n-3 Fatty acid derived endocannabinoids: a new link between fish oil and inflammation

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Abstract

Inflammatory processes are critical components of many illnesses, and dietary n-3 fatty acids have been shown to contribute to a reduction of the inflammatory status, both in *in vivo* and *in vitro* studies. The mechanisms underlying the modulation of inflammation are not completely understood, but it is clear that dietary n-3 fatty acids alter the eicosanoid metabolome profile, resulting in increased levels of n-3 fatty acid eicosanoids, whereas eicosanoids from other fatty acids are decreased. Until now, the anti-inflammatory properties of n-3 fatty acids had not been linked to an interaction with endocannabinoids/*N*-acyl ethanolamides (NAEs) levels before. This thesis describes a series of studies on the link between dietary fatty acids, endocannabinoids/NAEs, and inflammation.

Previous research indicated that dietary fatty acids alter the profile of endocannabinoids/NAEs rather than just affecting single compounds such as arachidonoyl ethanolamide (AEA) and 2-arachidonoyl glycerol (2-AG) as suggested before, and therefore a method based on liquid chromatography coupled to mass spectrometry (LC-MS/MS) to quantify a broad range of endocannabinoids/NAEs was developed. This method was used to demonstrate that n-3 fatty acids are converted to their endocannabinoid derivates by adipocytes *in vitro*. These n-3 derived NAEs, docosahexaenoyl ethanolamide (DHEA) and eicosapentaenoyl ethanolamide (EPEA), were shown to have anti-inflammatory properties in lipopolysaccharide (LPS)-stimulated adipocytes by reducing interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) excretion. Further studies showed that serum free fatty acid levels and plasma NAE levels are correlated under both fasting and post-prandial conditions in women, and demonstrated that plasma AEA and oleoyl ethanolamide (OEA) correlated with body mass index (BMI).

Considering the complexity of endocannabinoid and eicosanoid metabolism, it is likely that their concentrations are dynamic over time and tissue-specific during inflammation. So far, most studies had focused on limited numbers of endocannabinoids and eicosanoids in restricted numbers of tissues or plasma, and the effect of inflammation on DHEA and EPEA levels had not been studied before. Therefore, an animal study was conducted which investigated in detail the time-dependent effects of *i.p.* LPS on the levels of lipid derived mediators (endocannabinoids/NAEs and eicosanoids) in plasma, liver, ileum and adipose tissue in mice fed with a diet rich in fish oil. The results demonstrated that both

DHEA and EPEA levels were increased after LPS treatment, but also time- and tissue dependent effects were observed. Based on these data, another study was performed which investigated the combined effect of different fish oil diets and inflammation on the profiles of endocannabinoids and eicosanoids using the same multi-compartment targeted lipidomics approach. The data indicated that that dietary n-3 fatty acids and inflammation alter both the endocannabinoid and eicosanoid metabolomes towards higher levels of n-3 derived metabolites at the expense of metabolites derived from other fatty acids. Multivariate data analysis revealed that under normal conditions the diet groups were primarily separated based on decreased levels of other than n-3 derived metabolites. However, during inflammation, the separation was primarily explained by increases in n-3 derived compounds. Finally, additional analyses demonstrated that plasma and erythrocytes contain significant levels of esterified NAEs. The esterified levels were approximately 20-60 fold higher than the free NAE levels, and their profiles resembles the free NAE profiles.

In conclusion, (dietary) n-3 fatty acids increased the levels of DHEA and EPEA, and these metabolites displayed anti-inflammatory properties. Although the n-3 fatty acids are likely to be converted to a variety of other metabolites, the work in this thesis suggests that 'fish oil-derived' endocannabinoids are a new link between fish oil and its anti-inflammatory properties. Further research is needed to relate nutrition-based modulation of endocannabinoid profiles to more specific effects on health and disease.

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Chapter 1

General introduction

The interface between nutrition and pharmacology: an emerging scientific field

Nutrition belongs to the most important factors determining (human) health, and 'unhealthy' nutritional habits are linked to the development of various illnesses like diabetes, cancer, cardiovascular diseases, and deficiency syndromes. On the other hand, several food components are associated with positive health effects, like anti-oxidants, vitamins, calcium, probiotics, certain proteins, or omega-3 fatty acids. For many of these compounds, their normal physiological role is obvious at first glance; bone consists largely of calcium salts, so it is not surprising that the National Health Council advises people at risk for bone-degenerative diseases, like osteoporosis, to ensure sufficient intake of calcium [1]. However, several food components are more than just 'building blocks' for cells and tissues. Many molecules in our diet are capable of binding to macromolecules, including cellular receptors and enzymes, thereby evoking specific biological responses or modulating (patho-) physiological processes. For example, caffeine from coffee and other sources increases alertness by antagonizing the brain adenosine receptors [2], and thus represents a well-known example of a dietary compound with pharmacological properties. Compared to the 'classical' pharmacological approach, where the 'one target–one drug' concept has been a central principle, nutrition shows a more subtle and broader mode of action in influencing health, acting at multiple sites and producing smaller effects [3]. In their search for products with additional beneficial health effects, many food companies have engaged themselves in pharma-like strategies to discover nutrients and other bio-actives with biological effects that go beyond nutritional properties. During the last decades different nutritional product categories have evolved which claim to provide some form of specific health benefit. In the European Union (EU) for instance, a 'health claim' is any message or representation that states, suggests or implies that a relationship exists between a food category, a food or one of its constituents and health. A 'reduction of disease risk' claim is defined as any health claim that states, suggests or implies that the consumption of a food category, a food or one of its constituents significantly reduces a risk factor in the development of a human disease. In the EU, the European Food Safety Authority (EFSA; httt://www.efsa.europa.eu) is responsible for verifying that health claims made on a food label are substantiated by scientific evidence. In addition to these

food products, functional foods or supplements with health claims, there is an increasing activity in the area of clinical and medical nutrition, *i.e.* nutritional strategies with therapeutic claims. For example in diabetes, cardiovascular disease or cancer, some people benefit from more intensive forms of nutritional support as part of the therapy [3]. It is expected that better, healthier nutrition will be part of future advances in the prevention and treatment of disorders mentioned before. However, it is clear that more research is needed to better understand the often very complex and subtle mechanisms of action of nutrients or food products and to evaluate their biological effectiveness.

Inflammation: more than just a defense mechanism

Inflammation is an organisms' response to a noxious stimulus, such as invasion by micro-organisms or traumatic tissue damage [4]. Its purpose is to limit ongoing damage, and to initiate the processes which should lead to repair or regeneration of the damaged tissue. Inflammation involves vascular effects and the action of different cells of the (innate) immune system, including macrophages and granulocytes. The communication between these various cells is regulated by signaling molecules such as interleukins, prostaglandins and leukotrienes. Normally, inflammation is considered to be beneficial as it helps organisms to overcome *e.g.* pathogenic infection. However, several diseases are associated with dysregulated inflammatory processes, such as allergies, rheumatoid arthritis, inflammatory bowel disease, and obesity [5]. These diseases can greatly affect the quality of life of people suffering these, and together represent a significant burden on health care economics. For several conditions, pharmacological therapy is used to treat the symptoms of inflammation [6], and a wide range of anti-inflammatory agents is currently available, as over-the-counter or prescription drugs. Although these agents are generally effective, adverse effects associated with their (chronic) use could limit their applicability.

Over the last years, it has become evident that inflammation can be regarded as an overarching process involved in the development, progression and resolution of many pathological conditions. Depending on the situation, modulation of inflammation can be clinically advantageous. For example, patients suffering from solid tumors displayed prolonged survival when treated with anti-inflammatory drugs [7]. Furthermore, blockade of the cytokine interleukin-6 (IL-6) was shown to improve metabolic parameters in an experimental model of cancer cachexia [8].

Cachexia is commonly observed in advanced stages of cancer, and is associated with *e.g.* reduced effectiveness of anticancer therapies, and increased incidence of side-effects [3]. In addition to treatment with medication, nutrients like omega-3 fatty acids and amino acids are also effective against cancer cachexia, and (early) nutritional support has been associated with an improved response to therapy and quality of life [9, 10].

Type 2 diabetes (T2D) represents another example of a complex disease in which inflammation is involved as one of the key pathological processes [11, 12]. Insulin resistance or T2D can be part of a larger constellation of metabolic abnormalities known as the *metabolic syndrome*. There are several definitions of the metabolic syndrome, and numbers on its prevalence may differ as different definitions are used. The US National Cholesterol Education Program Adult Treatment Panel III (NCEP – ATP III) definition includes criteria for central obesity, dyslipidemia, hypertension and hyperglycemia. But also other definitions are used, including criteria for insulin sensitivity or body mass index (BMI). Nevertheless, it is clear that the metabolic syndrome is characterized by metabolic disturbances at the systemic level, and multiple sites like liver, pancreas, skeletal muscle and adipose tissue are involved in its etiology.

In a publication by Weisberg *et al*, a link between obesity and inflammation was established for the first time since obesity was related to increased macrophage infiltration in adipose tissue of mice [13]. Adipose tissue is capable of secreting pro-inflammatory compounds like tumour necrosis factor-α (TNF-α), IL-6 and monocyte chemotactic protein-1 (MCP-1), which are delivered to the bloodstream and can act on distant targets like liver and skeletal muscle [14-17]. This might in part lead to the increased plasma IL-6 and MCP-1 levels which are observed in obesity, and it is now well established that obesity is associated with a systemic low grade inflammation. MCP-1 is involved in the recruitment of macrophages to extra-vasal sites, including adipose tissue, which can lead to further macrophage infiltration of adipose tissue [17]. *In vitro* experiments showed that there is cross-talk between macrophages and adipocytes, leading to increased synthesis of inflammatory cytokines when they are allowed to make physical contact [18]. IL-6 and TNF-α are pro-inflammatory proteins, but also reduce insulin sensitivity in skeletal muscle cells, linking obesity to the development of insulin resistance and eventually type 2 diabetes (T2D) and the metabolic syndrome [11, 12]. Anti-inflammatory treatments, such as inhibiting the IL-1 receptor or inhibiting the NF-

κB pathway, are effective in reducing blood sugar levels and systemic inflammation, thereby reducing insulin resistance in diabetes [11].

Modulation of inflammation by dietary fatty acids

Nutrition provides an alternative way to attenuate inflammation and its complications. Dietary intake of the omega-3 'fish-oil' fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) is associated with positive health effects, including improvement of metabolic parameters in obesity and anti-inflammatory effects [5, 19-21]. The mechanisms underlying the immune-modulating properties of omega-3 fatty acids are not completely understood, but it is clear multiple receptors and pathways are involved (see Figure 1.1 for an overview of established pathways).

One of these proposed pathways involves binding of DHA and EPA to peroxisome proliferator-activated receptor-γ (PPAR-γ), which results in anti-inflammatory effects [22]. More recently, also the orphan receptor GP120 was indicated as a mediator of the anti-inflammatory properties of DHA and EPA in RAW264.7 and primary intraperitoneal macrophages [23]. At the same time, GP120 was also implicated in the insulin sensitizing effects of omega-3 fatty acids in obesity, again attesting to the mechanistic relation between inflammation and insulin resistance.

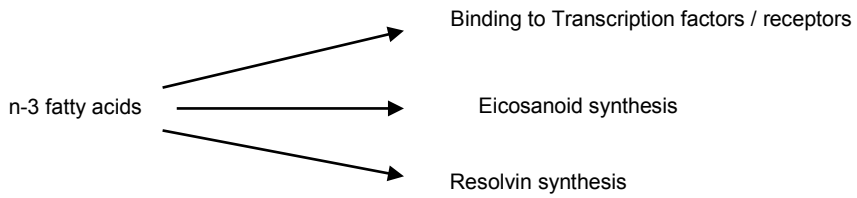

Figure 1.1: *Established mechanisms through which n-3 fatty acids modulate inflammatory processes.*

Another line of evidence points to a link with the synthesis of lipids with biological activity ('bioactive lipids'), such as the eicosanoids. For example, arachidonic acid (ARA; 20:4 omega-6), can be converted by cyclo-oxygenase (COX), lipo-oxygenase (LOX), and/or cytochrome P450 (CYP) to form prostaglandins (PGs), leukotrienes (LTs), and epoxyeicosatrienoic acids (EETs), respectively (see Figure 1.2 for an

overview) [5, 24-26]. These classes of compounds are involved in inflammatory signaling and are generally (except the EETs) assumed to be pro-inflammatory. The synthesis of these compounds starts within the cell membrane, were fatty acids are released from membrane phospholipids and are eventually converted to the above mentioned COX or LOX metabolites. Dietary intervention studies have clearly demonstrated the link between dietary fatty acid intake, cell membrane fatty acid composition, and subsequent prostaglandin levels [27]. Increasing dietary intake of n-3 fatty acids reduced the levels of ARA in plasma membrane phospholipids. At the same time, *ex vivo* stimulated immune cells produced lower quantities of the ARA-derived metabolite PGE_2 and cytokines, but higher levels of n-3 fatty acid derived eicosanoids were found. These compounds, also referred to as the '3-series' of prostaglandins (see Figure 1.3 for a full overview of n-3 fatty acid derived metabolites), are less potent inflammatory molecules than ARA-derived metabolites, thus indicating that manipulation of dietary fatty acid intake shifts the profile of released prostaglandins to a 'less inflammatory' phenotype.

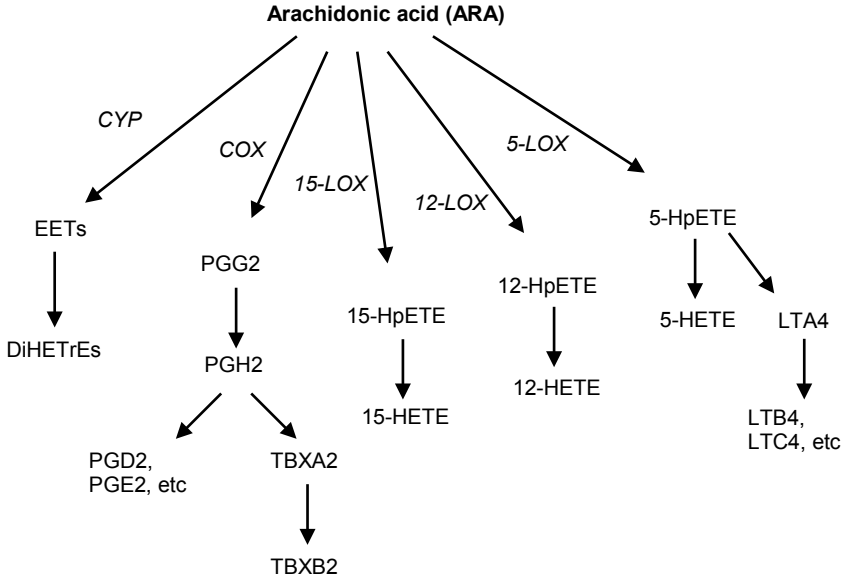

Figure 1.2: *Overview of the enzymatic pathways involved the metabolism of arachidonic acid (ARA) to bioactive lipids, and their principal metabolites. Several enzymes, e.g. COX, CYP and LOX, can metabolize ARA, yielding a variety of bioactive lipids.*

Recently, evidence has emerged that n-3 fatty acids can be converted to a class of compounds called *resolvins*, which have anti-inflammatory properties and could also contribute to the anti-inflammatory effects which are related to dietary intake of n-3 fatty acids [28]. Resolvins are synthesized from the COX-2 and LOX pathways when aspirin is present. Their anti-inflammatory properties have been demonstrated in a murine model of inflammatory bowel disease, where *e.g.* pretreatment with certain resolvins protected against wasting, inflammatory cell infiltration, and colonic damage [22]**.**

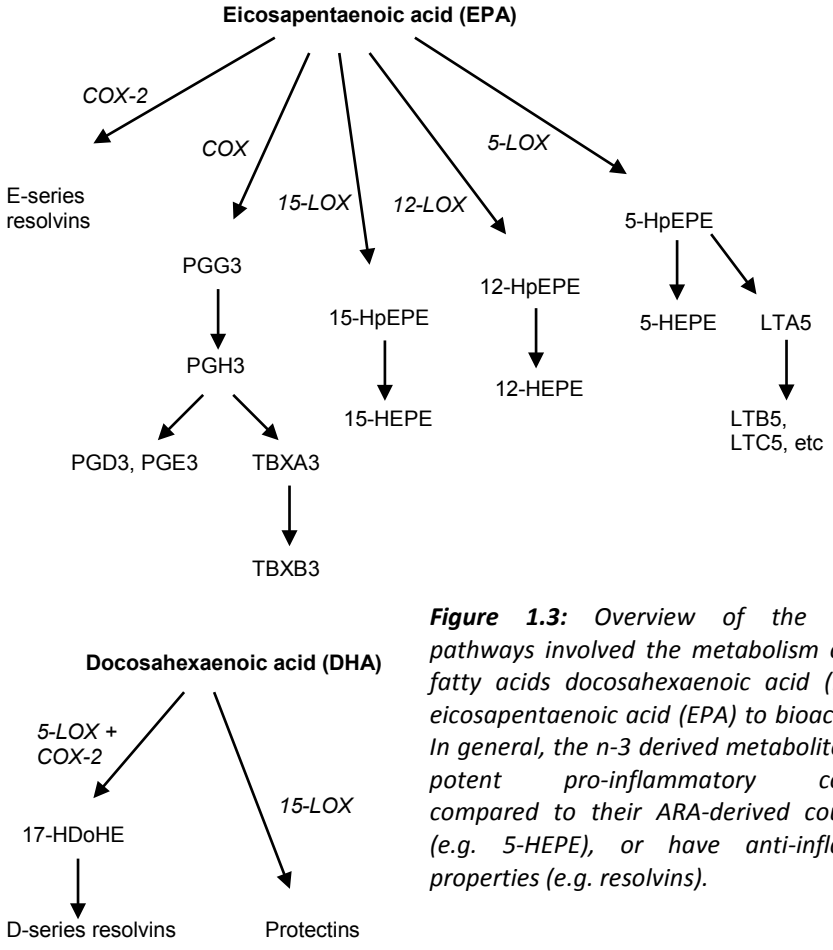

Figure 1.3: *Overview of the enzymatic pathways involved the metabolism of the n-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) to bioactive lipids. In general, the n-3 derived metabolites are less potent pro-inflammatory compounds compared to their ARA-derived counterparts (e.g. 5-HEPE), or have anti-inflammatory properties (e.g. resolvins).*

Finally, fatty acids also serve as precursors for the synthesis of the so-called endocannabinoids, another class of compounds known for its role in metabolism and inflammation. The potential relation between dietary n-3 fatty acids, endocannabinoids, and inflammation has not received much attention yet. The endocannabinoid system will be introduced in more detail in the next section.

The endocannabinoid system

Over the last years, the so-called endocannabinoid system (ECS) has received much interest for its role in metabolism and inflammation. Studies into the effects of smoking marijuana led to the discovery of the cannabinoid receptor type 1 (CB1) in brain [29]. CB1 was found to be responsible for mediating the effects of Δ-9 tetrahydrocannabinol, one of the major active compounds released from smoking *Cannabis sativa*. Later, the endogenous ligands for CB1, the so-called *endocannabinoids*, were identified. The first identified endocannabinoid was arachidonoyl ethanolamide (AEA), which is also known as *anandamide* (Sanskrit for *'inner bliss'*) and belongs to the class of fatty acid *N*-acyl ethanolamines (NAEs) [30]. AEA does not only bind to CB1, but also to other receptors, such as PPAR-α and PPAR-γ, and the ligand-gated ion channel transient receptor potential vanilloid-1 (TRPV-1) [31-33]. Subsequently, another 'classic' endocannabinoid 2-arachidonoyl glycerol (2-AG), and a second endocannabinoid receptor, CB2, were identified [34]. In addition to these two established cannabinoid receptors, there is now data suggesting that there are more cannabinoid receptor types, with GPR18 [35] and GPR55 [36] being candidates to be recognized as cannabinoid receptors, and other orphan receptors might follow. Other currently known endocannabinoids are *N*-arachidonoyl dopamine (NADA), 2-arachidonoyl glycerol ether (2-AG ether, noladin ether), *O*-arachidonoyl ethanolamide (O-AEA; virodhamine), dihomo-γ-linolenoyl ethanolamide (DLE) and docosatetraenoyl ethanolamide (DEA) [30, 34, 37-39] (see Figure 1.4 for these endocannabinoids and chemically related structures). Together, the endocannabinoids, the enzymes involved in their metabolism, and the cannabinoid receptors, are referred to as *the endocannabinoid system* (ECS). The endocannabinoid system is widely present throughout the body and is involved in a variety of physiological processes, including the regulation of food intake, metabolism and inflammation. The synthesis of endocannabinoids, their roles in metabolism and inflammation, and the relation with dietary fatty acids will be discussed in the next sections.

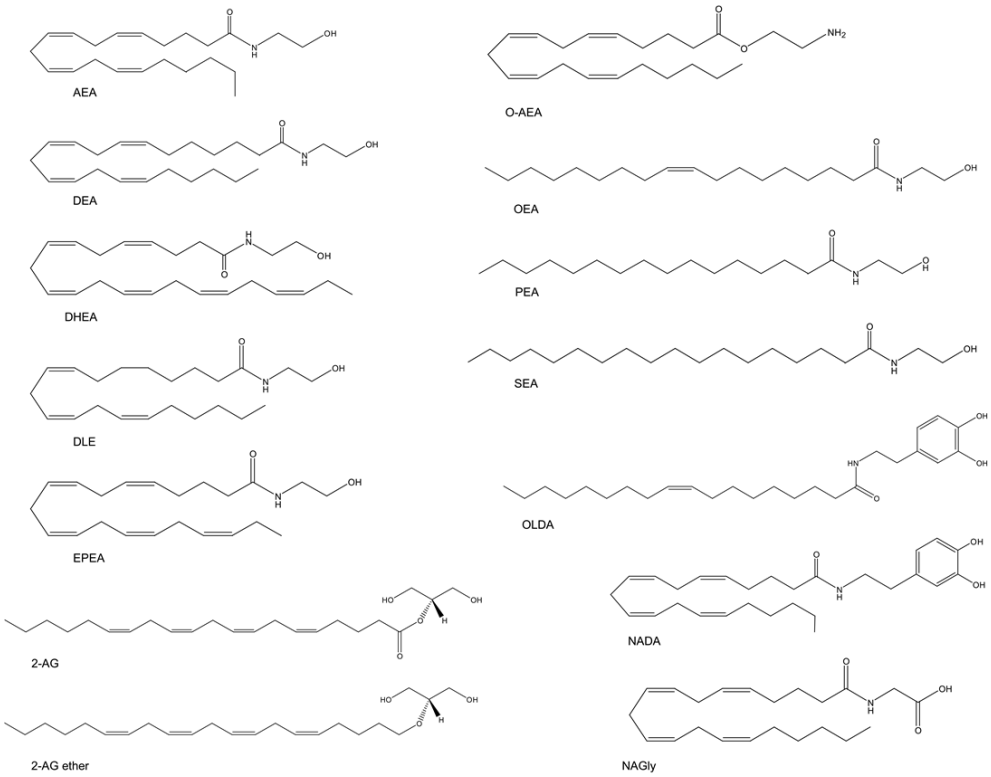

Figure 1.4*: Overview of several endocannabinoids and related structures. Anandamide (AEA) is the best studied endocannabinoid and belongs to the group of N-acyl ethanolamines. Other known compounds with affinity for either CB1 and/or CB2 in this figure include DHEA, EPEA, DLE, 2-AG, O-AEA, and NADA. Other structures, such as OEA, PEA and SEA, do not display any significant affinity for CB1 or CB2, but can bind to other receptors, including PPARs or TRPV-1, which are also targets for e.g. AEA.*

Synthesis and breakdown of endocannabinoids

Similar to the prostaglandins, the endocannabinoids are synthesized from fatty acids which are initially esterified to membrane phospholipids. NAEs are synthesized on demand, and it is likely that their levels are regulated by enzymes which are responsible for their release and breakdown. Several mechanisms are described through which AEA and 2-AG can be synthesized, involving the action of

multiple enzymes and the formation of several lipid intermediates [34, 37, 40]. For NAEs, the transacylation-phosphodiesterase pathway is the most studied route. Here, NAEs are released in a two-step process [37]. First, Ca^{2+}-dependent *N*-acyltransferase (Ca-NAT) transfers the fatty acyl chain from the *sn*-1 position of a glycerophospholipid to the ethanolamine moiety of phosphatidylethanolamine (PE), to form *N*-acylphosphatidylethanolamine (NAPE). In the second step, NAPE is broken down by NAPE-hydrolyzing phospholipase D (NAPE-PLD), which releases phosphatidic acid and the NAE. Several alternatives to this pathway have been described in literature, requiring the action of other enzymes such as phospholipase A2 (PLA2) which yield other distinct lipid intermediates [40].

