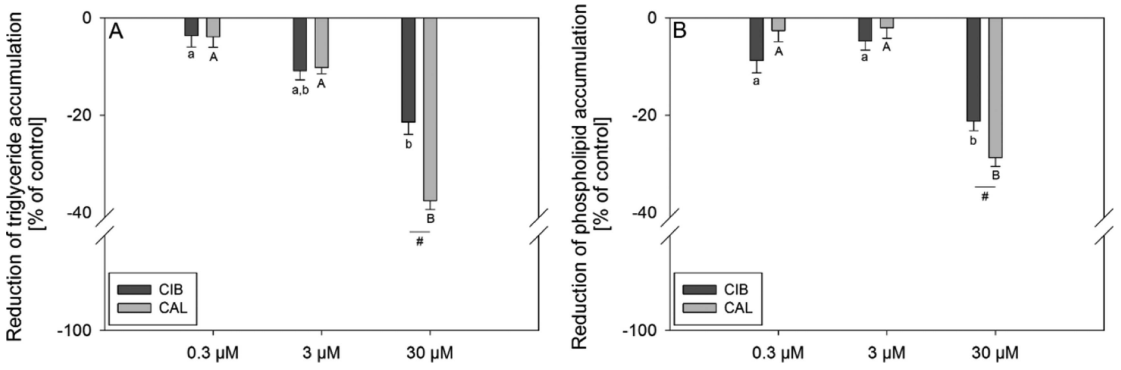

Figure 2. Reduction of lipid accumulation in % of control (0.1% ethanol; set to 0%) after addition of 0.3−30 μM CAL or CIB during differentiation and maturation of 3T3-L1 cells. Triglycerides (A) and phospholipids (B) in fully mature adipocytes were stained 12 d after initiation of differentiation with nile red. Data are displayed as mean ± SEM. N = 4−5 (tr = 3−6). Significant differences between control and treatments are tested with one-way ANOVA on ranks followed by Dunn's method or one-way ANOVA followed by the Holm−Sidak post hoc test, and significant differences between treatments are tested with two-way ANOVA followed by the Holm−Sidak post hoc test. Significant differences between control and treatments are marked with different letters (a, A = control) and differences between treatments are marked with #$p \leq 0.05$.

Table 1. BODIPY-C_{12} Fatty Acid Uptake after a 30 min Pretreatment with CAL and CIB in Concentrations of 0.3−300 μM[a]

	CIB (%)	CAL (%)
0.3 μM	105 ± 13.7	98.8 ± 8.72
3 μM	102 ± 13.9	99.6 ± 4.90
30 μM	97.0 ± 14.2	92.1 ± 4.71
300 μM	98.5 ± 16.0	88.3 ± 7.82

[a]Values are displayed as mean ± SD in percent compared to the control of 100 ± 14.1% (buffer with 0.1% ethanol). n = 4−7 (tr = 1−3).

2.4. Impact of CIB and CAL on PPARγ, C/EBPα, C/EBPβ, FABP4, and FAS mRNA Levels. To further examine and compare the antiadipogenic effect of CAL and CIB on 3T3-L1 cells, the impact of both test compounds in a concentration of 30 μM was tested on the gene expression levels of selected transcription factors and markers associated with adipogenic pathways. As depicted in Figure 3A,B, the mRNA levels after CAL and CIB treatment over a period of 3h up to 12 days were studied in a time-dependent manner and revealed a regulation of the mRNA expression for all adipogenic transcription factors PPARγ, C/EBPα, and C/EBPβ as well as markers FABP4 and FAS over the course of the differentiation and maturation process. Compared to the solvent control, 30 μM CAL treatment revealed a regulation of the C/EBPα mRNA expression after 3 h, 24 h, and 7 days, whereas 30 μM CIB showed an effect on C/EBPα expression levels after 24 h, 2 d, 5 d, and 7 d treatment. C/EBPβ mRNA levels were downregulated after 12 and 24 h CAL treatment. Similarly, CIB treatment revealed C/EBPβ downregulation after 12 h, 24 h, and 5 days. Furthermore, PPARγ mRNA levels were regulated after 12 and 24 h CAL treatment as well as after 12 h CIB treatment. Gene expression levels of the adipogenic marker FAS were downregulated after 3 h, 24 h, and 7 d CAL

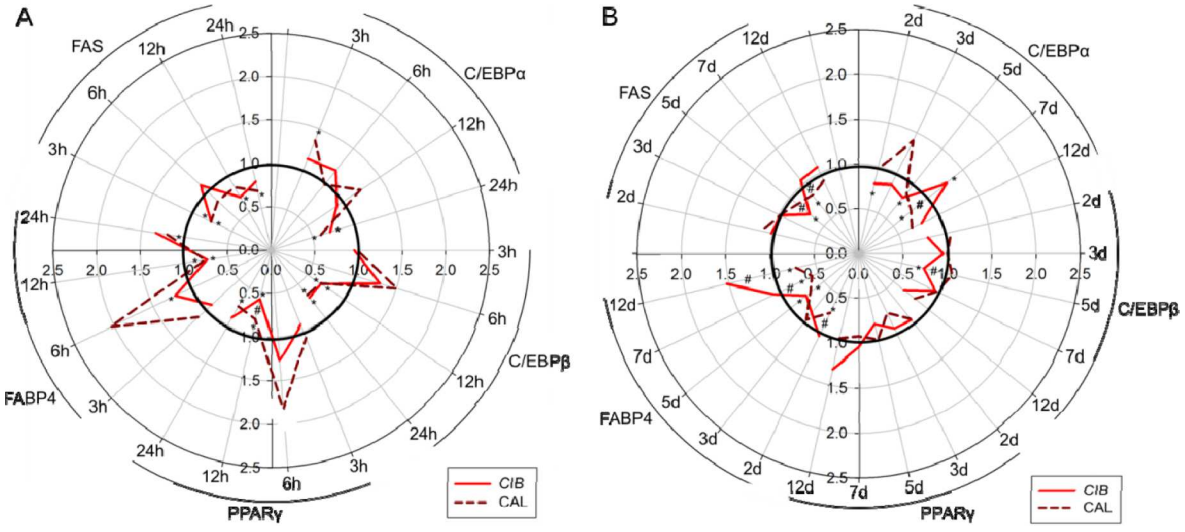

Figure 3. Gene expression levels for C/EBPα, C/EBP, PPARy, FABP4, and FAS after treatment with 30 μM CAL and CIB (A) after 3 h, 6 h, 12 h, and 24 h and (B) after 2 d, 3 d, 5 d, 7 d, and 12 d. Data are shown as mean fold change compared to the controls (buffer with 0.1% ethanol) of 1.00 with SEMs of 0.00−0.06%; n = 3−4 (tr = 1−3). Significant differences are tested with one-way ANOVA followed by the Holm−Sidak post hoc test or Kruskal−Wallis one-way analysis of variance on ranks followed by Dunn's Method or Tukey Test. Significant differences between treatments and controls were marked with *$p \leq 0.05$, and significant differences between different treatments were marked with #$p \leq 0.05$.

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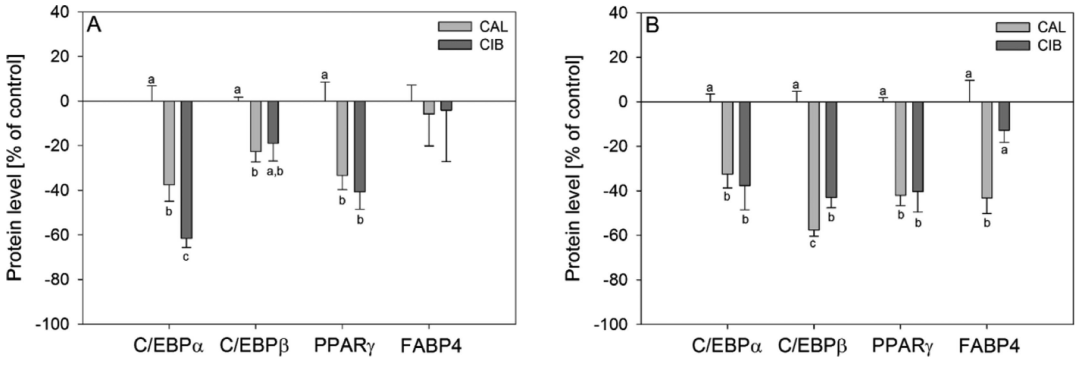

Figure 4. Protein levels for C/EBPα, C/EBPβ, PPARγ, and FABP4 after treatment with 30 μM CAL and CIB after (A) 24 h and (B) 12 d. Data are shown as mean ± SEM in % compared to the controls (buffer with 0.1% ethanol, set to 0%). n = 3−5 (tr = 1−2). Significant differences are tested with one-way ANOVA followed by the Holm−Sidak post hoc test and Kruskal−Wallis one-way analysis of variance on ranks followed by a Tukey test or Dunn's method and marked with different letters.

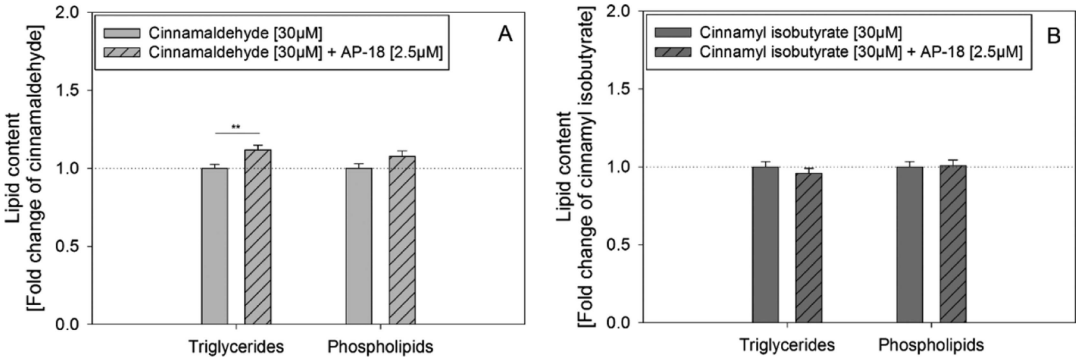

Figure 5. Lipid content (triglycerides and phospholipids) after addition of 30 μM CAL (A) or CIB (B) during differentiation and maturation of 3T3-L1 cells alone (set to 1) and after cotreatment with TRPA1 inhibitor AP-18 [2.5 μM]. AP-18 was added 20 min prior to the test compounds. Values are presented as mean ± SEM compared to CAL or CIB alone (set to 1); n = 4−5 (tr = 3−8). Significant differences between treatments are tested with Student's t-test and marked with $^{**}p \leq 0.01$.

treatment. CIB treatment led to FAS downregulation after 3 h, 12 h, and 5 days. Finally, FABP4 mRNA levels were altered after 12 h, 24 h, 2 d, 5 d, 7 d, and 12 d CAL treatment as well as after 6 h, 12 h, and 5 day CIB treatment. A stronger PPARy downregulation could be determined after 12 h CIB compared to CAL treatment. Additionally, CIB more strongly decreased FAS mRNA levels after 5 d treatment as well as C/EBPb mRNA levels after 5 d treatment compared to CAL. CAL showed a stronger effect on FABP4 downregulation after 2 d, 7 d, and 12 d treatment and a stronger FAS downregulation after 7 d treatment compared to CIB, as shown in Figure 3.

2.5. Impact of CIB and CAL on PPARγ, C/EBPα, C/EBPβ, FAS, and FABP4 Protein Levels. To additionally verify the CAL- and CIB-mediated impact on factors of the differentiation process, PPARγ, C/EBPα, C/EBPβ, and FABP4 protein levels were analyzed 24 h and 12 days after initiation of differentiation with or without compound treatment in a concentration of 30 μM by means of ELISA (Figure 4A,B). Treatment of 3T3-L1 cells with CAL for 24 h as well as 12 days decreased PPARγ (24 h: −33.3 ± 6.38%; 12 d: −42.1 ± 4.51%), C/EBPα (24 h: −37.5 ± 7.42%; 12 d: −32.6 ± 6.19%), and C/EBPβ (24 h: −22.6 ± 4.57%; 12 d: −57.6 ± 2.72%) levels compared to their untreated controls. Similarly, CIB treatment reduced PPARγ levels by 40.7 ± 7.69% and C/EBPα levels by 61.5 ± 4.13% after 24 h as well as PPARγ (−40.4 ± 9.15%), C/EBPα (−37.6 ± 10.9%), and C/EBPβ (−43.0 ± 4.61%) levels after 12 days. A CAL-induced lowered protein level could also be determined for FABP4 after 12-day treatment (−43.2 ± 6.97) compared to the control and CIB treatment, whereas CIB treatment over a 12-day differentiation period did not reduce the FABP4 expression.

