UNIVERSITY OF CRETE
DEPARTMENT OF SCIENCES OF HEALTH
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Crosstalk of inflammation and metabolism: the role of Akt1 kinase

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Αλληλεπίδραση φλεγμονής και μεταβολισμού: ο ρόλος της Akt1 κινάσης

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Abstract

Inflammatory responses are orchestrated and fine-tuned by a plethora of mechanisms at multiple levels. Macrophages are important players in innate immunity who can be classically or alternatively activated and exhibit a pro-inflammatory or an anti-inflammatory phenotype respectively. Metabolic inflammation is a state of chronic, low-grade inflammation and both immune cells and adipose tissue are implicated. Excess nutrients provide the primary induction of metabolic inflammation, while the crosstalk between adipocytes and immune system is what maintains it. There are several studies exploring the effects of dietary metabolites in inflammation, but their exacts effects and mechanisms of action in metabolic inflammation are not fully understood. The present study is focused on studying the effects of gut diet-derived short chain fatty acids on macrophage polarization and metabolism and identifying Akt1 kinase's role in this effect, so that it will be possible to understand macrophage-mediated intestine microbiome alterations in obesity. We also explore the potential anti-adipogenic effects of terpenoids from sea algae, in the context of adipogenesis causing/deteriorating metabolic inflammation. Overall, this study adds to the current knowledge on the regulation of metabolic inflammation by dietary metabolites and the role of Akt1 kinase in it.

Περίληψη

Οι φλεγμονώδεις αποκρίσεις συντονίζονται από μια πληθώρα μηχανισμών σε πολλά επίπεδα. Τα μακροφάγα είναι σημαντικοί συμμετέχοντες στην έμφυτη ανοσία οι οποίοι μπορούν να ενεργοποιηθούν κλασσικά ή εναλλακτικά και εμφανίζουν προφλεγμονώδη ή αντιφλεγμονώδη φαινότυπο αντίστοιχα. Η μεταβολική φλεγμονή είναι μια κατάσταση χρόνιας, χαμηλού βαθμού φλεγμονής στην οποία εμπλέκονται τόσο κύτταρα του ανοσοποιητικού όσο και κύτταρα του λιπώδους ιστού. Η περίσσια θρεπτικών στοιχείων αποτελεί την πρωταρχική επαγωγή της μεταβολικής φλεγμονής, ενώ η επικοινωνία μεταξύ λιποκυττάρων και ανοσοκυττάρων είναι αυτή που τη συντηρεί. Υπάρχουν πολλές μελέτες που μελετούν τις επιδράσεις διατροφικών μεταβολιτών στη φλεγμονή, αλλά οι ακριβείς μηχανισμοί δράσης τους στη μεταβολικού φλεγμονή δεν είναι γνωστοί. Η παρούσα μελέτη εστιάζει στην μελέτη των επιδράσεων των λιπαρών οξέων κοντής αλυσίδας του εντέρου, που προέρχονται από την τροφή, στην πολικότητα και τον μεταβολισμό των μακροφάγων και εξετάζει το ρόλο της Ακτ1 κινάσης σε αυτές. Με αυτόν τον τρόπο θα μπορέσουμε να καταλάβουμε καλύτερα τις αλλαγές του μικροβιώματος του εντέρου που προκαλούνται από τα μακροφάγα κατά την παχυσαρκία. Ερευνούμε επίσης τις πιθανές αντι-λιπογενετικές δράσεις τερπενίων από θαλάσσια φύκη, στα πλαίσια της λιπογένεσης που προκαλεί/επιβαρύνει τη μεταβολική φλεγμονή. Συνολικά, αυτή η μελέτη προσθέτει στην υπάρχουσα γνώση για τη ρύθμιση της μεταβολικής φλεγμονής από διατροφικούς μεταβολίτες και το ρόλο της Ακτ1 κινάσης σε αυτή.

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Chapter 1

Introduction

1.1 Mechanisms of inflammation

Inflammation is a protective response initiated after infection or injury. Through inflammation the organism essentially attempts to eliminate the inciting stimulus and/or repair the damage and in the case of infection, establish memory such that the host mounts a faster and more specific response upon a future encounter [1]. Inflammation is characterized by pain, heat, redness, swelling, and loss of function.

The inflammatory response consists of inducers, sensors, mediators and effectors. Inducers signal the initiation of the inflammatory response and activate specialized sensors, which in turn induce specific sets of mediators to be produced. Mediators then through modifications of the functional states of tissues and organs (which are the effectors of inflammation) lead them to adjust to the conditions indicated by the particular inducer of inflammation [2].

An inflammatory inducer can be either endogenous or exogenous. Exogenous inducers of inflammation can be classified as microbial or non-microbial. Pathogen-associated molecular patterns (PAMPs) and virulence factors are characteristic microbial inducers of inflammation. Non-microbial inducers of inflammation include allergens, irritants, foreign bodies, and toxic compounds etc. Endogenous inflammatory inducers refer to signals released from malfunctioning or dead cells and from damaged tissues, the formation of crystals by endogenous compounds and the breakdown of extracellular matrix (ECM) products.

Inflammatory mediators are produced by specialized leukocytes (mainly tissue-resident macrophages and mast cells), are preformed and stored in the granules of mast cells, basophils and platelets, or are preformed and circulate as inactive precursors in the plasma. They are classified into seven groups: vasoactive amines, vasoactive peptides, fragments of complement components, lipid mediators, cytokines, chemokines and proteolytic enzymes. Histamine and serotonin, which are vasoactive amines, are preformed and stored in mast cells, basophiles and platelets and are the first mediators released in acute inflammation. Vasoactive peptides are either stored in an active form, or circulate as inactive precursors in plasma, while the complemets fragments are produced directly in response to stimulation by specific inducers of inflammation. Lipid mediators (eicosanoids and platelet-activating factors) are derived from phospholipids, such as phosphatidylcholine. Cytosolic phospholipase A_2, after activation by intracellular Ca^{2+} ions, generates arachidonic acid and lysophosphatidic acid. Arachidonic acid is metabolized to form eicosanoids by cyclooxygenases (COX1 and COX2) and prostaglandins and thromboxanes are generated. It can also be metabolized by lipoxygenases which generates leukotrienes and lipoxins. Prostaglandins PGE^2 and PGI^2 cause vasodilation, pain and fever [3]. Lipoxins inhibit inflammation and promote resolution of inflammation, and tissue repair [4]. Inflammatory cytokines are produced by many cell types mainly macrophages and mast cells and activate the endothelium and leucocytes and induce the acute-phase response. Chemokines are produced by various cell types in response to inflammatory inducers to control leukocyte extravasation and chemotaxis to the infected tissue. Proteolytic enzymes have diverse roles in inflammation, in part through degrading ECM and basement-membrane proteins.

The effectors of an inflammatory response are the tissues and cells that are specifically affected by the inflammatory mediators. Responsiveness to certain inflammatory mediators such as tumor necrosis facrot alpha (TNFα) and interleukin 1 (IL1) is almost ubiquitous, however they affect

differently the tissues and cell types they act.

Resolution of inflammation and the return of tissues to homeostasis are essential. A critical requirement for the inflammatory response to switch off is the elimination of the injurious agents that initiated it in the first place. The successful removal of the inciting stimulus signals the pro-inflammatory mediator catabolism and levels of cytokines, chemokines, eicosanoids, cells adhesion molecules etc. return back to that expressed during the pre-inflamed state [1]. Next, recruited monocyte-derived macrophages commit to the phagocytosis/efferocytosis of apoptotic cells. After phagocytosis is complete most macrophages leave the inflamed site while some may die locally by apoptosis [1].

1.2 The role of macrophages in the regulation of inflammation

The mononuclear phagocyte system consists of closely related cells of bone marrow origin, including blood monocytes, and tissue macrophages. Macrophages which are professional phagocytic cells are a major component of this system. Monocytes migrate from the blood into various tissues and transform macrophages. Macrophages are cells that undergo many transcriptional, translational and metabolic adaptations in order to co-ordinate of the acute phase of inflammation and control its resolution. They have three major functions in inflammation: antigen presentation, phagocytosis and immunomodulation through production of various cytokines and growth factors [5]. Thus, they play an important role in the initiation, maintenance and resolution of inflammation. During the inflammatory process, cytokines (interferon gamma-IFNγ, TNFα), bacterial lipopolysaccharide (LPS), ECM proteins and other chemical mediators signal through Toll-like receptors (TLR) and cytokine receptors of macrophages and activate them. Upon activation, transcriptional and epigenetic changes lead to production of proinflammatory cytokines and chemokines, migration, and elimination of the insult. At later stages of inflammation, activated macrophages are deactivated by anti-inflammatory cytokines (IL10 and transforming growth factor beta-TGF-β) and cytokine antagonists that are mainly produced by macrophages, contributing to the resolution of inflammation, and prohibit prolonged inflammatory responses that may lead to tissue injury. For example, prolonged macrophage activation results in diminished response which is described as endotoxin tolerance when the inflammatory stimulus derives from Gram-negative bacterial LPS [6]. Therefore, macrophages are part of the autoregulatory loop in the inflammatory process.

Depending on the activation stage and stimuli macrophages can be classified into two distinct groups: the classically activated (M1) and the alternatively activated (M2) macrophages. This distinction is described as M1 and M2 polarization [7]. M1 macrophages arise in inflammatory settings and are activated by LPS, IFNγ and TNF. M2 macrophages are found in settings dominated by T_H2 responses and are activated by IL4, IL13, IL10, immune-complexes and glycocorticoids [8, 9]. M1 macrophages downstream signaling leads to transcriptional activation of genes like induced Nitrogen Oxide Synthase (iNOS), that uses L-arginase as substrate to produce nitric oxide, production and secretion of proinflammatory cytokines, such as IL1, IL6, IL12 and TNF and NADPH derived reactive oxygen and nitrogen species (ROS, RNS). Moreover, they are characterized by downregulated production of antiinflammatory cytokines like IL10. Consequently, M1 cells are related to T_H1 pathologies, have microbicidal activity, control the acute phase of infection and have a role of protection against exogenous opportunistic pathogens [9]. On the other hand, M2 macrophages bare immunomodulatory functions useful for inflammation resolution, but are also related to diseases like cancer, diabetes and arthritis when the above mentioned stimuli are abundant [10, 11]. M2 macrophages produce low levels of IL12 and high levels of IL10 and TGF-β and express high levels of Arginase 1 enzyme that, in contrast with iNOS, converts L-arginine to L-ornithin and urea [9]. It is generally accepted that M1 macrophages are responsible for initiating and sustaining inflammation while M2 macrophages are associated with the resolution of inflammation. So, macrophages have been classified as classically activated macrophages (M1) or alternatively activated (M2) based on their cytokine secretory patterns and pro-inflammatory versus immune-regulatory activity but in reality there are not two completely distinguished static states but rather a dynamic spectrum of polarization. Plasticity of polarization is orchestrated by epigenetic modifications and multiple signalling cascades and enables macrophages to adjust to host demands [12, 13]. For example, the coincident presence of T cells producing IL-4 or IFN-γ would tilt polarization toward M2 or M1, respectively, depending on the amount of cytokine, time

of exposure, and the competition for cytokine [8].