After release, NAEs are immediately exposed to enzymes that are able to break them down, thereby terminating their action. The primary NAE degrading enzyme is fatty acid amide hydrolase (FAAH), which is located at the surface of the endoplasmatic reticulum membrane [41]. FAAH hydrolyses the NAE to its corresponding fatty acid and ethanolamine. Due to the intracellular location of FAAH, the NAE must get back into the cell through an as yet not completely understood process. There is debate on the existence of the 'anandamide membrane transporter' (AMT), which is predicted to exist based on experimental data that point to a transporter-dependent mechanism. Several inhibitors of this alleged transporter have been described, contributing to this idea. However, so far, such a membrane transporter has not been identified yet, and evidence supporting a simple transmembrane diffusion model is growing. Anandamide was shown to diffuse across membranes in a transporter-independent fashion [42]. In addition, several purported transport inhibitors in fact inhibit FAAH, thereby preventing the development of a concentration gradient across the membrane and thus stopping the NAE transmembrane movement [43]. Future research should establish if the AMT truly exists or whether data supporting its existence are in fact pointing to FAAH as the principal bottleneck in NAE hydrolysis.

Recently, FAAH-2, a second NAE degrading enzyme, was discovered. FAAH-2 is localized on cytoplasmic lipid droplets, and displayed slower NAE hydrolysis rates compared to FAAH [41]. Interestingly, FAAH-2 is also expressed in heart and ovary tissue, where FAAH is not detected [44].

Finally, next to enzymatic breakdown, NAEs are also substrates for COX-2, LOX and CYP, yielding *e.g.* prostamides and *n*-hydroperoxyanandamides [45, 46], representing another pathway to clear NAEs.

Endocannabinoids and related compounds as important regulators of metabolism

In addition to AEA, DEA and DLE, other fatty acids can also couple to ethanolamine to form a NAE. Known examples include oleoyl ethanolamide (OEA), palmitoyl ethanolamide (PEA), and stearoyl ethanolamide (SEA), which are present in higher concentrations in blood that AEA [47] (see Figure 1.4). These compounds do not have any significant affinity for either CB1 or CB2, but can bind to other receptors which are also targets for *e.g.* AEA, including PPARs and TRPV-1. Moreover, it has been hypothesized that PEA can potentiate the action of AEA due to competition at the breakdown level; increasing relative PEA levels would result in reduced breakdown of AEA, elevating its levels and potentially leading to increased CB1 stimulation.

OEA is known for reducing appetite, body weight gain and mesenteric fat deposition [48, 49]. Gut levels of OEA are decreased by fasting and increased after eating, and *i.p.* administration of OEA results in acute reduction of food intake. These effects are in part mediated by PPAR-α, TRPV-1 and possibly GPR119, but it is likely that other receptors are also involved as these effects were not completely blocked with antagonists or knock-out models. The effects of OEA are in contrast to the appetite-inducing properties of AEA [50]. When in the fasting state, endocannabinoid levels are increased in the limbic forebrain, and are decreased upon eating [51]. Exogenous administration of 2-AG in the brain stimulated appetite, which was attenuated with co-administration of the CB1 inverse agonist SR141716 (also known as Rimonabant; see below for further details) [51]. This relates to the sudden strong desire for food (*'munchies'*) which is observed after the use of marijuana. AEA levels are increased in the small intestine upon food deprivation, and normalized after eating, and this might be involved in gut-to-brain signaling.

In addition to effects on food intake and satiety, endocannabinoids are also active in other tissues which are involved in energy homeostasis. Levels of AEA and 2-AG display peak concentrations just before differentiation in 3T3-F442A adipocytes, with 2-AG levels remaining relatively high [52], and pharmacological stimulation of CB1 increased lipid droplet formation in differentiating adipocytes. Interestingly, PPARs are also involved in adipocyte differentiation and energy homeostasis, linking both endocannabinoids and non-endocannabinoid NAEs to adipose tissue biology [53].

CB1 receptors are also present in pancreatic tissue, skeletal muscle and liver [54, 55]. In the liver, endocannabinoids stimulate lipogenesis and are believed to be involved in the development of steatosis. In skeletal muscle, the endocannabinoid system affects oxidative metabolism by decreasing glucose uptake and metabolism, and increased levels of endocannabinoids might interfere with insulin signaling.

The ECS in relation to metabolic diseases and inflammation

Dysregulation of the endocannabinoid system is associated with metabolic diseases such as obesity. Increased plasma levels of AEA and 2-AG were observed in obese subjects [56, 57], leading to the hypothesis that the ECS is 'overactivated' in obesity. Another study from Côté and coworkers, investigating correlations between plasma endocannabinoids and cardiometabolic risk factors in obese men, demonstrated that plasma 2-AG levels correlated positively with BMI, waist girth, intra-abdominal adiposity (IAA), plasma triglyceride and insulin levels, but correlated negatively with high-density lipoprotein (HDL) cholesterol and adiponectin [58]. Visceral adipose tissue of obese subjects had increased levels of 2-AG. Interestingly, no relation between AEA and BMI, waist circumference, HDL cholesterol, or adiponectin was found. To the contrary, AEA was negatively related to IAA. Work from Di Marzo and coworkers showed that a life style intervention in obesity improved cardiometabolic risk factors in parallel to decreases in plasma AEA and 2-AG levels [59].

The involvement of CB1 in food intake and energy metabolism led to the hypothesis that CB1 would be a target for the treatment of obesity, leading to the development of CB1 blockers. Rimonabant (Acomplia® by Sanofi-Aventis) was the first CB1 inverse agonist to be approved as an adjunctive therapy in addition to life style interventions against obesity. One-year use of Rimonabant combined with caloric restriction resulted in increased weight loss and HDL cholesterol levels, and decreased plasma triglycerides and fasting insulin levels compared to placebo [60]. However, after its market approval in Europe, several reports emerged indicating that the use of Rimonabant was associated with depression or even suicidal thoughts in certain sub-populations of patients [61]. This eventually led to the withdrawal of Rimonabant from the European market in 2008, and it is currently no longer produced by Sanofi-Aventis. Despite this, the search for CB1 antagonists with actions restricted to peripheral tissues is ongoing.

In addition to a link with energy homeostasis, endocannabinoids and related structures are also important in inflammation. In this respect, PEA is the best studied compound, with demonstrated anti-inflammatory properties using *in vitro* models of human adipocytes [62] and anti-inflammatory and anti-nociceptive effects in animal models of *in vivo* inflammation, including carrageenan-induced joint inflammation [63]. Anti-inflammatory or immune-modulatory properties of AEA and 2-AG have also been described in several *in vitro* models using *ex vivo* immune cells, typically demonstrating reduced cytokine synthesis, decreased proliferation, or decreased chemotactic capacity after stimulation with *e.g.* lipopolysaccharide (LPS) or mitogenic substances [32, 64, 65]. The mono-unsaturated compound OEA displayed anti-nociceptive properties in animal models of visceral and joint inflammation [66]. However, another report, using a paw edema model of inflammation using other parameters, observed less reliable anti-edema effects of OEA [67]. Finally, SEA displayed anti-edema effects in a murine model of skin allergy [68].

Dietary fatty acids, endocannabinoids, and their analogues – do nutrition and pharmacology meet?

As with the prostaglandins, manipulation of dietary fatty acid intake could also represent a way to modify the *in vivo* NAE profile, and some studies have already demonstrated this link [69]. Supplementing AA to milk fed to piglets increased brain AEA levels, but feeding a milk formula deficient in AA led to decreased AEA and 2-AG brain levels [70]. In a study published by Artmann *et al.*, the effect of different fatty acid diets on NAE levels in rat tissues was investigated systematically [71]. This study showed that *e.g.* a fish-oil rich diet decreased jejunal levels of AEA, OEA and PEA, but at the same time increased the levels of the fish-oil derived NAEs docosahexaenoyl ethanolamide (DHEA) and eicosapentaenoyl ethanolamide (EPEA). This showed that diet altered the profile of endocannabinoids rather than individual compounds. Another dietary study from Batetta *et al.* used Zucker rats to demonstrate that dietary n-3 fatty acids reduced liver triglyceride levels and the macrophage inflammatory response, and that this was associated with reduced tissue AEA and 2-AG levels, but no data on DHEA or EPEA was reported [21]. More recently, similar findings were reported by Banni *et al.* in obese human subjects, were krill oil consumption resulted in decreased plasma 2-AG levels [72]. Again, levels of DHEA and EPEA were not

reported. Taken together, it seems that *in vivo* endocannabinoid levels are a reflection of the relative abundance of fatty acids in the diet, thus altering the endocannabinoid profile rather than individual compounds.
Considering the biological properties of endocannabinoids, their modulation by *e.g.* dietary n-3 fatty acids could affect processes like energy metabolism and inflammation, it has been hypothesized that endocannabinoids are the link between dietary n-3 fatty acids and their health effects [21]. Interestingly, a role for the fish oil derived DHEA and EPEA in this relation has barely received attention as most publications focused on levels and effects of the 'classical' endocannabinoids AEA and 2-AG. Similar to the above-mentioned resolvins and n-3 derived eicosanoids, DHEA and EPEA could potentially have anti-inflammatory properties, but until the start of this research project no reports on the biological effects of DHEA or EPEA were available in scientific literature. In addition to understanding their biological properties, data on the levels of DHEA and EPEA were limited, underlining the importance of an analytical technique which is capable of quantifying a broad range of endocannabinoids, including DHEA and EPEA

Quantification of endocannabinoids and eicosanoids using mass spectrometry

Plasma and tissue levels of endocannabinoids, NAEs and eicosanoids are generally low, and their quantification requires adequate technology. A triple-quad mass spectrometry-based technique is usually the first choice because this allows the simultaneous quantification of multiple compounds. A mass spectrometer (MS) can differentiate between compounds based on their molecular weight. This is used for identification of molecules, since the molecular weight can be regarded as its 'fingerprint'. The MS is mostly coupled after a liquid chromatography system (LC) to separate compounds based on their physical properties (*e.g.* hydrophobicity). The MS can only detect molecules which are electrically charged, thus requiring an ionization step. For example, AEA has a molecular weight of 347 Da, and the ionization step (attraction of one proton under the appropriate conditions) gives AEA a mass-to-charge (*m/*z) ratio of 348 ((347 + 1)/1).
Different types of MS are available, such as high-resolution MS, iontrap MS, and triple-quadrupole MS. For the analysis of low-abundant compounds like

endocannabinoids, triple-quadrupole MS would be the first choice due to its superior sensitivity.

A typical triple-quad MS system contains three quadrupoles (*'quads'*) Q1, Q2, and Q3, through which ions are accelerated. A quadrupole consists of four parallel metal rods, over which a voltage is applied. This induces a complex electrical field within the quad, causing ions flowing through them to oscillate. Depending on the quad voltage and the ion's *m/z* value, an ion will either have a stable flight through the quad and should reach the detector, or an unstable trajectory. In the last case, the ions will (eventually) collide to the rods and are therefore lost. By carefully choosing the rod voltage, a quadrupole can serve as an ion filter, only allowing ions of a certain *m/z* value to be detected by the MS.

In a triple-quad, three quads are connected in linear series. Q1 and Q3 serve as *m/z* filters, and Q2 is used as a collision cell to fragment the ions, that are pre-selected in Q1. These pre-selected ions (parent ions) are fragmented in Q2 with *e.g.* argon gas, and all fragments are passed on to Q3, which can be set to filter specific fragments. Fragmentation dissociation analysis gives additional structural information, increasing the specificity of the MS analysis. In case of AEA, several dissociation fragments (daughter ions) are found, but the most dominant fragment is at *m/z* 62, which represents the ethanolamine moiety of AEA. The dissociation pattern, combined with the retention time, makes triple-quad MS a highly sensitive and specific technique. Triple-quadrupole MS is also known as tandem MS or MS/MS.

Several factors can negatively influence the performance of MS analysis. Among these, ion suppression is a well-known phenomenon. Ion suppression refers to the sub-optimal ionization of analytes, thereby hampering the detection since non-charged molecules are not detected by the MS. Ionization is a saturable process, and the presence of compounds other than the analytes of interest could potentially cause ion suppression. Therefore, adequate sample clean-up is of great importance when using the extracts for MS analysis. The extraction procedure should ensure maximum recovery of the analytes, but minimize co-extraction of other compounds that could potentially cause ion suppression. There are several ways to extract endocannabinoids and eicosanoids from plasma or tissue, using organic solvents, solid phase extraction (SPE) or a combination of these.

MS method development should also include optimizing chromatography conditions, such as choice of eluents, chromatographic gradient and analytical column, to ensure good peak shape and separation of (all) extracted compounds. In addition, other variables such as (long term) stability and choice of labware (glass, plastic) should also be investigated, since certain compounds can be prone to oxidation or are known to adhere to glass or plastic.
In literature, several analysis techniques are described for the quantitation of endocannabinoids/NAEs in a variety of biological matrices, often using LC-MS or LC-MS/MS based methods [73-80]. However, few of the published methods are developed and validated for the simultaneous detection of a broad range of endocannabinoids and related structures. As described above, concentrations of a certain NAE might depend on levels of other NAEs, and different NAEs can activate the same receptors, making it of biological relevance to have a LC-MS/MS method which is capable of simultaneously quantifying multiple endocannabinoids and eicosanoids. Such methods are also known as *'targeted metabolomics'* platforms.

Dealing with large datasets: multivariate data analysis helps out!

Targeted metabolomics platforms can quantify dozens of compounds from one sample, which results in large datasets, especially when multiple platforms are used in parallel. Advanced statistical tools, such as multivariate data analysis, are useful for extracting relevant information, *e.g.* differences in metabolic profiles between intervention groups, from large amounts of data. A well-established multivariate data analysis approach is *'principal component discriminant analysis'* (PCDA), which assesses separation of intervention groups and identifies variables which are responsible for this. The PCDA algorithm summarizes all variables into a single variable (the principal component) which accounts for the variance in the data, also considering the original group designation of the individual samples. As a result, it generates lists of compounds which account for the separation of groups, ranking the compounds based on their relevance for the group separation. As such, PCDA is useful for identifying and ranking compounds which are affected by fatty acids.

Aims and outline of this thesis

As discussed above, dietary intake of n-3 fatty acids is associated with beneficial effects on metabolism and inflammation. The molecular mechanisms underlying this are not completely understood, but it is clear that there is a link with fatty acid derived bioactive lipids, such as endocannabinoids and eicosanoids. However, so far very little data on 'fish oil-derived' endocannabinoids has been presented, and their biological properties have not been described in literature.

In this thesis, the relation between dietary fatty acids and endocannabinoids is investigated, with special attention for 'fish oil-derived' endocannabinoids and inflammation. First, we developed a LC-MS/MS based analytical method for the simultaneous quantification of 12 endocannabinoids and related compounds, with special attention for SPE clean-up and sample evaporation, which will be presented in **chapter 2** of this thesis. Next, we investigated the formation of DHEA and EPEA from their fatty acids, DHA and EPA, respectively, and possible immune-modulating properties. For this, we used 3T3-L1 murine adipocytes, an established model of *in vitro* endocannabinoid biosynthesis. The findings of this work are described in **chapter 3**.

In **chapter 4** we investigated the relation between serum free fatty acid levels and plasma NAE levels under both fasting and postprandial conditions.

The findings from chapters 2 and 3 prompted us to study the relation between dietary fatty acid intake and endocannabinoid levels in an *in vivo* setting, investigating the effect of dietary fatty acids on eicosanoid and endocannabinoid levels. Here, we also investigated the effect of inflammatory stress on this relation. In **chapter 5**, we report on a pilot study in which we explore the time-dependent effects of a single *i.p.* dose of LPS or saline on plasma and tissue eicosanoid and endocannabinoid levels in C57Bl/6 mice. We used this work for a second *in vivo* study, in which we compared the effect of different fish-oil containing diets on plasma and tissue eicosanoid and endocannabinoid balance, after a single *i.p.* dose of either LPS or saline. The results of this study are presented in **chapter 6**. Finally, we demonstrate in **chapter 7** that plasma contains previously ignored pools of esterified NAEs, which closely resembled patterns observed for free NAE levels.

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Chapter 2

Development and validation of a quantitative method for the determination of 12 endocannabinoids and related compounds in human plasma using liquid chromatography-tandem mass spectrometry

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Abstract

A sensitive and specific LC-MS/MS method for the quantification of the endocannabinoids and related structures anandamide, 2-arachidonoyl glycerol, 2-arachidonyl glycerol ether, O-arachidonoyl ethanolamide, dihomo-γ-linolenoyl ethanolamide, docosatetraenoyl ethanolamide, N-arachidonoyl dopamine, N-arachidonyl glycine, N-oleoyl dopamine, oleoyl ethanolamide, palmitoyl ethanolamide, and stearoyl ethanolamide in human plasma was developed and validated. Compounds were extracted using acetonitrile followed by solid-phase extraction. Separation was performed on a XTerra C8 column using gradient elution coupled to a triple-quadrupole MS. LLOQ levels ranged from 0.02-1.75 µg/mL, LODs ranged from 0.0002-0.1266 ng/mL, and accuracies were > 80% (except stearoyl ethanolamide at lowest spike level) at all spike levels.

Introduction

Almost two decades after the identification of the cannabinoid type 1 (CB1) receptor in 1990 as mediator of the psychotropic effects of marijuana, research on the endocannabinoid system has increased spectacularly [1]. In the meantime a second cannabinoid (CB2) receptor has been characterized and probably more receptors, including GPR55, will follow [2]. A significant body of evidence now suggests that the endocannabinoid system plays an essential role in many physiological processes, including energy metabolism and neurological functioning, and in several pathological conditions, including inflammation, cardiovascular diseases, cancer, neurological disorders, obesity, and the metabolic syndrome [3-7]. Anandamide (arachidonoyl ethanolamide, AEA) was the first endocannabinoid discovered. It is a fatty acid amide, thought to be formed from phospholipid-esterified arachidonic acid and phosphatidylethanolamine [8]. In addition to anandamide the search for endogenous ligands has revealed a number of so-called endocannabinoids. These include 2-arachidonoyl glycerol (2-AG), 2-arachidonyl glycerol ether (noladin ether, 2-AG ether), *O*-arachidonoyl ethanolamide (virodhamine, O-AEA), dihomo-γ-linolenoyl ethanolamide (DLE), docosatetraenoyl ethanolamide (DEA), and N-arachidonoyl dopamine (NADA) [8-12] (see Figure 2.1 for structures). In addition, compounds structurally related to AEA and NADA have been found, including N-arachidonyl glycine (NAGly), N-oleoyl dopamine (OLDA), oleoyl ethanolamide (OEA), palmitoyl ethanolamide (PEA), and stearoyl ethanolamide (SEA) [3, 5-7]. These compounds were found to lack significant affinity for CB1 or CB2, but may potentiate the effects of the endocannabinoids by competing for hydrolysis by the membrane-bound fatty-acid amide hydrolase (FAAH) or possess affinity for other receptors, such as TPRV1, PPAR-α or PPAR-γ [4, 8, 10, 13].

Some fatty acid amides have been shown to have significant biological activity. For example, PEA and SEA are known for their anti-inflammatory properties [14, 15]. Oleoyl ethanolamide has been described to have potent anorexogenic properties [16]. A similar activity has been proposed for SEA, but less is known about this [17].

Taken together, amides, esters and ethers of fatty acids and amines represent an important group of bio-active molecules. These compounds are considered to be synthesized “on demand” in various tissues and are rapidly broken down by FAAH and monoacylglycerol lipases (MAGLs) [3, 4, 9, 18]. The combination of biogenic

amines with fatty acids can be regarded as a form of “natural combinatorial chemistry” since it may lead to the formation of numerous different compounds [19]. As many of these compounds are produced in specific tissues only, and due to their instability, their biological role has often not been clarified yet and their presence in plasma not been confirmed. However, for many other compounds, including those described in this paper, an association between (patho-) physiological factors and plasma concentration has either been proven or seems at least likely. The latter refers to the fatty acid amides of common fatty acids like oleic acid, palmitic acid, and stearic acid. A number of GC-MS or LC-MS based methods to quantify endocannabinoids and related compounds have been described. Often, these methods are limited to detecting AEA and 2-AG in *e.g.* brain tissue, adipose tissue or plasma [20-25]. Only few methods are designed to quantify a broader range of endocannabinoids, but only in brain tissue, liver, small intestine or adipose tissue [26-30]. Furthermore, extensive validation data are often lacking in these publications.

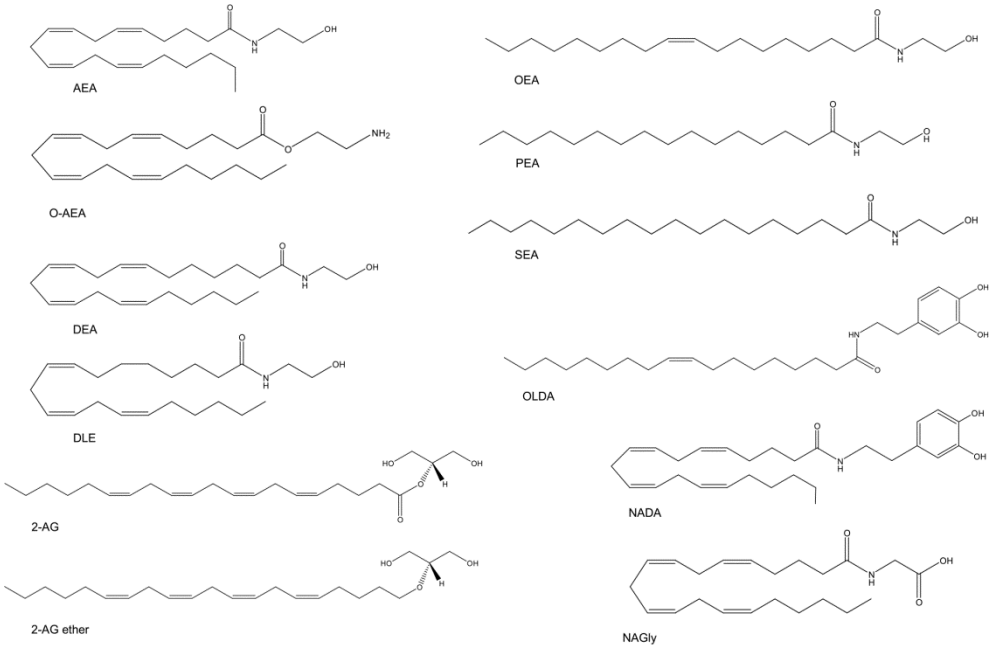

Figure 2.1*: Structures of the endocannabinoids and related compounds described in this chapter.*

To the best of our knowledge, no method is published which is capable of detecting a wide range of structurally differing endocannabinoids and related compounds in human plasma using a single clean-up procedure. Here we report a validated sample clean-up procedure and quantitative LC-ESI-tandem MS based method for the analysis of AEA, 2-AG, 2-AG ether, O-AEA, NADA, DEA, DLE, NAGly, OLDA, OEA, PEA, and SEA in human plasma.