2.6. TRPA1 Involvement in CAL- and CIB-Mediated Decrease in Lipid Accumulation. CAL has been shown to be a potent activator of TRPA1 channels.[25−27] In order to investigate if TRPA1 channels might play a role in the CAL- and CIB-induced inhibition of lipid accumulation during the adipogenesis, coincubation experiments using the TRPA1 inhibitor AP-18 were carried out. As presented in Figure 5, 12-day cotreatment with CAL (30 μM) and AP-18 (2.5 μM) reversed the 30 μM CAL-induced decrease in triglyceride accumulation (CAL: 1.00 ± 0.03 vs coincubation: 1.12 ± 0.03). No effect could be determined on the level of phospholipids. Lipid accumulation after 12-day coincubation with CIB (30 μM) and AP-18 (2.5 μM) also did not differ from the CIB-mediated decrease.

3. DISCUSSION

CAL, one of the major aroma compounds in cinnamon bark oil, has been shown to exert antiobesity properties by inhibiting body weight gain in mice after long-term supplementation in a concentration of 250 mg/kg body weight as well as adipogenesis and lipid accumulation in vitro after 4-day treatment with 10−40 μM CAL.[4,7,9] Ongoing research indicates that CAL, however, might not be the only bioactive cinnamon-derived aroma compound associated with antiadipogenic activity.[10,11] Moreover, its unique cinnamon flavor

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characteristics and nociceptive sensations might limit its application. Therefore, the less spicy CIB, a cinnamic ester and structurally related, naturally occurring cinnamon constituent, was examined for its antiobesity potential in the present study. We aimed to investigate the impact of CIB on the adipogenesis of 3T3-L1 pre-adipocytes as well as its potential effect size compared to CAL.

As hypothesized, the structurally related CIB also exhibited a reduced lipid accumulation after 12-day treatment with 30 μM of the test compound during the differentiation and maturation phase of 3T3-L1 cells, pointing to an antiadipogenic effect of CIB as well. However, in contrast to the CAL-mediated decrease in triglyceride accumulation by approximately 38%, which is comparable to the CAL-induced effect sizes reported in the literature,[4,9] CIB decreased triglyceride accumulation by 21%. CAL and CIB treatment decreased not only the content of triglycerides as determined by nile red as well as oil red O staining, but also that of phospholipids, which has been found to increase during adipogenesis as well and has been suggested to be required for membrane biosynthesis.[28] Again, CAL exhibited a more pronounced effect of approximately 7.5% compared to CIB. Interestingly, whereas CIB showed the same effect on triglyceride and phospholipid accumulation, in the case of CAL, a more pronounced effect on triglycerides compared to phospholipids was demonstrated. This result might point to an additional modulating impact of CAL on the lipid accumulation during the maturation phase of adipogenesis. Taken together, these results suggest that, although the cinnamyl ester CIB has antiadipogenic potential as well, the aldehyde CAL is more effective concerning the inhibiting impact on lipid accumulation. As bioactivities of naturally occurring compounds highly depend on their bioavailability and metabolization and numerous cinnamyl compounds have been shown to metabolize quickly to cinnamic acid and cinnamic acid derivatives in vivo, biotransformation of CIB and/or CAL in adipocytes needs to be investigated in future studies. Also, the stability of the test compounds has to be taken into account, making it difficult to specify exactly if the lipid accumulation-reducing effect of CIB is caused by the ester itself or a degradation product. In vivo, fast enzymatic hydrolyzation of aromatic esters has been reported, whereas CAL was also found in small doses in lipid tissue of animal models.[29−37] Because of a possible hydrolyzation of the cinnamic ester into its respective components, it cannot be excluded that its derivatives cinnamic acid or cinnamyl alcohol might also be involved to a greater or lesser extent in the demonstrated decreased lipid accumulation in 3T3-L1 cells. Both have been reported to decrease triglyceride accumulation by approximately 20−25% when applied in similar concentrations as CIB.[10,11] However, altogether, the net effect of CIB on lipid accumulation was still less than that of CAL.

Next, we examined whether a reduced short-term fatty acid uptake might also play a role in decreasing the lipid accumulation in mature adipocytes. Interestingly, however, no effect could be demonstrated for either test compound, further pointing to a stronger effect of CAL and CIB on the development of pre-adipocytes to adipocytes.[11]

For further verification of the CAL- and CIB-induced effect on markers of the differentiation process, protein levels of PPARγ, C/EBPα, and C/EBPβ as well as FABP4 were examined after selected time points. Protein levels were examined 24 h and 12 days after induction, selecting a time point in the early phase of adipogenesis and a time point after the differentiation process has been completed. Treatment with CAL led to reduced PPARγ, C/EBPα, and C/EBPβ levels after 24 h and 12 days, confirming the CAL-mediated downregulation of the transcription factors on the gene expression level. CIB treatment also led to reduced PPARγ and C/EBPα levels after 24 h and reduced PPARγ, C/EBPα, and C/EBPβ levels after 12 days. A stronger effect on C/EBPα levels could be shown after 24 h CIB treatment, whereas a stronger downregulation of C/EBPβ levels could be determined after 12 d CAL treatment. Altogether, these results suggest that CIB and CAL treatment, to a similar extent, affect key adipogenic transcription factors, which play a role especially in the earlier adipogenic phase. However, even though key transcription factors of adipogenesis, such as PPARγ, C/EBPα, and C/EBPβ were decreased after CIB treatment over a 12-day differentiation period, FABP4 protein levels were not reduced. In contrast, after 12-day CAL treatment, less FABP4 protein was detected in the fully matured cells. In accordance with the CAL-mediated bigger effect size in lipid accumulation detected by nile red and oil red O staining, these results further emphasize the stronger impact of CAL on diminishing the development to fully matured adipocytes and support the finding that CAL is the more potent antiadipogenic compound as compared to CIB.

On a mechanistic level, CAL has been proposed to exert its antiobesity effect via (i) inhibiting the differentiation of pre-adipocytes to mature adipocytes,[4] (ii) modulating lipolysis and lipid biosynthesis of adipocytes,[9] as well as (iii) activating thermogenesis and metabolic reprogramming.[26,38] However, CAL is also known as a potent agonist of TRPA1 channels, constituting nonselective thermosensitive cation channels, that have been identified in a variety of neuronal and nonneuronal cell types.[25−27] Multiple TRPA1-dependent actions for CAL have been reported over the last decades, such as immunomodulatory[39] and vasodilatory[40] actions as well as the secretion of hormones such as serotonin,[27] ghrelin,[12] and PYY.[41] Additionally, the role of TRP channels in the physiological processes of adipogenesis has grown as a topic of extensive research.[42] Activation of these multimodal receptors through physical and mechanical stimulation on the one hand and a wide range of endogenous and exogenous agents on the other hand is associated with altered intracellular Ca^{2+} concentrations and, therefore, it has the potential to regulate various cellular processes.[27] With regard to the lipid metabolism, involvement of calcium signaling in the adipogenic process has been suggested. In 3T3-L1 cells, for instance, elevated intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) have been reported to block early stages of the adipocyte differentiation process by inhibiting the post-confluent mitotic phase and modulating the expression of c-myc genes.[43] It was also found, however, that, in later stages of the adipogenesis, elevated $[Ca^{2+}]_i$ actually increased markers of differentiation in human adipocytes.[44]

We hypothesized a potential TRPA1 dependency in the CAL-mediated decrease in lipid accumulation, which was investigated by cotreatment of 3T3-L1 cells with CAL and the competitive TRPA1 inhibitor AP-18 for 12 days during the differentiation and maturation phase. The results showed an increased triglyceride accumulation compared to the effect of CAL alone, pointing to involvement of TRPA1 in the antiadipogenic effect of CAL. In contrast, no TRPA-1 involvement in the antiadipogenic effect of CIB could be determined, which might explain the smaller impact of CIB on

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lipid accumulation compared to CAL. Consistently, it was shown by Lieder et al. (2020) that TRPA1-mediated Ca^{2+} mobilization in transiently hTRPA1-transfected HEK293 cells is reduced after stimulation with cinnamon derivatives such as cinnamic acid, ferulic acid, or CIB compared to cinnamyl aldehyde.[45] As mentioned above, it has been suggested that apart from directly inhibiting the differentiation process,[4] CAL also modulates lipolysis and lipid biosynthesis in mature adipocytes.[9] However, based on our data, it could not be distinguished whether the TRPA1 dependency in the CAL-mediated effect on the reduced lipid accumulation only plays a role in the early and intermediate differentiation phases or if a TRPA1-dependent effect of CAL is also involved in the subsequent terminal differentiation and maturation phase of the adipogenesis. As it was reported that the trigeminally active *trans*-pellitorine demonstrated a TRPA1-dependent antiadipogenic effect only in early to intermediate stages of adipogenesis, despite its continuing lipid accumulation reducing effect during maturation phase,[25] CAL-mediated TRPA1 activation in the early differentiation might be hypothesized as well. Additionally, the time-dependent, biphasic regulatory effect of $[Ca2^{2+}]_i$ on adipogenesis[44] could point to the fact that a CAL-mediated Ca^{2+} influx via TRPA1 might only be the case in early phases of the differentiation process. However, it cannot be excluded that CAL, as an exogenous inhibitory agent, regulates adipogenesis, its downstream cascade of transcription factors and lipid accumulation through different signaling pathways, especially because a modulating impact of CAL on lipolysis and lipid biosynthesis in adipocytes was also suggested.

In conclusion, analyzing and comparing the impact of the structural analogs CIB and CAL on adipogenesis in 3T3-L1 cells demonstrated the aldehyde to be the more potent antiadipogenic candidate as evidenced by a stronger inhibition in lipid accumulation and a stronger decrease in the expression of differentiation marker FABP4. This stronger effect size of CAL might be explained by its potential to activate TRPA1 channels, as TRPA1 dependency was found in the CAL-mediated decrease in triglyceride accumulation. The CIB- and CAL-induced decrease in lipid accumulation was further accompanied by a similar downregulation of the key adipogenic transcription factors PPARγ, C/EBPα, and C/EBPβ on a gene and protein level, indicating a compound-mediated effect on the signaling cascade of the adipogenic differentiation program.

4. MATERIALS AND METHODS

4.1. Chemicals. All chemicals and reagents were purchased from Sigma-Aldrich (Vienna, Austria), unless stated otherwise. The murine fibroblast cell line 3T3-L1 was purchased from ATCC.

4.2. Cell Culture. 3T3-L1 pre-adipoycte cells were cultured in Dulbecco's modified eagle's medium (DMEM) with the addition of 10% fetal bovine serum, 4% L-glutamine, and 1% penicillin/streptomycin at 37 °C and 5% CO_2 in a humidified atmosphere. Cells were harvested and seeded after reaching a confluence of 70−80% and used between the passages 4 and 15. To induce the differentiation of pre-adipoyctes into mature adipocytes, cells were treated with differentiation medium containing growth medium with the addition of dexamethasone (1 μM), 3-isobutyl-1-methyl-xanthine (0.5 mM), and insulin (10 μg/mL) 2 days after reaching confluence (day 0), according to the protocol described by Riedel et al. (2012).[46] After 2 days, the differentiation media were substituted with maturation medium comprising growth medium supplemented with 10 μg/mL insulin for additional 48 h. Cells were subsequently cultivated using normal growth medium for 5 more days and used for fatty acid uptake experiments on day 9.

Stock solutions of the test compounds CAL, CIB, and AP-18 were dissolved in ethanol or dimethyl sulfoxide (DMSO). Final ethanol and DMSO concentrations never exceeded 0.1% on the cells.