1.3 Contribution of the PI3K/Akt axis in the regulation of macrophage responses

1.3.1 The PI3K-Akt pathway

Phosphoinositide-3-kinase (PI3K)-Akt/PKB is a highly conserved pathway that is crucial for macrophage activation. PI3K/Akt pathway has a vital role in cellular homeostasis since it participates in many different processes including cell growth, proliferation, differentiation, metabolism, survival and cell cycle progression [14, 15]. Due to its multiple functions, slight deregulations of its components can have harmful consequences. For example, mutations that inappropriately activate the PI3K/Akt/mTORC1 pathway are associated with various malignancies, including cancers [16].

PI3Ks are a family of lipid kinases that phosphorylate the 3-hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns) lipids in the plasma membrane [17]. There are three classes of PI3Ks: Class I, Class II and Class III, which differ in structure and lipid substrate preference. Class I PI3K is the most studied group because it plays an important role in cancer. Class I PI3Ks are heterodimers of a catalytic subunit and a regulatory subunit [18] and this group of kinases is further divided into Class IA and Class IB, according to the ability to bind p85 [19].

PI3K is activated by ligands such as growth factors, cytokines and hormones (Fig. 1.1). When a stimuli such as epidermal growth factor (EGF) and insulin [20] binds to the N-terminal extracellular region of its corresponding transmembrane receptor tyrosine kinases (RTKs), the tyrosine residues on the cytoplasmic regions of the RTKs and in linker molecules are autophosphorylated [18]. This phosphorylation recruits the PI3K to the RTKs through interactions between the p85 SH2 domains and phospho-Tyr residues on members of RTK complex, and PI3K is allosterically activated. PI3K can also be activated by G-protein-coupled receptors and small GTPases such as Ras and RAB5, directly and indirectly [21].

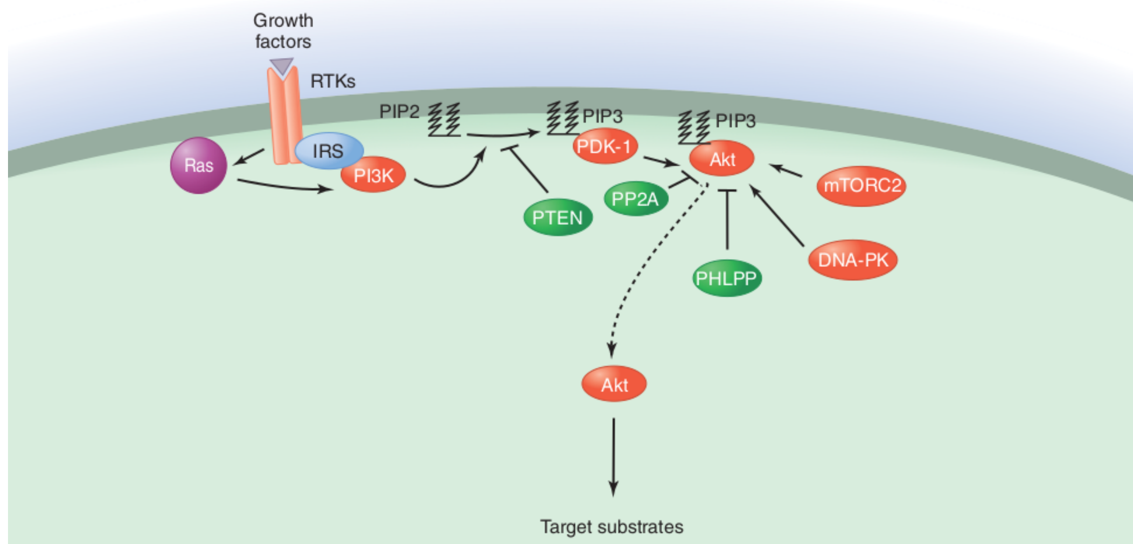

Figure 1.1: Akt/PKB activation via the PI3K pathway. (Image taken from [14])

Activated Class I PI3K phosphorylates phosphatidylinositol (3,4)-bis-phosphate (PIP_2) lipids to phosphatidylinositol (3,4,5)-tris-phosphate (PIP_3) on the inner cell membrane, which propagates activation signals to downstream molecules [14] (Fig. 1.1). PIP_2 is synthesized by Class II PI3Ks using PIP as substrate [22]. Phosphatase and tensin homolog (PTEN) acts as a negative regulator of the PI3K-Akt/PKB pathway by dephosphorylating PIP_3 to generate PIP_2 [23]. PIP_3 triggers the activation of protein Akt through the actions of two intermediate protein kinases, phosphoinositide-dependent kinase-1 (PDK1) and Rictor/mTOR [24]. Akt/PKB is recruited to the plasma membrane through high-affinity binding of its pleckstrin homology (PH) domain to PIP_3 produced by activated PI3K [25]. Akt is not activated directly by PIP_3, but instead PIP_3

appears to recruit Akt to the plasma membrane and to alter its conformation to allow subsequent phosphorylation by the PDK1 [26].

There are three isoforms of Akt/PKB that differ in serine/threonine residues Akt1, Akt2 and Akt3. All three isoforms have a broad tissue distribution, although Akt1 is the most ubiquitously expressed. However, Akt2 predominates in insulin-responsive tissues, such as skeletal muscle, adipose tissues and liver, and Akt3, which is expressed at the lowest level, in testes and brain [27]. All three isoforms contain three domains: a PH domain, a central kinase domain and a a regulatory carboxy-terminal domain of which PH domain regulates the membrane Akt translocation [27].

After Akt is recruited to the plasma membrane, PDK1 accesses and phosphorylates T308 (Akt1) in the "activation loop", which leads to partial Akt/PKB activation [28]. Akt acquires full activity by phosphorylation at S473 (Akt1) in the carboxy-terminal hydrophobic motif through mTOR complex 2 (mTORC2) [23, 29], which is activated by a PI3K-dependent mechanism. Similar phosphorylation events are observed at corresponding residues in Akt2 (T309 and S474) and Akt3 (T305 and S472) [30]. Fully active Akt is responsible for additional substrate-specific phosphorylation events in both the cytoplasm and nucleus, mediating this way a number of cellular functions including growth, metabolism, proliferation, survival, protein synthesis, transcription, apoptosis and angiogenesis [14]. Figure 1.2 summarizes the important role of this pathway in regulating countless subsequent pathways and adjusting cellular responses.

1.3.2 Akt kinases in macrophage activation and polarization

As described above the PI3K/Akt pathway, among others, regulates macrophage survival, proliferation and orchestrates their response to different metabolic and inflammatory signals. Except from cytokine and chemokine receptors that are already mentioned, TLR4 and other pathogen receptors and Fc receptors also activate the PI3K/Akt pathway [31, 32, 33, 34], modulating downstream signals that control cytokine production. Tuberous sclerosis complex (TSC) 1/2 is one of the target molecules of Akt, which is phosphorylated and inactivated. This inhibition leads to mTORC1 activation, via Ras homolog enriched in brain (Rheb) suppression [31, 33]. So, PI3K/Akt pathway in TLR-stimulated macrophages restricts proinflammatory, while it promotes anti-inflammatory responses [35] and negatively regulates TLR and NF-κB signaling [32, 33]. Specifically, macrophages exhibit reduced activation by LPS, when PI3K or Akt kinases are activated or overexpressed, whereas NF-κB activation and inducible NO synthase (iNOS) expression are augmented in TLR-activated cells when PI3K signaling is inhibited [36, 37, 38]. Inhibition of PI3K signaling promotes M1-type macrophages responses, since Akt activation is required for M2 activation of macrophages. Akt1 and Akt2 isoforms contribute oppositely in macrophage polarization. Deletion of Akt1 promotes upregulation of iNOS and IL12 (M1 polarization) and enhanced bacteria clearance, increased responses to LPS and abrogation of endotoxin tolerance [6]. On the contrary, Akt2 deficient macrophages present an M2 phenotype, expressing elevated levels of the M2 transcription factor C/EBPβ and the M2 markers Arg1, Ym1, Fizz1 [39].

1.4 Composition of the adipose tissue

Adipose tissue correct functionality is crucial for systemic energy balance and metabolic homeostasis [40]. Human adipose tissue is divided into brown adipose tissue, which is made by multilocular adipocytes with abundant mitochondria that express high amounts of uncoupling protein 1 (UCP-1), responsible for the thermogenic activity of this tissue and white adipose tissue. White adipose tissue (WAT) is responsible for accumulating excess of energy deriving from diet intake in the form of triglycerides and in phases of high energetic request such as fasting, exercise, and stress to promptly release fatty acids, and glycerol [41]. WAT is composed by several distinct group of cells: adipocytes, which represent mature fat cells able to store triglycerides, and an the inter-adipocyte stromal-vascular fraction, which consists of pre-adipocytes, endothelial cells, immature adipocyte precursors, different types of immune cells, and ECM with dispersed fibroblasts [42]. White adipocytes are spherical cells with a single large lipid droplet formed by triglycerides that is $>$90% of the cell's volume. The thin cytoplasm contains the nucleus, characteristically squeezed in a peripheral position by the large lipid vacuole, an abundant smooth endoplasmic reticulum, few mitochondria and free ribosomes, an usually under-developed Golgi apparatus, and a reduced rough endoplasmic reticulum [43]. Their size varies and it depends on the size of the lipid droplet