Experimental

Chemicals and reagents

Endocannabinoid stock solutions including deuterated standard solutions for 2-AG, 2-AG-d8, 2-AG ether, AEA, AEA-d8, DEA, DLE, NADA, NADA-d8, NAGly, NAGly-d8, OLDA, OEA, PEA, PEA-d4, and SEA were purchased from Cayman Chemical (Ann Arbor, MI, USA). O-AEA (virodhamine) was obtained from Tocris Cookson Ltd. (Bristol, UK). Acetonitrile (HPLC-S grade) was purchased from Biosolve (Valkenswaard, The Netherlands). Methanol was from Riedel-de-Häen (Steinheim, Germany). Milli-Q water (Milli-Q Advantage unit, Millipore, Amsterdam, The Netherlands) was used in all analyses. Phenylmethanesulfonyl fluoride (PMSF), a serine protease inhibitor used to prevent enzymatic endocannabinoid breakdown by FAAH, was purchased from Fluka (Steinheim, Germany). Stock solution (100 mM) of PMSF was prepared in isopropanol and stored at 4-8°C. Trifluoro acetic acid (TFA) was from Sigma. Human pooled EDTA-plasma was obtained from Bioreclamation Inc. (Westburry, NY, USA).

LC-MS/MS system

The LC-MS/MS measurements were conducted on a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer (San Jose, CA, USA) coupled to a Thermo Finnigan Surveyor autosampler and a Surveyor MS pump using an electrospray ionization source. Liquid chromatography was performed on a XTerra MS C8 guard column (particle size 3.5 µm, column dimensions 2.1 x 10 mm) coupled to a XTerra MS C8 analytical column (particle size 3.5 µm, column dimensions 2.1 x 150 mm). Both columns were from Waters (Milford, MA, USA). LCquan software® (version 2.5, Thermo Electron) was used for data acquisition and processing.

LC-MS/MS conditions

The autosampler temperature was set at 4 °C and the columns were maintained at 40°C during the whole analysis. Gradient elution was applied with a constant flow of 150 µL/min, starting with 40% A (1 g/L ammonium acetate and 0.1% v/v formic acid in 95:5 v/v milli-Q water-methanol), 40% B (1 g/L ammonium acetate and 0.1% v/v formic acid in methanol) and 20% C (1 g/L ammonium acetate and 0.1% v/v formic acid in 95:5 v/v acetonitrile-milli-Q water) for the first two minutes followed by a linear increase towards 70% B and 30% C which is achieved in 6 minutes. At minute 16 the gradient changed linear to the initial setting which is achieved after 5 minutes. This gradient allows the chromatographic separation of AEA and O-AEA, which have identical masses and transition characteristics (see Figure 2.1).

The electrospray interface was operated in positive ion mode and an ion-spray voltage of 4.5 kV was applied. Capillary temperature was set at 350 °C. Other analysis parameters, such as skimmer offset and argon-mediated collision-induced dissociation (CID) energy, were optimized for each endocannabinoid separately by direct infusion of standard solution into the MS to ensure maximum product ion formation for each endocannabinoid. See Table 2.1 for details concerning precursor and product ion *m/z* values and CID values.

Plasma extraction

Different extraction protocols were investigated to determine optimal conditions for maximum endocannabinoid recovery and detection in human EDTA plasma. The effect of different solvents (acetonitrile, methanol, isopropanol, ethylacetate), evaporation techniques (freeze-drying, evaporation under nitrogen, vacuum concentrator) lab ware materials (glass, polypropylene) and various SPE (C18, C8, silica) protocols on recovery and matrix effect were investigated. In the final protocol, 1 mL plasma containing 100 µM PMSF was extracted in a polypropylene tube by adding 4 mL acetonitrile spiked with the internal standards 2-AG-d8, AEA-d8, NADA-d8, and NAGly-d8 and 100 µM PMSF. The extract was centrifuged for 5’ at 3000 x *g* and 15 mL water containing 0.133% TFA was added to the supernatant to dilute the acetonitrile prior to SPE clean-up.

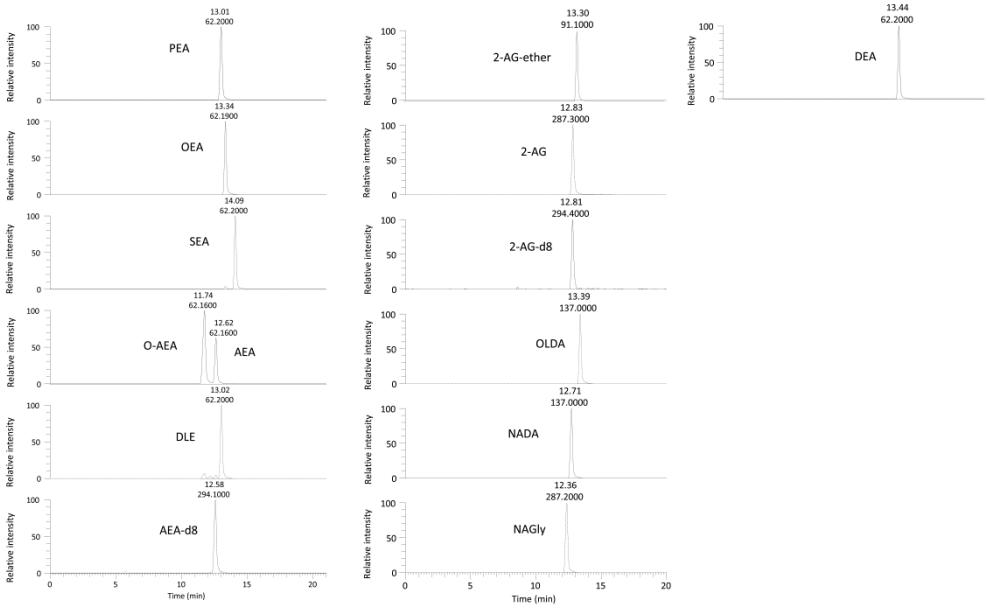

Figure 2.2a*: Base peak chromatograms for all endocannabinoids and related compound from direct injection of standard solutions. Retention time (RT) and product ion m/z value are depicted for each peak.*

SPE was performed using Bond Elute C8 (Varian Incorporated, Lake Forest, CA, USA) by activating the column first with 1 mL methanol followed by a washing step with 1 mL water. The whole diluted extract (20 mL) was subsequently applied on the column and washed with 2 mL 20% v/v acetonitrile in water containing 0.1% TFA. Finally the endocannabinoids were eluted from the column using 2 mL 80% v/v acetonitrile in water containing 0.1% TFA and this endocannabinoid containing fraction was evaporated to dryness using a vacuum concentrator (MAXI Dry Plus, Heto-Holten, Denmark) and stored at -80 °C until further analysis. Immediately before analysis, the pellet was reconstituted in 100 µL PEA-d4 spiked acetonitrile with 0.1% TFA, mixed thoroughly using an ultrasonic bath and vortexer and transferred to a glass autosampler vial. Finally 5 µL was injected on the analytical column for analysis.

Validation

The validation procedure consisted of the preparation of one batch per day for three consecutive days. Each batch included two seven-point calibration curves

prepared in plasma and in acetonitrile, quality controls at three different levels in five-fold, and miscellaneous quality control samples, including stability- and freeze-thaw control samples. Peak identification and quantification was performed using LCquan 2.5 software®. Data processing was performed using in-house programmed calculation Excel sheets for regression analysis, calculation of accuracy, and inter- and intra-batch variation (MS Excel, Microsoft Corporation, Redmond, WA, USA).

Linearity

Quantification of endocannabinoid levels was performed using linear regression on the response ratios (peak area analyte/peak area internal standard) from the calibration curve as a function of the corresponding endocannabinoid concentration. Duplicate calibration curves were analyzed to obtain one regression equation. Data was $1/x^2$-weighted and linear regression was used since this resulted in the higher accuracy of back-calculated concentrations than 1/x weighting or quadratic regression. Deviations from the actual concentrations within 20% for all calibration points were considered acceptable. If necessary, correction for the endogenous presence of endocannabinoids in the reference plasma was performed in regression analysis of the calibration curves. The lower limit of quantification (LLOQ) was defined as the lowest calibration point that would result in an accuracy of more than 80% compared to the actual concentration. The limit of detection (LOD) was defined as the concentration that resulted in a peak having a greater than 3:1 signal-noise ratio as obtained from direct injection of standards prepared in acetonitrile.

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	CID-energy (eV)
2-AG	396	287	15
2-AG ether	382	91	27
AEA	348	62	15
DEA	376	62	15
DLE	350	62	15
NADA	440	137	26
NAGly	362	287	10
O-AEA	348	62	15
OLDA	418	137	28
OEA	326	62	16
PEA	300	62	15
SEA	328	62	15
2-AG-d8	404	294	12
AEA-d8	356	294	7
NADA-d8	448	137	27
NAGly-d8	370	294	10
PEA-d4	304	62	15

***Table 2.1**: m/z Values for precursor- and product ions and used collision-induced dissociation (CID energies for the MS/MS analysis of the 17 endocannabinoids and related compounds.*

Accuracy and precision

Accuracy and precision of the method were determined using quality control (QC) samples spiked at three concentrations levels of endocannabinoids (see Table 2 for concentrations). QC samples were prepared by spiking reference plasma containing 100 µM PMSF with 10 µL acetonitrile containing endocannabinoids prior to plasma extraction as described. The QC's were analyzed in five-fold and multiple batch analysis (n=3) was performed. Endocannabinoid concentrations in the QC samples were calculated using the plasma and acetonitrile calibration curve that was prepared on the same day and analyzed in the same analytical run. A deviation from the actual concentration of less than 20% was considered acceptable, otherwise concentrations should be corrected for the deviation determined by analyzing QC samples. Inter- and intra-batch precision was expressed as the variation coefficient (CV) within (n=5) and between the batches (n=3), respectively. The inter- and intra-batch precision was considered acceptable when the CV was less than 20%.

Stability

Stability was evaluated using QC samples after storage at -80 °C for one week and two months, after three freeze-thaw cycles, and for processed samples after one week in the autosampler at 4 °C.

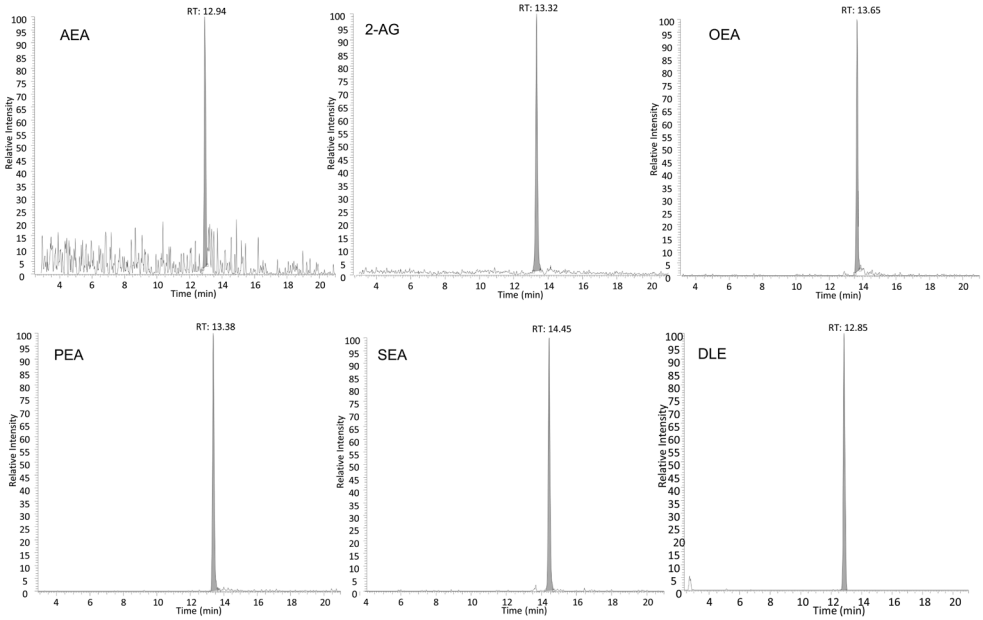

***Figure 2.2b**: Base peak chromatograms for AEA, 2-AG, DLE, OEA, PEA, and SEA in human EDTA plasma. Non-spiked plasma (1 mL) was extracted using 4 mL of acetonitrile, subsequently subjected to SPE sample clean up, and the endocannabinoid containing fraction was evaporated to dryness using a vacuum concentrator. Dried extracts were reconstituted in 100 µL acetonitrile containing 0.1% v/v TFA and 5 µL was injected on column. MS settings were as described in experimental section. Retention times are depicted for each peak.*

Human plasma samples

The levels of endocannabinoids and related compounds in human plasma were analyzed in 23 healthy postmenopausal female volunteers in order to study the appropriateness of the validated method. Blood samples were obtained (after

informed consent) from the anticubital vein of the forearm and collected in ice-chilled tubes containing Potassium Ethylene Diamine Tetra Acid (K_3EDTA) for plasma (Vacutainer Systems, Becton Dickinson, Plymouth, UK). Blood was centrifuged for 15 minutes at 2.000 *g* at 4 °C, within 15-30 minutes after collection and stored at -80 °C. PMSF was added to plasma prior to storage (final concentration of 100 µM). Endocannabinoids and related compounds were extracted from 1 mL plasma and measured using the final protocol.

Results and discussion

MS/MS- and LC optimisation

Conditions for MS/MS detection were optimized for maximum product ion formation and detection by direct infusion of standard solution into the MS. The product ion masses shown in table 2.1 correspond with loss of ethanolamine (*m/z* 62), glycerol (*m/z* 91), dopamine (*m/z* 137), and the arachidonic acid moiety (*m/z* 287 or 294 for deuterated standards).

Several protocols for quantifying endocannabinoids are described in literature, including reversed-phase silver chromatography and several SIM and SRM-based methods [20-30]. Silver ions are thought to overcome inefficient protonation of 2-AG and 2-AG ether in reversed-phase chromatography which is due to their lack of structural elements with a high proton affinity. However, in our hands this method was not successful. The intensities of 2-AG and 2-AG ether were indeed enhanced, however we encountered problems with clogged transfer tubes due to silver deposits. Therefore, an alternative reversed-phase chromatography method was developed using methanol and acetonitrile in the gradient elution of endocannabinoids. With the XTerra C8 columns and the specified gradient a good separation of AEA and O-AEA was obtained while maintaining optimal peak intensities. However, this gradient did not separate 2-AG from its isomer 1-AG (data not shown). It is not known to what extend 2-AG isomerizes to 1-AG during the extraction procedure or whether 1-AG is already present in human plasma. Moreover, protic solvents are believed to enhance the rate of isomerization, and in this sample pre-treatment acetonitrile is used, which should keep possible isomerization limited. We used the combined peak for determining 2-AG as is described by other authors [29, 31]. See Figure 2.2a for base peak chromatograms obtained from direct injection of standard solutions prepared in acetonitrile and

Figure 2.2b for blank human EDTA plasma after sample pre-treatment as described in section experimental using the final protocol.

Sample pre-treatment

Different sample pre-treatment protocols were tested to optimize recovery for all specified endocannabinoids and yield clean samples to minimize ion suppression and prolong column life. To this extend, several extraction procedures were tested, including different solvents, SPE protocols, and evaporation techniques.

For liquid-liquid extraction of plasma, the following solvents acetonitrile, isopropanol, methanol, acetone, ethylacetate, and chloroform-methanol were investigated. The aim was to maximize recovery of the low-abundant endocannabinoids while minimizing co-extraction of the high-abundant triglycerides and cholesterol esters which cause interfering ion suppression. Acetonitrile proved to be the best solvent since the least proteins, triglycerides and cholesterol esters are maintained in solution while providing optimal recovery of endocannabinoids (see Figure 2.3a). Other solvents used in previously published reports, such as isopropanol and methanol, are more hydrophobic and thus able to dissolve more triglycerides and cholesterol esters. These compounds may increase ion suppression or contaminate the column and LC-MS/MS system.

To further clean up acetonitrile extracts, several SPE protocols were investigated using C18-based and mixed-phase cation exchange columns from various manufacturers. Unfortunately, none of these columns proved to recover all 12 endocannabinoids satisfactory. Especially O-AEA, NAGly, NADA, and OLDA showed variable results and low recoveries (see Figure 2.3b). These differences were not consistent between the C18 columns obtained from different manufacturers; some endocannabinoids showed good retention and elution on a particular column while performing insufficiently on a column from another manufacturer. Finally we obtained good results with the Bond Elut® C8 (200 mg, 3 mL, Varian Inc.). Acceptable recoveries (>75%) were obtained for all endocannabinoids, including NAGly, NADA and OLDA. It is difficult to compare the performance of the C8 column to other published SPE protocols since validation data are often lacking or other methods are not optimized for recovery of a broad range of endocannabinoids.

Another cause of endocannabinoid loss during sample preparation was the evaporation step. Evaporation under a gentle stream of nitrogen, freeze dryer and

using a vacuum concentrator were investigated. The recoveries obtained with freeze drying and the nitrogen method were obviously lower compared to the vacuum concentrator. Again, NADA and OLDA showed to be critical structures (Figure 2.3c).

The vacuum concentrator yielded recoveries of typically >90% for all endocannabinoids and related structures. Evaporation under nitrogen should therefore be avoided, but is used in many other publications [21-23, 25, 29]. A drop of glycerol could be useful but was not miscible in acetonitrile. Moreover, regular glassware should be avoided since this causes NADA and OLDA to be lost (data not shown). The use of polypropylene-based plastics showed improved recoveries of all endocannabinoids.

In conclusion, several critical factors for the extraction of a broad range of endocannabinoids (including dopamine-containing structures) from human plasma were identified during sample pre-treatment optimization, such as choice of solvent, SPE-column and evaporation technique.

Validation

The analytical performance of the final protocol was investigated by performing a comprehensive validation. Linearity, accuracy, precision, LLOQ, LOD, and stability under different conditions were determined. Due to the endogenous presence of some endocannabinoids, it was necessary to correct the calibration curves prepared in plasma and QCs for the levels in blank plasma. Detector responses for each endocannabinoid were corrected for the appropriate internal standard. Correction for detector responses using different internal standards was investigated and proved that structurally-related internal standards provide the most stable response ratio. Therefore, all ethanolamides were corrected using AEA-d8, NAGly-d8 was used for the glycine-containing endocannabinoid NAGly, 2-AG-d8 was used for the correction of glycerol-containing compounds (2-AG and 2-AG ether), and NADA-d8 for the dopamine-containing compounds (NADA and OLDA).

	R^2 (±SD, n=3)	Slope (±SD, n=3)	QC spiked (ng)	Accuracy (%, n=15) plasma	Accuracy (%, n=15) acetonitrile	Intra-batch variation (% CV, n=15)	Inter-batch variation (% CV, n=3)
2-AG	0.976 (±0.016)	10.473 (±2.15)	35.11	156	130	18	25
			140.43	141	118	18	27
			561.71	139	120	20	23
2-AG ether	0.971 (±0.024)	4.263 (±0.80)	25.87	102	72	18	18
			103.48	96	65	15	15
			413.9	103	69	17	20
AEA	0.983 (±0.006)	132.494 (±20.91)	2.54	181	222	12	13
			10.16	177	212	9	11
			40.65	187	229	11	15
DEA	0.983 (±0.010)	55.778 (±3.15)	0.88	103	62	16	16
			3.51	100	58	14	14
			14.04	101	59	11	11
DLE	0.970 (±0.030)	308.571 (±33.97)	0.88	101	177	14	16
			3.51	106	187	10	12
			14.04	105	188	12	12
NADA	0.978 (±0.005)	127.060 (±52.93)	1.20	115	470	11	30
			4.81	103	552	8	11
			19.22	99	580	8	8
NAGly	0.988 (±0.011)	2.264 (±0.18)	12.8	107	86	11	27
			51.18	100	82	13	13
			204.73	102	82	8	9
O-AEA	0.990 (±0.001)	35.398 (±2.97)	2.36	83	117	18	22
			9.42	84	116	8	8
			37.69	85	119	12	14
OLDA	0.970 (±0.015)	83.327 (±22.75)	2.22	106	538	13	22
			8.87	106	574	7	13
			35.48	112	596	8	9
OEA	0.971 (±0.018)	328.686 (±47.40)	0.88	97	104	25	25
			3.51	105	109	12	12
			14.04	110	117	12	13
PEA	0.970 (±0.021)	478.844 (±86.91)	0.88	83	139	40	40
			3.51	106	184	13	13
			14.04	107	175	13	16
SEA	0.953 (±0.034)	857.468 (±221.31)	0.43	69	153	50	57
			1.74	118	199	12	22
			6.95	98	209	13	24

Table 2.2*: Linearity (n=3), accuracy and intra- and inter-batch variation of the analytical method. Values in column 'QC spiked' represent the amount of analyte added to 1 mL plasma prior to sample clean-up. Accuracies were calculated using a calibration curve*

spiked in plasma or prepared in acetonitrile and results for both are depicted. Intra- and inter-batch variations are only shown for results obtained after calculating with the calibration curve spiked in plasma.

Linearity

Data concerning the linearity of the method are shown in Table 2.2. The results show that the method offers good linearity for all structures which was found to be reproducible between the batches. Higher R^2 values could be obtained when 1/x weighing was applied, but this also resulted in higher deviations in back-calculated concentrations compared to the actual concentration, which was undesirable.

Values for LLOQ and LOD are presented in Table 2.3. The LLOQ for the endocannabinoids was set at the lowest calibration point when spiked to plasma, except for NADA and OLDA (second calibration point) due to lack of accuracy at the lowest calibration point. It is difficult to validate the method for lower endocannabinoid levels due to basal levels of these compounds in plasma. Using an calibration curve in acetonitrile it is possible to determine the concentration of endocannabinoids at lower levels, however correction for accuracy is necessary due to differences in matrix effect (see also Table 2.2 and section 'accuracy and precision'). The presented values here are difficult to compare to other published methods due to differences in matrix, sample-pre treatment and LC-MS conditions. The LOD values for AEA, 2-AG, NAGly, O-AEA, OEA and PEA obtained with our method are lower than reported for other methods [20, 21, 29, 30].