4.3. Cell Viability. The impact of the applied concentrations of the test compounds CAL (0.3−300 μM), CIB (0.3−300 μM), and AP-18 (2.5 μM) as well as combinations thereof on metabolic activity was examined using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazoliumbromide) assays as described before.[47]

4.4. Nile Red Staining. Lipid accumulation was analyzed using nile red (9-diethylamino-5*H*-benzo [α] phenoxazine-5-one), a fluorescent lipophilic dye, which allows for distinction between neutral lipids and polar lipids through transition of emission from red to yellow based on the lipid hydrophobicity.[48] For nile red staining, 3T3-L1 cells were seeded in 48-well plates at a density of 1.5×10^4. After initiating the differentiation as stated above, cells were cultured in maturation media for 10 days. Addition of the test compounds started at day 0. On day 12, cells were washed with 750 μL PBS, stained with nile red solution at a final concentration of 4 μg/mL, and incubated for 20 min at room temperature. Subsequently, fluorescence was measured at 485 nm excitation and 572 nm emission to determine triglyceride accumulation as well as 530 nm excitation and 635 nm emission for determination of the phospholipids using a Tecan plate reader (Tecan infinite M200, Tecan Austria). Lipid content after substance treatment was calculated as % to the untreated control cells. As a comparison, lipid staining was also performed using the oil red O staining protocol reported by Riedel et al. (2012).[46]

4.5. Fatty Acid Uptake. The uptake of free fatty acids in fully matured 3T3-L1 adipocytes was examined in 96-well plates applying the QBT fatty acid uptake kit (Molecular Devices Germany GmBH, Germany), which was used following manufacturers' instructions. As described elaborately by Holik et al. (2016),[49] cells were seeded and used for analysis on day 9 post-differentiation. After 30 min pretreatment of 3T3-L1 adipocytes with 0.3−300 μM CIB or CAL diluted in HBSS/HEPES, the BODIPY-C_{12} containing loading dye was added. BODIPY-C_{12} uptake was measured for 60 min with an excitation wavelength of 485 nm and emission wavelength of 515 nm. For quantification, the area under the curve (AUC) from the respective signal/time plots was determined using SigmaPlot and assessed relative to untreated control cells (100%).

4.6. Quantitative Real-Time Polymerase Chain Reaction. The gene expression of peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer binding protein α (C/EBPα) and β (C/EBPβ), fatty acid binding protein 4 (FABP4), and fatty acid synthase (FAS) was examined at different time points over 12 days, applying quantitative real-time polymerase chain reaction (PCR). RNA extraction using the MasterPure Complete DNA & RNA Purification Kit (Biozym) according to the manufacturer's protocol was performed after 3, 6, 12, and 24 h as well as after 2, 3, 5, 7, and 12 days post-differentiation with or without 30 μM CIB or CAL treatment, which was added to the differentiation and maturation medium. Following a reverse

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Table 2. Sequence of Primers Used in qRT-PCR Experiments

target	forward primer	reverse primer
HPRT[47]	GAGAGCGTTGGGCTTACCTC	ATCGCTAATCACGACGCTGG
ACTB[50]	TCTTTGCAGCTCCTTCGTTG	CATTCCCACCATCACACCCT
PPARγ[47]	GTGCCAGTTTCGATCCGTAGA	GGCCAGCATCGTGTAGATGA
C/EBPα[47]	GCCCCGTGAGAAAAATGAAGG	ATCCCCAACACCTAAGTCCC
C/EBPβ[51]	CGCCTTATAAACCTCCCGCT	TGGCCACTTCCATGGGTCTA
FABP4[47]	TTTGGTCACCATCCGGTCAG	TGATGCTCTTCACCTTCCTGTC
FAS[52]	CACAGATGATGACAGGAGATGG	TCGGAGTGAGGCTGGGTTGAT

transcription applying the high capacity cDNA Kit (Life Technology, Carlsbad, CA, USA), qRT-PCR analysis was carried out in triplicates on a StepOnePlus device by means of SYBR Green MasterMix (Life Technology, Carlsbad, CA, USA). The individual hypothetical starting mRNA levels were determined using LinRegPCR v.2012.2 and normalized to HPRT[47] and ACTB[50] as reference genes. Primers sequences are listed in Table 2.

4.7. PPARγ, C/EBPα, C/EBPβ, and FABP4 ELISA. Analysis of PPARγ, C/EBPα, C/EBPβ, and FABP4 protein expression was carried out 24 h as well as 12 days after initiation of differentiation with or without compound treatment (30 μM), applying specific ELISA kits (mouse PPARγ and C/EBPβ, Cloud-Clone Corp., USA; mouse C/EBPα and FABP4, ELISA Genie, United Kingdom). For sample preparation, 3T3-L1 cells were washed twice with ice-cold PBS and collected in lysis buffer (RIPA buffer) with the addition of 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium ortho-vanadate, and a protease inhibitor cocktail, as described by Rohm et al. (2015).[47] After homogenization and subsequent agitation (30 min, 4 °C), the lysate was centrifuged for 19 min at 4 °C and 16,900g. The PPARγ, C/EBPα, C/EBPβ, and FABP4 protein content in the supernatant was determined by using the respective ELISA following the manufacturer's instructions and normalized to the protein content of each sample assessed by means of Bradford.

4.8. Statistical Analysis. Data from the in vitro experiments are presented as mean ± SD, unless indicated otherwise, or as fold change (treated over control: T/C) from at least three biological and two technical replicates. Outliers were identified and removed from statistical analysis according to the Nalimov outlier test. To test significant differences in treated versus untreated cells and in time course experiments, Student's t-test, one-way ANOVA followed by the Holm−Sidak post hoc test or Kruskal−Wallis one-way analysis of variance on ranks followed by a Tukey test or Dunn's Method were applied. Significant differences between different treatments and test concentrations were tested with two-way ANOVA followed by the Holm−Sidak post hoc test. To test a significant difference between the effect of CAL or CIB alone versus coincubation, Student's t-test was performed. Statistical analysis was carried out using SigmaPlot 11.0.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c05083.

MTT data (PDF)

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Author Contributions

J.K.H., Barbara Lieder, J.P.L., J.H., and V.S. designed and established conditions for the experiments. J.K.H, Beatrix Liebisch, and C.C performed experiments and data analysis. The manuscript was written by J.K.H. and revised by Barbara Lieder, J.P.L., J.H., and V.S.

Notes

The authors declare the following competing financial interest(s): The authors J. Hans and J.P. Ley are employees at Symrise AG, Holzminden, Germany.

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■ ABBREVIATIONS USED

CAL, cinnamaldehyde; CIB, cinnamyl isobutyrate; C/EBPα, CCAAT/enhancer binding protein α; C/EBPβ, CCAAT/enhancer binding protein β; PPARγ, peroxisome proliferator-activated receptor; FABP4, fatty acid binding protein 4; FAS, fatty acid synthase; TRPA1, transient receptor potential channel A1

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3.4 Structure-Dependent Effects of Cinnamaldehyde Derivatives on TRPA1-Induced Serotonin Release in Human Intestinal Cell Models

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In this publication, the TRPA1-dependent impact of cinnamaldehyde and 17 further naturally occurring structural analogues on serotonin release in differentiated Caco-2 cells and enterochromaffin QGP-1 cells was investigated. Additionally, the molecular structural characteristics of the tested aroma compounds were analyzed regarding their ability to stimulate or inhibit serotonin release.

The PhD candidate analyzed intracellular calcium mobilization in TRPA1 transfected HEK293 cells after stimulation with selected cinnamaldehyde derivatives and performed data analysis in this study.

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Article

Structure-Dependent Effects of Cinnamaldehyde Derivatives on TRPA1-Induced Serotonin Release in Human Intestinal Cell Models

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ABSTRACT: Activation of the transient receptor potential (TRP) channel TRPA1 by cinnamaldehyde has been shown to stimulate serotonin release in enterochromaffin QGP-1 cells. However, the impact of cinnamaldehyde on serotonin release in enterocytes is less well understood. In addition, since the neurotransmitter serotonin plays a regulatory role in a large variety of gastrointestinal and metabolic functions, it is of interest to study which structural characteristics determine cinnamaldehyde-induced serotonin release by enterocytes. Thus, the present study analyzed serotonin release in differentiated Caco-2 cells as a model for enterocytes in comparison to enterochromaffin QGP-1 cells after stimulation with cinnamaldehyde and 17 naturally occurring structurally related compounds by means of a serotonin ELISA. Stimulation with cinnamaldehyde induced a dose-dependent increase in serotonin release starting from 0.5 mM in both cell lines, with a larger effect size in Caco-2 enterocytes compared to that in QGP-1 enterochromaffin cells. Serotonin release in Caco-2 cells induced by additional 17 structurally related compounds correlated with serotonin release in QGP-1 cells, showing the highest effects for coniferylaldehyde with a 15.84 ± 3.23-fold increase in Caco-2 cells, followed by the parent compound cinnamaldehyde (13.45 ± 2.15), cinnamyl alcohol (6.68 ± 1.08), and α-methyl-cinnamaldehyde (6.59 ± 0.93). Analysis of structural and molecular characteristics that modulate serotonin release in Caco-2 enterocytes revealed that the ability of a compound to activate TRPA1, demonstrated by means of HEK293 cells transiently expressing hTRPA1, is a decisive factor to stimulate serotonin release in Caco-2 enterocytes, preferring small, electrophilic compounds with a lower polar surface area. In addition, blocking of TRPA1 using 30 μM AP-18 significantly reduced the cinnamaldehyde-induced serotonin release by 30.0 ± 5.24%, confirming a TRPA1-dependent component in serotonin release by Caco-2 cells.

KEYWORDS: *cinnamaldehyde, coniferylaldehyde, Caco-2, QGP-1, serotonin, TRPA1*

INTRODUCTION

Activation of the transient receptor potential (TRP) non-selective cation channel TRPA1 has been previously demonstrated to potently stimulate serotonin release in the enterochromaffin cell model QGP-1.[1] TRPA1 has been shown to be not only activated by noxious cold (<17 °C)[2] but also by a number of naturally occurring bioactive aroma compounds present in the human diet. For example, compounds present in ginger, clove oil, mustard oil as well as in cinnamon oil have been shown to be potent ligands for the TRPA1 channel.[3] Only recently, cinnamaldehyde, the main ingredient and key aroma compound of cinnamon oil, has been demonstrated to effectively increase serotonin release and block serotonin reuptake more potently than the other tested cinnamon-derived compounds cinnamic acid, cinnamyl alcohol, and cinnamyl isobutyrate in differentiated Caco-2 cells.[4] The effects of cinnamaldehyde on serotonin release have been demonstrated to be at least partly allocated to the activation of TRPA1.[4]

Serotonin, a neurotransmitter and hormone, plays a regulatory role in a large variety of gastrointestinal and metabolic functions. Earlier studies focused on the role of central serotonin in the brain and largely ignored the peripheral occurring serotonin, although about 95% of the total serotonin is located in the gut.[5] Therefore, several functions of peripheral serotonin have been unraveled only in the past years. For example, serotonin is nowadays recognized to be a hormone, neurotransmitter, and growth factor that is involved in the regulation of gastrointestinal motility during digestion, liver regeneration, bone formation, intestinal mucosal growth, and intestinal inflammation.[6] Serotonin is biosynthesized by a multistep pathway. First, 5-hydroxytryptophan (5-HTP) is generated in the gut from the amino acid tryptophan by the enzyme tryptophan hydroxylase (TPH-1), whereas enteric and central neurons express TPH-2. Serotonin is then synthesized from its precursor 5-HTP by the aromatic L-amino acid decarboxylase (AAAD).[7] The vast majority of peripheral serotonin is produced by enterochromaffin cells[8] and may be released in response to nutrients, tastants, and olfactants, which in turn stimulate contraction via vagal afferent fibers to facilitate digestion.[9−11] Inactivation of 5-HT in the mucosa of the bowel is executed by enterocytes that express serotonin reuptake transporters (SERT).[12] Dysregulation of

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Table 1. Primer Pairs Used for the qPCR Analysis of *TRPA1* Gene Expression in QGP-1 and Caco-2 Cells

gene	primer (forward)	primer (reverse)	ref
TRPA1	GCAGCCAGTTATGGGCGTAT	TTTGCTGCCAGATCGAGAGG	Rohm et al.[30]
HPRT1	CCTGGCGTCGTGATTAGTGA	CGAGCAAGACGTTCAGTCCT	Riedel et al.[18]

this process is associated with gastrointestinal disorders like irritable bowel syndrome.[7] Thus, understanding the regulation of serotonin release by gastrointestinal cells in response to compounds present in our diet is of interest.