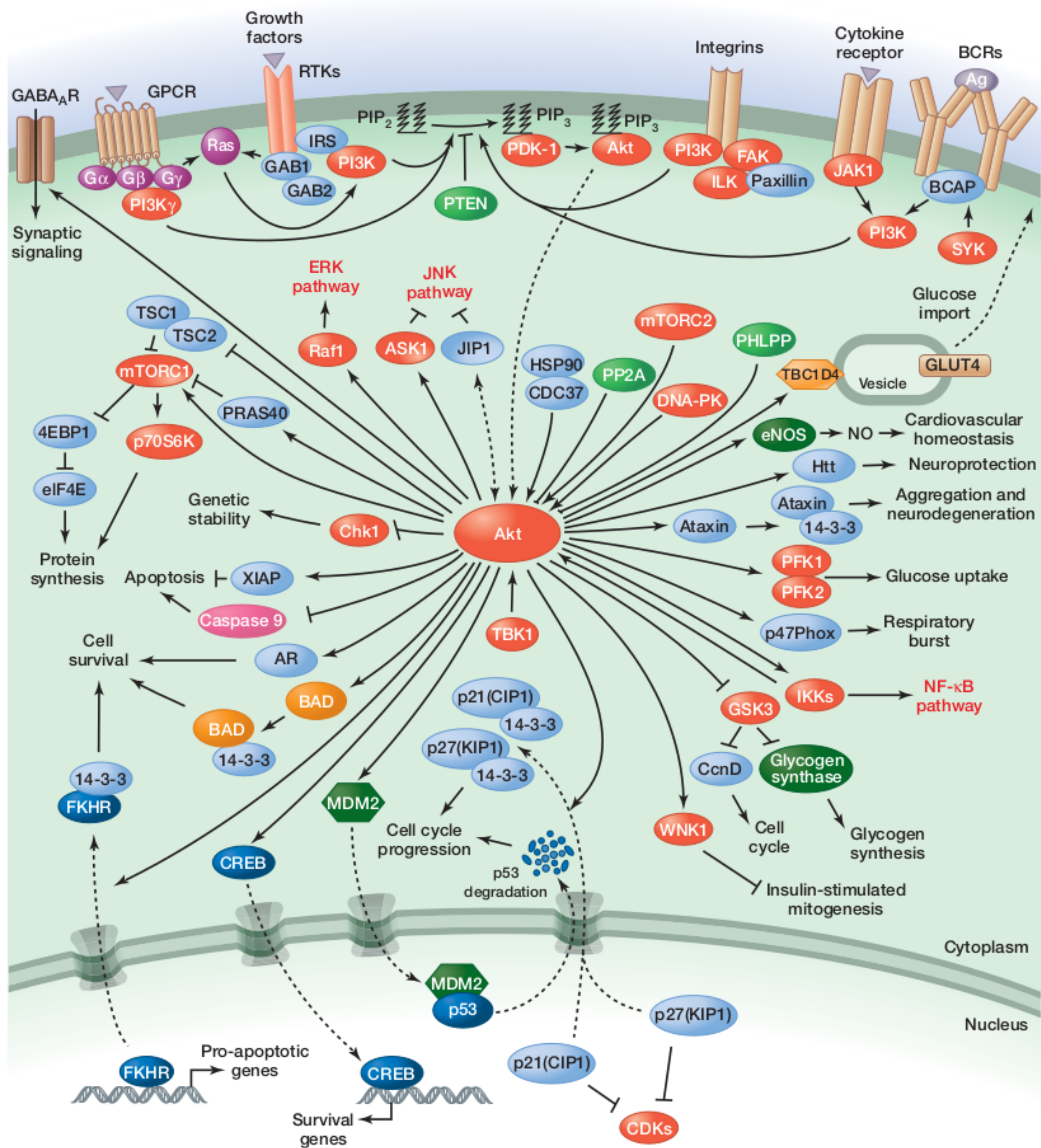

Figure 1.2: Signaling events activating Akt/PKB and cellular functions regulated by Akt/PKB. (Image taken from [14])

stored in them. The lipid droplet is separated from the thin cytoplasm by a non-membranous electron-dense barrier that contains functionally important proteins such as perilipin [44].

1.4.1 Mechanisms of adipogenesis

Adipogenesis is the development of adipose derived stem cells into mature lipid containing adipocytes. Insulin, by its specific receptors, stimulates adipogenesis, resulting in triglycerides synthesis by glycerol esterification with fatty acids. The process of adipogenesis also involves changes in cell morphology, induction of insulin sensitivity and changes in secretory caparacity of cells [45]. Adipocyte differentiation is characterized by the activation of specific adipogenic genes and fine epigenetic regulation. These early markers of commitment include the transcription factor CCAAT/enhancer binding protein (C/EBP) gene family and peroxisome proliferator-activated receptor γ (PPARγ) a member of the nuclear receptor superfamily, which encodes an adipocyte-specific nuclear hormone receptor. These marker genes, in turn, activate the adipose-specific fatty acid-binding protein 4

(FABP4) [also called adipocyte Protein 2 (aP2)] gene which codes for a carrier protein for fatty acids and lipoprotein lipase (LPL), another important player involved in fatty acid metabolism. Therefore, C/EBP and PPARγ regulate the expression of intermediate and late adipose phenotype-markers, as well as triglycerides accumulation [46]. There are also several factors that are capable of inhibiting adipogenic differentiation in different cell lines. An example is the preadipocyte factor-1 (Pref-1), a transmembrane protein highly expressed in preadipocytes. It prevents lipid accumulation and expression of adipocyte transcription factors such as PPARγ and C/EBPα and other late adipocyte markers such as FABP4 [47].

The course of adipocyte differentiation has been well studied using 3T3-F422A and 3T3-L1 cells, two cell lines that are definitely committed to the adipocyte lineage. In the presence of a hormonal cocktail consisting of insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX), 3T3-L1 and 3T3-F422A preadipocytes can differentiate into mature adipocyte cells, expressing specific adipocyte genes and accumulating triacylglycerol lipid droplets [48].

1.4.2 Adipocyte-Immune system crosstalk

Adipose tissue-resident immune cells represent approximately two thirds of the stromal-vascular fraction and they include almost the full spectrum of immune cell types, playing important roles in tissue housekeeping, removal of molecular debris and apoptotic cells, and tissue homeostasis maintenance [49]. In obesity, adipose tissue can account for up to 50% of total body mass and represents a major compartment of the immune system capable of influencing systemic inflammation. Therefore, the extensive expansion of adipose tissue during obesity increases its ability to act as an immunological tissue and control systemic inflammation and metabolism [50]. The excessive fat accumulation and following adipose tissue dysfunction that characterize obesity lead to essential changes in the amount, distribution and polarization of immune cells. Macrophages, mast cells, neutrophils and T- and B- lymphocytes are increased, while eosinophils and some subsets of T-lymphocytes are reduced [51]. Inflammatory changes are typically observed in visceral adipose tissue that becomes infiltrated by macrophages, which switch to a pro-inflammatory phenotype because of free fatty acid and endogenous signals released from stressed adipocytes [52], as will be discussed in the next section.

1.5 Metabolic Inflammation

Metabolic inflammation is a low-grade, chronic inflammation state orchestrated by metabolic cells in response to excess nutrients and energy. It is an established pathological condition that contributes to the development of obesity, insulin resistance and type 2 diabetes (T2D) and it depends on multiple signalling events. It is known that there is a cross talk between canonical immune and metabolic pathways. While malnutrition compromises proper immune responses, overnutrition activates them [53]. Immune modulators such as lipid-induced TLR [54, 55] and PPAR [56] contribute to nutrient sensing . On the other hand, cytokines such as TNFα and IL1β attenuate insulin receptor signalling, inhibiting this way insulin-mediated anabolic functions in different organs. Chronic overnutrition causes accumulation of lipids and essentially expansion of the white adipose tissue (WAT), which is reflected by obesity. Several lipid species are particularly prone to enhance inflammation and as a result the WAT is primarily predisposed to the induction of the above signalling networks. Pro-inflammatory cytokines are not only secreted by WAT-resident immune cells, but also white adipocytes themselves [57].

WAT-resident macrophages play an important role in establishing low-grade metabolic inflammation during obesity. In normal conditions, WAT mainly contains M2 macrophages, which secrete anti-inflammatory cytokines such as IL10 and Arg1 and keep adipose inflammation low. The M2 phenotype is maintained by the T_{H2} cytokines IL4 and IL13, which bind to their receptor and stimulate the Janus-associated kinase (JAK)/STAT6 pathway. The phosphorylation of the STAT6 trascription factor, results in its nuclear translocation and the induction of STAT6-mediated transcription [58]. PPARγ and PPARβ/δ are targets of STAT6. They interact with it and PPARγ coactivator 1β (PGC-1β) to enhance the transcription of fatty acid oxidation and mitochondrial biogenesis genes, upregulating oxidative metabolism and to maintain the transcription of M2 anti-inflammatory genes (Fig. 1.3).

In obesity, saturated fatty acids (SFAs) released by adipocytes through lipolysis, signal to macrophages through TLRs and inflammatory intracellular pathways such as the c-Jun N-terminal

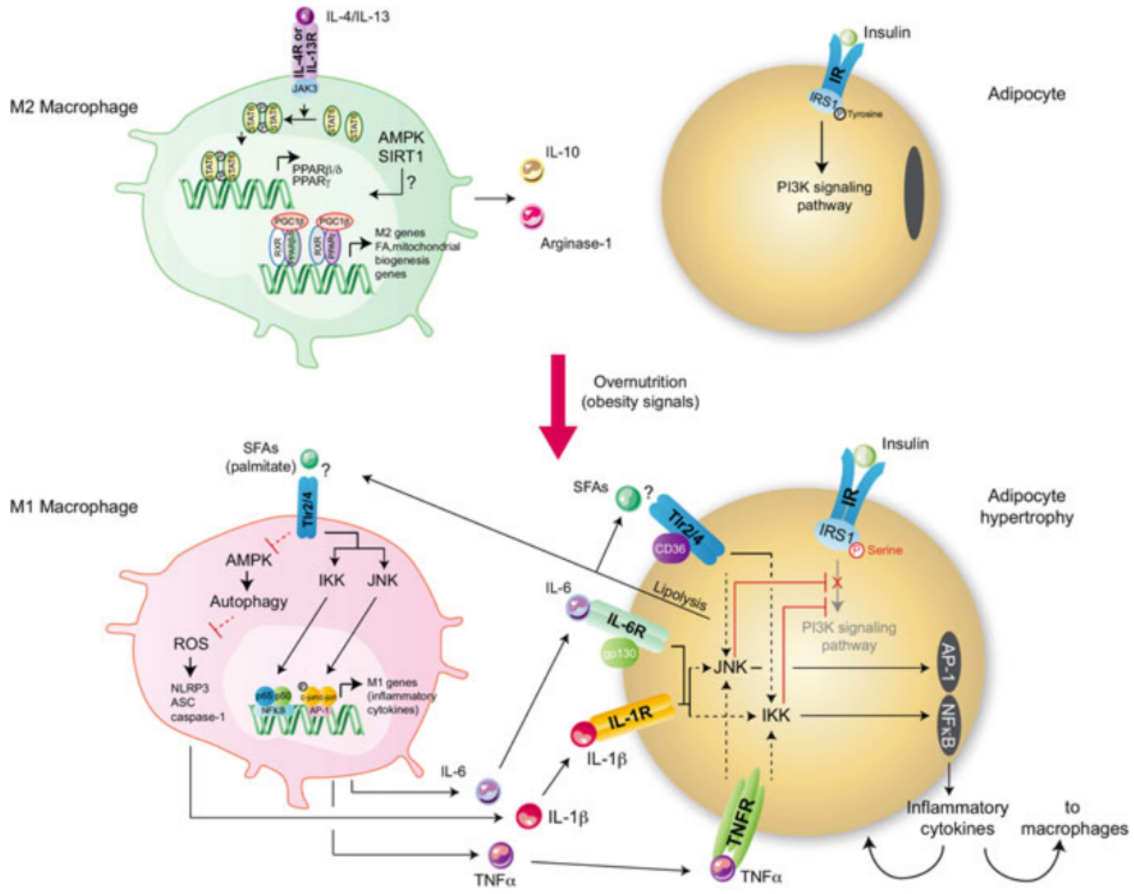

Figure 1.3: Metabolic inflammation at the molecular level. (Image taken from [59])