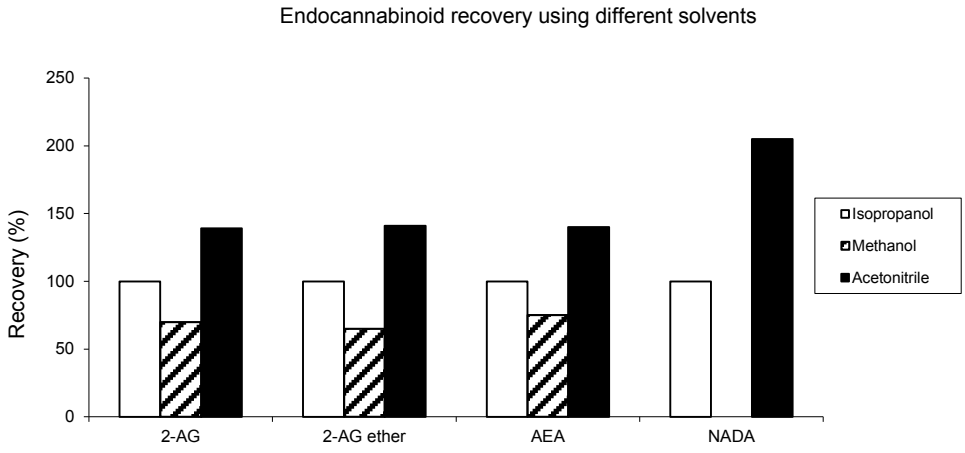

***Figure 2.3a**: Effect of solvent on endocannabinoid recovery. Non-spiked plasma (100 µL) was extracted with different solvents and the recovery for isopropanol was set at 100%.*

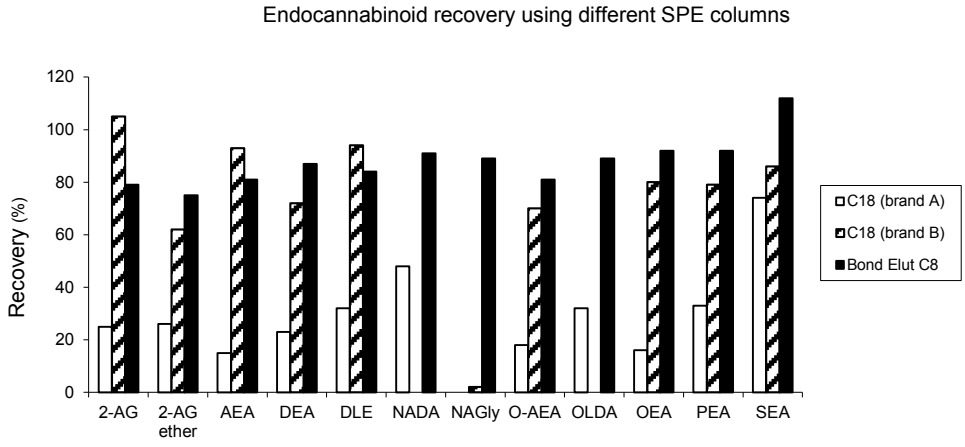

***Figure 2.3b**: Effect of type of SPE column on endocannabinoid recovery. The recovery is expressed as percentage response compared to direct injection of standard.*

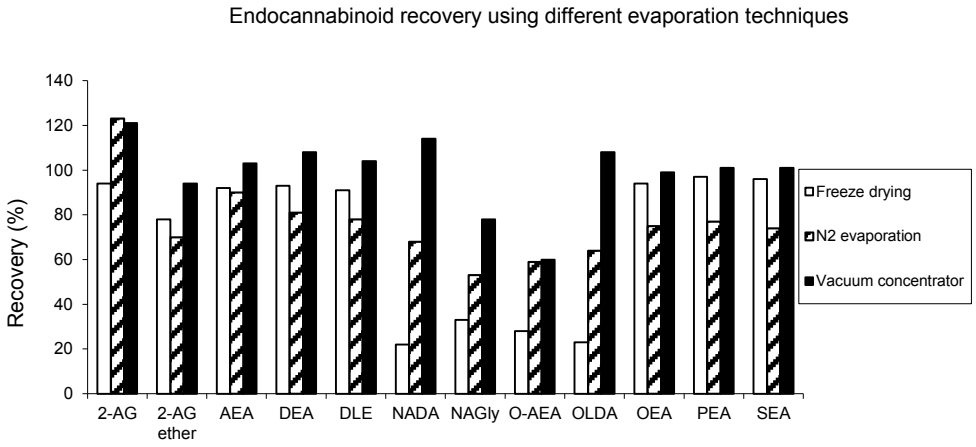

***Figure 2.3c**: Effect of evaporation technique on endocannabinoid recovery. The recovery evaporation technique is expressed as percentage response compared to direct injection of standard.*

	LLOQ plasma (pg on column)	LOD standard (fg on column)
2-AG	878	14
2-AG ether	647	73
AEA	64	4
DEA	22	36
DLE	22	6
NADA	60	10
NAGly	320	633
O-AEA	59	21
OLDA	111	13
OEA	22	2
PEA	22	2
SEA	11	1

***Table 2.3**: Lower limit of quantification (LLOQ) and limit of detection (LOD) values. The lowest point from the calibration curve that allowed back-calculation of the determined concentration within 20% deviation from the actual concentration was accepted as LLOQ. LODs were calculated from detector responses obtained from direct injection of endocannabinoid standard.*

Accuracy and precision

The results in Table 2.2 show that the method provides good accuracy and precision for the specified endocannabinoids when using a calibration curve prepared in plasma. The initial validation criteria for accuracy and precision were not met in some occasions. However, deviations were only minor and in addition, the correction for background endocannabinoids in the reference plasma may have introduced an additional source of variation which is dominant especially at low concentrations. This effect was most prominent for AEA and 2-AG. The accuracies were much higher than 100% but consistent, which makes correction for the observed deviation in accuracy justified.

Due to the endogenous presence of some endocannabinoids it is difficult to prepare a calibration curve in plasma with the correct concentration range for unknown samples. The best option is to prepare a calibration curve in 'endocannabinoid-free' plasma, however this 'blanc' plasma is unavailable. The second best option is to use a calibration curve prepared in acetonitrile. It must be noted that calculating the accuracies using a calibration curve prepared in acetonitrile will result in higher deviations with respect to calculations using the calibration curve in plasma (see Table 2.2). It is therefore necessary to correct the endocannabinoids concentration calculated with the calibration curve in acetonitrile for this matrix effect using QC samples.

Ion suppression

Ion suppression was calculated by dividing the peak area of the deuterated standard in blanc plasma sample after clean up and reconstitution in acetonitrile containing deuterated standards by the area of deuterated standard in standard solution in acetonitrile. The ion suppression was 64.8%, for AEA-d8, 45.9% for NADA-d8, 65.4% for NAGly-d8, and 61.3% for PEA-d4. For 2-AG-d8, ion enhancement was observed and was quantified at 77.7%. It is clear from these results that matrix effects are significant, thus providing a good argument for preparing calibration curves in plasma.

	Pre- preparative (n=3)			Post-preparative (n=3)
	Day 1	1 week	2 months	1 week
2-AG	-6.9	20.1	43.2	-8.9
2-AG-ether	-3.7	-2.2	-12.2	5.4
AEA	62.8	54.3	21.6	-2.4
DEA	3.9	-0.1	11.8	13.2
DLE	4.2	1.5	7.6	-6.8
NADA	-10.2	3.4	-	8.8
NaGly	1.2	8.4	-0.4	-0.1
O-AEA	-15.8	-15.9	22.6	2.7
OEA	2.4	1.0	38.4	8.9
OLDA	-1.3	12.0	-	-7.5
PEA	2.6	3.1	51.2	12.9
SEA	-0.5	6.7	48.9	3.9

Table 2.4*: Pre- and post-preparative stability of endocannabinoids in human plasma. Deviations (%) of endocannabinoid levels in stored plasma (1 day, 1 week and 2 months) at -80 °C with respect to actual concentration from freshly prepared samples. - = not detectable.*

Stability

Storage of plasma samples for one day and one week revealed only slight deviations in recovery except for AEA; see Table 2.4. Short term storage of spiked plasma showed higher deviations compared to freshly prepared samples. Especially for 2-AG, PEA, and SEA the concentrations deviated more than 20% from the actual concentration. Therefore, it is recommended to process the plasma samples as soon as possible. Moreover, PMSF was added to plasma to prevent enzymatic breakdown by FAAH. It is recommended to immediately separate plasma from blood after venipuncture since endocannabinoids are released *ex vivo* [24], add PMSF to 100 µM in plasma and store at -80 °C or extract immediately.

Post-preparative stability of reconstituted extracts showed only minor deviations between first and second injection of the samples. Therefore it is concluded that endocannabinoids in reconstituted plasma extracts are stable for at least one week when stored at 4 °C.

Freeze-thaw cycle analysis revealed that 2 cycles results in deviations larger than 20% for almost all endocannabinoids (data not shown). It is therefore recommended to avoid freeze-thaw cycles.

Human plasma samples

The presented method was used to determine endocannabinoid levels in plasma from 23 healthy female volunteers using a calibration curve prepared in acetonitrile. Plasma (1 mL) was extracted, evaporated and reconstituted in 100 µL acetonitrile which was analyzed on the LC-MS/MS system according to the final protocol. Concentrations were corrected for each sample using its corresponding QC which was prepared and analyzed on the same day.

AEA, 2-AG, DLE, OEA, PEA and SEA were detected in all samples but no 2-AG ether, O-AEA, DEA, NAGly, NADA, or OLDA was observed. Mean plasma concentrations were 0.24 ± 0.11 ng/mL for AEA, 7.09 ± 12.39 ng/mL for 2-AG, 1.39 ± 0.36 ng/mL for PEA, 0.04 ± 0.02 ng/mL for DLE, 1.37 ± 0.43 ng/mL for OEA, and 0.52 ± 0.18 ng/mL for SEA. The mean plasma AEA concentration reported here is in the same order of magnitude compared to observations by other authors [21, 23, 32, 33]. However, it must be noted that it is difficult to compare concentrations in real life samples reported in literature due to differences in subjects, intervention and sample pre-treatment.

Conclusion

A method for the simultaneous detection of twelve endocannabinoids and related compounds in human plasma has been developed and validated. The method was shown to be linear, accurate, and precise. However, some validation criteria were mildly exceeded, most likely due to the endogenous presence of some endocannabinoids in plasma. To our knowledge no validation criteria have been defined for the analysis of endogenous compounds using methods as described in this paper. It is difficult to validate this method at low concentrations due to the lack of ‘blank’ sample materials and therefore it is difficult to determine an accurate LLOQ.

Since sample clean-up and chromatographic separation were optimized to recover structurally different compounds, the quantitative analysis of other endocannabinoids or related compounds can easily be integrated in this method. Moreover, this method has been modified and successfully used to quantify the

level of endocannabinoids in other matrices, such as adipose tissue, brain tissue, cell culture media and cell homogenates. This supports the conclusion that the method presented here is also able to detect components which have not been found in human plasma so far. A detailed discussion about the physiological significance of a plasma concentration for each specific compound falls beyond the scope of this paper. Due to their tissue-specific production and instability, some compounds may not appear in plasma in significant amounts under normal conditions but could be present under other circumstances. Indeed, NADA was not found in the real samples analyzed so far. On the other hand the flexibility of the method has been shown to allow detection of other fatty acid amides that may be formed, for example those of n-3 polyunsaturated fatty acids after their intake.

To the best of our knowledge, no other validated method has been published that offers quantification of this broad range of endocannabinoids and related acyl-amides in human plasma.

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Chapter 3

Docosahexaenoic acid and eicosapentaenoic acid are converted by 3T3-L1 adipocytes to *N*-acyl ethanolamines with anti-inflammatory properties

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Abstract

n-3 PUFAs have beneficial health effects which are believed to be partly related to their anti-inflammatory properties, however the exact mechanisms behind this are unknown. One possible explanation could be via their conversion to *N*-acyl ethanolamines (NAEs), which are known to possess anti-inflammatory properties. Using fatty acid precursors we showed that 3T3-L1 adipocytes are indeed able to convert docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) to their NAE derivatives docosahexaenoyl ethanolamine (DHEA) and eicosapentaenoyl ethanolamine (EPEA), respectively. This synthesis took place on top of an apparent background formation of these NAEs in standard culture medium. In addition we were able to demonstrate the presence of DHEA, but not of EPEA, in human plasma. DHEA and EPEA were found to decrease LPS induced adipocyte IL-6 and MCP-1 levels. Results of combined incubations with PPAR-γ and CB2 antagonists suggest a role of these receptors in mediating the reduction of IL-6 by DHEA. Our results are in line with the hypothesis that in addition to other pathways, formation of N-acyl ethanolamines may contribute to the biological activity of n-3 PUFAs. Different targets, including the endocannabinoid system, may be involved in the immune-modulating activity of these “fish oil-derived NAEs”.

Introduction

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) such as docosahexaenoic acid (DHA; C22:6; n-3) and eicosapentaenoic acid (EPA; C20:5; n-3) have been linked to several positive health effects, such as a reduced risk for cardiovascular diseases, potentially cancer and certain mental illnesses [1]. Moreover, consumption of DHA, EPA or fatty fish containing high levels of n-3 PUFAs has been shown to reduce the inflammatory component and improve other characteristics of the metabolic syndrome [2, 3]. The exact mechanisms behind these effects are unknown, but evidence suggests that DHA and EPA can be converted to anti-inflammatory eicosanoids known as resolvins [3, 4]. Fatty acids can also be directly converted to *N*-acyl ethanolamines (NAEs). Several members of this class possess anti-inflammatory properties [5-8]. NAEs can be rapidly synthesized in membranes, released on demand and broken down again by the enzyme fatty acid amide hydrolase (FAAH) [9-11]. Therefore, the local availability of a specific type of fatty acid precursor may determine product formation and hence bio-activity. This suggests a link with dietary intake, since it is well known that incorporation of fatty acids into membranes can be modulated by their proportional abundance in the diet [4]. Recently, Artmann *et al.* demonstrated a correlation between NAE patterns in various organs and tissues of rats and fatty acid composition of their diets [12]. Such a shift in balance can also explain the reported decrease in the levels of the endocannabinoids anandamide (arachidonoyl ethanolamine; AEA) and 2-arachidonoylglycerol (2-AG) following exposure to DHA or EPA [13, 14].

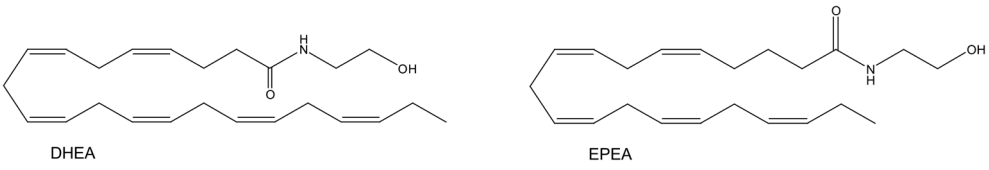

Figure 3.1: *Structures of the N-acyl ethanolamines from DHA and EPA, DHEA and EPEA respectively.*

Obesity is characterized by low-grade systemic inflammation and increased infiltration of activated macrophages in the adipose tissue [15]. Interestingly, the adipocytes themselves can also produce pro-inflammatory cytokines such as

interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1), which are delivered to the blood stream to act on distant organs such as liver and skeletal muscle [16-20]. Although it is not clear what initiates this inflammation, current evidence suggests that these inflammatory processes in the adipose tissue are the link to co-pathologies observed with the metabolic syndrome, such as hypertension, decreased insulin sensitivity and artherosclerosis [19]. Therefore, attenuating the inflammatory process in adipose tissue is now considered as a promising target to reduce complications of obesity.

The present study addresses the question whether NAEs formed from DHA and EPA, docosahexaenoylethanolamine and eicosapentaenoylethanolamine (DHEA and EPEA, Figure 3.1) could be involved in suppressing an inflammatory response in 3T3-L1 adipocytes. Formation of NAEs was studied at different precursor concentrations and time points and deuterated substrates were used to follow product formation. We also demonstrated the presence of DHEA in human plasma. Effects of DHEA and EPEA on the release of the pro-inflammatory cytokines IL-6 and MCP-1 were studied after stimulating the adipocytes with lipopolysaccharide (LPS).

Material and methods

Chemicals and materials

AEA-d8, DHEA, DHA, DHA-d5, EPA, EPA-d5, GW9662 (PPAR-γ antagonist) and SR144528 (CB2 antagonist) were from Cayman Chemical (Ann Arbor, MI, USA). Acetonitrile (ACN) was from Biosolve (Valkenswaard, The Netherlands). Isopropanol (IPA) and ethanol were from JT Baker (Deventer, The Netherlands). Trifluoro acetic acid (TFA) and thiazolyl blue tetrazolium bromide (MTT) were from Sigma (Steinheim, Germany). Phenylmethylsulfonyl fluoride (PMSF) was from Fluka (Steinheim, Germany) and was diluted to 100 mM in IPA and stored at 4 °C. Bond Elut SPE C8 columns (200 mg, 6 mL) were from Varian Inc (Lake Forest, CA, USA). Dulbecco's Modified Eagle Medium (DMEM) supplemented with GlutaMAX I, penicillin-streptomycin (pen-strep), Newborn Calf Serum (NCS), and Fetal Calf Serum (FCS) were from Gibco (Auckland, New Zealand). Insulin, isobutyl-1-methylxanthine (IBMX), dexamethasone and LPS (O111:B4) were from Sigma. ELISA kits for IL-6 and MCP-1 were purchased from R&D Systems (Minneapolis, MN, USA).

Enzymatic synthesis of EPEA

Since EPEA is not commercially available yet, it was synthesized using our method described earlier [21]. In short, EPA was coupled to ethanolamine using *Candida antarctica* lipase and purified with preparative HPLC to yield highly concentrated EPEA extracts. To confirm the identity of the product EPEA, the purified extract (> 98% pure) was subjected to high-resolution MS/MS analysis on a Thermo-Finnigan LTQ linear ion trap mass spectrometer coupled to a high-resolution Thermo-Finnigan Orbitrap mass spectrometer. Mass Frontier 5.0 software (HighChem, Slovak Republic) was used to predict the fragmentation pattern for the NAE structure from EPA.

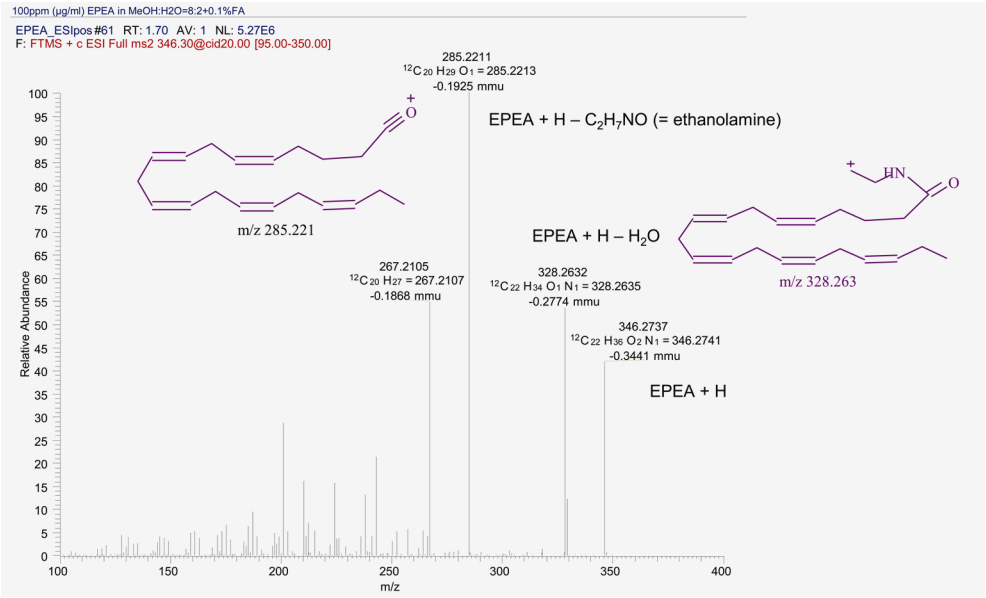

Figure 3.2: *High resolution MS/MS spectrum of EPEA. Compound was subjected to MS-MS analysis at 20% collision energy. Peaks were observed at m/z 346.27 (intact EPEA+H), 328.26 (-18, loss of H_2O, see structure) and 285.22 (-61, loss of ethanolamine, see structure), which are consistent with the EPEA structure and not its esterified isomer, which should reveal loss of the ammonium terminus but not loss of water. The m/z 328.26 and 285.22 peaks were also predicted by Mass Frontier. The m/z 267.21 peak is consistent with loss of ethanolamine and water from EPEA.*

As shown in Figure 3.2, the high resolution MS/MS dissociation analysis of EPEA (*m/z* 346.27) yielded fragments at *m/z* 328.26 (-18; loss of H_2O) and 285.22 (-61; loss of ethanolamine), which were also predicted by Mass Frontier. An additional peak at m/z 267.21 was found and is consistent with loss of H_2O and ethanolamine from the parent compound. The peaks which were consistent with loss of water and ethanolamine confirmed that the synthesis product is the NAE structure of EPA.

LC-MS/MS analysis of NAE levels in human plasma and cell culture medium

To determine whether the NAEs DHEA and EPEA are detectable in human plasma, blood samples were taken from 3 healthy volunteers after obtaining informed consent. Blood was collected in EDTA containing tubes (Beckton Dickinson). Plasma was immediately separated, processed and analyzed using LC-MS/MS as described earlier [22] with inclusion of scan events for DHEA (*m/z* 372.3 → 62.2) and EPEA (*m/z* 346.3 → 62.2). In short, 1 mL plasma was extracted with 4 mL ACN containing AEA-d8 as an internal standard. After centrifugation, the supernatants were diluted with 15 mL MQ water containing 0.13% TFA, loaded on activated SPE C8 columns (Bond Elut C8, Varian Inc, Lake Forest, CA, USA), washed with 2 mL 20% v/v ACN in MQ containing 0.1% TFA. NAEs were finally eluted with 2 mL 80% v/v ACN in MQ containing 0.1% TFA, evaporated to dryness using a vacuum concentrator (MAXI Dry Plus, Heto-Holten, Denmark) and stored at -80°C until further analysis. Before LC-MS/MS analysis, dried extracts were reconstituted in 100 µL ACN containing 0.1% TFA and 5 µL was injected on column for quantification. NAE levels were determined on a Thermo Finnigan Surveyor autosampler coupled to a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer (San José, CA, USA) operated in selective reaction mode (SRM). The interface between LC and MS consisted of an electrospray ionization source operating in positive ion mode. Peaks were indentified using LCquan version 2.5.5 software (Thermo). Concentrations NAEs were calculated using an external calibration curve of each NAE individually using the peak ratio of the compound divided by the internal standard (AEA-d8).

Cell culture medium was transferred to a 2 mL eppendorf tube and PMSF was added to a final concentration of 100 µM. Ten µL internal standard solution containing AEA-d8 was added and the tubes were vortexed. Subsequently, TFA was added to a final concentration of 0.1%, samples were vortexed and

centrifuged for 5 minutes at 14k rpm. Supernatants were subsequently extracted and analyzed using the solid phase extraction (SPE) procedure and LC-MS/MS method mentioned earlier with additional wash steps using 2 mL 10% v/v ACN in MQ with 0.1% TFA and 2 mL 40% v/v ACN in MQ containing 0.1% TFA in the SPE procedure. Dried extracts were stored at -80 °C prior to analysis or immediately analyzed for AEA, DHEA, EPEA, oleoyl ethanolamine (OEA), palmitoyl ethanolamine (PEA), and stearoyl ethanolamine (SEA) with the LC-MS/MS method described above.

Cell culture

3T3-L1 pre-adipocytes (kind gift from Dr. E. Kalkhoven, University Medical Center Utrecht, The Netherlands) were cultured in DMEM supplemented with 10% NCS and 1% pen-strep at 37 °C and 5% CO_2 and were split every 2-3 days. Cells were not allowed to grow to confluence prior to the differentiation step.

Differentiation of 3T3-L1 adipocytes

3T3-L1 pre-adipocytes were seeded in 6-wells plates ($8*10^3$ cells/mL; 2 mL/well) and grown to confluence in five days at 37 °C and 5% CO_2 with the medium being refreshed every 2-3 days. At three days post confluency, differentiation was initiated with 1 µM insulin, 0.5 mM IBMX and 1 µM dexamethasone in DMEM supplemented with 10% FCS and 1% pen-strep (DMEM-FCS) for 3 days followed by fresh insulin medium (1 µM insulin in DMEM-FCS) every 2-3 days, which was continued during experiments. Differentiation was confirmed with light microscopy at eight days after initiation of differentiation, revealing cells with the typical adipocyte phenotype characterized by increased cell volume, round morphology, and the presence of lipid droplets. Experiments were conducted with fully differentiated cells at 8-10 days post induction of differentiation.

Biosynthesis of DHEA and EPEA from DHA and EPA by 3T3-L1 adipocytes

Differentiated adipocytes were exposed to 10, 30 and 50 µM of DHA or EPA in insulin medium for 24 or 48 hrs. Insulin medium and solvent vehicle in insulin medium were used as controls. DHA stock solution was prepared in IPA, EPA was prepared in ethanol. After incubation, medium content of DHEA and EPEA was determined as described above. Cytotoxicity assays based on LDH release showed that the used concentrations and solvents are not cytoxic (data not shown).