In this context, also cell types other than enterochromaffin cells have been shown to be able to synthesize and release serotonin upon stimulation, e.g., parietal cells of the stomach.[13] Also, Nakamura et al.[14] showed TPH expression not only in enterochromaffin cells but also in brush border cells of adult rat intestines. In addition, they demonstrated that the widely used intestinal cell model Caco-2 has the enzymes to synthesize and degrade serotonin, and also detected smaller amounts of serotonin in the cells,[14] although data from human enterocytes are lacking so far. In a previous study by our group, we confirmed the usage of Caco-2 cells as a model for peripheral serotonin release by intestinal cells as well.[15]

However, since serotonin is involved in the regulation of various gastrointestinal and metabolic functions,[5] it is of interest to understand which structural characteristics of cinnamaldehyde are important for the stimulation of serotonin release in intestinal cells. Cinnamaldehyde is the key aroma compound of cinnamon oil, reaching concentrations of around 8 mg/g in commercially available cinnamon.[16] Thus, following the ingestion of 6 g of cinnamon powder, a concentration of around 400 μM could theoretically reach enterocytes in the gastrointestinal tract,[4] demonstrating the nutritional relevance of higher concentrations of cinnamaldehyde.

In the present study, we aimed to investigate which molecular structural characteristics of cinnamaldehyde and related compounds are advantageous for stimulating or inhibiting serotonin release by enterocytes. We analyzed serotonin release in two intestinal cell models, QGP-1 cells as a model for human enterochromaffin cells and differentiated Caco-2 cells as a model for human enterocytes, after incubation with 500 μM cinnamaldehyde and additional 17 naturally occurring compounds that are structurally related to cinnamaldehyde. The selected concentration was chosen based on dose−response experiments carried out in the cell models.

MATERIAL AND METHODS

Chemicals. All chemicals (purity ≥ 95%), reagents, media, and media supplements were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), unless stated otherwise.

Cell Culture. QBP-1 cells were a kind gift from Prof. Dr. Massimo Donadelli (University of Verona, Italy) and cultivated as described before[17] in RPMI medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, Austria), 1% (v/v) penicillin/streptomycin at 37 °C at 5% CO_2 in a humidified atmosphere. Caco-2 and HEK293 cells were obtained from CLS Cell Lines Service GmbH, Eppelheim, Germany and cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated FBS (Thermo Fisher Scientific, Austria), 1% (v/v) penicillin/streptomycin at 37 °C at 5% CO_2 in a humidified atmosphere. Caco-2 cells were differentiated to an enterocyte-like phenotype within 21 days as described before.[18,19] Previous studies by our group showed that this method results in an intact monolayer, confirmed by transepithelial electrical resistance (TEER) values >350 Ω*cm^2.[18,19]

Cellular Vitality. Negative effects of treatment with the test compounds were excluded using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid) assays as a measure of cellular proliferation. MTT assays were performed as described in detail before.[19] In brief, the different cell lines were treated according to the respective assay conditions before the MTT reagent (5 mg/mL in PBS), diluted in 5 parts serum-free DMEM, was added to the cells. After 15−20 min incubation at 37 °C, the MTT solution was aspirated and the formed purple formazan salt was dissolved in dimethyl sulfoxide (DMSO) before measuring the absorption at 570 nm by a Tecan infinite M200 device (Tecan, Männedorf, Switzerland).

Serotonin Release Assay. Serotonin release assays are based on the serotonin levels in the cell supernatant and were basically performed as described previously.[15] Confluent Caco-2 cells were differentiated for 21 days in 12-well plates, whereas 250 000 cells per well of QGP-1 cells were seeded in 24-well plates, and the serotonin release assay was performed after 72 h.[17] The test compounds were dissolved in DMSO as stock solutions (1000×) and applied to the cells diluted in Krebs−Ringer−HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer supplemented with 0.1% (v/v) ascorbic acid (incubation solution) for 5 min. The final concentration of DMSO in the incubation solution was 0.1% (v/v), or 0.2% (v/v) in the co-incubation assays with AP-18. The incubation solutions (cellular supernatants) were then collected in tubes and briefly centrifuged for 5 min at 1000 rpm and 4 °C to remove cellular debris. The supernatants were stored at −80 °C for a maximum of two weeks until final analysis using a Serotonin High Sensitive ELISA kit (DLD Diagnostica, Hamburg, Germany) according to the manufacturer's protocol with a test volume of 50 μL. The sensitivity of the used test kit is 0.39 pg per sample and the intra-assay variation is <9% according to the manufacturer.

Transfection of HEK293 Cells and Analysis of Intracellular Calcium Levels. HEK293 cells were seeded in poly-L-ornithine-coated 96-wells plates (50 μL/well of a 20 μg/mL solution) to a density of 12 500 cells per well. Transfection was started 24 h after plating by the addition of Viromer One Red (Bio-Rad, Austria) with 10 ng/well pCDN3 vector (Thermo Fisher Scientific, Germany) with human TRPA1 (kindly provided by Prof. H. Hatt, Ruhr University Bochum, Germany). The transfection rate was typically between 80 and 95%, which was monitored by a GFP control using a pCDNA3 backbone (kindly provided by Prof. H. Hatt) at 10 ng/well, and analyzed on an EVOS FL digital microscope (Thermo Fisher Scientific, Austria) with 10× magnification.

Intracellular calcium mobilization in transfected HEK293 cells was carried out according to the protocol of Luo et al.[20] using a Fluo-4 dye on a Flex-Station III equipped with SoftMax Pro 7.0.2 software (Molecular Devices, Biberach, Germany). In brief, transfected HEK293 cells were loaded for 1 h at 37 °C with 50 μL of loading dye solution (2 μM Fluo-4, 0.04% (w/v) pluronic acid) and 0.1% (w/v) bovine serum albumin dissolved in Hank's balanced salt solution (HBSS #H6648, Sigma-Aldrich, Austria), containing 10 mM HEPES, with the pH adjusted to 7.4 using NaOH. After washing with assay buffer (2 mM probenecid in HBSS), cells with 80 μL of assay buffer/well were transferred to a Flex-Station instrument and the Ca^{2+} signal was monitored in Flex mode every 1.5 s for a total of 240 s at 494 nm excitation and 525 nm emission after stimulation with 40 μL of 3× concentrated test compound dissolved in assay buffer. A second application with a final concentration of 10 μM ionomycin was used to assess cell vitality. For analysis, the data range (maximum−baseline) for each compound was calculated after the subtraction of the blank and subsequently treated over its solvent control.

RT-qPCR Detection of TRPA1. To confirm the expression of *TRPA1* in the cell lines, a RT-qPCR experiment was performed. RNA

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was isolated from fully differentiated Caco-2 cells and QGP-1 cells using the Epicentre Masterpure complete DNA and RNA purification kit (Lucigen, Madison, WI, USA) and reverse-transcribed to cDNA using the High-Capacity RNA-to-cDNA kit (Applied Biosystems, Thermo Fisher Scientific, Austria). PCR was subsequently performed using Fast Master Mix (Applied Biosystems via Thermo Fisher Scientific, Austria) on a Step-One Plus Device (Applied Biosystems via Thermo Fisher Scientific, Austria). The primer pairs used during the reaction can be found in Table 1.

Statistical and Computational Analyses. MS Excel 2013 and SigmaPlot 13 were used for statistical analysis of the data. All data are shown as the mean fold change ± standard error of mean (SEM) calculated from at least three independent experiments with multiple technical replicates each. Outliers ($P < 0.001$) were identified with the Nalimov outlier test and excluded from the final calculation. Data were tested for normality using the Shapiro−Wilk test. Differences between two groups were tested using Student's *t*-test. Two-way analysis of variance (ANOVA) with the Holm−Sidak posthoc test was used to compare treatments and dose-dependent effects. Significant differences were assumed at P values < 0.05.

The calculation of physicochemical descriptors of each test molecule was carried out using RDKit node for the KNIME Analytics Platform 3.7.0. A number of topological descriptors were calculated, but only those displaying values with considerable difference between the tested molecules and less than 5% zero-values were used for further correlation analysis as indicated in the Results section. Electrostatic potential maps of selected compounds were drawn using the software 'Flare for academics' (Cresset, U.K.). Matched-molecular pair analysis was carried out using Vortex (Version 2019.04, Dotmatics Ltd., U.K.) including single-atom changes and nonring fragmentations (with a maximum fragment size of eight and a minimum core size of eight atoms), using both experimental readouts of TRPA1 activation and serotonin secretion by Caco-2 enterocytes as parameters of interest.

RESULTS

Cellular Proliferation Using the MTT Assay. All test compounds were tested for their impact on cellular proliferation as a measure for toxicity in QGP-1 and Caco-2 cells at the concentrations and incubation times applied at the serotonin release experiments (Figure S1). Cinnamaldehyde applied at the highest concentration of 5 mM reduced the cellular proliferation to 69.8 ± 5.34% in QGP-1 cells (Figure S2). Since this value is below the cutoff level of 70% according to ISO 10993:5,[21] 2.5 mM was chosen as the highest test concentration in QGP-1 cells. In Caco-2 cells, no negative effects on cellular proliferation were detected after applying the compounds in the described assay conditions (Figure S1 and S2).

Dose-Dependent Serotonin Release by QGP-1 Cells and Caco-2 Induced by Cinnamaldehyde. Treatment of QGP-1 cells with final concentrations of 0.05−2.5 mM cinnamaldehyde dose-dependently stimulated serotonin release, starting with a 5.10 ± 0.71-fold increase at a concentration of 0.5 mM and up to 13.8 ± 2.20-fold increase at 2.5 mM (Figure 1). Due to the negative effects on cellular toxicity as described above, higher test concentrations were not applied and no saturation of serotonin release was reached. An EC_{50} value is therefore not presented.

Similarly, Caco-2 cells were treated with final concentrations ranging from 0.05 up to 5 mM cinnamaldehyde, which led to a dose-dependent stimulation of serotonin release as well. Also here, a significant stimulation of serotonin release started at 0.5 mM with a fold change of 15.2 ± 1.92, reaching up to 71.6 ± 7.62-fold change at a test concentration of 5 mM (Figure 1). Due to its limited solubility in aqueous solutions, cinnamaldehyde was not tested in concentrations exceeding 5 mM. An EC_{50} value for the effects in Caco-2 cells is therefore not presented as well.

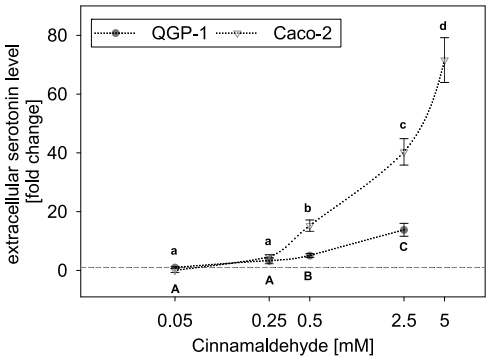

Figure 1. Concentration-dependent serotonin release in QGP-1 enterochromaffin cells (gray circles) and Caco-2 enterocytes (white triangles) after stimulation with 0.05−2.5 or 5 mM cinnamaldehyde, respectively. Data are presented as mean fold change ± SEM calculated from four independent experiments with two technical replicates each. Significant differences between the treatments and the cell models were tested by two-way ANOVA with the Holm−Sidak posthoc test; the concentration-dependent effects are marked by distinct letters in the figure.

A comparison of the stimulating effect of cinnamaldehyde in the two cell models revealed a more pronounced effect in Caco-2 enterocytes than in QGP-1 cells (Two-way ANOVA, $P < 0.001$).

Serotonin Release by Structural Analogues of Cinnamaldehyde. To determine which structural characteristics are important for the serotonin-releasing potential of a compound, additional 17 naturally occurring compounds that are structurally related to cinnamaldehyde were selected and tested in Caco-2 and QGP-1 cells at a concentration of 0.5 mM, namely, coniferylaldehyde, cinnamyl alcohol, vanillyl methyl ketone, sinapinaldehyde, caffeic acid phenethylester (CAPE), eugenol, 2-methoxy-cinnamaldehyde, α-methyl-cinnamaldehyde, hydrocinnamaldehyde, caffeic acid, isoeugenol, homovanillic acid, ferulic acid, cinnamic acid, cinnamyl isobutyrate, 2-phenylpropionaldehyde, and phenylacetaldehyde (see Figure 2 for an overview and the corresponding structures). The concentration of 0.5 mM was chosen as the lowest concentration that significantly increased serotonin release after stimulation with cinnamaldehyde in both tested cell models. The results of the comparison are displayed in Figure 3A. In both cell lines, coniferylaldehyde was the most potent compound, followed by the lead aroma compound cinnamaldehyde. Moreover, the serotonin-releasing potential of the test compounds in Caco-2 cells correlated with the serotonin-releasing potential of the test compounds in QGP-1 cells (Pearson's product-moment correlation, $R = 0.809$, $P < 0.001$, $n = 18$, see Figure 3B).