kinase (JNK) and the inhibitor of κ kinase (IKK) pathways are triggered, resulting in the NFκB- and AP-1-mediated transcription of pro-inflammatory genes, respectively. This means that macrophages acquire an M1 phenotype (Fig. 1.3). These M1 macrophages express pro-inflammatory molecules including IFNγ, IFNγ receptor, iNOS, IL1β, TNFα and IL6 [60]. Pro-inflammatory cytokines and lipids signal through respective receptors, employing the same canonical pro-inflammatory kinases in white adipocytes to interfere with the insulin/PI3K signalling pathway. As adipocytes become hypertrophied, they activate a pro-inflammatory gene programme by themselves and they produce and secrete pro-inflammatory cytokines like TNFα, IL1β, IL6 and the chemokine (C–C motif) ligand 2 (CCL-2) [61, 62], providing this way another important source of pro-inflammatory cytokines. Over time, this low-grade inflammation induces the infiltration and activation of many professional immune cells in metabolically active tissues. While macrophages, mast cells and various T cell populations are increased, the pro-inflammatory response is enhanced and the metabolic cell function is inhibited. Maintenance of the inflammation induced by excess nutrients and continuous reciprocal reinforcement of the inflammatory pathways result in a chronic and unresolved inflammatory state [62] (Fig. 1.4).

1.6 Dietary metabolites affect inflammation

1.6.1 The role of short chain fatty acids in regulating immune responses

The gastrointestinal tract is colonized by a high density of commensal bacteria and is a major site of pathogen entry [63], so it requires a robust barrier function. Gut bacteria require as an energy source carbohydrates that exist in host diet. Specifically, bacteria from the phyla *Bacteroidetes* and *Firmicutes* produce short chain fatty acids (SCFAs) by anaerobic fermentation of non-digestible dietary carbohydrates in the colonic lumen. The SCFAs butyrate, propionate, and acetate promote intestinal epithelial barrier function and regulate the host mucosal immune system

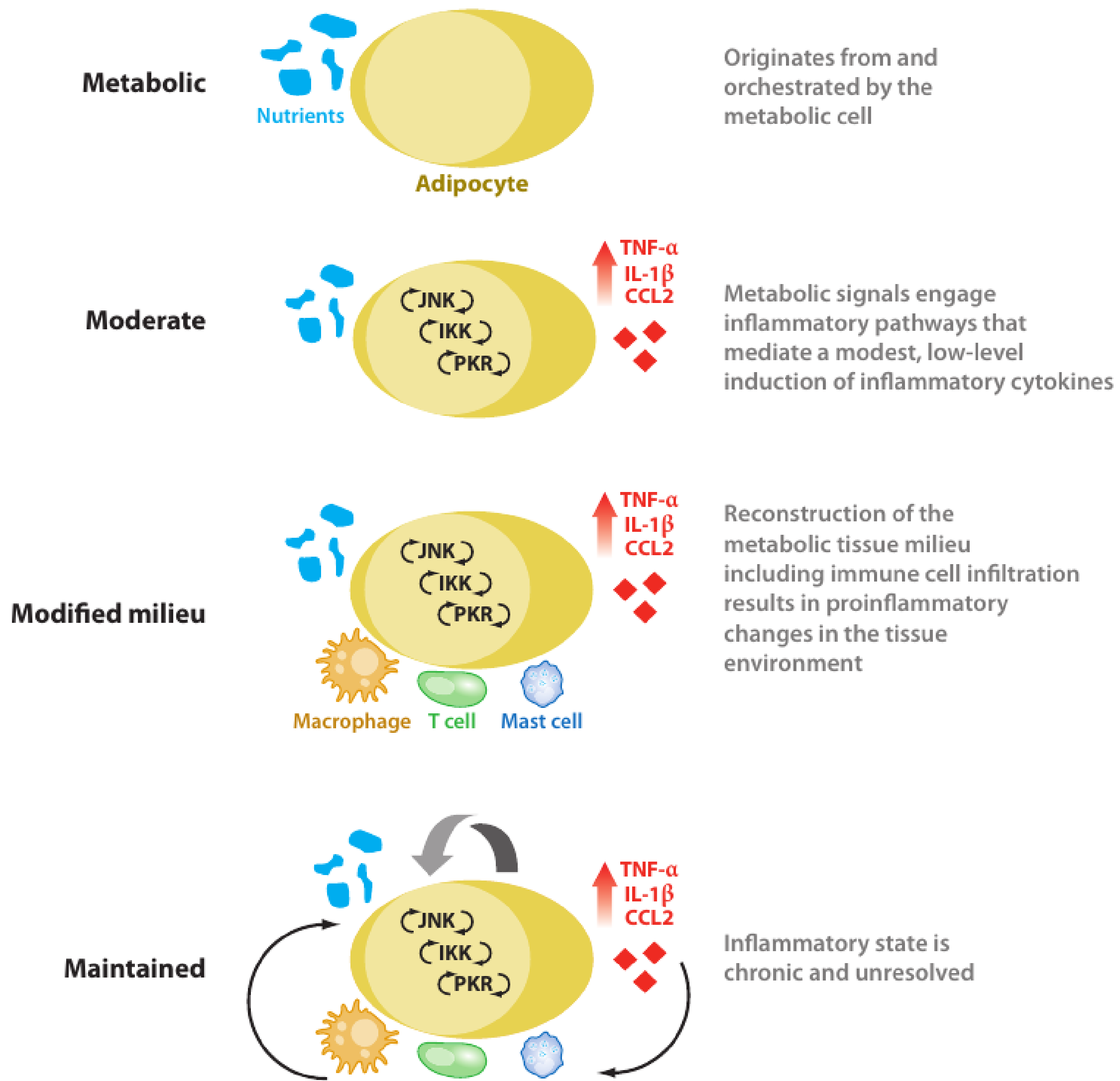

Figure 1.4: Events leading to metabolic inflammation. (Image taken from [62])

[64], by acting as signal molecules in the intestines, including macrophages. SCFAs can be found at high concentrations in the large intestine (e.g. 20mM butyrate in colonic lumen) [65]. Patients with inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn's disease, have reduced numbers of bacteria that produce SCFAs [66] and it seems that SCFA-containing enemas are clinically beneficial for some patients with colitis [67]. Previous studies suggest that SCFAs have anti-inflammatory properties that may contribute to their positive effects. For example, butyrate regulates stem cell turnover in intestinal epithelial crypts and also promotes regulatory T cells in the colon [68]. Furthermore, exposure of peripheral blood mononuclear cells such as neutrophils, macrophages, and dendritic cells to SCFAs or other histone deacetylases (HDAC) inhibitors, such as trichostatin (TSA), inhibits inflammatory cytokine production [69, 70, 71]. Thus, SCFAs modulate immune responses by macrophages by regulating the secretion of cytokines and macrophages, in turn, control the pro- or anti-inflammatory state of the intestines [69].

1.6.2 The role of terpenoids in regulating immune responses

Natural products derived from plants are an interesting and promising source for isolating and developing therapeutic molecules to fight against various diseases, including inflammatory ones [72]. It is well known that plant-derived terpenoids possess a wide range of biological functions, such as antioxidative, anti-inflammation, and anticancer properties, which are able to counteract endogenous and exogenous biological stimuli. For example, avicins, pentacyclic triterpenoids

derived from *Acacia victoriae*, inhibit activation of NFκB, which attributes to them an anti-inflammatory property [73, 74]. Pristimerin, a natural triterpenoid, inhibits induction of iNOS in murine macrophages [75], promoting an anti-inflammatory macrophage phenotype. Another natural triterpenoid, maslinic acid from *Olea europaea*, has shown to have anti-inflammatory effects in rat astrocyte cultures by inhibiting the production of NO, TNFα, COX-2 and iNOS, suggesting that maslinic acid can potentially reduce neuroinflammation by inhibiting NFκB signal transducer pathway [76]. Many more plant-derived terpenoids have exhibited anti-inflammatory properties, suppressing iNOS, TNFα, NFκB, COX-2 expression and other inflammatory agents [77, 78, 79, 80, 81, 82, 83]. More specific different natural products derived from marine organisms have been studied and seem to exhibit a broad spectrum of pharmacological activity, including anti-inflammatory effects [84, 85]. Red algae is a rich source of different secondary metabolites the majority of which consists of acetogenins, halogenated diterpenes and sesquiterpenes [86, 87, 88]. After noticing that total extracts from different algae display anti-inflammatory effects both *in vivo* [89, 90] and *in vitro* [91, 92], purified molecules were isolated to study and address these effects. Neorogioltriol (1), a brominated diterpene, was isolated from the organic extract of the red algae *Laurencia glandulifera* [93] and neorogioldiol (2), also a diterpene, and O^{11},15-cyclo-14-bromo-14,15-dihydrorogiol-3,11-diol (3), a prenylbisabolane, were isolated from red seaweed *Laurencia microcladia* [94]. This metabolites were studied both *in vivo* and *in vitro* and potent anti-inflammatory activity was attributed to them [95]. Macrophages exposed to these compounds acquired an M2-like anti-inflammatory phenotype, and also metabolites 2 and 3 have strong *in vivo* anti-inflammatory properties, which suppresses colon inflammation and tissue damage in the model of DSS-induced colitis [95].

The role of marine terpenoids in affecting adipogenesis

As earlier described, excess nutrients cause adipocytes to accumulate fat in the form of triacylglycerols that result in enhanced adipogenesis, increased adipose tissue mass, and consequently obesity. In addition to fat storage, adipose tissue is a major endocrine and metabolic organ secreting adipocytokines, cytokines, growth factors, and hormones that are involved in host immunity, energy homeostasis, systemic insulin sensitivity, and tissue regeneration. In obesity, dysfunction of adipose tissue contributes to abnormal cytokine and hormone production in adipocytes and results in metabolic dysfunction. The expansion and dysfunction of adipose tissue leads to metabolic inflammation. Thus, adipogenic differentiation is an important event for fat formation in obesity. For that reason natural compounds that inhibit adipogenic differentiation are frequently screened to develop therapeutic drugs for treating obesity.