Effects of DHEA and EPEA on LPS- induced cytokine production

Differentiated adipocytes potentially synthesize pro-inflammatory cytokines, e.g. after stimulation with LPS. To investigate the immune-modulating properties of DHEA and EPEA, differentiated 3T3-L1 adipocytes were pre-incubated for 1 hr with 1 nM – 10 µM of DHEA or EPEA in insulin medium. Subsequently, cells were exposed for 8 hr to medium containing DHEA and EPEA to which 50 ng/mL of LPS had been added. To investigate the possible roles of the CB2 or PPAR-γ receptors in these effects, GW9662 (PPAR-γ antagonist) and/or SR144528 (CB2 antagonist) were used in a final concentration of 100 nM. Medium aliquots of 100 µL were collected and immediately stored at -20 °C until analysis. Medium levels of IL-6 and MCP-1 were analyzed using ELISA according to the manufacturer's instructions. Cytotoxicity assays based on LDH release and MTT conversion showed that the used concentrations and solvents are not cytoxic (data not shown).

Statistical analysis

Data are presented as mean ± standard deviation where appropriate. Comparisons between groups were made with one-way analysis of variance (ANOVA) followed by Dunnett's *t* test using SPSS Statistics 17.0 software. $P < 0.05$ was considered as significant level of difference.

Results

DHEA, but not EPEA, is detectable in human plasma

Plasma from three healthy volunteers after an overnight fast was analyzed for DHEA and EPEA (see Figure 3.3 for SRM chromatogram). DHEA levels were on average 0.17 +/- 0.16 ng/mL. EPEA was below detection limits in human plasma.

Exposure of 3T3-L1 adipocytes to DHA and EPA leads to increased levels of DHEA and EPEA in cell culture medium

Exposure of 3T3-L1 cells for 24 hr to 10 µM DHA resulted in increased DHEA levels up to 42 pg/mL whereas in vehicle control medium (insulin medium with solvent) 21 pg/mL was found ($P = 0.069$). Following incubation with 30 and 50 µM DHA, 90 and 143 pg/mL DHEA, respectively was found ($P < 0.001$ for both concentrations; see Figure 3.4a for bar graph and Figure 3.5 for chromatograms). Similar values were found when the cells were incubated for 48 hours.

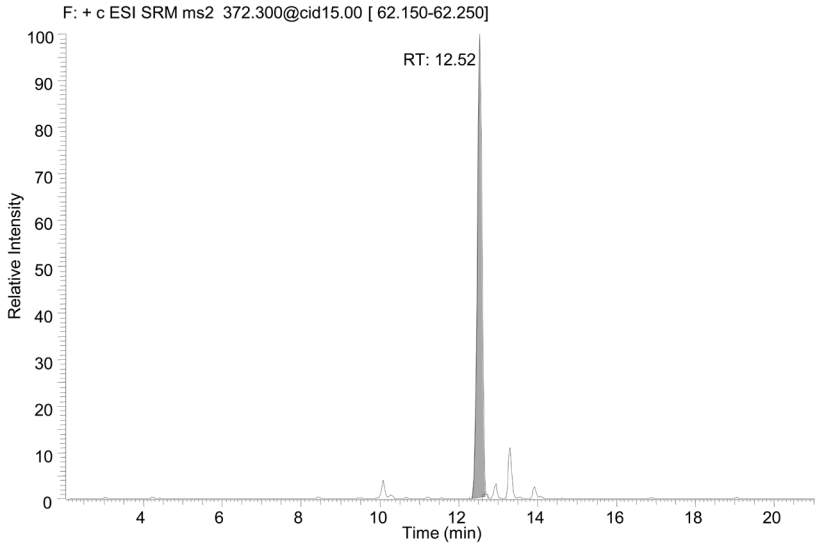

Figure 3.3: *SRM chromatogram of DHEA in human plasma. Extraction and LC-MSMS analysis was performed as described in section 'material and methods'. Human plasma contained DHEA, but EPEA was not detectable.*

EPEA levels were increased significantly from 509 pg/mL in vehicle control medium to 1796 pg/mL after 24 hr of exposure to 10 µM EPA ($P < 0.01$), whereas 30 and 50 µM EPA increased EPEA levels to 4027 pg/mL and 5556 pg/mL, respectively (both $P < 0.001$; see Figure 3.4b). Again, similar results were obtained after 48 hrs of incubation.

To ensure that the solvent did not affect NAE synthesis, insulin medium without solvent or PUFA was also included as a blanc control. Concentrations of DHEA and EPEA in vehicle control incubations did not significantly deviate from these incubations. In addition, to rule out the possibility of spontaneous condensation of the fatty acids with ethanolamine in the medium without the cells being involved, the same incubations were repeated in used medium in the absence of cells. Both DHA and EPA did not increase DHEA or EPEA levels in these controls, indicating that the adipocytes were needed for the increases in DHEA and EPEA as described above (data not shown). Medium levels of AEA, PEA, OEA, and SEA were not affected with 48 hr exposure to DHA, whereas EPA resulted in a small increase for only OEA and PEA (Figure 3.4c and 3.4d).

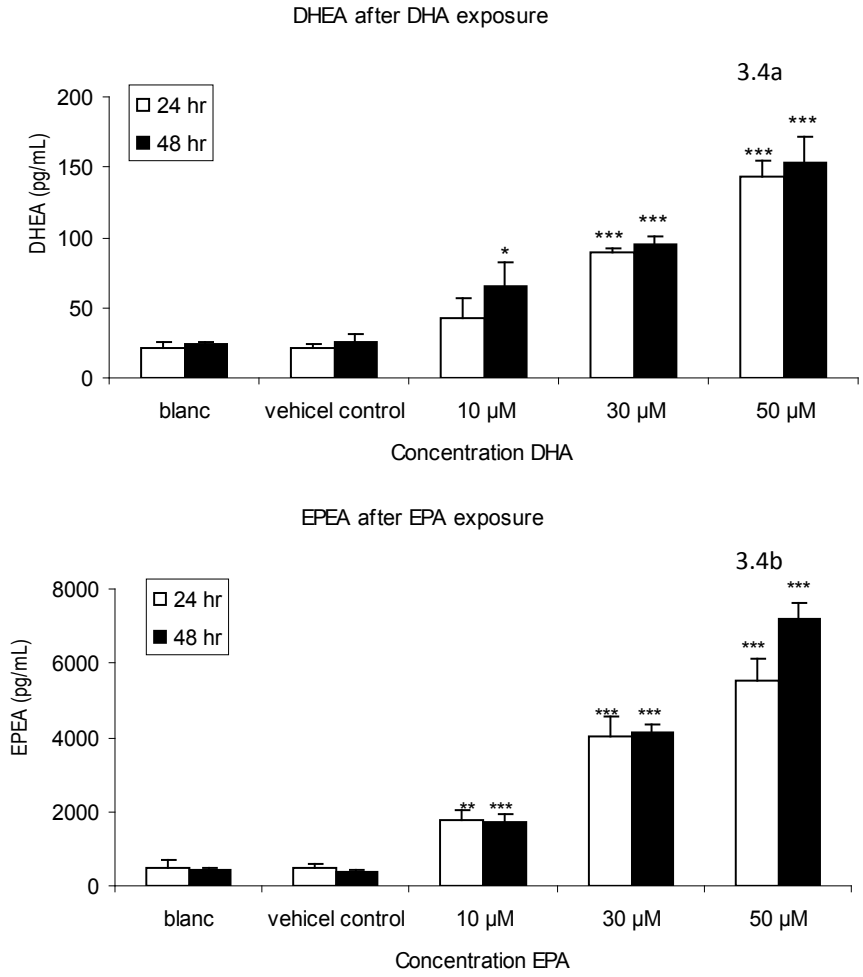

Figure 3.4a, b: *Medium levels of DHEA (a) EPEA (b) after exposure of 3T3 L1 adipocytes to DHA and EPA (n=3). Both DHEA and EPEA levels were increased after exposure to DHA or EPA, respectively. Legend: * P<0.05; ** P<0.01; *** P<0.001.*

In summary, 3T3-L1 adipocytes synthesize and release DHEA and EPEA in standard media, which can be further enhanced by adding their precursors DHA and EPA.

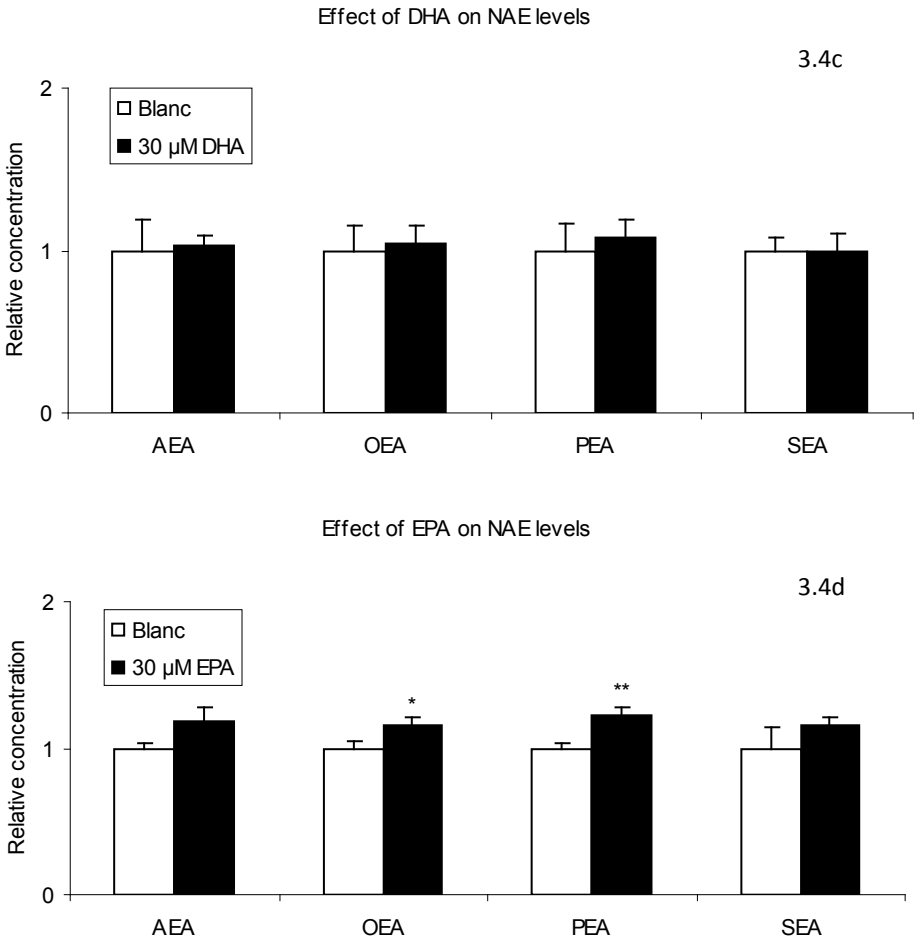

Figure 3.4c, d: *Medium levels of other NAEs (c and d) after exposure of 3T3 L1 adipocytes to DHA and EPA, respectively (n=3). Legend: * P<0.05; ** P<0.01.*

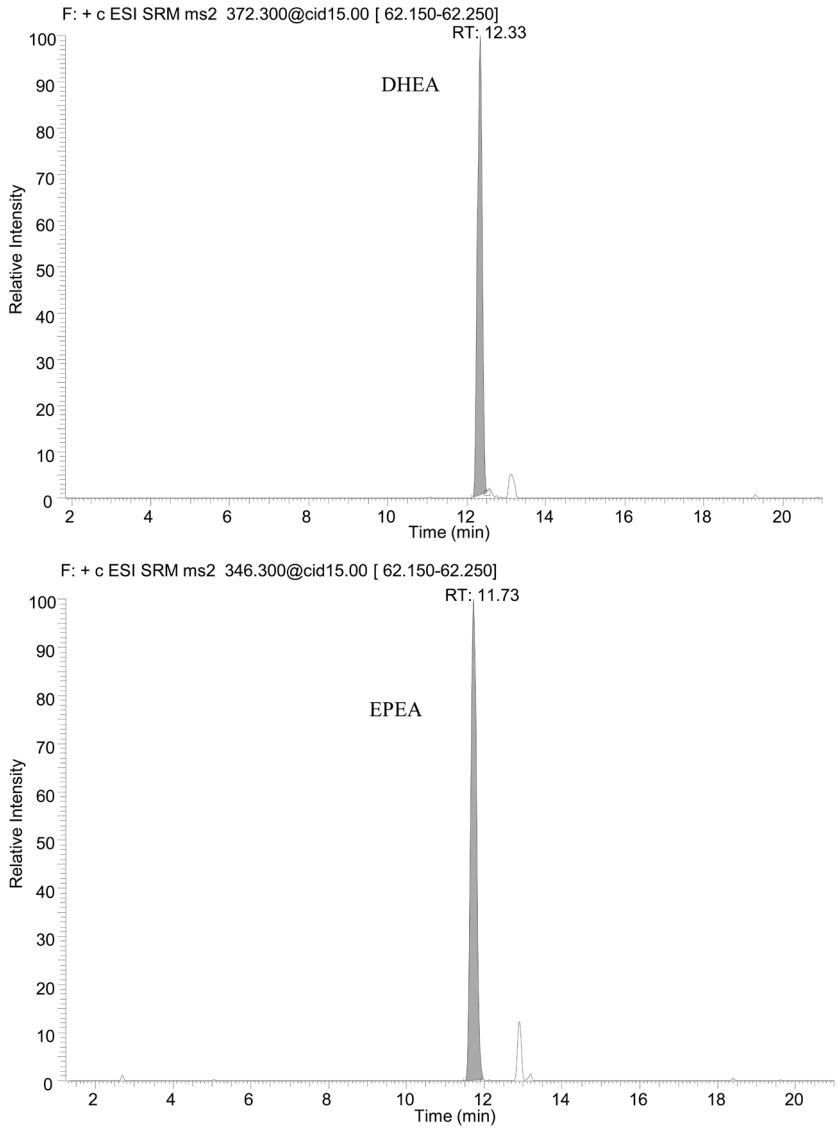

Figure 3.5: *SRM chromatogram for DHEA and EPEA in cell culture medium after 24 hr exposure to 50 µM DHA or EPA, respectively. Medium (2 mL) was extracted and analyzed for DHEA and EPEA levels as described in section 'material and methods'.*

The increase in DHEA and EPEA levels is directly related to conversion of fatty acids added to the medium

Exposure to DHA and EPA increases biosynthesis of DHEA and EPEA, but it is not known if this was mediated by direct conversion of the fatty acids added to the medium or if this was caused indirectly by mobilization of intracellular fatty acids which were subsequently converted. Therefore, adipocytes were exposed to the deuterium labelled DHA-d5 and EPA-d5 fatty acids, which are 5 Da heavier than the normally occurring structures. Therefore, this allows (semi-quantitative) tracking of these structures through metabolism using LC-MS/MS.

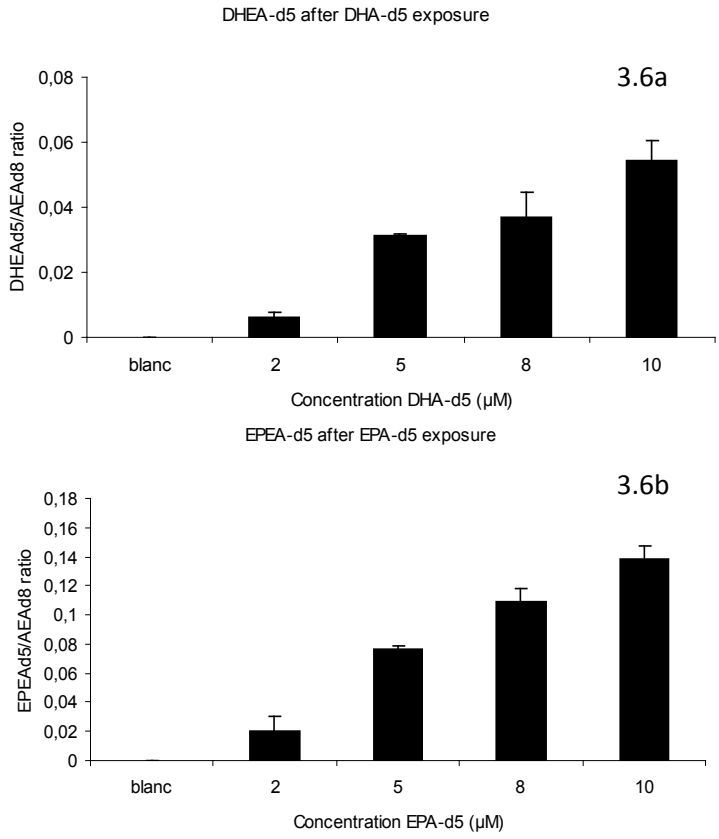

Figure 3.6: *Production of DHEA-d5 (a) and EPEA-d5 (b) in medium after exposure of 3T3-L1 adipocytes for 48 hr to DHA-d5 and EPAd-5, respectively (n=3). Exposure to DHA-d5 and EPA-d5 resulted in a dose-dependent increase in DHEA-d5 and EPEA-d5, respectively, whereas both ethanolamides were not detected in the blanc control incubation.*

Exposing differentiated 3T3-L1 adipocytes to 2-10 µM DHA-d5 and EPA-d5 for 48 hr resulted in a dose-dependent increase in both DHEA-d5 and EPEA-d5 levels (Figure 3.6a and 3.6b, respectively), whereas no DHEA-d5 or EPEA-d5 was observed in blanc control incubations. Therefore, it can be concluded that the increased levels of DHEA and EPEA after exposure to DHA and EPA are at least partly explained by direct conversion of these fatty acids from the medium.

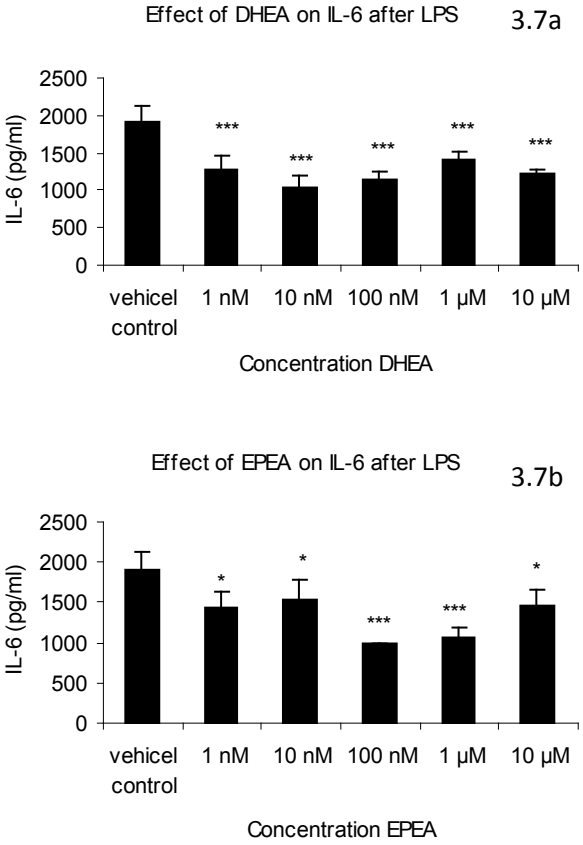

Figure 3.7 a, b: *Effect of DHEA (a) and EPEA (b) on IL-6 levels in medium of 3T3-L1 adipocytes after LPS stimulation (n=4). Both DHEA and EPEA reduced IL-6 levels in response to LPS stimulation at all concentrations tested. Legend: * P<0.05; *** P<0.001.*

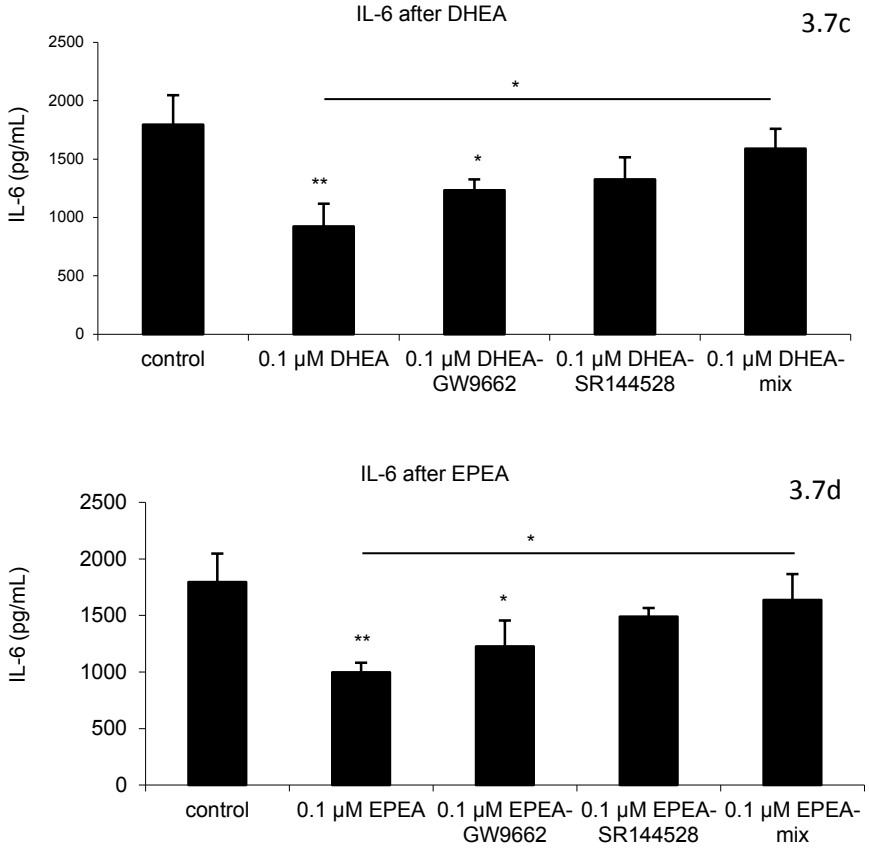

Figure 3.7 c, d: *Co-incubations of DHEA and EPEA with GW9662 (100 nM, PPAR-γ antagonist) and SR144528 (100 nM, CB2 antagonist) both restored IL-6 levels with a maximum and significant effect when GW9662 and SR144528 were simultaneously added at 50 nM. Legend: * P<0.05; ** P<0.01.*

DHEA and EPEA reduce adipocyte IL-6 and MCP-1 levels after LPS-stimulation

To investigate the possible interactions of DHEA and EPEA with an inflammatory response, differentiated 3T3-L1 cells were pre-incubated for 1 hr with 1 nM - 10 µM of DHEA or EPEA and subsequently stimulated with 50 ng/mL LPS while maintaining the same concentrations of DHEA or EPEA. Results are depicted in Figures 3.7 (IL-6) and 3.8 (MCP-1). Both DHEA and EPEA significantly reduced IL-6

levels at all concentrations tested (see Figure 3.7a and 3.7b for significance levels) compared to the vehicle control. The strongest reduction was approximately 50% and was found for 10 nM for DHEA and 100 nM for EPEA. Between the experiments (4 repetitions) there was a variation in the dose-response relation. Interestingly, this reduction became less when DHEA and EPEA were added in the micromolar range. The inhibitory effect of DHEA and EPEA almost completely disappeared in the presence of GW9662 or SR144528 (Figure 3.7c and 3.7d). Antagonizing effects of the combination of GW9662 and SR144528 (50 nM each) were more pronounced than those of the individual compounds at 100 nM.

In addition to IL-6, MCP-1 levels were also significantly reduced by DHEA and EPEA for all concentrations tested (see Figure 3.8 for significance levels) compared to the vehicle control. Similar to IL-6, MCP-1 levels were also less reduced when DHEA and EPEA were added in the micromolar range compared to the nanomolar range. DHEA and EPEA produced the strongest reduction of MCP-1 at 10 nM, corresponding to approximately 50% of the control value. Co-incubation with GW9662 and SR144528 did not affect MCP-1 levels for both DHEA and EPEA.

To confirm that the observed effects are mediated by NAEs and not by the fatty acids DHA and EPA that could be re-formed by hydrolysis, the experiments were repeated with the fatty acids mentioned above. DHA and EPA did not lower IL-6 and MCP-1 levels (data not shown).