Structural Determinants of Serotonin Release in Caco-2 Enterocytes. Since structural characteristics that determine serotonin release by enterocytes are largely unknown, the following matched pair analysis (see also Figure S1) focused on serotonin-releasing potential by cinnamaldehyde derivatives in Caco-2 enterocytes.

Aldehydes were more active than the corresponding acids: Stimulation with coniferylaldehyde induced the highest serotonin release (15.84 ± 3.23), in comparison to ferulic acid with a fold change of 1.39 ± 0.09 ($P < 0.01$). Similar to

https://dx.doi.org/10.1021/acs.jafc.9b08163
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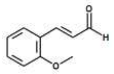
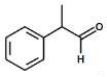
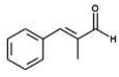
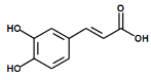
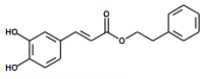
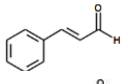
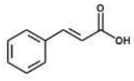
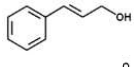
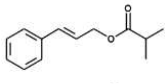
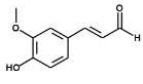
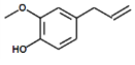
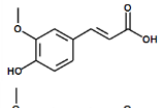
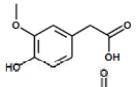
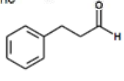
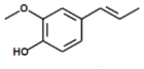
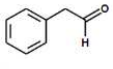
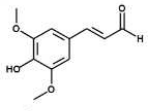
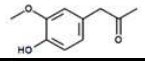
Compound	Chemical Structure	SlogP	SMR	Labute ASA	TPSA	MW	Rotab. Bonds	Hetero Atoms	Heavy Atoms	Num. Atoms
2-Methoxy-cinnamaldehyde		1.907	48.09	71.48	26.3	162.1	3	2	12	22
2-Phenylpropionaldehyde		1.989	40.92	60.69	17.07	134.1	2	1	10	20
α-Methyl-cinnamaldehyde		2.289	46.16	66.36	17.07	146.1	2	1	11	21
Caffeic acid		2.897	79.91	122.5	66.76	284.1	5	4	21	37
CAPE		1.196	46.44	74.38	77.76	180	2	4	13	21
Cinnamaldehyde		1.899	41.54	60	17.07	132.1	2	1	10	18
Cinnamic acid		1.784	43.11	64.79	37.3	148.1	2	2	11	19
Cinnamyl alcohol		2.215	38.1	54.27	20.23	120.1	1	1	9	17
Cinnamyl isobutyrate		3.082	51.7	73.68	9.23	162.1	3	1	12	26
Coniferylaldehyde		1.613	49.76	76.27	46.53	178.1	3	3	13	23
Eugenol		2.129	48.56	72.11	29.46	164.1	3	2	12	24
Ferulic acid		1.499	51.33	81.07	66.76	194.1	3	4	14	24
Homovanillic acid		1.028	46	75.39	66.76	182.1	3	4	13	23
Hydrocinnamaldehyde		1.818	40.83	60.69	17.07	134.1	3	1	10	20
Isoeugenol		2.434	49.37	72.11	29.46	164.1	2	2	12	24
Phenylacetaldehyde		1.428	36.21	54.32	17.07	120.1	2	1	9	17
Sinapinaldehyde		1.622	56.31	87.75	55.76	208.1	4	4	15	27
Vanillyl methyl ketone		1.532	49.04	76.96	46.53	180.1	3	3	13	25

Figure 2. Overview of the tested compounds in the alphabetical order, showing their chemical structure as well as the calculated molecular descriptors: Log of the octanol/water partition coefficient (SlogP); molecular refractivity (SMR); accessible surface area (Labute ASA); topological polar surface area (Å^2) (TPSA); molecular weight (MW); and the number of rotable bonds (Rotab. Bonds), atoms, heteroatoms, and heavy atoms.

that, the second most potent compound cinnamaldehyde is more potent than cinnamic acid, with a fold change of 13.45 ± 2.15 vs 1.02 ± 0.15 ($P < 0.001$). In addition, exchanging the α,β,-unsaturated carbonyl group of coniferylaldehyde with an allyl group, leading to the aroma compound eugenol, reduced the serotonin-releasing potential of the phenylpropanoids by 10.7. Out of the eight tested aldehydes, sinapinaldehyde, which has two methoxy and one hydroxyl group at the phenyl ring, was the least potent aldehyde with an effect size of 1.71 ± 0.22, which is in the range of the tested acids. The test compound 2-methoxy-cinnamaldehyde with one methoxy group at the phenyl ring was also less potent than cinnamaldehyde (4.30 ± 1.55, $P < 0.01$) and equally potent as sinapinaldehyde ($P > 0.05$).

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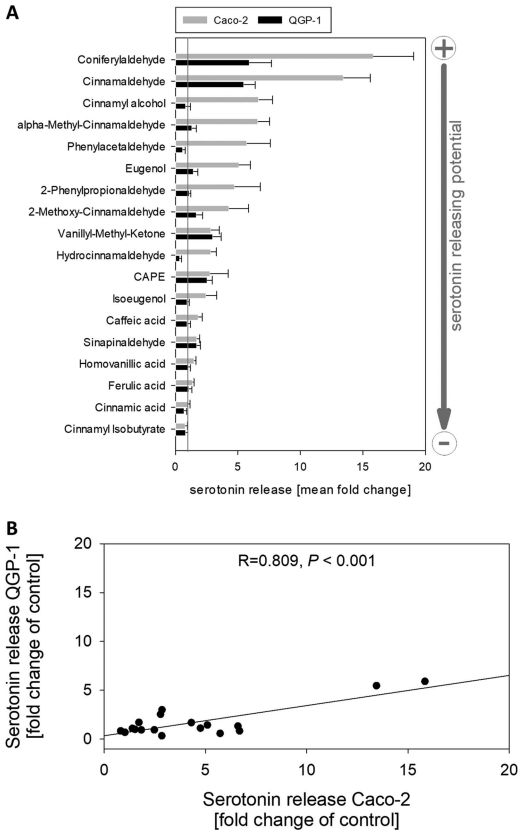

Figure 3. (A) Serotonin release in QGP-1 enterochromaffin cells (black bars) and Caco-2 enterocytes (light gray bars) induced by cinnamaldehyde and the 17 tested structural related compounds at a concentration of 0.5 mM. Data are presented as mean fold change ± SEM calculated from three to six independent experiments with two technical replicates each. Serotonin release induced by buffer with or without 0.1% DMSO is set to 1 and represented as a gray line. The SEM values for the controls are 0.11 (control) and 0.13 (DMSO control) for QGP-1 cells and 0.25 (control) and 0.06 (DMSO control) for differentiated Caco-2 cells, respectively. (B) Correlation analysis (Pearson's product-moment correlation) of serotonin release induced by 0.5 mM cinnamaldehyde or its structural analogues in QGP-1 enterochromaffin cells and Caco-2 enterocytes. Correlation coefficient (R) = 0.809, $P < 0.001$, n = 18.

Moreover, the four most potent compounds coniferylaldehyde, cinnamaldehyde, cinnamyl alcohol, and α-methyl-cinnamaldehyde, inducing a fold change of 5 or higher, are phenylpropanoids with an unsaturated side chain. A saturated side chain, as shown by stimulation with hydrocinnamaldehyde, led to a reduced serotonin release ($P < 0.05$) compared to the direct unsaturated analogue, cinnamaldehyde, with a 2.84 ± 0.44 fold increase for hydrocinnamaldehyde compared to a fold change of 13.45 ± 2.15 for cinnamaldehyde.

TRPA1 Dependency of Serotonin Release in Caco-2 Cells. The identified beneficial structural characteristics to induce serotonin release in Caco-2 cells have similarities to those described for potent TRPA1 activators.[22] In addition, for QGP-1 and Caco-2 cells, a stimulating effect of cinnamaldehyde on serotonin release was demonstrated to depend at least partly on TRPA1 stimulation.[1,4] Thus, a potential TRPA1-dependent component for serotonin release in Caco-2 cells was analyzed here as well.

Using RT-qPCR, we confirmed the gene expression of TRPA1 in QGP-1 cells and differentiated Caco-2 cells that were used for the experiments. In relation to the reference gene *HPRT1*, Caco-2 enterocytes expressed with 0.55 ± 0.28% significantly higher levels of *TRPA1* than QGP-1 cells (0.06 ± 0.02%, $P < 0.01$, data not shown in a figure). Moreover, pre-incubation for 20 min with 30 μM of the selective TRPA1 inhibitor AP-18[23] reduced the response of Caco-2 cells to cinnamaldehyde by 30 ± 13.9% (Figure 4A).

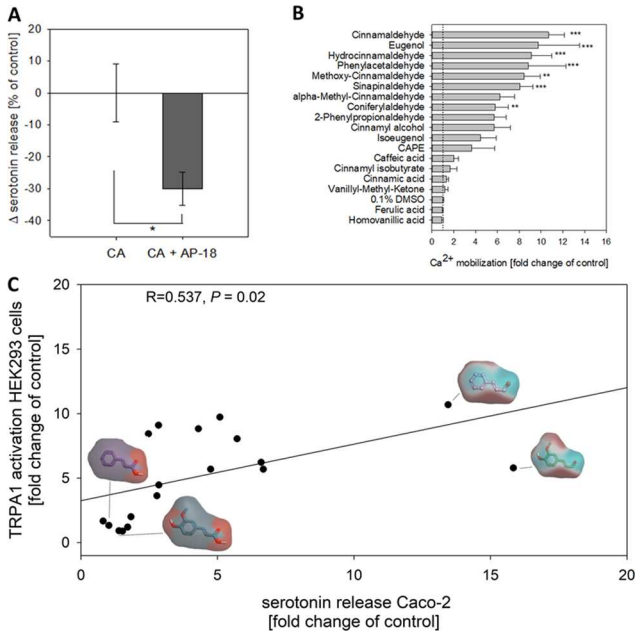

Figure 4. (A) Difference in serotonin release in Caco-2 enterocytes induced by 0.5 mM cinnamaldehyde (CA) solely and after 20 min pre-incubation with 30 μM of the specific TRPA1 inhibitor AP-18. Data are presented as mean fold change ± SEM calculated from three independent experiments with two technical replicates each. The significant difference (*$P < 0.05$) was tested using Student's t-test. (B) Maximum Ca^{2+} response after stimulation with cinnamaldehyde and the 17 tested structural related compounds at a concentration of 0.5 mM in transiently hTRPA1-expressing HEK293 cells. Data are presented as mean fold change of the control ± SEM calculated from four to six independent experiments with four technical replicates each. Significant differences were analyzed using one-way ANOVA and are marked by **$P < 0.01$, ***$P < 0.001$. Mock-transfected cells showed no increase in Ca^{2+} after stimulation with 0.5 mM cinnamaldehyde (data not shown in the figure). (C) Correlation analysis (Pearson's product-moment correlation) of serotonin release in Caco-2 enterocytes and the maximum Ca^{2+} response in transiently hTRPA1-expressing HEK293 cells induced by 0.5 mM cinnamaldehyde or its structural analogues. Correlation coefficient (R) = 0.537, P = 0.02, n = 18. Electrostatic potential maps visualizing areas of high (cyan) and low (red) electron densities are exemplarily shown for cinnamaldehyde and coniferylaldehyde as high-potency and cinnamic acid and ferulic acid as low-potency compounds.