There are many marine terpenoids that have been shown to possess anti-obesity effects by preventing adipogenesis and lipid accumulation. Fucoxanthin, a pigment from the chloroplasts of marine brown algae, inhibits lipid accumulation and ROS production during adipocyte differentiation by down-regulating the expression of adipogenic and lipogenic factors and ROS-generative factors [96]. Fucoxanthin-rich seaweed extract is equally effective with purified fucoxanthin in reducing adipose tissue weight and regulating lipid metabolism [97, 98]. Siphonaxanthin, a keto-carotenoid contained in several green algae, also inhibits lipid accumulation in mice WAT as well as adipogenesis in 3T3-L1 preadipocytes [99]. Another natural marine compound, phorbaketal A inhibits adipogenic differentiation by increasing the interaction of TAZ (transcriptional coactivator with PDZ-binding motif) and PPARγ to suppress PPARγ target gene expression [100].

Although the anti-obesity effects of some marine terpenoids have been explored, the anti-adipogenic properties that terpenoids from *Laurencia* spp. may have are not yet studied.

Chapter 2

Aims of the study

Given the established importance of inflammation and metabolic inflammation and the anti-inflammatory effects that dietary metabolites display, the present study focused on two aims:

- To identify how diet derived short chain fatty acids can affect macrophage polarization and metabolism upon LPS stimulation and whether these effects are Akt-mediated and
- To decipher whether adipocyte differentiation, and subsequently metabolic inflammation, is affected by terpenoids from red algae.

Chapter 3

Materials and Methods

Mice. Male and female mice of the C57BL/6J genetic background were used in this study. In detail, adult C57BL/6J mice and mice that lacked the expression of Akt1 kinase specifically in macrophage/monocytes cells (LysM^{Cre}Akt1$^{fl/fl}$ mice) were used for our experiments. Mice were housed in the animal facility of the University of Crete medical school at stable temperature 25°C and a 12-hour light-dark cycle. All procedures described below were approved by the Animal Care Committee of the University of Crete School of Medicine, Heraklion, Crete, Greece, by the Veterinary Department of the Heraklion Prefecture, Heraklion, Crete, Greece.

Cells culture. The preadipose cell line 3T3-L1 was used for cell differentiation experiments. Subcultures of 3T3-L1 cells were routinely cultured in Dulbecco′s Modified Eagle′s Medium DMEM, High Glucose 4.5g/l (GIBCO, Life technologies Corp, Carlsbad, CA), supplemented with 10% (v/v) heat inactivated Newborn Calf Serum (NBCS) and 1% penicillin/streptomycin (P/S) in a 37°C incubator at 5% CO_2. Cells were seeded in T-75 flasks at a density of $25x10^4$ cells/flask. Cultures never became completely confluent according to the manufacturer's instructions. They were passaged once they reached 70-80% confluency.

Elicitation of peritoneal macrophages with thioglycolate. Thioglycolate Broth (Brewer's Thioglycolate Medium) was diluted in normal saline 4% w/v and heated for 1-2 minutes to facilitate dissolving. The solution was then autoclaved, aliquoted and stored at 4°C in dark place (light-sensitive solution) for at least 1-2 months before use. Mice were immobilized and cleaned with 70% EtOH in the abdominal area to prevent any infections. Then 2mls of thioglycolate solution were injected intraperitoneally in each mouse. Four days after the initial injection, macrophages that concentrated in the peritoneal cavity were harvested by performing peritoneal lavage.

Peritoneal lavage for macrophage isolation. Four days after the injection of Thioglycolate, mice were sacrificed and the skin of the abdominal area was removed carefully, leaving the peritoneal membrane intact. Peritoneal lavage was performed by injecting 8-10mls of cell medium (Dulbecco′s Modified Eagle′s Medium DMEM, Low Glucose 1g/l, supplemented with 10% Fetal Bovine Serum (FBS) and 1% P/S) intraperitoneally at the lower abdominal area, using a 10ml syringe. After the injection, the abdomen of the mouse was gently palpated to achieve an even distribution of the fluid in it. Syringe was disconnected from the needle and the cell containing fluid coming out of it, was collected in a 50ml falcon. This procedure was repeated two times in total for each mouse. The solution was then centrifuged for 5 minutes at 1000rpm and the cell pellet was resuspended in fresh medium (DMEM, Low Glucose 10% FBS, 1% P/S).

Plate seeding of peritoneal macrophages and preadipocytes. Following isolation, macrophages were seeded in cell culture plates for performing the appropriate processes. For enzyme-linked immunosorbent assay (ELISA) and RNA extraction experiments wild type and LysM^{Cre}Akt1$^{fl/fl}$ peritoneal macrophages were seeded in 24-well, flat-bottom cell culture plates at a density of 4-$5x10^5$ cells per well, containing 0.8ml DMEM. For differentiation, 3T3-L1 cells were seeded in 24-well, flat bottom plates at a density of $6x10^4$ cells per well, containing 0.8ml DMEM.

Macrophage pretreatment and stimulation. The isolated macrophages were resuspended in fresh low glucose DMEM 10% FBS, 1% P/S and seeded to cell culture plates. After macrophages attached to the cell culture plates, they were pretreated with different SCFAs for 30 minutes. The SCFAs used were sodium butyrate, sodium propionate and sodium acetate at the following concentrations: 1000uM, 100uM, 50uM, 25uM, 1uM. After 30 minutes, macrophages were stimulated with E.coli-derived LPS (O111:B4; catalogue no. L2630; Sigma-Aldrich) at a final concentration of 100ng/ml. A group of wells left untreated and no LPS was added to them and in another group cells only were stimulated with LPS without a SCFA pretreatment. For ELISA and RNA extraction triplicates were created for each condition.

3T3-L1 cell differentiation. Preadipocytes were resuspended in fresh high glucose DMEM 10% NBCS, 1% P/S and seeded to cell culture plates. After 48h (day 1), cell differentiation was induced by changing the medium to high glucose DMEM containing 10% FBS, 1% P/S, 1ug/ml Insulin (Sigma I0516), 0.25uM Dexamethasone (GAP), 0.5mM IBMX (Sigma I5879) (=differentiation medium I, DMI). On day 3, the medium was changed to high glucose DMEM containing 10% FBS, 1% P/S, and 1ug/ml Insulin (=differentiation medium II, DMII). The following groups of wells were created: a group of wells with pre-adipocytes that were harvested two days after seeded and served as control group, a group of wells with cells that differentiated to adipocytes, a group of wells with cells induced to differentiate in the presence of 8uM neorogioltriol (1), a group of wells with cells induced to differentiate in the presence of 62.5uM neorogioldiol (2), a group of wells with cells induced to differentiate in the presence of 10uM O^{11},15-cyclo-14-bromo-14,15-dihydrorogiol-3,11-diol (3) and a group of wells with cells induced to differentiate in the presence of 0.1% carbowax that served as a control for the groups with the compounds, since they were diluted in Carbowax™ 400. Medium was changed every other day switching between DMI and DMII until day 14, when adipocytes were harvested.

Enzyme Linked ImmunoSorbent Assay (ELISA). Cytokine concentration (IL-6) in cell culture supernatants of control, LPS stimulated and both SCFAs pretreated and LPS stimulated macrophages after 16 hours of LPS stimulation was determined by ELISA using ELISA kit (Biolegend), according to the manufacturer's instructions. No normalization was needed since the same amount of cells was seeded in each well.

Total RNA isolation, cDNA synthesis and Real-Time RT-PCR. After removing supernatants for ELISA at the indicated time period, macrophages remained to the wells were used for RNA extraction. For the experiments conducted with macrophages and adipocytes the following protocol was used: 3T3-L1 were lysed directly on the culture dish. 1ml of the TRI Reagent per $10cm^2$ of culture plate surface area was used and total cellular RNA was extracted according to TRI Reagent® Protocol (Merck). The RNA content of samples was quantified using the Nanodrop micro-volume spectrophotometer. cDNA synthesis was performed by reverse transcription with PrimeScript™ RT reagent Kit; TaKaRa, (using 500ng isolated RNA as template) according to manufacturer's instructions. The cDNA produced by the above described procedure was used for RT-PCR analysis. Real-Time PCR reaction was performed with the KAPA SYBR ® FAST qPCR Master Mix (2X) Kit (Kapa Biosystems) according to manufacturer's instructions. Amplification was performed in a Applied Biosystems™ StepOnePlus™ Real-Time PCR System for a maximum of 40 cycles, as follows: Start steps: 3min at 95°C, Repeat steps: 15s at 95°C and 30s at 60°C. The primers used for macrophages metabolic genes were: RPS9 Forward: 5' – GCT AGA CGA GAA GGA TCC CC – 3' Reverse: 5' – CAG GCC CAG CTT AAA GAC CT – 3', iNOS Forward : 5' – TCC TGG AGG AAG TGG GCC GAA G – 3' Reverse: 5' – CCT CCA CGG GCC CGG TAC TC – 3', LDH Forward: 5' – CAT TGT CAA GTA CAG TCC ACA CT – 3' Reverse: 5' – TTC CAA TTA CTC GGT TTT TGG GA – 3', HK2 Forward: 5' – GAG AAC CGT GGA CTG GAC AA – 3' Reverse: 5' – GAC ACG TCA CAT TTC GGA GC – 3' , HK3 Forward: 5' – TGC TGC CCA CAT ACG TGA G – 3' Reverse: 5' – GCC TGT CAG TGT TAC CCA CAA – 3' . The primers used for adipocyte experiments were: Pref-1 Forward: 5' – AAG TGT GTA ACT GCC CCT GG – 3' Reverse: 5' – TGC AAG CCC GAA CGT CTA TT – 3', PPIB Forward: 5' – ACA GCA AGT TCC ATC GTG TCA T – 3' Reverse: 5' – GAA GAA CTG TGA GCC ATT GGT G – 3'. The amplification efficiencies were the same as the one of RPS9 for macrophages and PPIB for adipocytes as indicated by the standard curves of amplification, allowing us to use the following formula: fold difference = $2^{-(\Delta CtA - \Delta CtB)}$, where Ct is the cycle threshold. Reactions were

performed in triplicate or quadruplicate for statistical evaluation. The results of gene expression were analyzed using StepOne™ Software v2.3 and GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

Metabolic phenotype and metabolic potential. The metabolic profile and metabolic potential of pre-adipocytes induced to differentiate was measured using the Agilent Seahorse XFp Cell Energy Phenotype Test Kit (103275-100) according to the manufacturer's instructions (Agilent Technologies). 3T3-L1 cells were plated in the Agilent Seahorse XFp Cell Culture Miniplate two days prior to assay. Cells were seeded at a density of $3x10^4$ cells per well in fresh high glucose DMEM 10% NBCS, 1% P/S. One day prior to assay, medium was changed to DMI which additionally contained 0.1% carbowax or 62.5uM neorogioldiol (1) or 8uM neorogioltriol (2) or 10uM O^{11},15-cyclo-14-bromo-14,15-dihydrorogiol-3,11-diol (3). The assay was conducted on an Agilent Seahorse XFp Analyzer, using the Agilent Seahorse XF Cell Energy Phenotype Test template according to the manufacturer's instructions. Upon completion of the run, the data were exported from the Seahorse Wave Desktop Software in a MS Excel workbook and analyzed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

Statistical Analysis. Data are expressed as mean +/- SD of data obtained from independent experiments. Statistical analysis was performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Groups were compared using t-test. P-values <0.05 were considered statistically significant.