Discussion

"Fish oil" fatty acids DHA and EPA are associated with several positive health effects, including a lower risk for fatal heart disease, improvement of parameters associated with obesity and the metabolic syndrome, and anti-inflammatory properties [1-3]. Several mechanisms have been proposed to explain these effects, including conversion to anti-inflammatory eicosanoids and resolvins [3]. Interestingly, PUFAs also serve as precursors for NAEs, with some of them possessing anti-inflammatory properties or metabolic effects mediated through CB1, CB2, and PPAR receptors [8, 9, 11]. However, the contribution of PUFA-derived NAEs to the beneficial effects of PUFAs is not known. Here, we show for the first time that 3T3-L1 adipocytes can directly synthesize DHEA and EPEA from their corresponding n-3 fatty acid precursors.

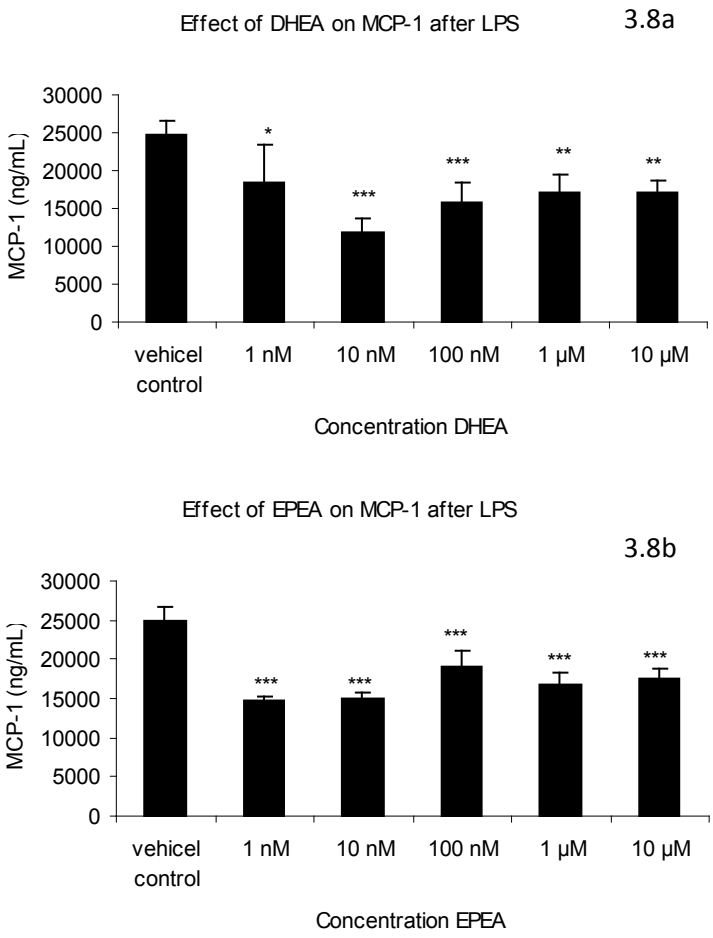

Figure 3.8: *Effect of DHEA (a) and EPEA (b) on MCP-1 levels in medium of 3T3-L1 adipocytes after LPS stimulation (n=4). Both DHEA and EPEA reduced MCP-1 levels in reponse to LPS stimulation at all tested concentrations. Legend: * P<0.05; ** P<0.01; *** P<0.001.*

As the endocannabinoid system is involved in many physiological and pathological processes, it is regarded as an important target for intervention by drugs or nutrients. Although only few studies show direct formation of NAEs from long-chain PUFA precursors, several other studies demonstrated that dietary intervention with n-3 fatty acids also indirectly affects the endocannabinoid

balance. In the study of Artmann *et al.,* decreased levels of all NAEs measured were found in livers of rats fed with LCPUFAs for 1 week, except for DHEA and EPEA [12]. Batetta *et al.* recently showed that diets rich in long-chain PUFAs decreased AEA and 2-AG levels in visceral fat of obese Zucker rats [13]. Together, these data suggest that NAE patterns tend to follow the relative abundance of the corresponding precursor lipids in the diet. Batetta *et al.* also showed that feeding n-3 PUFAs to Zucker rats leads to a reduction in ectopic fat deposition, lower liver triglycerides and LDL plasma levels, and a decreased tumor necrosis factor-α (TNF-α) secretion by macrophages [13]. The authors link these observations to the observed decrease of AEA and 2-AG concentrations in visceral adipose tissue, suggesting that this might be due to reduced stimulation of CB1, which is associated with increased adipogenesis and lipogenesis and negative crosstalk between adipose tissue and muscle [11, 23, 24]. In a study reported by Matias *et al.* [14], incubation of 3T3-F442A adipocytes with 100 µM of DHA for 72 h resulted in reduced intracellular AEA and 2-AG levels. However, no data on the formation of DHEA or EPEA were given in their paper. In our study levels of the other NAEs, AEA, OEA, PEA and SEA in the medium were not significantly changed upon exposure to DHA whereas EPA only increased OEA and PEA slightly. This may be due to the differences in experimental conditions. In addition, our data correspond to extracellular (medium) NAE levels, which might be different to the intracellular values measured by Matias *et al* [14]. Since NAEs act on adjacent cells, with their receptors being at least (CB1, CB2, TRPV1) located on cell membranes, levels of excreted NAE will be of biological relevance. The observation that values of NAEs other than DHEA and EPEA were not affected can also be due to the relatively slow incorporation of fatty acids in the cell membranes or a low conversion efficiency. Certainly, the formation of DHEA and EPEA, compared to the level of precursor added to the cells, is relatively low. When 30 µM DHA is added, only 90 pg/mL DHEA (= 0.24 nM) is found in the medium. The overall metabolic fate of most of the DHA and EPA added to the adipocytes is not known as yet, but could also be esterified to triglycerides or phospholipids in membranes or reside in the medium. Finally, they may be further metabolized by COX-2, which is also present in adipocytes [25, 26].

Remarkably, EPEA levels were higher than those of DHEA. This may be explained by differences between levels of esterified EPA compared to DHA in 3T3-L1 adipocytes, which is reported to exist for 3T3-F442A adipocytes [14]. To the best

of our knowledge, there is no data available concerning DHA and EPA background levels in the 3T3-L1 cells used in the work presented here.

Adipocytes communicate with distant tissues and cells to regulate metabolism through so-called adipokines, including IL-6, MCP-1, adiponectin, leptin and TNF-α [17-20]. Obesity is associated with increased serum levels of IL-6 and MCP-1, which are considered to contribute to the increase of insulin resistance and thereby to the further development of the metabolic syndrome. Previous work demonstrated that DHA and EPA have anti-inflammatory properties in macrophages and this was also reported for DHA in 3T3-L1 adipocytes, but at higher doses and longer exposures than tested in this study for their respective NAEs [27, 28]. Data from the present study show that both DHEA and EPEA decrease adipocyte IL-6 and MCP-1 levels after stimulation with LPS and suggest that both PPAR-γ and CB2 receptors are involved in the inhibition of IL-6. In another paper, we also demonstrated that DHEA has anti-inflammatory properties in macrophages which are mediated through an interaction with the CB2 receptor [29]. Furthermore, CB2 receptor mRNA expression was reported in human adipocytes [30]. Previously performed studies suggested that the n-pentyl chain in anandamide is necessary for optimal binding to cannabinoid receptors [31], but other work demonstrated that both DHEA and EPEA have affinity for CB1, although less than AEA [32]. It remains to be established whether increasing the ethyl chain to a pentyl chain will increase affinity for CB2.

In addition to CB2, DHEA also binds to other receptors including PPARs [12], underlining the complexity of NAE signaling. This may explain that the reduction of IL-6 and MCP-1 was strongest when NAEs were applied in the nanomolar range. Remarkably, no effects on MCP-1 levels were found using GW9662 and SR144528. More studies are needed to reveal the mechanism of inhibition of MCP-1 release.

Human plasma was shown to contain DHEA with no detectable levels of EPEA, which is similar to what was reported previously by others [33]. In line with previous observations, DHEA could potentially be a mediator of the beneficial effects of dietary n-3 PUFA in humans.

In conclusion, cultured adipocytes secrete increased amounts of DHEA and EPEA to the medium after exposure to DHA and EPA, and these ethanolamines have anti-inflammatory properties on adipocytes. The beneficial health effects of n-3 PUFAs could potentially be mediated by conversion to their ethanolamines.

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Chapter 4

Plasma anandamide and other *N*-acyl ethanolamines are correlated with their corresponding free fatty acid levels under both fasting and non-fasting conditions in women

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Abstract

N-acylethanolamines (NAEs), such as anandamide (AEA), are a group of endogenous lipids derived from a fatty acid linked to ethanolamine and have a wide range of biological activities, including regulation of metabolism and food intake. We hypothesized that i) NAE plasma levels are associated with levels of total free fatty acids (FFAs) and their precursor fatty acid in fasting and non-fasting conditions and ii) moderate alcohol consumption alters non-fasting NAE levels. In a fasting and non-fasting study we sampled blood for measurements of specific NAEs and FFAs. In the fasting study blood was drawn after an overnight fast in 22 postmenopausal women. In the non-fasting study blood was sampled before and frequently after a standardized lunch with beer or alcohol-free beer in 19 premenopausal women. Fasting AEA levels correlated with total FFAs ($r=0.84$; $p<0.001$) and arachidonic acid levels ($r=0.42$; $p<0.05$). Similar results were observed for other NAEs with both total FFAs and their corresponding fatty acid precursors. In addition, AEA ($r=0.66$; $p<0.01$) and OEA levels ($r=0.49$; $p<0.02$) positively related with BMI. Changes over time in non-fasting AEA levels were correlated with changes in total FFA levels, both after a lunch with beer ($r=0.80$; 95% confidence interval: 0.54-0.92) and alcohol-free beer ($r=0.73$; 0.41-0.89). Comparable correlations were found for other NAEs, without differences in correlations of each NAE between beer and alcohol free beer with lunch. In conclusion, i) in fasting and non-fasting states circulating anandamide and other *N*-acylethanolamines were associated with free fatty acid levels and ii) moderate alcohol consumption does not affect non-fasting NAE levels. This suggests that similar physiological stimuli cause the release of plasma *N*-acylethanolamines and free fatty acids in blood.

Trial registration: ClinicalTrials.gov ID no: NCT00524550 and NCT00652405

Introduction

N-acylethanolamines (NAEs) are a group of lipid mediators, derived from a fatty acid precursor linked to an ethanolamine moiety. The best studied NAE is the endocannabinoid arachidonoylethanolamide (anandamide; AEA). In addition to AEA, NAEs also comprise of other non-endocannabinoids such as palmitoylethanolamide (PEA), oleoylethanolamide (OEA) and stearoylethanolamide (SEA), for which palmitic acid, oleic acid and stearic acid serve as their respective precursor fatty acids (see Figure 4.1 for structures). NAEs have several biological effects, including regulation of food intake and energy metabolism [1, 2].

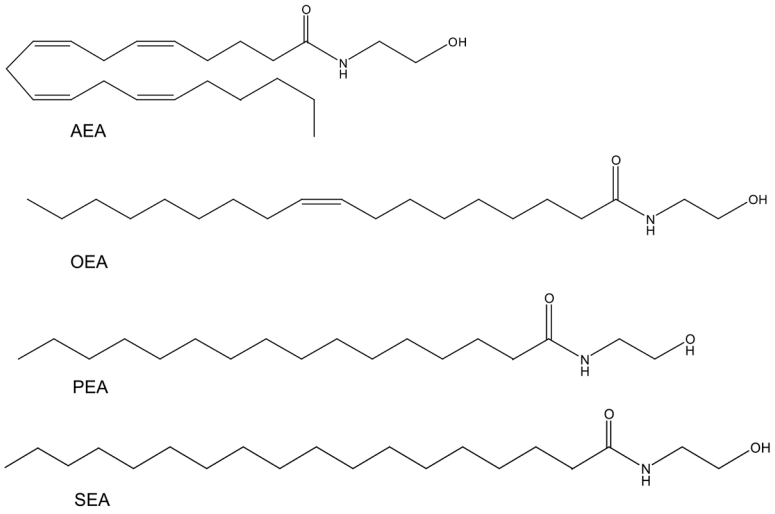

***Figure 4.1**: Structures of the N-acylethanolamines AEA, OEA, PEA and SEA.*

Several studies have shown that dietary fatty acids determine levels of corresponding NAEs in different tissues, suggesting a link between availability of precursor fatty acids and NAEs formation [3-5]. In obesity, a state characterized by elevated circulating free fatty acids (FFAs), levels of AEA are also increased [6, 7]. Conversely, when FFA levels decline after a meal, non-fasting levels of AEA are concomitantly decreased in normal-weight subjects [8]. This made us hypothesize

that their release into plasma is regulated by similar physiological stimuli and that plasma NAE levels parallel those of total FFAs and their precursor fatty acids.
Secondly, since AEA and OEA are involved in the regulation of appetite [9], we also wanted to study whether the well-known appetite-inducing effects of acute alcohol consumption [10] might be related to changes in NAEs. Therefore, we investigated the correlation of different NAEs with FFAs in both fasting and non-fasting conditions and the effect of moderate alcohol consumption on non-fasting NAE levels.

Subjects and Methods

Samples were analyzed from two studies (fasting and non-fasting) conducted at TNO Quality of Life, Zeist, the Netherlands. An independent centralized ethics committee (METOPP; Tilburg, the Netherlands) approved both protocols. Studies are registered at Clinical trials.gov: NCT00524550 and NCT00652405. Eligible women consumed between 5 and 21 units of alcohol per week, were apparently healthy, non-smokers and had no family history of alcoholism. They gave written informed consent.
For the fasting study we used samples from an intervention study completed earlier [11]. At the first day of the intervention subjects arrived at the premises after an overnight fast when blood was drawn.
The non-fasting study was part of a randomized, open label, crossover trial in which subjects consumed two cans of beer (~13g alcohol each) or two cans of alcohol-free beer (<0.1 g alcohol) (both: Amstel, Amsterdam, the Netherlands) daily for three weeks during dinner. Each three-week intervention period was preceded by a one-week wash-out. At the last day of each treatment, subjects came to the premises for a standardized lunch (1978 kJ; 16.1% protein, 21.0% fat and 62.9% carbohydrate). The two lunches were consumed around noon under similar conditions, 28 days apart. Treatment order (beer vs. alcohol-free beer with lunch) was randomized according to the intervention trial. A first can of study substance was consumed one hour before the start of the lunch, a second can during the lunch. Both cans were consumed within 15 minutes. Blood was sampled before and at several time points after lunch.
In both studies, venous blood was drawn in tubes containing silica as clot activator for serum (FFA analysis) or Potassium Ethylene Diamine Tetra Acid for plasma (NAE analysis). To inactivate fatty acid amide hydrolase (FAAH),

phenylmethanesulphonyl fluoride (final concentration: 100 µM) was added to the plasma samples. Total FFA determinations in blood were performed using Olympus analytical equipment and reagents. Plasma levels of NAEs were determined using a LC-MS/MS technique [12]. Specific serum FFA were measured using a high-resolution UHPLC-MS technique [13].

Variable	
Glucose (mmol/L)	5.45 ± 0.11
Insulin (pmol/L)	51.9 ± 5.8
Triglycerides (mmol/L)	1.47 ± 0.13
Free fatty acids (mmol/L)	0.59 ± 0.06
Anadamide (nmol/L)	6.8 ± 0.70
Oleoylethanolamide (nmol/L)	43.8 ± 3.3
Palmitoylethanolamide (nmol/L)	40.0 ± 3.7
Stearoylethanolamide (nmol/L)	16.3 ± 1.9

Table 4.1. *Fasting characteristics of the twenty-two postmenopausal women.*

Pearson's coefficient of correlation was calculated to assess correlations between fasting FFA and NAE. In the non-fasting study variables at baseline (t=-60 min before lunch) were compared between treatments with a mixed analysis of variance model. For the correlation between changes over time (non-fasting study), a Fisher's *z* transformation was applied on individual correlations to correct for deviations from the normal distribution and 95% confidence interval (CI) for each correlation coefficient were calculated [14]. SAS statistical software package (SAS version 9, SAS Institute, Cary, NC) was used to perform statistical analyses. Statistical significance was defined as $p<0.05$.

Results

The 22 postmenopausal women enrolled in the fasting study had a mean BMI of 26.3 kg/m^2 (range: 19.4-34.1) and a mean age of 55.8 years (range: 51-61). See Table 4.1 for fasting characteristics. Fasting AEA levels correlated positively with total FFA ($r=0.84$, $p<0.001$) and with arachidonic acid levels ($r = 0.42$; $p<0.05$).

	Alcohol-free beer	Beer	P value
Glucose (mmol/L)	4.97 ± 0.07	5.09 ± 0.07	0.18
Insulin (pmol/L)	44.0 ± 9.2	37.1 ± 9.2	0.55
Triglycerides (mmol/L)	1.17 ± 0.07	1.08 ± 0.07	0.17
Free fatty acids (mmol/L)	0.39 ± 0.04	0.44 ± 0.04	0.18
Anadamide (nmol/L)	5.90 ± 0.39	5.98 ± 0.39	0.86
Oleoylethanolamide (nmol/L)	52.7 ± 6.5	54.4 ± 6.5	0.74
Palmitoylethanolamide (nmol/L)	53.9 ± 4.1	57.8 ± 4.1	0.23
Stearoylethanolamide (nmol/L)	15.1 ± 0.9	15.2 ± 0.9	0.98

Table 4.2. *Fasting characteristics of the nineteen premenopausal women at baseline (t=-60 min before lunch) after the three weeks of consuming beer or alcohol-free beer.*

OEA, PEA and SEA levels also positively correlated with serum fasting total FFA levels (All $r>0.44$; $p<0.05$). Comparable correlations were observed between oleic acid and OEA ($r=0.71$; $p<0.001$), palmitic acid and PEA ($r=0.54$; $p<0.01$) and stearic acid and SEA ($r=0.77$; $p<0.0001$) (Figure 4.2). Furthermore, AEA ($r=0.66$; $p<0.01$) and OEA levels ($r=0.49$; $p<0.02$) were also positively related with BMI.

All 19 premenopausal (mean age 23.1 years; range 20-32) women in the non-fasting study had a normal weight (mean BMI: 22.2 kg/m^2; range: 19.8-24.7). No differences in FFA or NAE levels at baseline were observed between treatments after the three week intervention periods (see Table 4.2 for fasting characteristics). Curves for AEA, OEA, PEA and total FFA after both meals are shown in Figure 4.3. Non-fasting changes in total FFA were positively associated with changes in NAEs: ($r=0.73$; 95% confidence interval: 0.41-0.89) and ($r=0.80$; 0.54-0.92) for AEA; ($r=0.89$; 0.73-0.96) and ($r=0.91$; 0.78-0.97) for OEA and ($r=0.77$; 0.49-0.91) and ($r=0.76$; 0.47-0.90) for PEA after a meal either with nonalcoholic beer or beer respectively. Correlations over time of each NAE with total FFAs did not differ between lunches with or without alcohol consumption. In a subgroup of six women, comparable coefficients with NAEs and their specific fatty acids were found for both lunches (data not shown).

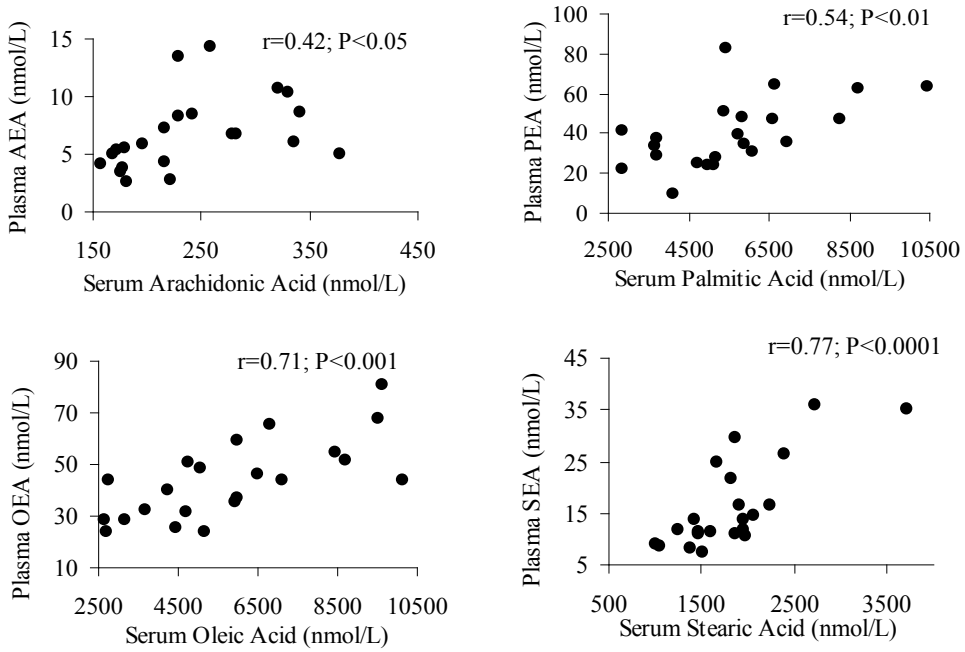

Figure 4.2: *Pearson correlations between plasma levels of several N-acylethanolamines and their corresponding serum levels of fatty acid in twenty-two postmenopausal women after an overnight fast.*

Discussion

The primary findings of this study are i) all fasting and non-fasting plasma NAEs investigated are positively associated with both serum total FFAs and their specific fatty acid precursor and ii) prolonged and acute moderate alcohol consumption does not alter non-fasting NAE levels. These findings imply that circulating NAEs are a reflection of plasma FFA levels. This suggests AEA levels to be altered in general under conditions characterized by changes in circulating FFAs. Indeed, increased levels of AEA are observed in obesity, diabetes, and eating disorders such as anorexia and binge-eating disorder [6, 8, 15]. Furthermore, increased levels of circulating AEA, OEA and PEA were found in patients with liver cirrhosis [16]. Besides the correlation with fasting FFAs, we also observed a strong correlation between AEA and OEA with BMI in postmenopausal women. This is in

line with previous work which observed higher levels of AEA among obese postmenopausal women compared with normal-weight counterparts [6].