Next, we aimed to compare the potency of the test compounds to activate TRPA1 with serotonin release in Caco-2 cells. For this purpose, HEK293 cells that transiently expressed hTRPA1 were stimulated with 0.5 mM of the test compound and the maximum Ca^{2+} mobilization was evaluated using Fluo-4. Mock-transfected cells were stimulated with 0.5 mM as a control and showed no increase in Ca^{2+} levels (data not shown). Cinnamaldehdyde, eugenol, hydrocinnamaldehyde, phenylacetaldehyde, and methoxy-cinnamaldehyde had the most pronounced effects, followed by other aldehydes

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(Figure 4B). The tested organic acids (caffeic acid, cinnamic acid, ferulic acid, and homovanillic acids) did not induce Ca^{2+} mobilization in TRPA1-expressing HEK293 cells (Figure 4B). Moreover, matched-molecular pair analysis of TRPA1 activation by the test compounds demonstrated that an α,β-unsaturated aldehyde or an allyl group is more active than the corresponding organic acid or ketone (Figure S3). The maximum Ca^{2+} response of hTRPA1-transfected HEK293 cells induced by stimulation with the structural analogues of cinnamaldehyde significantly correlated with the serotonin release in Caco-2 cells (Pearson's product-moment correlation, $R = 0.537$, $P = 0.02$, Figure 4C). An electrostatic potential map exemplarily shown for the most potent compounds cinnamaldehyde and coniferylaldehyde and their respective non-potent acids cinnamic acid and ferulic acid demonstrates the areas of high (cyan) or low (red) electron densities as a marker for their electrophilic and nucleophilic reaction sites.

Since serotonin release in Caco-2 cells is modulated by TRPA1 activation, molecular characteristics of the test compounds that are beneficial for TRPA1 stimulation were analyzed. For that purpose, several physical and surface descriptors, as well as atom and bond counts, were calculated using RDKit node for the KNIME Analytics Platform, using the R server as a backend. Using Pearson's product-moment correlation, the relevant descriptors (>95% nonzero values, differences in values between molecules) were correlated to their potential to stimulate intracellular Ca^{2+} mobilization in hTRPA1-transfected HEK293 cells. The descriptors tested were log of the octanol/water partition coefficient (SlogP); molecular refractivity (SMR); accessible surface area (Labute ASA); topological polar surface area ($Å^2$) (TPSA); molecular weight (MW); and the number of rotable bonds, atoms, heteroatoms, and heavy atoms. An overview of the compounds, structures, and descriptors can be found in Figure 2. The descriptors TPSA (-0.526, $P < 0.05$) and the number of heteroatoms (-0.495, $P < 0.05$) negatively correlated with the intracellular Ca^{2+} mobilization in hTRPA1-transfected HEK293 cells. In addition, there was a trend for a negative correlation of the MW (-0.477, $P = 0.06$) and TRPA1 activation.

DISCUSSION

The present study aimed to clarify which structural characteristics determine serotonin release induced by the cinnamon-derived aroma compound cinnamaldehyde in intestinal cells. Therefore, serotonin release induced by cinnamaldehyde and additional 17 structurally related compounds was analyzed in QGP-1 enterochromaffin cells and Caco-2 enterocytes.

As a first step, we compared dose-dependent serotonin release induced by cinnamaldehyde in QGP-1 cells as a model for human enterochromaffin cells and differentiated Caco-2 cells as a model for human enterocytes. In both cell models, cinnamaldehyde induced a dose-dependent increase in serotonin release starting from a concentration of 0.5 mM, although the effect size in Caco-2 cells was significantly higher than that in QGP-1 cells. Based on the limited solubility of cinnamaldehyde in water, the highest test concentration applied was 5 mM for Caco-2 cells. Due to negative effects on cellular viability, concentrations up to 2.5 mM of cinnamaldehyde were tested in QGP-1 cells. With the here-tested concentrations, no saturation point was reached and, thus, no EC_{50} values were calculated.

As a next step, we investigated which molecular structural characteristics are advantageous for stimulating or inhibiting serotonin release by analyzing the serotonin release in the two intestinal cell models after incubation with further 17 naturally occurring compounds that are structurally related to the lead aroma compound cinnamaldehyde. Based on the dose dependency of cinnamaldehyde, a concentration of 0.5 mM was chosen to investigate serotonin release after stimulation with aroma compounds. The concentrations chosen for the present study are, although relatively high, in the typically used range of concentrations for TRPA1-based mechanistic studies.[3] These concentrations do not necessarily reflect dietary doses of cinnamaldehyde, although a concentration of around 400 μM could theoretically reach enterocytes in the gastrointestinal tract following ingestion of 6 g of cinnamon powder.[4] The concentrations were selected to induce a strong serotonin response to reach higher effect levels, and consequently, larger differences to analyze characteristics that will lead to an increased serotonin release in gastrointestinal cell models.

The serotonin release induced by the test compounds in Caco-2 significantly correlated with the serotonin release in QGP-1, which points to a common mechanism for structure-dependent serotonin release in the two cell models. More specifically, the matched pair analysis used in the present study revealed the following structural characteristics to be advantageous to stimulate serotonin release by enterochromaffin cells and enterocytes: In both cell models, unsaturated phenylpropanoids led to the highest effect levels. An aldehyde group was more effective than a hydroxyl group and a ketone group, which was advantageous over a carboxyl group. Due to the electronic and steric profile of the compounds, aldehydes are generally more reactive toward nucleophilic substitutions than ketones.[24] This suggests electrophilic compounds with electron-withdrawing properties to be beneficial to stimulate serotonin release from intestinal cells, which is additionally suggested by the fact that the four most potent compounds are α,β-unsaturated aldehydes. Direct comparison of the efficacy of hydrocinnamaldehyde and cinnamaldehyde also confirms the importance of the double bond in the C3 side chains of phenylpropanoids. Due to the electronic profile, α,β-unsaturated aldehydes are especially prone to nucleophiles such as the $-SH$ group in cysteine residues[25] However, also, phenylacetaldehyde with a shorter side of C2 was among the most potent compounds. The substitution of the phenylethyl group with one methoxy and one hydroxyl group was beneficial, as shown for coniferylaldehyde. However, the introduction of an additional methoxy group, as demonstrated using sinapinaldehyde, largely reduced the serotonin-releasing potential. A reason for this could lie in the change in the steric profile of the compound.[26]

The here-identified structural characteristics are in accordance with characteristics of known TRPA1 ligands: Electrophysiological studies showed that several TRPA1 agonists are electrophilic molecules, for example, cinnamaldehyde, that activate the ion channel via covalent modification of conserved cysteine or lysine residues within the cytoplasmic N terminus.[22] In that context, aldehydes are more reactive than ketones due to the more pronounced polar nature of the carbonyl group.[27] Esters and carboxylic acids bear an additional oxygen that reduces the electrophilic reactivity of the respective carbonyl carbon,[26] which may explain the reduced serotonin-releasing potential of the tested acids and esters in comparison to aldehydes in Caco-2 cells. This

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hypothesis is illustrated by a graphical representation of the electrostatic potential map of the most potent compounds, namely, cinnamaldehyde, visualizing the electrophilic reactive site (red) of the molecules at the ß-carbonyl carbon, which is lacking at the respective acids. Likewise, we detected reduced TRPA1-dependent Ca^{2+}-mobilization after stimulation with the tested organic acids, namely, cinnamic acid, caffeic acid, homovanillic acid, and ferulic acid, and the esters, namely, CAPE and cinnamyl isobutyrate, in comparison to aldehydes using a transiently hTRPA1-transfected HEK293 cell model. This is also reflected by the finding that the compound's potential to induce serotonin release correlates with the potential to activate TRPA1 in transiently transfected HEK293 cells. In addition, using AP-18 as a specific TRPA1 inhibitor,[23] our results verify a TRPA1-dependent component in serotonin release, confirming the results from Doihara et al.[1] in QGP-1 cells and Hoi et al.[4] in Caco-2 cells. However, since only about 30% of the signal was blocked by AP-18, it cannot be excluded that also other pathways might play a role. These unknown additional pathways could also explain why, although there is a correlation between serotonin release in Caco-2 cells and the TRPA1 activation in a single-receptor model, there are differences in the order of the most effective compounds.

Moreover, serotonin release after stimulation with cinnamaldehyde was more pronounced in Caco-2 cells than in QGP-1 cells. The exact reason for that remains unknown and needs to be addressed in future studies. However, since serotonin release in the gastrointestinal tract is regulated by an extracellular Ca^{2+} influx mediated by voltage-gated Ca^{2+}-channels,[28] an influx of Ca^{2+} via TRPA1 may be responsible for the TRAP1-dependent component in serotonin release in Caco-2 cells. Thus, we hypothesize here that the higher effect size in Caco-2 cells compared to QGP-1 cells could be due to the higher *TRPA1*/*HPRT* ratio found in the fully differentiated Caco-2 enterocytes used in the present study. In contrast to our finding, Doihara et al.[1] showed a higher *TRPA1* gene expression in QGP-1 than in undifferentiated Caco-2 cells. Thus, an increase of *TRPA1* gene expression upon differentiation to an enterocyte model is assumed, which might largely influence serotonin release and reuptake behavior of the cells.

To further examine the underlying structural characteristics, several molecular descriptors, representing numerical properties of the molecules, were computed independently from the molecule's conformation based on their chemical 2D structure and compared to TRPA1 activation in transfected HEK293 cells. The descriptors' topological polar surface area, molecular weight, and the number of heteroatoms were shown to be negatively correlated with TRPA1 activation. This points to smaller molecules with a lower polar surface area to be beneficial. As a rule of thumb, molecules with a polar surface area higher than 140 $Å^2$ tend to have a lower cell membrane permeability.[27] Since electrophilic molecules such as cinnamaldehyde and related structures target intracellularly located cysteine residues of the TRPA1 channels, the tested compounds' ability to permeate the cell membrane is associated with the ability to activate the TRPA1 channel. Although the tested acids have a polar surface area below 140 $Å^2$, the carboxyl group leads to a higher polarity of the molecule,[26] which may reduce their potency to activate TRPA1. This is also in accordance with the observation that the tested organic acids did not induce a noteworthy response in hTRPA1-expressing HEK293 cells. However, also non-electrophilic compounds such as menthol or carvacrol can activate the TRPA1 cation channel without modifying cysteine residues,[2,29] opening the possibility for alternative pathways as well.

In transfected HEK293 cells, EC_{50} values for cinnamaldehyde of 61 ± 9 μM at 23 °C and 84 ± 9 μM at 35 °C for TRPA1 activation have been reported previously,[3] demonstrating also the temperature sensitivity of TRPA1 channels. The here-presented results were recorded at room temperature, which might also have an impact on the results. In addition to temperature sensitivity, in respect of concentration-dependent TRPA1 activation by the test compounds, it has to be taken into account that Caco-2 and QGP-1 models are native cells, which might react differently as a single-receptor model such as transfected HEK293 cells due to the possible synergistic or agonistic pathways.

It has to be noticed that, with the present study design, it cannot be fully distinguished between an increased serotonin release and a decreased activity of the serotonin reuptake transporter (SERT). Reduced activity of SERT would lead to a decreased reuptake of serotonin and thus to increased serotonin levels in the cellular supernatant. This might also play a role in data obtained with Caco-2 cells in the present study, as a recent study by Hoi et al.[4] showed that the application of cinnamaldehyde reduced the SERT activity while increasing serotonin levels in the cellular supernatant of differentiated Caco-2 cells.

In conclusion, the present study showed that the ability of a compound to activate TRPA1 is a decisive factor to stimulate serotonin release in Caco-2 enterocytes, preferring small, electrophilic compounds with a lower polar surface area. The results of the present study may serve as an important base for studying the prediction of peripheral serotonin release. Future studies are needed to show whether these results may be transferred to other compound classes as well and to confirm the results in vivo.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.9b08163.

Cell viability of Caco-2 and QGP-1 cells after treatment with 0.5 mM test compounds used in the present study assessed via the MTT assay (Figure S1); cell viability of Caco-2 and QGP-1 cells after treatment with 0.5−5 mM cinnamaldehyde assessed via the MTT assay (Figure S2); and graphical representation of the matched-molecular pair analysis carried out with Vortex software (version 2019.04, Dotmatics Ltd., U.K.) including single atom changes and nonring fragmentations (with a maximum fragment size of 8 and a minimum core size of 8 atoms), using both experimental readouts of TRPA1 activation and serotonin secretion by Caco-2 enterocytes as parameters of interest (Figure S3) (PDF)

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Notes

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■ ABBREVIATIONS

ANOVA, analysis of variance; CAPE, caffeic acid phenethylester; TRP, transient receptor potential; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

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4 Conclusion and perspectives

In view of the growing worldwide obesity prevalence and its consequential health challenges, interest and research is expanding to identify potential novel anti-obesity agents, specifically taking into account naturally occurring plant-derived substances. As an alternative to synthetic drugs, they might be of benefit in supplementing a balanced daily nutrition as a supporting measure in obesity therapy[24, 307]. In that regard, a variety of diverse positive health effects have been linked to herbs and spices, such as, among many others, cinnamon, which has been used as traditional remedy for centuries[315, 316]. However, the amount of cinnamon powder that can be consumed is limited due to its distinctive aroma and flavor characteristics, on the one hand, and potentially exceeding coumarin levels, on the other hand[331]. Furthermore, naturally occurring concentrations of relevant bioactive compounds might be insufficient for the anticipated beneficial health effects. Therefore, administering individual cinnamon constituents might be a safer and more effective alternative.