Chapter 4

Results

4.1 Butyrate and propionate have an Akt1-mediated anti-inflammatory effect on peritoneal macrophages

It is known that gut microbiota affects and is affected by the phenotype of instestine macrophages and also bacteria-derived SCFAs been shown to have anti-inflammatory properties and to inhibit inflammatory cytokine production by macrophages and other peripheral blood mononuclear cells but the exact molecular mechanism of regulation of cytokine production is not fully understood. Peritoneal macrophages were used to study the SCFAs anti-inflammatory effect to macrophages. To determine the optimal dose for induction of cytokine secretion by macrophages, peritoneal macrophages from wild type (wt,C57BL/6J) and LysM^{Cre}Akt1$^{fl/fl}$ mice were stimulated with LPS for 16 hours after being pretreated with different doses of SCFAs (sodium butyrate, sodium propionate and sodium acetate) for 30 minutes. Then the secreted amounts of the proinflammatory cytokine IL-6 were measured. Macrophages from LysM^{Cre}Akt1$^{fl/fl}$ mice have a potent M1 phenotype, enhanced inflammatory response to bacterial stimulus and enhanced bactericidal activity. The results showed that butyrate and propionate lowered the LPS-induced IL-6 secretion in wt macrophages at a concentration as low as 1uM, while in Akt$^{-/-}$ macrophages IL-6 secretion was affected at concentrations over 50uM for butyrate and over 100uM for propionate (Fig.4.1, top left and top right). However, acetate had no effect on the secreted amounts of IL6 by macrophages upon LPS (Fig.4.1, bottom). At a 25uM concentration butyrate and propionate mitigated the LPS response by wt macrophages, while they had no effect on Akt$^{-/-}$ macrophages (Fig. 4.2). For this reason the 25uM concentration was decided to be used for the subsequent experiments, since it is the lowest dose for both butyrate and propionate that differentially affects the two genotypes. These data suggest that butyrate and propionate have an anti-inflammatory effect on macrophages which is Akt1-mediated.

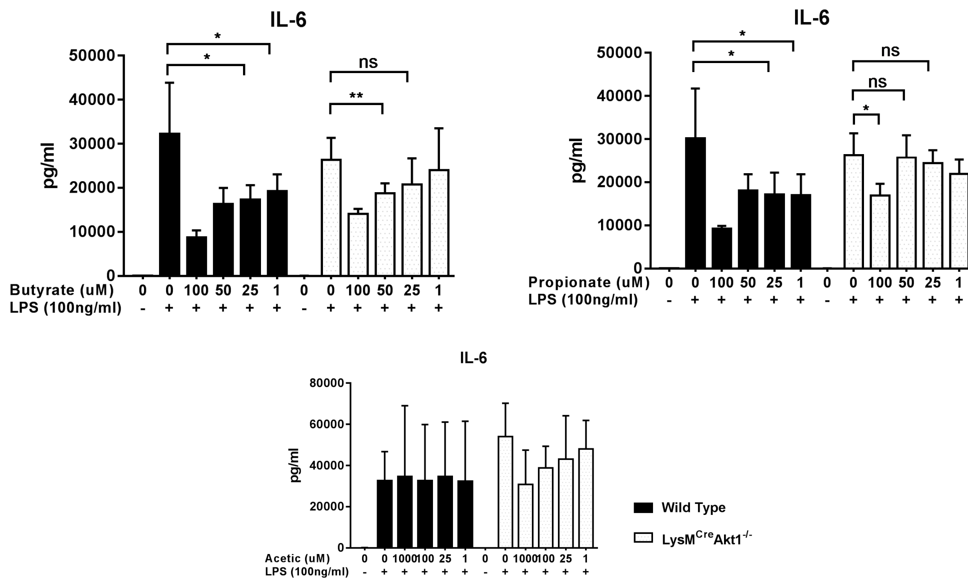

Figure 4.1: IL-6 secretion dose response by macrophages to SCFAs upon LPS stimulation. Butyrate and propionate inhibits IL-6 secretion in macrophages, while acetate doesn't have an effect. Wt and Akt$^{-/-}$ were stimulated with LPS (100ng/ml) for 16h after being pretreated with butyrate (1uM-100uM, top left), acetate (1uM-100uM, top right), or acetate (1uM-100uM, bottom). Cells supernatants were collected and analyzed by ELISA. Data are expressed as mean ± SD. *P $<$ 0.05; **P $<$ 0.01; n.s., not significant (unpaired Student's t test).

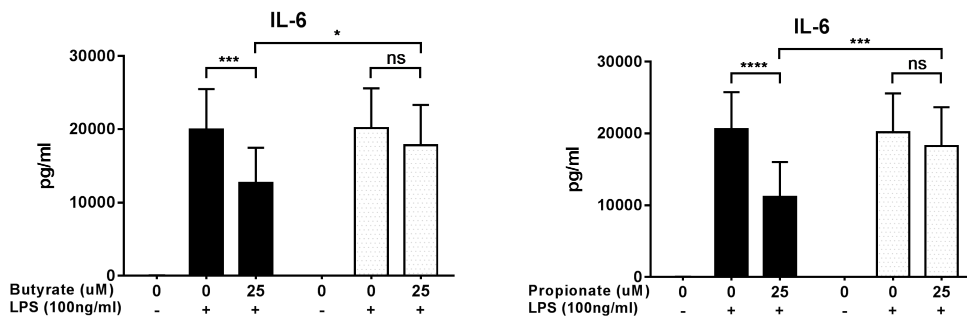

Figure 4.2: Butyrate and propionate lower the LPS-induced IL-6 secretion by wt macrophages, while when Akt1 is absent macrophages aren't affected by either of them. Data are representative of at least 3 independent experiments. Data are expressed as mean ± SD. *P $<$ 0.05; ***P $<$ 0.001; ****P $<$ 0.0001; n.s., not significant (unpaired Student's t test).

4.2 Butyrate and propionate affect metabolic genes expression in peritoneal macrophages

Gut microbiota also affects and is affected by macrophage metabolism, but the exact effect of gut bacteria-produced SCFAs on the expression of metabolic genes in macrophages remains unknown. After demonstrating the anti-infammatory effect of butyrate and propionate on macrophages, the effect of these SCFAs on macrophage metabolism was tested using the cells in whose supernatants IL-6 was measured.

Butyrate further increased the LPS-induced expression of the inflammatory marker iNOS in wt macrophages (Fig. 4.3, top left). While we noticed that when there is no Akt1 iNOS expression is more enhanced by LPS and even more when butyrate is added. Changes in the expression levels of Hexokinase 2 (HK2), which catalyzes the first step of glucose metabolism, exhibited no difference between wt and $Akt^{-/-}$ macrophages (Fig. 4.3, bottom left). The Hexokinase 3 (HK3) isoform expression was induced by butyrate beyond the LPS-induction in wt macrophages, whereas butyrate had no effect in HK3 expression in $Akt^{-/-}$ macrophages (Fig. 4.3, bottom right). Lactate dehydrogenase (LDH) is also a catalytic enzyme that also contributes to glucose metabolism. Butyrate had no effect in LDH mRNA levels when Akt1 was absent, while it further increased the LDH production when Akt1 was present (Fig. 4.3, top right). These results indicate that butyrate affects macrophage metabolism by inducing the expression of metabolic genes and that Akt1 mediates HK3 and LDH production upon LPS stimulation in macrophages.

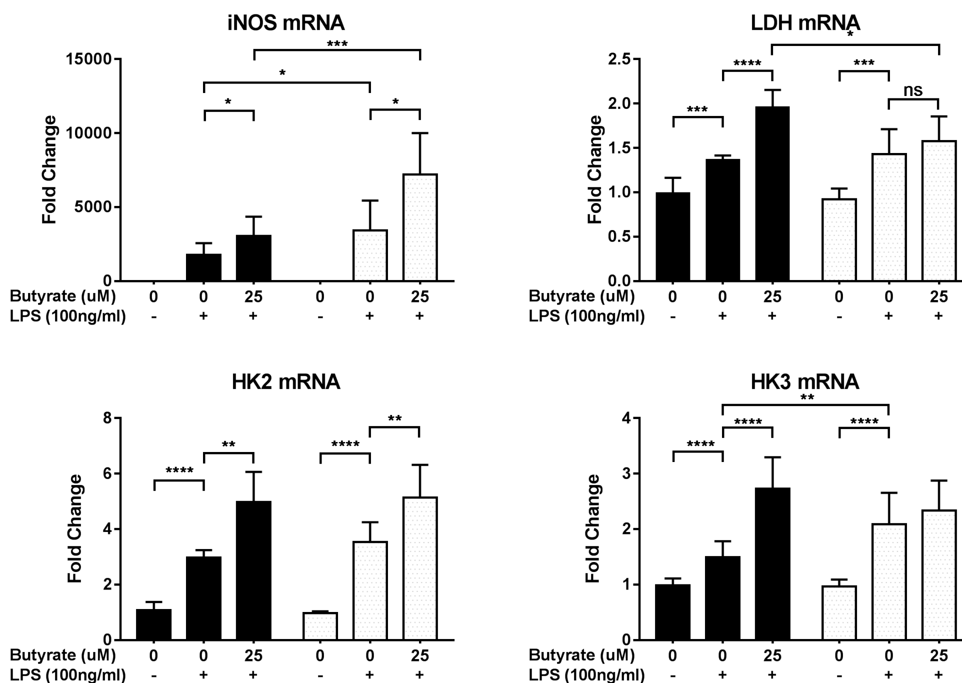

Figure 4.3: Butyrate affects the expression of metabolic genes. RNA extracts were prepared and analyzed for the expression of iNOS, LDH, HK2 and HK3. Results showed that butyrate induces the expression of metabolic genes in activated macrophages and Akt1 mediates this induction in LDH and HK3. mRNA levels measured using real time PCR. Data are expressed as mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; n.s., not significant (unpaired Student's t test).

Propionate affected the expression of these genes in a different way than butyrate in $Akt^{-/-}$ macrophages. It enhanced the LPS induction in wt macrophages but it attenuated it when Akt1 was absent (Fig. 4.4). This indicates that propionate too induces the expression of metabolic genes and that Akt1 is needed for propionate's action.