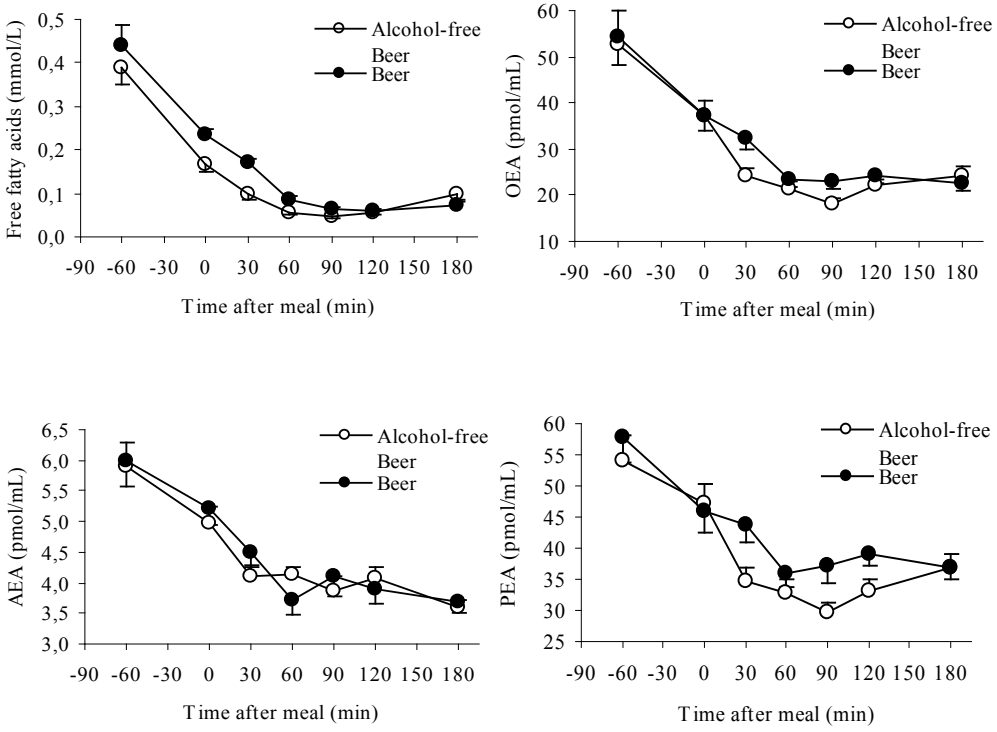

Figure 4.3: *Means (±SEM) of serum free fatty acid (FFA) concentrations and plasma concentrations of anandamide (AEA), palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) before and during three hours after a lunch with beer (●) or alcohol-free beer consumption (○) in 19 normal-weight premenopausal women.*

Although the release of NAEs into plasma may still be biologically relevant, it remains to be determined of the observed relation between FFA and NAE levels is also causal. Perhaps, the increase in FFA levels is paralleled by increased membrane phospholipid cleavage. The increased levels of AEA and 2-arachidonoylglycerol (2-AG), another arachidonic acid-derived endocannabinoid,

have led to the concept of the 'overactivated' endocannabinoid system in obesity in which FAAH expression is reduced [6, 7, 17]. However, our data suggest that increased levels of endocannabinoids and other NAEs in obesity could take place in parallel with increased FFA concentrations and does not necessarily reflect a functional change of the endocannabinoid system alone.

Only one other study investigated non-fasting AEA concentrations in humans, without reporting results on FFAs [8]. They found decreased AEA levels one hour after a lunch. Possibly the physiological stimuli involved in the decrease of FFA levels after consumption of a meal also contribute to a reduced release of NAEs from membrane phospholipids. However, a decreased activity of enzymes involved in NAE synthesis such as N-acylphosphatidylethanolamine hydrolyzing phospholipase (NAPE-PLD) or increased FAAH activity can not be excluded.

To our best knowledge, this is the first human study in which NAEs are reported after both prolonged and acute moderate alcohol consumption. Acute alcohol consumption is generally known to stimulate appetite [10]. In our study, circulating non-fasting NAEs did not differ between a lunch with or without alcohol consumption. It thus seems unlikely that the acute appetite-inducing effects of alcohol are caused by alterations in circulating NAEs.

The observed correlations between NAEs and FFAs are persistent for several NAEs in various populations (pre- and postmenopausal women) and under different conditions (fasting and non-fasting). However, some limitations warrant consideration. It remains to be established whether changes in plasma NAEs will also modify NAEs in target tissues such as adipose tissue, brain, liver and intestines as seen in animal studies [3] and to which extend these changes affect physiology. The exact site of NAE synthesis is unknown, but the liver, adipose tissue or blood cells could contribute to the changes in NAE levels. Furthermore, other regulators such as insulin may affect NAE levels [18]. Finally, we did not measure 2-AG, another important endocannabinoid, or other NAEs.

In conclusion, we provide evidence that in humans fasting peripheral NAEs are positively correlated with both serum total fasting FFA and their specific fatty acid precursor. Furthermore, we showed that non-fasting changes over time in AEA and related NAEs are positively correlated with non-fasting changes in free fatty acids, independent of alcohol consumption. This suggests that circulating *N*-acylethanolamines might be a reflection of free fatty acids in blood. The biological significance of these findings is still unknown and requires further investigation.

Acknowledgements

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Plasma NAE levels are correlated with free fatty acid levels

Chapter 5

Time-dependent effect of *in vivo* inflammation on eicosanoid and endocannabinoid levels in plasma, liver, ileum an adipose tissue in C57BL/6 mice fed a fish oil diet

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Abstract

Eicosanoids and endocannabinoids/*N*-acyl ethanolamines (NAEs) are fatty acid derived compounds with a regulatory role in inflammation. Considering their complex metabolism, it is likely that inflammation affects multiple compounds at the same time, but how lipid profiles change in plasma and other tissues after an inflammatory stimulus has not been described in detail. In addition, dietary fish oil increases levels of several n-3 fatty acid derived eicosanoids and endocannabinoids, and this may lead to a broader change in the profiles of bioactive lipids.

In the present study mice were fed a diet containing 3 % w/w fish oil for 6 weeks before receiving *i.p*. saline or 3 mg/kg lipopolysaccharide (LPS) to induce an inflammatory response. Eicosanoid and endocannabinoid/NAE levels (in total 61 metabolites) in plasma, liver, ileum, and adipose tissue were quantified using targeted lipidomics after 2, 4, 8, and 24 hrs, respectively.

Tissue- and time-dependent effects of LPS on bioactive lipids profiles were observed. For example, levels of CYP derived eicosanoids in the ileum were markedly affected by LPS, whereas this was less pronounced in plasma and adipose tissue. For some compounds, such as 9,10-DiHOME, opposing effects of LPS were seen in the plasma compared to the other tissues, suggesting differential regulation of bioactive lipid levels after an inflammatory stimulus.

Taken together, our results show that plasma levels do not always correlate with the effects found in tissues, which underlines the need to measure profiles and pathways of mediators involved in inflammation, including endocannabinoid-like structures, in both plasma and tissues.

Introduction

The initiation, amplification and resolution of inflammation is controlled by a variety of molecules, including eicosanoids [1]. The synthesis of eicosanoids starts in the cell membrane, were fatty acids are released from membrane phospholipids and subsequently shuttled into one or more metabolic pathways, involving enzymes like cyclooxygenase (COX), inducible cyclooxygenase (COX-2), cytochrome P450 (CYP), or lipooxygenases (LOX)-5, -12 or -15 [1-4]. These pathways result in distinct eicosanoids, yielding prostaglandins (PGs), thromboxanes (TBXs), epoxyeicosatrienoic acids (EETs), hydroxyeicosatetraenoic acids (HETEs), and leukotrienes (LTs) (see Figure 5.1a for an overview of eicosanoids). Their effects depend on target tissue, concentration, nature of stimulus, timing and presence of other mediators [1]. Arachidonic acid (ARA) is the best studied substrate in eicosanoid biosynthesis, partly because of its high abundancy in cell membranes of immune cells [5]. However, other fatty acids, such as the n-3 polyunsaturated fatty acids (PUFAs) docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are also substrates for the above-mentioned enzymes, yielding compounds like PGD_3, PGE_3, $TBXB_3$, and 5-HEPE [1, 6, 7] (Figure 5.1b). The n-3 derived eicosanoids are in general less potent pro-inflammatory mediators compared to their ARA counterparts, and this could contribute to the anti-inflammatory properties which are associated with dietary intake of n-3 PUFAs [8-10]. In addition to this, n-3 PUFAs are also converted into resolvins, a class of compounds with active anti-inflammatory and inflammation resolving properties [11, 12].

The *N*-acyl ethanolamines (NAEs) represent another class of lipid-derived mediators with a role in inflammation. Anti-inflammatory properties have been described for several NAEs, including endocannabinoid arachidonoyl ethanolamide (AEA; also known as anandamide) and palmitoyl ethanolamide (PEA) [13, 14]. Anti-inflammatory properties have been described for the n-3 fatty acid derived NAEs docosahexaenoyl ethanolamide (DHEA) and eicosapentaenoyl ethanolamide (EPEA) (see Figure 1a-b), and these compounds were more potent than AEA in inhibiting nitric oxide release from macrophages [15, 16]. Increased levels of NAEs during inflammation have been described in several *in vitro* and animals models [17-19], and are observed in parallel with decreased expression of the primary NAE degrading enzyme fatty acid amide hydrolase (FAAH) [18]. Furthermore, pharmacological inhibition of FAAH or monoacyl glycerol lipase

(MAGL) reduced disease symptoms in several models of inflammation [20, 21], suggesting that NAEs/endocannabinoids are part of a protective response against inflammatory stress.

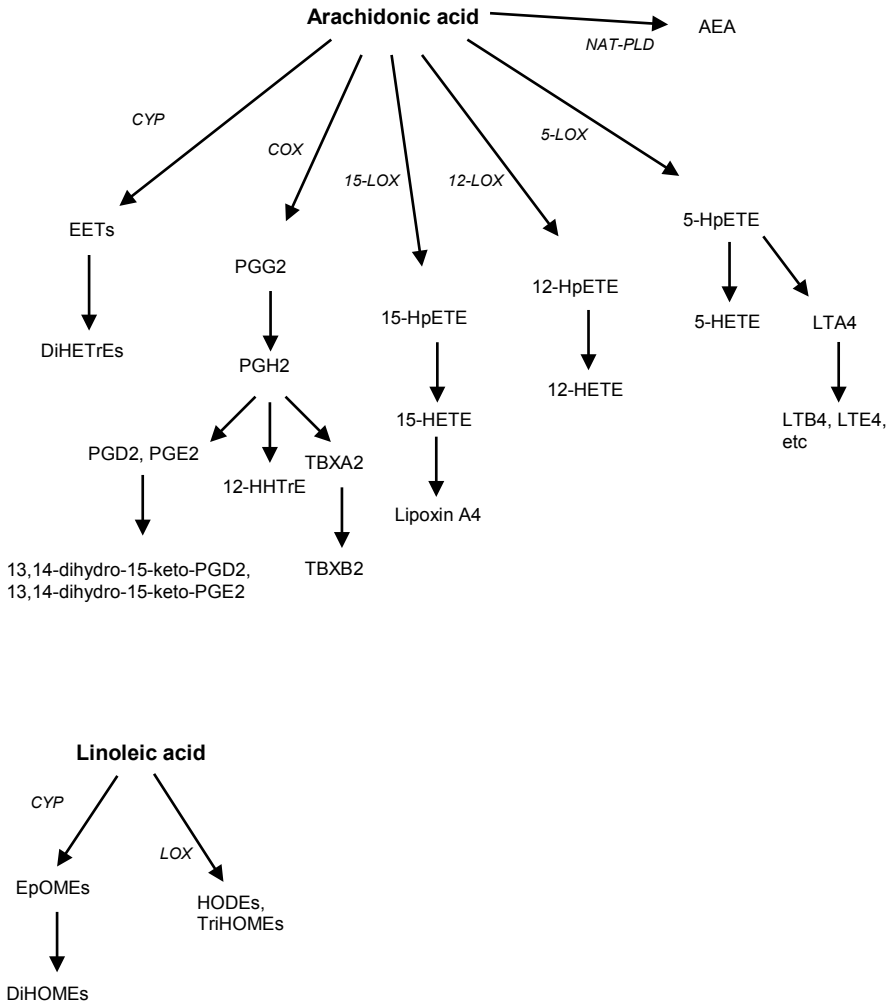


Figure 5.1a: *Overview of enzymatic pathways involved in eicosanoid and NAE synthesis. Arachidonic acid (ARA) is the best studied precursor for eicosanoid synthesis, but linoleic acid (LA) can also serve as substrates for different enzymes, including COX, CYP and the LOX isoenzymes, yielding distinct eicosanoids and intermediates.*

It is likely that multiple bioactive lipids are affected by inflammation considering their complex metabolism, but most studies only report effects on a limited number of (established) compounds, such as PGE_2 and AEA. In addition, studies on

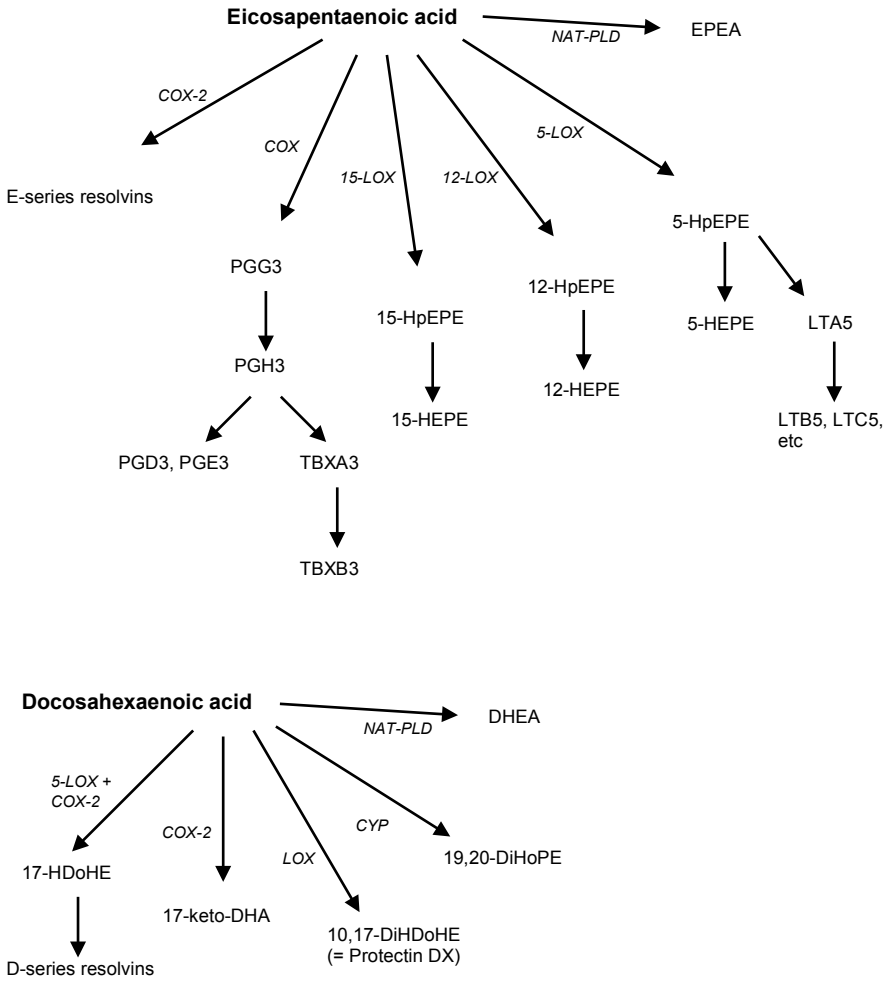


Figure 5.1b: *Overview of enzymatic pathways involved in eicosanoid and NAE synthesis. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can also serve as substrates for different enzymes, including COX, CYP and the LOX isoenzymes, yielding distinct eicosanoids, intermediates, and NAEs.*

time-dependent effects of *e.g.* LPS on bioactive lipid levels in plasma and peripheral tissues are scarce, and the formation of n-3 fatty acid derived bioactive lipids after various diets in these tissues needs further exploration. To better understand the role of bioactive lipids in the various phases of inflammation, it is important to know how they react to an inflammatory stimulus, specifically how their levels change over time and to what extent effects are comparable between plasma and peripheral tissues. In the present study, we investigated in detail the time-dependent effect of *i.p.* LPS on the levels of bioactive lipids (endocannabinoids/NAEs and eicosanoids) in plasma, liver, ileum and adipose tissue in mice fed with a diet rich in fish oil by using a targeted lipidomics approach. The results showed time- and tissue dependent effects of LPS on bioactive lipid profiles, and more importantly effects on plasma were not always a reflection of bioactive lipid levels in tissues.

Materials and methods

Chemicals

Lipopolysaccharide (0111:B4; LPS), indomethacin, paraoxon, butylated hydroxytoluene (BHT), and trifluoro acetic acid (TFA) were from Sigma (Steinheim, Germany) and phenylmethylsulfonyl fluoride (PMSF) was from Fluka (Steinheim, Germany). Milli-Q water (Milli-Q Advantage unit, Millipore, Amsterdam, The Netherlands) was used in all analyses. ULC-grade acetonitrile (ACN) and formic acid (FA) were obtained from Biosolve (Valkenswaard, The Netherlands). LC-MS grade methanol was from Riedel-de-Häen (Steinheim, Germany). Isopropanol and ethanol were from JT Baker (Deventer, The Netherlands). 12-[(tricyclo[3.3.1.13,7]dec-1-ylamino)carbonyl]amino]-dodecanoic acid (AUDA) and all analytical and internal standards were from Cayman (Ann Arbor, MI, USA). Standard stock solutions were prepared in ethanol (eicosanoids) or acetonitrile (endocannabinoids/NAEs), aliquoted and stored at -80 °C until analysis. HLB SPE columns (Oasis, 60 mg, 3 mL) were obtained from Waters (Etten-Leur, The Netherlands). C8 SPE columns (Bond Elut; 200 mg, 3 mL) were from Varian Inc (Lake Forest, CA, USA). ELISA kits were from R&D Systems (Minneapolis, MN, USA).

Animal experiment

Wild type male C57BL/6 mice were obtained from Harlan (Horst, The Netherlands) and housed two or three mice per cage in a temperature controlled environment with a 12 hour light-dark cycle (light at 6.00-18.00). The mice, 4 weeks old at arrival, had free access to a standard run-in diet (AIN93-M, with a 4% w/w fat as soy bean oil) for two weeks, after which all animals switched to a fish oil diet (AIN-93-M, containing 1% soy bean oil and 3% Marinol®). The diets were obtained from Research Diet Services (Wijk bij Duurstede, The Netherlands) and the Marinol® was a kind gift from Lipid Nutrition (Wormerveer, The Netherlands). Diets were stored in air-tight bags at -20 °C until just before feeding, and fresh food was provided two times per week to minimize oxidation of the fatty acids in the diet. GC-MS based analysis of the diets confirmed that the correct amounts of DHA and EPA were present, and that the DHA and EPA levels were stable for at least 4 weeks under the described conditions. Food consumption and animal weight were measured two times per week.

After six weeks, animals were divided into two groups and received either *i.p.* saline or 3 mg/kg LPS. At t=0, and after 2, 4, 8, and 24 hrs respectively, four animals from both the saline and the LPS groups were anesthetized (only four saline treated animals at t=0), blood was collected from the orbital sinus, captured in 1.3 mL EDTA coated tubes (Sarstedt; Etten-Leur, The Netherlands) and put on ice until centrifugation (10', 10,000 rpm at 4 °C). After centrifugation, plasma was aliquoted. For eicosanoid analysis, 200 µL plasma was stored in 1 mL methanol containing paraoxon, BHT, AUDA, indomethacin, and PMSF to prevent eicosanoid oxidation and breakdown. For endocannabinoid/NAE analysis, 100 µL plasma was stored in the presence of 100 µM PMSF and 100 µM URB602 (inhibitor of 2-AG hydrolysis). Subsequently, the animals were sacrificed by cervical dislocation after which liver, ileum and epididymal adipose tissue was collected and immediately snap-frozen in liquid nitrogen. All plasma and tissue samples were stored at -80 °C until further analysis. Analysis of plasma interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) levels confirmed that LPS had triggered an inflammatory response by showing strongly increased IL-6 and MCP-1 levels in LPS-treated mice (data not shown). From the 24 hr LPS group, one animal died just before the end of the experiment; not enough plasma could be obtained from two other animals in the 24 hr LPS group to perform endocannabinoid analysis.

The study was conducted according to the Netherlands Law on Animal Experiments, and approved by the local Animal Experiments Committee of Wageningen University.

Extraction of endocannabinoids/NAEs from plasma

Plasma (100 µL) was thawed and 400 µL extraction mixture containing 100 µM PMSF and internal standards (AEA-d8, 2-AG-d8 and OEA-d4) in ACN was added while the sample was gently vortexed. After subsequent centrifugation (5' at 13,000 rpm and RT), the supernatant was transferred to a clean eppendorf tube and evaporated to dryness in a vacuum concentrator (Scanvac; Lynge, Denmark). The dried extracts were reconstituted in 100 µL ACN containing 0.1% TFA and used for LC-MS/MS analysis.

Extraction of endocannabinoids/NAEs from tissues

Endocannabinoid/NAE were extracted from freeze-dried liver and ileum using a method adapted from a previously published protocol for plasma [22]. Approximately 50 mg freeze-dried liver or 10 mg freeze-dried ileum were extracted by adding 1 mL extraction mixture (ACN) and sonication. The samples were centrifuged (5' at 14,000 rpm), the supernatant was transferred to a clean 15 mL tube, and this was repeated once. The pooled ACN fractions were diluted with MQ water containing 0.13% TFA until the final ACN concentration was 20% prior to SPE clean-up as described before [22]. In short, columns were washed with 20% v/v ACN in MQ water containing 0.1% TFA, eluted with 80% v/v ACN in MQ water containing 0.1% TFA and evaporated to dryness using vacuum centrifugation. The dried extracts were reconstituted in 100 µL ACN containing 0.1% TFA and used for LC-MS/MS analysis.

For adipose tissue, approximately 100 mg 'wet' tissue was extracted with 1 mL extraction solution (ACN) by sonication. The samples were centrifuged for 5' at 14,000 rpm and RT, the supernatant was transferred to a clean 2.0 mL eppendorf tube, and the ACN extraction was repeated once. The 2 mL ACN extract was subsequently evaporated to dryness, reconstituted in 100 µL ACN containing 0.1% TFA and used for LC-MS/MS analysis.

LC-MS/MS analysis of endocannabinoids/NAEs

Two LC-MS/MS systems were used for endocannabinoid/NAE analysis. Plasma extracts were analyzed by UPLC coupled to a Xevo TQ-S mass spectrometer (Waters; Etten-Leur, The Netherlands) because high sensitivity was essential for adequate quantification in extracts obtained from 100 µL plasma samples. Liver, ileum and adipose tissue were analyzed on a Surveyor HPLC coupled to a TSQ Quantum Discovery mass spectrometer (Thermo Finnigan; Breda, The Netherlands) using a method adapted from a previous publication [22].

For the UPLC-Xevo system, 3 µL plasma extract was injected on a Acquity C8 BEH UPLC column (2.1 x 100 mm, 1.7 µm) and was separated using gradient elution with a stable flow of 500 µL/min. The gradient started with 100% A (40:40:20 v/v/v of MQ water : methanol : ACN with 0,1% FA) which was maintained until 0.35 minutes, followed by a linear increase to 100% B (7:3 v/v methanol : ACN with 0.1% FA) which was achieved at 7.0 minutes and was maintained until 9.0 minutes. Finally, the column equilibrated for 3 minutes at 100% A. The column was maintained at 60 °C during analysis, and the samples were kept at 10 °C. The MS was operating in selective reaction mode using electrospray ionization in positive ion mode, with a capillary voltage of 1.5 kV, a source temperature of 150 °C and a desolvation temperature of 500 °C. Cone voltage and collision energy were optimized for each compound individually (see Table 5.1a for parent and product *m/z* values). Peak identification and quantification was performed using MassLynx software version 4.1. Calibration curves were run in duplicate from which one regression equation was generated.

For the analysis of liver, ileum and adipose tissue, a TSQ Quantum Discovery was used as described before [22]. Five µL extract was separated on an Xterra C8 MS column (2.1 x 150 mm, 3.5 µm) using gradient elution with a constant flow of 150 µL/min. The same solutions were used as with the Xevo system, but now 1 g/L ammonium acetate was added (the most dominant parent for 2-AG in this MS is the ammonium adduct). The gradient started with 100% A which was maintained until 2.0 minutes, followed by a linear increase to 100% B which was achieved at 8.00 minutes and maintained until 16.0 minutes, and the column was left to equilibrate for 5 minutes at 100% A. The column was maintained at 40 °C during analysis and the samples were cooled at 4 °C. The MS was operating in selective reaction mode using electrospray ionization in positive ion mode, with a capillary voltage of 4.5 kV and a capillary temperature of 350 °C. Cone voltage and collision

energy were optimized for each compound individually. Peak identification and quantification was performed using LCquan software version 2.5.5. Calibration curves were run in duplicate from which one regression equation was generated.