The major bioactive cinnamon constituent cinnamaldehyde, in particular, has been emphasized for its hypoglycemic, hypolipidemic, anti-adipogenic and possibly also satiating potential in animal studies following a long-term administration[23, 318, 329]. Not only was it shown to reduce body weight gain in mice after chronic supplementation of 0.2% CAL to a high fat chow[318], but also to decrease cumulative short-term food intake in mice following a bolus administration of 10 and 250 mg per kg bw[318, 327]. Due to its nociceptive attributes and its specific odor and spicy flavor qualities, however, CAL might also be, to some extent, restricted in its use. Whereas, however, anti-obesity and anti-diabetic activities have been reported numerously for cinnamaldehyde, minor cinnamon constituents are scarcely studied in terms of their anti-obesity and anti-diabetic potential. In the present thesis, firstly (i) a short-term impact of CIB, as a further, structurally related, but less spicy cinnamon constituent, on food intake and endogenous satiety signal was hypothesized *in vivo.* For this purpose, a crossover human intervention study was performed, where a bolus dose of 0.45 mg CIB in a glucose solution (75 g per 300 ml water) was administered to moderately overweight men in order to determine its effect on energy consumption from a standardized breakfast and on selected plasma hormone levels as short-term markers of satiety (*Cinnamyl Isobutyrate Decreases Plasma Glucose Levels and Total Energy Intake from a Standardized Breakfast: A Randomized, Crossover Intervention*). As indicated by our results, CIB administration indeed resulted in a decrease of short-term *ad libitum* energy intake compared to the control treatment by approximately 4 %. This small, yet significant decline in energy intake could be demonstrated, even though CIB was given at a dose of 0.45 mg to healthy male subjects showing a body weight index between 25 and 32 kg x m^{-2}. This dose was much lower than those applied as bolus doses in animal models, varying between 10 to 250 mg per kg bw[318, 327]. However, since estimated food records analyzed in the human intervention study of this theses revealed no changes in the cumulative caloric intake over 24 hours past intervention, long term effects of CIB

on food intake still have to be demonstrated. Additionally, the hypothesized satiating impact of CIB could not be determined by visual analogue scale (VAS). At this point, it remains elusive, why CIB did not reduce subjective hunger perceptions in the present study. However, in general, findings in humans regarding an effect of cinnamon on satiety are conflicting, despite the strong *in vivo* animal evidence regarding an impact of cinnamon and constituents on food intake and maintenance of body weight[23, 321, 327].

As regulatory effects of glucose in food intake have been discussed[370], a potential postprandial effect of CIB was also analyzed as a secondary outcome measure. Since anti-diabetic properties of cinnamon and its major constituent CAL have been reported repeatedly, a potential hypoglycemic effect of 0.45 mg CIB was hypothesized and substantiated in the present study by decreased delta AUC glucose levels compared to control treatments without simultaneously altering insulin responses. As possible modes of action after long-term cinnamon treatment, delayed effects on insulin sensitivity[323] and improved glucose utilization[371] have been proposed. A delay in gastric emptying[318] or an impact on glucose uptake at a cellular level might explain the more rapid effects of cinnamon and its constituents, such as CAL or possibly also CIB, on postprandial glucose levels, which however, still warrants further investigation.

Whereas, on the one hand, a mean 4 % reduction in food consumption could be assessed in the here presented human intervention study, GLP-1, $PYY_{3–36}$, ghrelin and peripheral serotonin levels, on the other hand, were not changed after CIB administration. However, a trend for a serotonin increase ($p = 0.076$) two hours after the 0.45 mg CIB bolus administration was calculated compared to the control, putting forth the argument, that future studies might detect if higher or periodical CIB supplementation, resulting in a more pronounced decrease, reveal a regulation on anorexigenic and orexigenic hormonal level. Food consumption not only depends on nutritional or hormonal cues from the gut. Also, hedonic influences, such as palatability, social or environmental stimuli can affect food intake without the requiring calories[29, 372, 373]. Hence, hunger perceptions must be differentiated from appetite, which characterizes the desire to eat and is triggered by the presence of food and satisfaction of eating[29, 374]. Consequently, an effect of CIB on energy intake and appetite might be hypothesized in contrast to a long-term satiating effect of CIB, which could not be demonstrated by our data. In conclusion, our study findings might suggest a possible short-term satiating effect of 0.45 mg CIB. However, studies are warranted examining the long-term application of CIB as a potential anti-obesity agent, which might reveal a regulating effect on hormonal level as well. Moreover, taking into account the small sample size, which is considered a limitation of this study, larger intervention trials are required as well.

Despite consistent evidence, revealing regulating effects of cinnamon and its constituents, like CAL or, as it was also determined in the present work, CIB, on energy intake or plasma nutrient levels, not much is known regarding their impact on macronutrient uptake on a cellular level,

especially concerning lipids and fatty acids. Since reducing lipid absorption from the gastrointestinal tract might be a strategy to decrease excessive caloric intake and a potential anti-obesity effect of cinnamon-derived compounds such as CAL has been proposed in the literature[23], the focus of the following mechanistic studies was placed on lipid metabolism. Although cinnamon and CAL have been shown to decrease plasma levels of free fatty acids and triglycerides *in vivo*[23], a direct impact on intestinal lipid absorption as first point of contact has not been examined so far. Therefore, this work secondly (ii) hypothesized an inhibiting impact of CAL, the most abundant constituent of cinnamon bark oil, and selected cinnamon-derived structural analogues and potential metabolites on intestinal lipid absorption and aimed to analyze possible underlying mechanistic pathways. For this purpose, the influence of CAL, CIB, CAL-C and CAC on fatty acid uptake in differentiated Caco-2 cells as an *in vitro* model for the intestinal barrier, were studied (*Identification of Cinnamaldehyde as Most Effective Fatty Acid Uptake Reducing Cinnamon-Derived Compound in Differentiated Caco-2 Cells Compared to Its Structural Analogues Cinnamyl Alcohol, Cinnamic Acid, and Cinnamyl Isobutyrate*). Indeed, according to our hypothesis, pre-treating Caco-2 cells with the test compound CAL dose-dependently and most potently reduced fatty acid uptake into the cells by up to 58.8 ± 8.83 %. Structural relatives CAC and C-ALC also decreased fatty acid uptake in higher concentrations, whereas no altered fatty acid uptake could be detected after CIB treatment. This detected lipid-absorption-modulating effect is consistent with further anti-obesity effects, that have already been reported for CAL. In animal models, CAL administration, in varying concentrations varying from 10-40 mg/kg, was shown to diminish body weight gain and visceral fat reservoirs[329], to possibly regulate lipolytic activity[329] and to reduce plasma triglyceride and free fatty acid levels compared to a control group[23], pointing to a hypolipidemic effect as well. More recently, also CAC and C-ALC have been discussed in terms of anti-adipogenic activity[311, 339].

To screen for potential pathways which might be involved in this inhibitory effect on lipid absorption, customized whole genome DNA microarrays were applied in this thesis as a first mechanistic approach. Interestingly, pathway analyses primarily revealed a notable effect of CAL and, to a lesser extent, the other test compounds on serotonin receptor regulation. Hence, an effect of the test compounds on serotonin release was hypothesized, which in turn might modulate fatty acid uptake. For that, we aimed to investigate the secreted serotonin levels in the cell supernatant by means of an ELISA assay. As it was shown before in enterochromaffin and pancreatic endocrine cells[156, 366], in the human enterocyte Caco-2 cell model presented here, the secretion of serotonin into the cellular supernatant was stimulated by CAL as well. Additionally, an effect on serotonin release was found for C-ALC, but not for CAC and CIB. At the same time, CAL and C-ALC reduced the uptake of a neurotransmitter imitating fluorophore into the cell, suggesting not only a stimulating effect on serotonin secretion, but also a reducing effect on the serotonin reuptake into the cells. Also, extracellular serotonin concentrations depend on the activity of the

serotonin reuptake transporter (SERT), which has been identified in Caco-2 cell before. Based on the results of this study, it cannot be specified, however, if CAL of CALC directly inhibit SERT, interact with SERT trafficking processes or whether high extracellular serotonin concentration themselves affect neurotransmitter uptake into the cells. Moreover, an influence on other biogenic amine transporters cannot be excluded. As multifunctional neurotransmitter, serotonin is known to play, among many others, an important role in the regulation of food intake, generating feelings of satiety and leading to suppression of energy consumption[177-180, 182]. Not being able to pass the blood-brain barrier, circulating serotonin has been associated with reduced food intake and earlier onset of satiety possibly via activation of vagal afferents[192-196]. Although effects of cinnamon and CAL on peripheral serotonin metabolism have not been reported yet, both have been linked repeatedly to modulating effects on satiety-related parameters, such as GLP-1 or PYY plasma levels, food intake or expression of anorectic hypothalamic genes in humans or animal models[327, 329, 330]. Collectively, our data show elevated extracellular levels of peripheral serotonin after CAL or C-ALC stimulation, and revealed a regulating effect on gene expression level of several serotonin receptors associated with satiety related mechanisms.

In order to identify modes of actions that might be involved in the test compound-mediated decrease in fatty acid uptake, the potential contribution of different mechanistic pathways have been examined. Firstly, a regulating impact of increased extracellular serotonin concentrations on fatty acid uptake in Caco-2 cells could not be substantiated. Secondly, a potential decrease in fatty acid uptake through modifications of the paracellular transport could be excluded as main mode of action. Even though transepithelial electrical resistance (TEER) was strongly increased after prolonged exposure of Caco-2 cells to CAL, suggesting a potential CAL-mediated impact on tight junction permeability, no significant TEER increase was found at the earlier time point when fatty acid uptake was decreased. Thirdly, an impact on intestinal fatty acid transporter CD36 was proposed. To examine this, stimulation via downstream signaling was attempted, based on findings demonstrating AMP-activated protein kinase (AMPK)-induced and CD36-dependent changes in fatty acid uptake in different cell types[375, 376]. However, an impact on fatty acid uptake via modulating effects on AMPK and calcium/calmodulin-dependent protein kinase kinase (CaMKKβ) could not be determined. Interestingly, fatty acid uptake was not affected by CD36 inhibitor SSO (Sulfo-N-succinimidyl oleate) as well, suggesting that CD36 in general does not play an important part in lipid absorption in differentiated Caco-2 cells. An impact of the test compounds on other fatty acid transporters such as FATPs or FABP4 cannot not be excluded and needs to be investigated in studies to come. Lastly, CAL, being a known potent TRPA1 activator, lead to the question of a potential TRPA1-dependency in the determined fatty acid uptake inhibition[362, 363]. TRPA1 agonists such as CAL and other unsaturated carbonyl-containing compounds, as electrophilic substances are believed to activate this channel by covalently modifying the intracellular

lysine and cysteine residues[362]. The mean 9.97 ± 9.57% % attenuated inhibition of fatty acid uptake determined after applying TRPA1 inhibitor AP-18 suggests, that TRPA1 activation might in fact play a minor role in the CAL-mediated inhibition of fatty acid uptake in differentiated Caco-2 cells, whereas no TRPA1 activation could be shown for C-ALC and CAC. The relatively small effect size, however, indicates the involvement of other modes of action as well. Additionally, TRPA1-dependency was examined in the test compound-mediated increase in serotonin release, which was in fact confirmed by an approximately 31 % decrease in the CAL-stimulated serotonin release after TRPA1 inhibition. This TRPA1-induced effect of CAL on serotonin release could further be indicated by examining structure-dependent effects of different CAL-derivatives on TRPA1-mediated serotonin release, revealing a correlation between the potential to induce serotonin release with the potential to stimulate TRPA1 in transiently hTRPA1 expressing HEK293 cells (*Structure-dependent effects of cinnamaldehyde-derivatives on TRPA1- induced serotonin release in human intestinal cell models*). However, as the impact of CAL was not entirely blocked, an involvement of additional signaling pathways might be assumed. Since TRPA1 activation is linked to Ca^{2+} influx, and the increase of intracellular Ca^{2+} concentration is known to play a major part in inducing exocytosis pathways, we propose at least in part a Ca^{2+}-mediated serotonin release in Caco-2 cells similar to endocrine cells[147, 156, 377, 378]. This, however, and the participation of potential additional cellular pathways need to be examined in more detail in further studies. Collectively, analysis and comparison of the selected cinnamon-obtained aroma test compounds demonstrated their potential as bioactive dietary substances to target fatty acid uptake and secretion of serotonin in the gut with CAL exhibiting the most pronounced effects, including an impact on TRPA1-dependent routes.