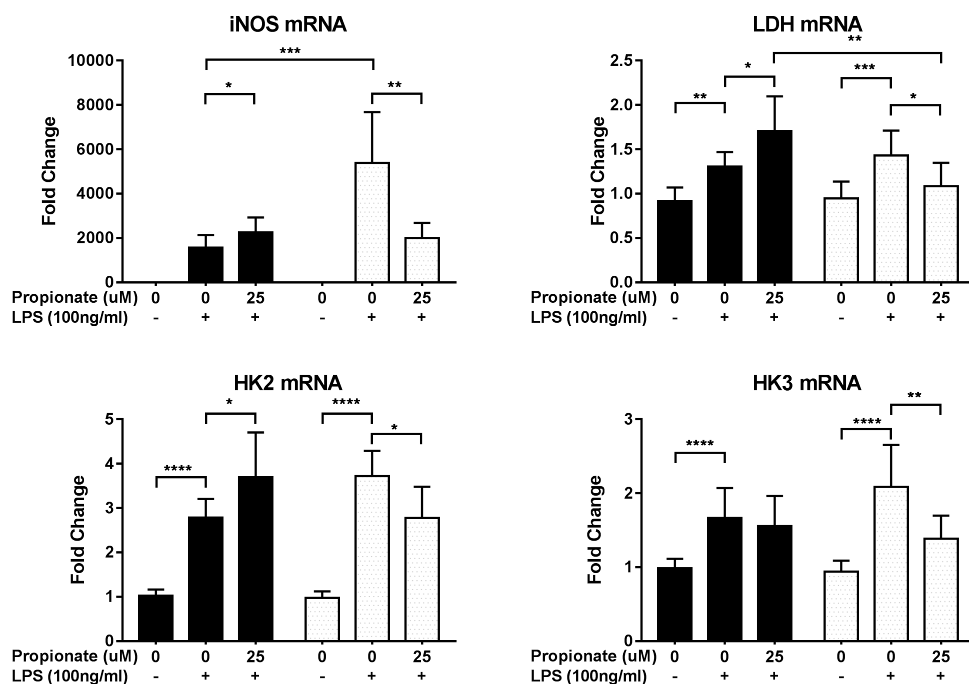

Figure 4.4: Propionate enhances the expression of iNOS, LDH, HK2 and HK3 through Akt1 in activated macrophages.

4.3 Marine terpenoids effect on adipogenesis

It is known that different compounds from sea algae extracts exhibit an anti-inflammatory effect on macrophages and that in metabolic inflammation macrophages are in close cross talk with adipocytes, but the direct effect of these compounds on adipocytes remains unknown. Preadipocytes of the 3T3-L1 cell line were used to study the effect of neorogioltriol (1), neorogioldiol (2), and O^{11},15-cyclo-14-bromo-14,15-dihydrorogiol-3,11-diol (3) in adipogenesis. Preadipocytes induced to differentiate in the presence of these compounds for 14 days and the adipogenesis was assessed based on the expression of the preadipocyte marker Pref-1 and the adipocyte marker FABP4. As expected, Pref-1 expression was decreased in differentiated cells, while FABP4 was increased (Fig. 4.5).

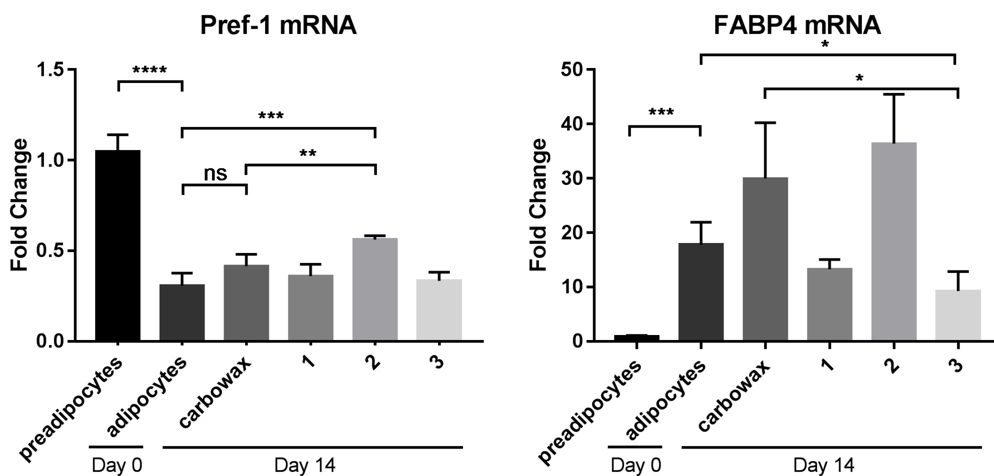

Figure 4.5: Effect of metabolites 1-3 on the expression of the preadipocyte marker Pref-1 and the adipocyte marker FABP4.

When cells induced to differentiate in the presence of compound 2 Pref-1 expression did not decrease as much as in cells that differentiated without this compound. Compounds 1 and 3 didn't seem to have an effect on Pref-1 expression (Fig. 4.5, left). FABP4 mRNA levels were decreased when cells induced to differentiate in the presence of compound 3 as compared with FABP4 mRNA levels in the absence of compound 3, while compounds 1 and 2 didn't cause any significant decrease on FABP4 expression. These results suggest that compounds 2 and 3 negatively affect adipogenesis.

4.4 Marine terpenoids effect on adipogenesis metabolism

Another aspect that was studied regarding the effect of metabolites 1-3 on adipogenesis was the metabolic changes they may cause. The metabolic potential of preadipocytes exposed to differentiation stimulus was assessed and compared with the metabolic potential of preadipocytes that were induced to differentiate in the presence of compounds 1-3. 3T3-L1 cells were stimulated with DMI and the compounds for 24hrs before measuring their basal and stressed oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). OCR is a measure of the mitochondrial respiration and ECAR is a measure of the rate of glycolysis of the cells. Together these metrics are used to assess the cells' ability to meet an energy demand via respiration and glycolysis also known as the metabolic potential. Figure 4.6 illustrates the percentage increase of stressed OCR over baseline OCR, and stressed ECAR over baseline ECAR. Compounds 1 and 2 seem to decrease the metabolic potential of differentiating preadipocytes by decreasing their OCR, while they have no significant effect on their ECAR. Compound 3 exhibited no effect on cell metabolic potential.

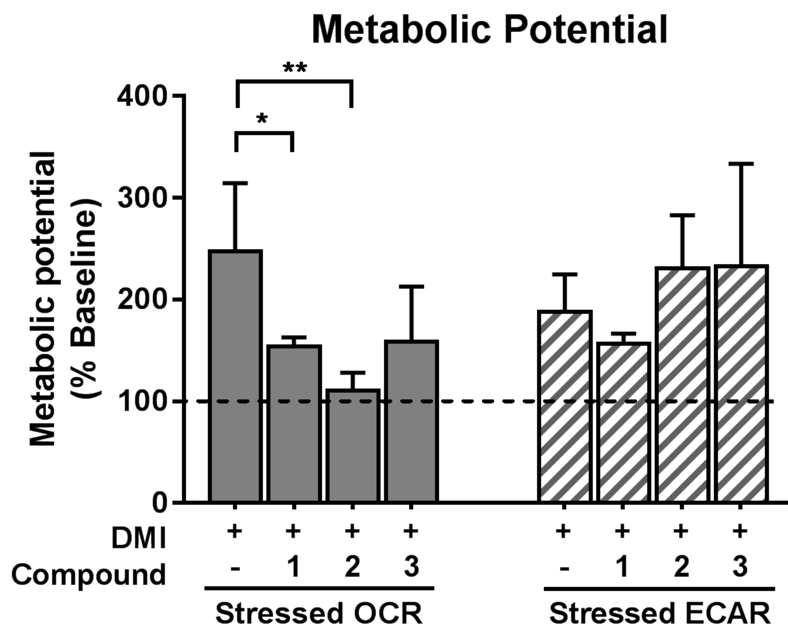

Figure 4.6: Preadipocytes induced to differentiate. The presence of metabolites 1 and 2 decreased their aerobic potential as shown by the difference in stressed OCR between the control (DMI) and treated (DMI+compounds) values.

Taking together OCR and ECAR into account the cell energy potential is measured (Fig. 4.7). The results showed that metabolites 1, 2 and 3 change differentiating preadipocytes phenotype towards a less aerobic-energetic phenotype. While metabolite 2 pushed cells to a less aerobic but more glycolytic phenotype.

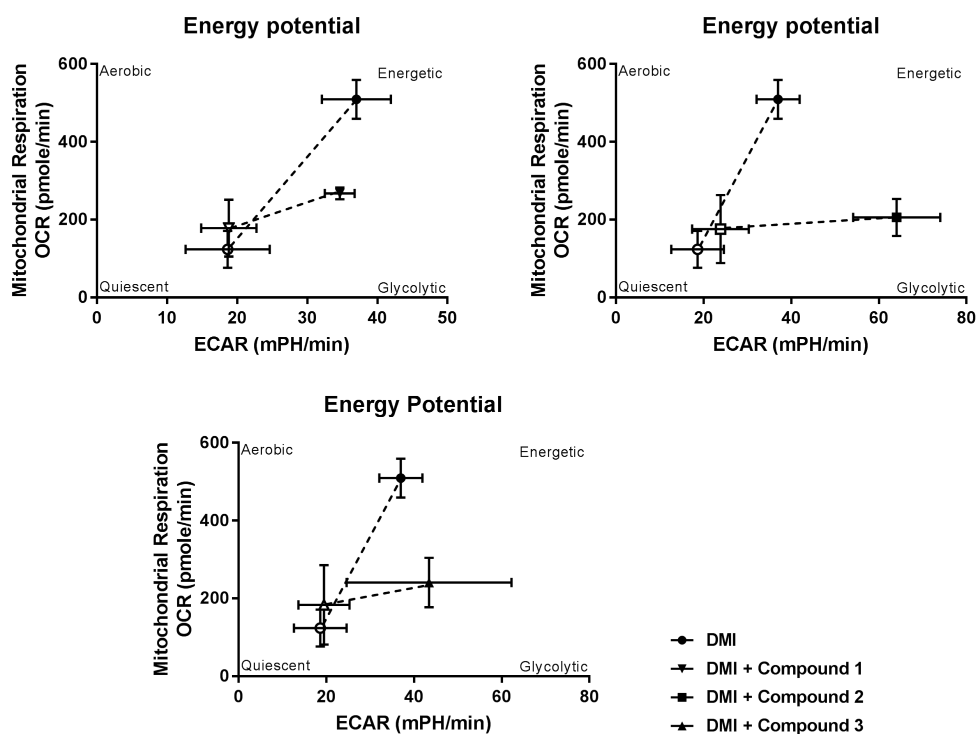

Figure 4.7: Preadipocytes induced to differentiate. Exposure of the stimulated to differentiate 3T3-L1 preadipocyte cell line to metabolites 1-3 caused no significant change in baseline activity (open symbols) but metabolites 1-3 decreased utilization of mainly the mitochondrial respiration pathway, while compound 2 pushed cells to a less aerobic but more glycolytic phenotype in response to mitochondrial stressors (closed symbols).

Chapter 5

Discussion

Inflammatory responses are orchestrated and fine-tuned by a plethora of mechanisms at multiple levels. The immune system can be stimulated by a variety of stimuli and subsequently different mechanisms, molecular pathways and cell types are set in motion, depending on the type of the inciting stimulus. Macrophages have a key role in innate immunity together with neutrophils, mast cells etc. After recognizing a noxious stimuli, via a variety of receptors that they possess, they become activated and trigger downstream signalling pathways that initiate responses towards homeostasis restoration. Depending on their activation state macrophages are categorized as classically activated M1 and alternatively activated M2. Akt kinases have an important role in this process, since deletion of Akt1 promotes an M1 phenotype with upregulated iNOS and IL12 [6] and absence of Akt2 tilt macrophage polarization towards an M2 phenotype [39].

Metabolic inflammation is a chronic state of low-grade inflammation, which is orchestrated by immune cells as well as adipose tissue. The crosstalk between adipocytes and immune system, especially macrophages, is culpable for the maintenance of the metabolic inflammation induced by excess nutrients. Part of this vicious circle of chronic and unresolved inflammatory state is the adipogenesis that causes adipose tissue to expand.

There are several studies exploring the effects of dietary metabolites in inflammation. Dietary metabolites can originate either from anaerobic fermentation of non-digestible dietary carbohydrates by commensal bacterial of the intestine, or from digestion of edible organisms, such as sea algae.

Despite the extensive work and research that has already been conducted on the effect of dietary metabolites in inflammation as well as metabolic inflammation, there are still a lot of questions that remain unanswered. Although many dietary metabolites have been identified, their exact effects and mechanisms of action in metabolic inflammation are not fully understood.

Butyrate and propionate affect macrophage polarization and metabolism

Bacteria from the phyla *Bacteroidetes* and *Firmicutes* produce SCFAs (butyrate, propionate) by anaerobic fermentation of non-digestible dietary carbohydrates in the colonic lumen. In obesity macrophage response potential in metabolic signaling from bacteria metabolites is altered, which may results in prevalence of different bacteria phyla. At the first part of the current study, we studied how diet-derived SCFAs affect macrophage polarization and whether this effect is Akt1 mediated and also how they affect macrophage metabolism. In this context, we sought to investigate whether macrophages isolated from LysM^{Cre}Akt1$^{fl/fl}$ mice responded differently to SCAFs upon LPS stimulation compared with wild type macrophages. We clearly demonstrated that butyrate and propionate have a dose dependent anti-inflammatory effect, while acetate has no effect on LPS-stimulated macrophages. While butyrate and propionate reduced the IL-6 secretion upon LPS, moderating the M1 phenotype in wild type macrophages, they had no effect in Akt1$^{-/-}$ macrophages. These results indicate that the anti-inflammatory effect of butyrate and propionate on macrophages occurs through the Akt molecular pathway. Next aspect to investigate was the effect of butyrate and propionate on the transcription of metabolic genes in LPS-stimulated macrophages. The expression of the inflammatory marker iNOS was induced more in Akt1$^{-/-}$ macrophages compared with wt, which confirms the knowledge that depletion of Akt1 in macrophages promotes an M1 pro-inflammatory phenotype. Butyrate further increased this induction in wt and significantly more in Akt1$^{-/-}$ macrophages. Regarding genes of the metabolism,

HK2 expression was increased by LPS and even more with butyrate, but had no difference between the two genotypes, but HK3 expression followed the iNOS expression pattern, except from Akt1$^{-/-}$ macrophages that butyrate had no effect. Butyrate also had no effect in LDH expression in Akt1$^{-/-}$ macrophages. These results suggest that butyrate affect macrophage metabolism increasing the expression of genes that participate in glucose metabolism making them more glycolytic. Specifically, HK2 and HK3 are two hexokinase isoforms which catalyze the ATP-dependent phosphorylation of glucose to glucose-6-phosphate (G6P), that is the rate-limiting and first obligatory step of glucose metabolism [101, 102]. LDH catalyzes the conversion of lactate to pyruvate and back, as it converts NAD+ to NADH and back. Thus it facilitates glycolysis by preventing pyruvate to build up and thus generation of ATP to slow down and by allowing the generation of NAD+ from NADH through anaerobic respiration. NAD+ is required when glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oxidizes glyceraldehyde-3-phosphate (GADP) in glycolysis, which generates NADH. By upregulating the expression of these genes butyrate promotes glycolysis in M1 macrophages and for LDH and HK3 this upregulation occurs through Akt1, since in its absence butyrate's effect is abolished. Propionate also increased the expression of iNOS, HK2 and LDH beyond the LPS induction in wt macrophages, but it diminished their LPS-elevated expression in Akt1$^{-/-}$ macrophages. This indicates that propionate has two opposing functions in the presence and absence of Akt1. When there is Akt1 it promotes glycolysis, like butyrate, but when there is no Akt1 it obstructs it. Collectively, these primary data reveal that butyrate and propionate affect macrophages in multiple levels and with a variety of ways. Macrophages in obesity are more inflammatory and respond differently to gut bacteria, which results in prevalence of different bacteria phyla. Thus, intestinal microbiome is altered, the size of bacteria populations changes, and the production of SCAFs changes. Which, as shown in this thesis, alter macrophage polarization and metabolism supporting mechanistically the reciprocal relationship between macrophages and microbiome in the intestine. Thus, the exhibited SCAFs effects on macrophages can explain the intestine microbiome changes taking place in obesity. In order to be able to take full advantage of butyrate and propionate potential beneficial effects on regulating immune responses, further experiments have to be performed. Their exact effect on macrophage metabolism needs to be studied and to do so, more metabolic markers need to be tested. Their effects on proteins upstream and downstream of Akt need to be tested, for the role of Akt1 in their mechanism of action to be unraveled. Moreover, to draw more complete conclusions it would be interesting to explore the effect of butyrate and propionate in Akt2$^{-/-}$ macrophages, studying how they affect macrophages of the M2 phenotype and what is the role of the Akt2 isoform as well in their mechanism of action.

Terpenoids affect adipocyte differentiation

For the second part of this study we wanted to investigate the possible anti-adipogenic properties of terpenoids from *Laurencia* spp.. The terpenoids tested were neorogioltriol (1), neorogioldiol (2) and O^{11},15-cyclo-14-bromo-14,15-dihydrorogiol-3,11-diol (3). The results showed that, regarding differentiation, neorogioldiol limited the reduction of the expression of the preadipocyte marker Pref-1, decreasing adipogenesis. The other two terpenoids didn't seem to affect adipocyte differentiation. The expression of FABP4, a terminal differentiation marker, was downregulated by the compounds 1 and 3 but their respective control group carbowax exhibited a strong effect on FABP4 mRNA levels, thus we cannot draw a safe conclusion from this experiment and it needs to be repeated. In addition to the expression of preadipocyte and adipocyte markers, the adipogenesis needs to be assessed with determination of lipid accumulation by oil red O staining and the expression of ROS-generative factors. We also studied the effect of these marine terpenoids on adipogenesis metabolism. Neorogioltriol and neorogioldiol reduced the metabolic potential of preadipocytes induced to differentiate by downregulating the mitochondrial respiration. Together with measurements of glycolysis rates the results showed that neorogioldiol changed the cells energy potential tilting them to a more glycolytic and less aerobic phenotype which is reasonable since we observed that it decreases adipogenesis. However, it has been shown that 7 days of adipogenic induction are required to stimulate cells to consume more oxygen and increase mitochondrial activity, indicating organelle maturation and a transition from glycolytic to oxidative energy metabolism [103]. Thus, the 24hrs treatment of preadipocytes wasn't enough for big significant differences to be created between the control group and the groups treated with the compounds. Although, there are a lot yet to be investigated regarding the anti-adipogenic properties of the marine terpenoids from *Laurencia* spp., we already have some indications that they possibly affect adipogenesis. After proving they can inhibit adipocyte differentiation at some extend at least, their mechanism

of action will be needed to be elucidated and a good starting point would be to test their effect on iNOS, TNFα, NFκB, COX-2 expression and other inflammatory agents that have been proved to be suppressed by terpenoids from other organisms. Moreover, their possible lipolytic effect should be explored to be able to use this compounds to their full potential. The anti-adipogenic properties of marine terpenoids could be exploited for the development of therapeutic drugs for treating obesity, but also sea algae could be part of the diet of healthy people, regardless obesity. The unraveling of the pathways that these compounds affect will provide us with a list of targets to use for manipulating adipogenesis and developing therapeutic drugs beyond natural products.

Overall, the present study contributes to the knowledge on the regulation of metabolic inflammation by dietary metabolites and the role of Akt1 kinase in it. More research is required to unravel all possible anti-inflammatory properties of dietary metabolites. Dissecting molecular mechanisms of their action will ultimately lead to novel therapeutic approaches and strategies for obesity and metabolic diseases.

Future perspectives

Metabolic diseases such as obesity and type II diabetes are characterized by chronic, low-grade inflammation, known as metabolic inflammation. This chronic inflammation has been shown to have pivotal role in the pathophysiology of these diseases. Herein, it was studied how diet metabolites affect inflammation. SCAFs were found to tilt macrophage polarization to an M1 phenotype and alter their metabolism through Akt1. While, marine terpenoids exhibited potential anti-adipogenic properties.

As future perspectives, it will be essential to explore in detail the effect of butyrate and propionate on the Akt pathway. This includes not only the proteins upstream and downstream of Akt, but also the effect of these SCAFs on Akt2 kinase. Future experiments include treatment of Akt2$^{-/-}$ macrophages with butyrate and analysis of cytokine secretion and metabolic genes expression. It would also be revealing if the same experiments will be performed in macrophages lacking both Akt1 and Akt2 kinases. Regarding intestine microbiome, microbiome analysis should be performed before and after feeding these different mice genotypes with high fat diet in order to detect the microbiome composition changes.

A limitation on the work presented here on adipogenesis is that it is exclusively conducted in a cell line. Although cell line preadipocytes are useful, it would be of point to study the effect of marine terpenoids in preadipocytes isolated from mice embryos (mouse embryonic fibroblasts MEFs). To study their possible lypolytic properties after treating differentiated 3T3-L1 cells with them, marine terpenoids could be used on adipose tissue (mouse, human). In addition, future experiments could be conducted to define their effect on adipogenesis. For example, more RNA analysis experiments would reveal the inflammatory agents whose expression is altered by marine terpenoids signaling. Moreover, the potential role of Akt kinases in these compounds action should be examined, since Akt is a critical node for many cellular functions.

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