Following the analysis of the anti-obesity effects of cinnamon test compounds in the intestinal cell model and due to anti-adipogenic effects that have been reported for CAL, C-ALC and CAC, (iii) a potential anti-adipogenic effect of CIB in lipid tissue was hypothesized in the present thesis as well. To target this hypothesis, long-term lipid accumulation during 3T3-L1 cell adipogenesis as well as possible underlying mechanistic pathways were examined in a comparative study applying CIB and CAL (*The TRPA1 agonist cinnamaldehyde decreases adipogenesis in 3T3-L1 cells more potently than the non-agonist structural analog cinnamyl isobutyrate*). As its structural cinnamon derived relatives, 30 µM CIB also revealed an anti-adipogenic potential, decreasing lipid accumulation after a 12-day treatment during differentiation and maturation phase of 3T3-L1 cells by 21.4±2.56 %, albeit to a lesser extent than CAL. Applying nile red staining allowed further differentiation between triglyceride and phospholipid staining, confirming an inhibiting impact of CAL and CIB on both, polar and neutral lipid accumulation. Since CAL-treatment showed a stronger effect on triglyceride than phospholipid accumulation, an additional regulating effect of CAL in later phases of the adipogenesis might be suggested as well. Based on the pos-

sible rapid hydrolyzation of the cinnamyl ester, it cannot be specified exactly if CIB or a degradation product might have caused the lipid accumulation inhibiting effect. Possible short-term effects of CIB and CAL by examining fatty acid uptake in fully developed adipocytes could not be determined.

Mechanistically, CAL has been suggested to facilitate its lipid-accumulation inhibiting effect via suppressing the differentiation process into mature adipocytes[23], altering lipolytic and lipid biosynthetic pathways[329] in adipocytes as well as to affect thermogenesis and metabolic reprogramming[340, 344]. Furthermore, involvement of AMPK signaling has been proposed in the adipogenic program as well[379]. The exact CAL-mediated signaling routes, however, are not completely understood yet. However, since the reduction of triglyceride accumulation after CAL-treatment is linked to a downregulation of essential adipogenic transcription factors, such as PPARγ and C/EBPα, a modulating impact on the signaling cascade has been suggested as possible underlying mode of action for CIB as well[23]. As hypothesized, CIB treatment also revealed a downregulation of the adipogenic transcription factors PPARγ, C/EBPα and C/EBPβ on gene as well as protein levels at different time points of the adipogenic process, indicating a substance-modulated impact on the adipogenic signaling cascade on a molecular level as well. Beside a CAL-induced downregulation of PPARγ mRNA levels, also a specific inhibitory effect on PPARγ transcriptional activity has been reported[23]. Interestingly, in the here presented work, FABP4 protein levels, which have been found to increase in the progressing differentiation process, were not significantly reduced after a 12 day CIB treatment contrary to CAL, which might support the lower anti-adipogenic potential of CIB compared to CAL.

Due to the known TRPA1 agonistic activity of cinnamaldehyde, a potential receptor involvement was examined in the CAL-mediated anti-adipogenic effect in 3T3-L1 cells[156, 344, 380]. Results revealed a significantly elevated triglyceride accumulation compared to the effect of CAL solely, suggesting a TRPA1-dependency in the CAL-modulated decreased lipid accumulation. With regard to these results, the exact time point at which the TRPA1-dependency might be involved in the adipogenetic process, could not be pinpointed, since the TRPA1-inhibitor was applied during differentiation as well as maturation phase. As another trigeminally active compound, *trans*-pellitorine, was found to TRPA1-dependently affect only earlier differentiation phases[380], a CAL-induced TRPA1 activation in early adipogenesis rather than adipocyte maturation might be hypothesized as well. Also, a modulating impact of $[Ca^{2+}]_i$, which was reported to biphasically modulate adipogenesis in a time dependent manner, might indicate a CAL-induced TRPA1-activation only in early adipogenic stages[368]. However, an additional CAL-induced regulation in lipolytic and lipid biosynthetic pathways of mature adipocytes might be suggested based on the here presented data as well.

In conclusion, in the search of potentially bioactive naturally occurring plant-derived substances, which might beneficially supplement conventional preventive or therapeutic strategies to counteract the rising prevalence of overweight and obesity associated with high caloric western diets, cinnamon-derived CIB might be a further interesting bioactive candidate. As it was shown to decrease short-term energy intake and postprandial plasma glucose levels, and might potentially even exhibit a regulation on satiety hormone level such as serotonin, if applied chronically or in higher concentration, CIB might pose an alternative to nociceptive structural relative CAL. Moreover, in the here presented comparative analysis of the selected naturally derived cinnamon aroma compounds CIB, cinnamaldehyde, C-ALC and CAC in cell models representing the intestinal barrier as well as the adipose tissue, revealed primarily cinnamaldehyde to be a potent anti-obesity candidate. Even though CIB administration was found to exhibit less pronounced effects on the lipid accumulation, in general, it might still be an interesting anti-obesity agent due to its hypoglycemic and food intake-modulating effects, that could be applied to minimize the sole use of nociceptive cinnamaldehyde without losing bioefficacy, or even used in addition to other naturally derived anti-obesity agents to potentiate their effects.

5 Abstract

Considering the globally increasing obesity prevalence and its concomitant health challenges, the search of potential new anti-obesity agents is growing. As alternative to synthetic drugs, naturally occurring compounds of plant origin might be used as a supplementary measure to benefit balanced diets in obesity prevention and therapy. A wide range of favorable health effects have been associated with herbs and spices, including, among many others, cinnamon. Its major bioactive constituent cinnamaldehyde (CAL) is known for exhibiting anti-diabetic, anti-adipogenic, hypolipidemic and possibly also satiating effects, but at the same time also shows nociceptive and unique flavor characteristics. In this work, firstly (i) a short-term impact of structurally related and less spicy constituent cinnamyl isobutyrate (CIB), on food intake and outcome measures of satiety was analyzed *in vivo*, revealing a bolus dose of 0.45 mg CIB to decrease *ad libitum* energy intake and postprandial plasma glucose levels, accompanied by a trend ($p = 0.076$) for increased peripheral serotonin. Albeit modulating effects of cinnamon constituents, such as CAL or CIB, on energy intake and plasma nutrient profiles have been shown, little is known regarding their impact on macronutrient uptake on cellular level, especially the uptake of fatty acids. Thus, the present work secondly (ii) investigated the impact of CAL as well as structural analogues CIB, cinnamyl alcohol (C-ALC) and cinnamic acid (CAC) on fatty acid uptake in differentiated Caco-2 cells as an *in vitro* model for the intestinal barrier. In fact, it could be demonstrated, that 300 µM CAL, most potently, decreased the uptake of free fatty acids into the cells by 58.8 ± 8.83 %, but also C-ALC and CAC revealed a fatty acid uptake-reducing effect. Whereas a TRPA1-associated increased release of satiety-related hormone serotonin could be shown at same time, serotonin itself did not change fatty acid uptake. Involvement of TRPA1-dependency could also be determined in the CAL-mediated decreased fatty acid uptake. Subsequent to analyzing anti-obesity effects of the selected cinnamon-derived test compounds in an intestinal cell model, a potential anti-adipogenic effect in adipose tissue, which has already been reported for other cinnamon-derived structural relatives, was hypothesized for CIB as well (iii). A comparative analysis studying the impact of CIB and CAL on adipogenesis and lipid accumulation, revealed a CIB-mediated anti-obesity potential, lowering lipid accumulation during the adipogenic process of 3T3-L1 cells. Both, CIB- and CAL-induced inhibition of triglyceride and phospholipid accumulation was accompanied by downregulation of key transcription factors in the adipogenic signaling cascade. Moreover, a TRPA1-dependency could be determined in the CAL-, but not the CIB-mediated anti-adipogenic effect. In conclusion, CIB might be a further naturally occurring bioactive candidate to help maintaining a healthy body weight by targeting mechanisms associated with decreasing short-term energy intake. Even though administration of CIB was found to display less potent effects on lipid metabolism compared to CAL, it might still provide an alternative or supplemental anti-obesity agent to the limited application of nociceptive CAL.

6 Zusammenfassung

Angesichts der weltweit zunehmenden Prävalenz an Fettleibigkeit und damit einhergehenden gesundheitlichen Herausforderungen, ist das Interesse an neuen, natürlich vorkommenden anti-adipogenen Wirkstoffen pflanzlichen Ursprungs groß. Als Alternative zu synthetischen Medikamenten könnten sie in Prävention und Therapie von Fettleibigkeit als ergänzende Maßnahme zu ausgewogener Ernährung eingesetzt werden. Viele Gewürze, wie etwa Zimt, wurden bereits mit einer Vielzahl gesundheitsfördernder Wirkungen in Verbindung gebracht. Insbesondere seinem Bestandteil Zimtaldehyd (CAL) werden antidiabetische, antiadipogene, hypolipidämische und möglicherweise sättigende Wirkungen zugeschrieben. Allerdings könnten dessen Geschmacks- und nozizeptive Eigenschaften auch einen limitierenden Faktor seiner Aufnahme darstellen. In dieser Arbeit wurde daher (I) der kurzfristige Einfluss des strukturell verwandten und weniger würzigen Cinnamylisobutyrat (CIB) auf Nahrungsaufnahme und Sättigungsparameter *in vivo* analysiert, wobei eine Bolusgabe von 0.45 mg CIB ad-libitum-Energieaufnahme und postprandiale Plasma-Glukosespiegel verringerte. Zusätzlich zeigte sich ein Trend ($p = 0.076$) einer gesteigerten peripheren Serotoninfreisetzung. Obwohl modulierende Effekte von Zimtbestandteilen wie CAL oder CIB auf Energieaufnahme und Plasma-Nährstoffprofile gezeigt wurden, ist wenig über ihre Auswirkung auf Makronährstoffaufnahme auf zellulärer Ebene bekannt, insbesondere jener der Fettsäuren. Daher wurde in dieser Arbeit (II) der Einfluss von CAL und seiner Strukturanaloga CIB, Zimtalkohol (C-ALC) und Zimtsäure (CAC) auf Fettsäureaufnahme in differenzierten Caco-2-Zellen als in-vitro-Modell für die Darmbarriere untersucht. Es konnte gezeigt werden, dass in erster Linie CAL, aber auch C-ALC und CAC Fettsäureaufnahme in Zellen verringerten. Während eine TRPA1-modulierte erhöhte Freisetzung des Sättigungshormons Serotonin nachgewiesen werden konnte, veränderte Serotonin selbst die Fettsäureaufnahme aber nicht. Eine geringe TRPA1-Abhängigkeit konnte zusätzlich in der CAL-vermittelten verminderten Fettsäureaufnahme festgestellt werden. Nachfolgend wurde (III) die potenzielle anti-adipogene Wirkung, die bereits für strukturverwandte Zimtverbindungen nachgewiesen wurde, auch für CIB untersucht. Eine vergleichende Analyse, die den Einfluss von CIB und CAL auf die Adipogenese testete, zeigte, dass auch CIB die Lipidakkumulierung während des adipogenen Prozesses von 3T3-L1-Zellen senkte. Sowohl die CIB- als auch CAL-induzierte Hemmung der Lipidakkumulierung ging mit einer Herunterregulierung adipogener Transkriptionsfaktoren einher. Darüber hinaus konnte eine leichte TRPA1-Abhängigkeit in der CAL-, nicht jedoch in der CIB-vermittelten antiadipogenen Wirkung festgestellt werden. Zusammenfassend könnte CIB also ein weiterer bioaktiver Kandidat sein, um kurzfristige Energieaufnahme und postprandialen Glukosespiegel zu senken. Und obwohl die Verabreichung von CIB im Vergleich zu CAL weniger starke Auswirkungen auf den Lipidstoffwechsel zeigte, könnte CIB dennoch ein alternatives oder ergänzendes anti-adipöses Mittel zu dem in seiner Verwendung limitierten CAL darstellen.

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