Compounds	*Parent (m/z)*	*Product (m/z)*
AEA	348	62
2-AG (NH_4 adduct)	379 (396)	287
DHEA	372	62
EPEA	346	62
DLE	350	62
OEA	326	62
PEA	300	62
SEA	328	62
Internal standards		
AEA-d8	356	63
OEA-d4	330	66
2-AG-d8 (NH_4 adduct)	387 (404)	294

Table 5.1a: *m/z Values of parent and product ions of endocannabinoids/NAEs used in the LC-MS/MS method. For tissue, 2-AG analyses (performed on TSQ Quantum MS), the ammonium adduct was the most dominant parent ion. For plasma 2-AG analyses (performed on Xevo TQ-S MS), the molecular ion (M+H) was the most dominant parent.*

Extraction of eicosanoids from plasma

Internal standards were added to the plasma samples which were already precipitated with methanol (see section 'animal experiment'), and the samples were put on ice for 30 minutes. Samples were subsequently centrifuged (5', 3000*g* and 4 °C) and the supernatant was transferred to a glass tube. Just before loading on activated HLB columns, 4.75 mL MQ water containing 0.1% v/v FA was added to the methanol extract, diluting the extract to 20% methanol. After loading, the columns were washed with 2 mL 20% methanol in MQ water containing 0.1% FA, and the columns were allowed to dry for 15 minutes. The SPE columns were eluted with 2 mL methanol and the samples were captured in tubes already containing 20 µL of 10% glycerol and 500 µM BHT in ethanol. The tubes were placed in a water bath at 40 °C and the methanol was evaporated under a gentle stream of nitrogen, after which the samples were reconstituted in 100 µL ethanol containing another internal standard (CUDA) and immediately used for LC-MS/MS analysis.

Extraction of eicosanoids from tissues

The extraction of eicosanoids from liver, ileum and adipose tissue was similar to plasma eicosanoid extraction. Approximately 100 mg liver and adipose tissue, and 50 mg ileum was extracted with 1 mL methanol containing internal standards and sonication. After centrifugation (5', 3000*g* and 4 °C), the supernatants were transferred to clean tubes and the methanol extraction was repeated once. Just before loading on HLB SPE columns, 8 mL MQ water containing 0.1% FA was added to the methanol extracts. For the SPE procedure and further, see section 'Plasma extraction of eicosanoids'.

LC-MS/MS analysis of eicosanoids

All eicosanoid analyses were performed on a UPLC coupled to a Xevo TQ-S mass spectrometer (Waters). Five µL extract was injected on a Acquity C18 BEH UPLC column (2.1 x 100 mm, 1.7 µm) and was separated using gradient elution with a stable flow of 600 µL/min. The gradient started with 95% A (MQ water with 0.1% FA) and 5% B (ACN with 0.1% FA) followed by a linear increase to 70% A and 30% B which was achieved at 5.0 minutes. This was followed by a linear increase towards 50% B which was achieved at 11.25 minutes and maintained until 13.25 minutes. The system was subsequently switched to 100% B, which was achieved at 15.75 minutes and maintained until 16.75 minutes, after which the column was left to equilibrate at 5% B for approximately 3 minutes. The column was maintained at 50 °C during analysis, and the samples were kept at 10 °C. The MS was operating in selective reaction mode using electrospray ionization in negative ion mode, with a capillary voltage of 3.3 kV, a source temperature of 150 °C and a desolvation temperature of 600 °C. Cone voltage and collision energy were optimized for each compound individually (see Table 5.1b for parent and product *m/z* values). Peak identification and quantification was performed using MassLynx software version 4.1. Calibration curves were run in duplicate from which one regression equation was generated. During data analysis, 5 peaks of unknown identity were found to be influenced by diet or LPS treatment, and these compounds are listed UK1 to UK5. These peaks were visible in the transitions *m/z* 295.2 > 195.2 and *m/z* 295.2 > 171.1. ARA, DHA and EPA were also determined using this method.

Compounds	*Parent (m/z)*	*Product (m/z)*
12-HHTrE	279.0	179.3
13-HODE	295.1	195.0
9-HODE	295.1	171.0
EPA	301.1	257.2
ARA	303.1	259.2
12,13-DiHOME	313.2	183.0
9,10-DiHOME	313.2	201.0
15-deoxy-d-12,14-PGJ_2	315.0	271.1
12-HEPE	317.2	179.0
5-HEPE	317.2	115.0
11,12-EET	319.1	167.0
12-HETE	319.1	179.2
5-HETE	319.1	203.1
14,15-EET	319.1	219.2
5,6-EET	319.1	191.3
11-HETE	319.1	167.0
8,9-EET	319.1	167.0
15-HETE	319.2	219.1
20-HETE	319.2	275.3
2,3-dinor-8-iso-$PGF_{2\alpha}$	325.1	237.2
DHA	327.1	283.1
9,10,13-TriHOME	329.2	171.1
9,12,13-TriHOME	329.2	211.1
PGB_2	333.2	174.9
LTB_4	335.1	194.8
14,15-DiHETrE	337.1	207.0
11,12-DiHETrE	337.1	166.9
5,6-DiHETrE	337.1	144.8
8,9-DiHETrE	337.1	127.0
17-keto-4(z),7(z),10(z),13(z),15(e),19(z) DHA	341.5	111.0
17-HDoHE	343.1	281.4
PGE_3	349.0	269.1
PGD_3	349.1	269.1
PGD_2	351.1	271.1
Lipoxin A_4	351.1	114.9
13,14-dihydro-15-keto-PGD_2	351.1	175.0
13,14-dihydro-15-keto-PGE_2	351.1	175.1
PGE_2	351.1	271.2
13,14-dihydro-15-keto-$PGF_{2\alpha}$	353.1	113.1
8-iso-$PGF_{2\alpha}$	353.1	193.0
11β-$PGF_{2\alpha}$	353.1	193.0
$PGF_{2\alpha}$	353.1	193.0
$PGF_{2\beta}$	353.2	193.1
10,17-DiHDoHE	359.1	152.9
Maresin	359.6	177.1
19,20-DiHDoPE	361.1	272.7
$TBXB_3$	367.1	168.9
$TBXB_2$	369.1	169.0
Resolvin D_2	375.1	175.0
Resolvin D_1	375.1	140.8
LTE_4	438.0	351.0
n-acetyl LTE_4	480.0	351.1
LTD_4	495.1	142.9

Table 5.1b: *m/z Values of parent and product ions of eicosanoids used in the LC-MS/MS analysis. Compounds are ranked based on their parent m/z value.*

Internal standards	*Parent (m/z)*	*Product (m/z)*
13-HODE-d4	299.2	198.2
ARA-d8	311.2	267.2
20-HETE-d6	325.1	281.3
15-HETE-d8	327.2	226.1
14,15-EET-d11	330.2	268.3
PGB_2-d4	337.1	178.9
CUDA	339.1	214.1
LTB_4-d4	339.1	197.1
8,9-DiHETrE-d11	348.2	127.0
PGE_2-d4	355.1	275.1
13-14-dihydro-15-keto-$PGF2_\alpha$-d4	357.1	187.0
8-iso-$PGF_{2\alpha}$-d4	357.1	196.9
11β-$PGF_{2\alpha}$-d4	357.1	313.4
$PGF_{2\alpha}$-d4	357.1	313.4
PGD_2-d9	360.3	280.1
$TBXB_2$-d4	373.1	173.0
LTD_4-d5	500.0	142.9

Table 5.1b (cont.): *m/z Values of parent and product ions of eicosanoids used in the LC-MS/MS analysis. Compounds are ranked based on their parent m/z value.*

Data analysis

Univariate analysis was performed with SAS version 9.1 (2002-2003 by SAS Institute Inc, Cary, NC, USA). Time-dependent effects of inflammation were analyzed using ANOVA. ANOVA assumptions were checked for each variable. If these assumptions were not met, rank transformation was applied for that particular variable. Partial tests were performed using Tukey-Kramer multiple comparison correction. Benjamini and Hochberg false discovery rate correction was applied to correct for false positives. In all statistical tests performed, the null hypothesis (no effect) was rejected at the 0.05 level of probability (α = 5%).

Only data from animals from which all analyses were completed were included in the univariate model. From one of the animals in the 8 hr saline group, the liver endocannabinoid analysis was not completed. The data from the t=0 saline treated animals was duplicated to make a t=0 LPS treated group to be analyzed in the univariate model. The animal experiment also contained 48 hr groups, but LPS treatment resulted in high mortality in these groups, for which the data from these groups is not presented in this paper.

Results

The metabolism of eicosanoids and endocannabinoids is complex, and only few studies have explored the effect of inflammation on their formation in detail. In this study, mice fed a fish oil diet were treated *i.p.* with saline or LPS, and the change in levels of 61 eicosanoids and endocannabinoids in plasma, liver, ileum and adipose tissue was followed during a 24 hrs time course.

The eicosanoids and endocannabinoids which were significantly altered by LPS treatment are listed in Table 5.2, with time curves presented in Figures 5.2-5 to illustrate the effect of LPS. Univariate data analysis was used to evaluate significant differences of LPS, and if necessary rank transformation was applied prior to ANOVA.

LPS treatment affected the plasma profiles of both eicosanoids and endocannabinoids

Treatment of mice with LPS had a time-dependent effect on the profile of eicosanoids in plasma (Table 5.2 and Figure 5.2). In general, LPS increased the levels of 16 eicosanoids, but different patterns were observed. For example; some compounds displayed a relatively delayed effect of LPS (*e.g.* 5,6-DiHETrE and LTE_4) with only significant effects after 24 hrs LPS, whereas others showed a relatively early increase followed by normalization (*e.g.* PGE_2). The n-3 fatty acid counterpart of PGE_2, PGE_3, however, was increased by LPS at all time points, which was most pronounced at 24 hrs after LPS injection. Furthermore, the time curves for PGE_2 and its metabolite 13,14-dihydro-15-keto-PGE_2 showed considerable overlap. The eicosanoids formed by the CYP pathway, such as 14,15-DiHETrE, 12,13-DiHOME and 11,12-DiHETrE were still increasing in the LPS treated mice at 24 hrs. In addition, also the fatty acids ARA and EPA were significantly increased by LPS after 4 hrs (ARA) and 8 hrs (ARA & EPA) respectively. $TBXB_2$ and 12-HHTrE were decreased bij LPS at all time points.

Plasma levels of the NAEs DHEA, EPEA, OEA, PEA and SEA were increased by LPS, typically from 4 hrs after LPS with a sustained or even stronger effect until 24 hrs for DHEA.

Fatty acids & n-3 derived metabolites		2 hr	4 hr	8 hr	24 hr
ARA	plasma	-	↑	↑	-
	liver	-	-	-	-
	ileum	↑	↑	↑	-
	adi. tiss.	-	↑	↑	↑
EPA	plasma	-	-	↑	-
	liver	-	-	-	-
	ileum	↑	↑	↑	-
	adi. tiss.	-	-	-	-
PGE_3	plasma	↑	↑	↑	↑
	liver	-	-	-	-
	ileum	-	-	-	-
	adi. tiss.	-	-	-	-
10,17-DiHDoHE	plasma	ND	ND	ND	ND
	liver	ND	ND	ND	ND
	ileum	-	-	-	-
	adi. tiss.	↑	↑	↑	↑
17 keto-DHA	plasma	↓	↓	↓	↓
	liver	↓	↓	↓	↓
	ileum	-	-	-	-
	adi. tiss.	↑	↑	↑	↑

		2 hr	4 hr	8 hr	24 hr
DHA	plasma	-	-	-	-
	liver	-	-	-	-
	ileum	↑	↑	↑	↑
	adi. tiss.	-	-	-	-
12-HEPE	plasma	-	-	-	-
	liver	-	-	-	-
	ileum	-	-	-	-
	adi. tiss.	↑	↑	↑	↑
$TBXB_3$	plasma	-	-	-	-
	liver	-	-	-	-
	ileum	↑	↑	↑	↑
	adi. tiss.	↑	↑	↑	↑
19,20-DiHoPE	plasma	↑	↑	↑	↑
	liver	-	-	-	-
	ileum	-	↑	↑	-
	adi. tiss.	-	↑	↑	↑

Table 5.2 (1/5): *Effect of LPS treatment on in vivo eicosanoid and endocannabinoid levels in mice. Only statistically significant effects are listed, with ↑ representing an increase and ↓ a decrease by LPS, respectively. ND indicates that the compound was not detected in the particular matrix, and '-' indicates that no statistical differences were observed. Adi. tiss.= adipose tissue.*

CYP metabolites		2 hr	4 hr	8 hr	24 hr
5,6-EET	plasma	ND	ND	ND	ND
	liver	ND	ND	ND	ND
	ileum	ND	ND	↑	-
	adi. tiss.	↑	↑	↑	↑
8,9-EET	plasma	ND	ND	ND	ND
	liver	-	-	-	-
	ileum	↑	↑	↑	↑
	adi. tiss.	ND	ND	ND	ND
11,12-DiHETrE	plasma	↑	↑	↑	↑
	liver	-	-	-	-
	ileum	↑	↑	↑	↑
	adi. tiss.	-	-	-	-
9,10-DiHOME	plasma	-	-	↑	↑
	liver	↓	↓	↓	↓
	ileum	-	-	-	-
	adi. tiss.	-	-	-	-

		2 hr	4 hr	8 hr	24 hr
5,6-DiHETrE	plasma	-	-	-	↑
	liver	-	-	-	-
	ileum	↑	-	↑	-
	adi. tiss.	-	-	-	-
8,9-DiHETrE	plasma	-	-	-	-
	liver	-	-	-	-
	ileum	↑	↑	↑	↑
	adi. tiss.	↑	↑	↑	↑
14,15-DiHETrE	plasma	-	↑	-	↑
	liver	-	-	-	-
	ileum	↑	↑	↑	↑
	adi. tiss.	-	-	-	-
12,13-DiHOME	plasma	-	-	↑	↑
	liver	-	-	-	-
	ileum	-	-	-	-
	adi. tiss.	-	-	-	-

Table 5.2 (2/5): *Effect of LPS treatment on in vivo eicosanoid and endocannabinoid levels in mice. Only statistically significant effects are listed, with ↑ representing an increase and ↓ a decrease by LPS, respectively. ND indicates that the compound was not detected in the particular matrix, and '-' indicates that no statistical differences were observed. Adi. tiss.= adipose tissue.*

COX metabolites		2 hr	4 hr	8 hr	24 hr
PGE_2	plasma	↑	↑	-	-
	liver	-	-	-	-
	ileum	-	-	-	-
	adi. tiss.	↑	↑	↑	↑
13,14-dihydro-15-keto-PGE_2	plasma	↑	↑	↑	↑
	liver	ND	ND	ND	ND
	ileum	-	-	-	-
	adi. tiss.	-	-	-	-
$TBXB_2$	plasma	↓	↓	↓	↓
	liver	-	-	-	-
	ileum	-	-	-	-
	adi. tiss.	-	-	-	-

		2 hr	4 hr	8 hr	24 hr
12-HHTrE	plasma	↓	↓	↓	↓
	liver	-	-	-	-
	ileum	-	-	-	-
	adi. tiss.	↑	-	-	-
13,14-dihydro-15-keto-PGF_{2a}	plasma	-	-	-	-
	liver	↑	↑	↑	↑
	ileum	-	-	-	-
	adi. tiss.	-	-	-	-

Table 5.2 (3/5): *Effect of LPS treatment on in vivo eicosanoid and endocannabinoid levels in mice. Only statistically significant effects are listed, with ↑ representing an increase and ↓ a decrease by LPS, respectively. ND indicates that the compound was not detected in the particular matrix, and '-' indicates that no statistical differences were observed. Adi. tiss.= adipose tissue.*

LOX metabolites		2 hr	4 hr	8 hr	24 hr
13-HODE	plasma	↑	↑	↑	↑
	liver	-	-	-	-
	ileum	-	-	-	-
	adi. tiss.	↑	↑	↑	↑
Lipoxin A4	plasma	-	-	-	-
	liver	-	-	-	-
	ileum	-	-	-	-
	adi. tiss.	-	-	↑	-
9,12,13-TriHOME	plasma	-	-	-	-
	liver	-	-	-	-
	ileum	-	-	-	-
	adi. tiss.	↑	↑	↑	↑

		2 hr	4 hr	8 hr	24 hr
LTE_4	plasma	ND	ND	ND	↑
	liver	ND	ND	ND	ND
	ileum	ND	ND	ND	ND
	adi. tiss.	↑	↑	↑	↑
5-HETE	plasma	ND	ND	ND	ND
	liver	-	-	-	-
	ileum	↑	↑	↑	↑
	adi. tiss.	↑	↑	↑	↑

Table 5.2 (4/5): *Effect of LPS treatment on in vivo eicosanoid and endocannabinoid levels in mice. Only statistically significant effects are listed, with ↑ representing an increase and ↓ a decrease by LPS, respectively. ND indicates that the compound was not detected in the particular matrix, and '-' indicates that no statistical differences were observed. Adi. tiss.= adipose tissue.*

NAEs		2 hr	4 hr	8 hr	24 hr			2 hr	4 hr	8 hr	24 hr
AEA	plasma	-	-	-	-		DHEA	↑	↑	↑	↑
	liver	↑	↑	↑	↑			↑	↑	↑	↑
	ileum	↑	↑	↑	↑			-	-	-	-
	adi. tiss.	-	-	-	-			-	-	-	-
EPEA	plasma	-	↑	↑	-		OEA	-	↑	↑	↑
	liver	-	↑	↑	-			↑	↑	↑	↑
	ileum	↑	↑	↑	↑			↑	↑	-	-
	adi. tiss.	-	-	-	-			-	-	↑	↑
PEA	plasma	-	↑	↑	↑		SEA	-	↑	↑	↑
	liver	-	-	-	-			-	-	-	-
	ileum	↑	↑	↑	↑			↑	↑	↑	↑
	adi. tiss.	-	-	-	-			-	-	-	-

Table 5.2 (5/5): *Effect of LPS treatment on in vivo eicosanoid and endocannabinoid levels in mice. Only statistically significant effects are listed, with ↑ representing an increase and ↓ a decrease by LPS, respectively. ND indicates that the compound was not detected in the particular matrix, and '-' indicates that no statistical differences were observed. Adi. tiss.= adipose tissue.*

LPS treatment had minor effects on liver eicosanoid levels

In contrast to plasma, only minimal effects of LPS treatment on liver eicosanoid levels were observed, with only 3 compounds significantly altered (Table 5.2 and Figure 5.3). Levels of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ were significantly increased by LPS over all time points. Levels of 17-keto-DHA and 9,10-DiHOME were decreased by LPS at all time points.

Liver levels of AEA, DHEA and OEA were increased by LPS at all time points, whereas EPEA was only increased after 4 and 8 hrs followed by normalization at 24 hr.

LPS treatment affected CYP-derived eicosanoids and NAE levels in ileum

Out of all components quantified, 12 eicosanoids were significantly increased by LPS (Table 5.2 and Figure 5.4) in the ileum. Most of the eicosanoids were increased at all time points, including three DiHETrE compounds, 8,9-EET, $TBXB_3$, 5-HETE, and the fatty acids (ARA, DHA and EPA). Interestingly, 5,6-EET was increased after 8 hrs in the LPS group and its metabolite 5,6-DiHETrE was increased after 2 and 8 hrs, whereas the other EETs and DiHETrEs were increased over a broader time range.

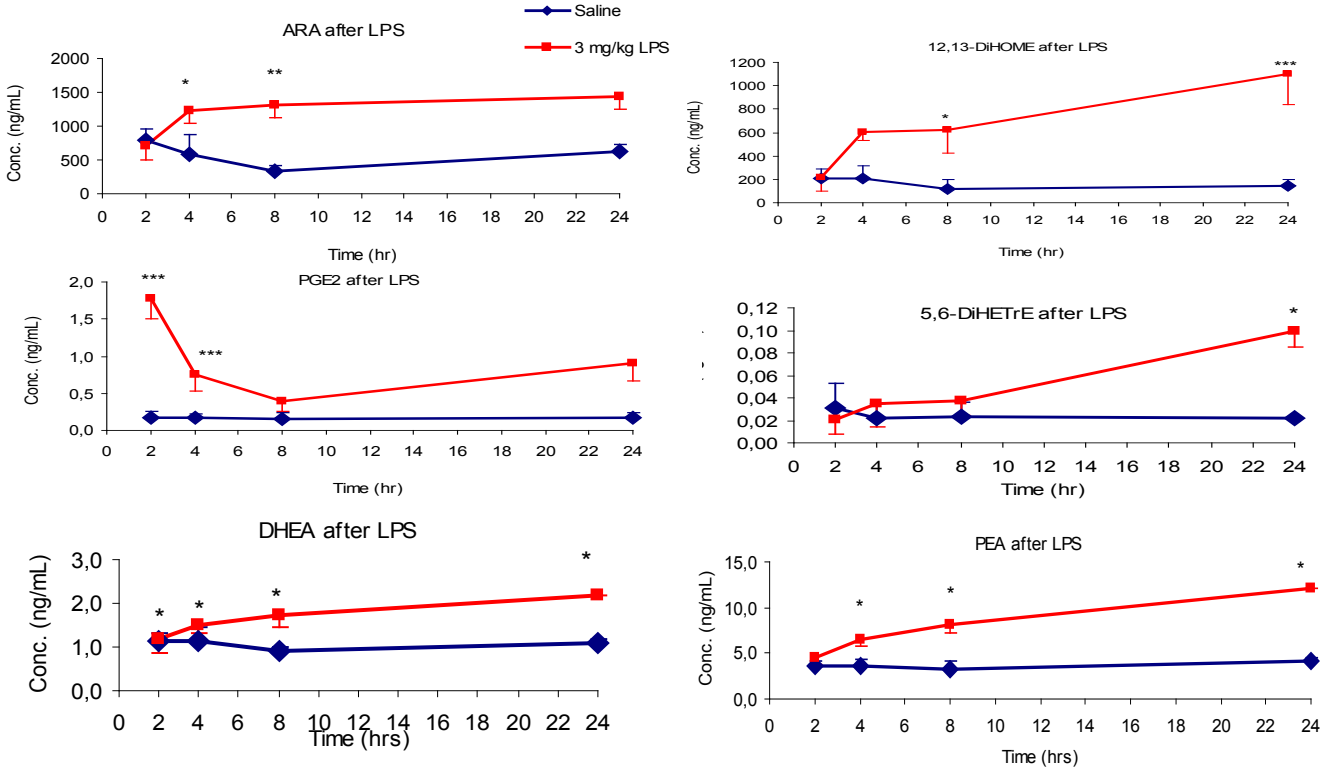
Levels of AEA, EPEA, OEA, PEA and SEA were increased 2 hrs after LPS stimulation and remained high at least until 4 hrs (OEA), but in general until 24 hrs after LPS stimulation.

Adipose tissue eicosanoid levels were differentially affected by LPS

In adipose tissue, 15 eicosanoids were significantly increased after LPS treatment, all belonging to several branches of the eicosanoid pathway (Table 5.2 and Figure 5.5). Higher levels of PGE_2, $TBXB_3$, 5-HETE, 5,6-EET, 8,9-DiHETrE, and the n-3 fatty acid derived compounds, 12-HEPE and 10,17-DiHDoHE, were observed at all time points. Levels of 19,20-DiHoDE, another n-3 derived compound, were also increased by LPS, but only from 4 hr and further. Other eicosanoids showed also different time effects, for example; 12-HHTrE only increased after 2 hrs of LPS, 10,17-DiHDoHE showed peak levels at 8 hrs, and ARA levels were still increasing at 24 hrs after LPS treatment. Adipose tissue levels of DHA and EPA were not affected by LPS.

From the NAEs, only OEA levels were increased at 8 and 24 hrs after LPS treatment.

Figure 5.2: *Time curves of mean plasma eicosanoid and endocannabinoid concentration levels in mice after treatment with either saline or LPS. Data indicate mean ± standard deviation (n=1 for 24 hr LPS plasma endocannabinoid analysis; n=3 for 24 hr LPS eicosanoid analysis; n=4 for all other analyses). Time points with a significant LPS effect are marked (* P < 0.05; ** P < 0.01; *** P < 0.001).*



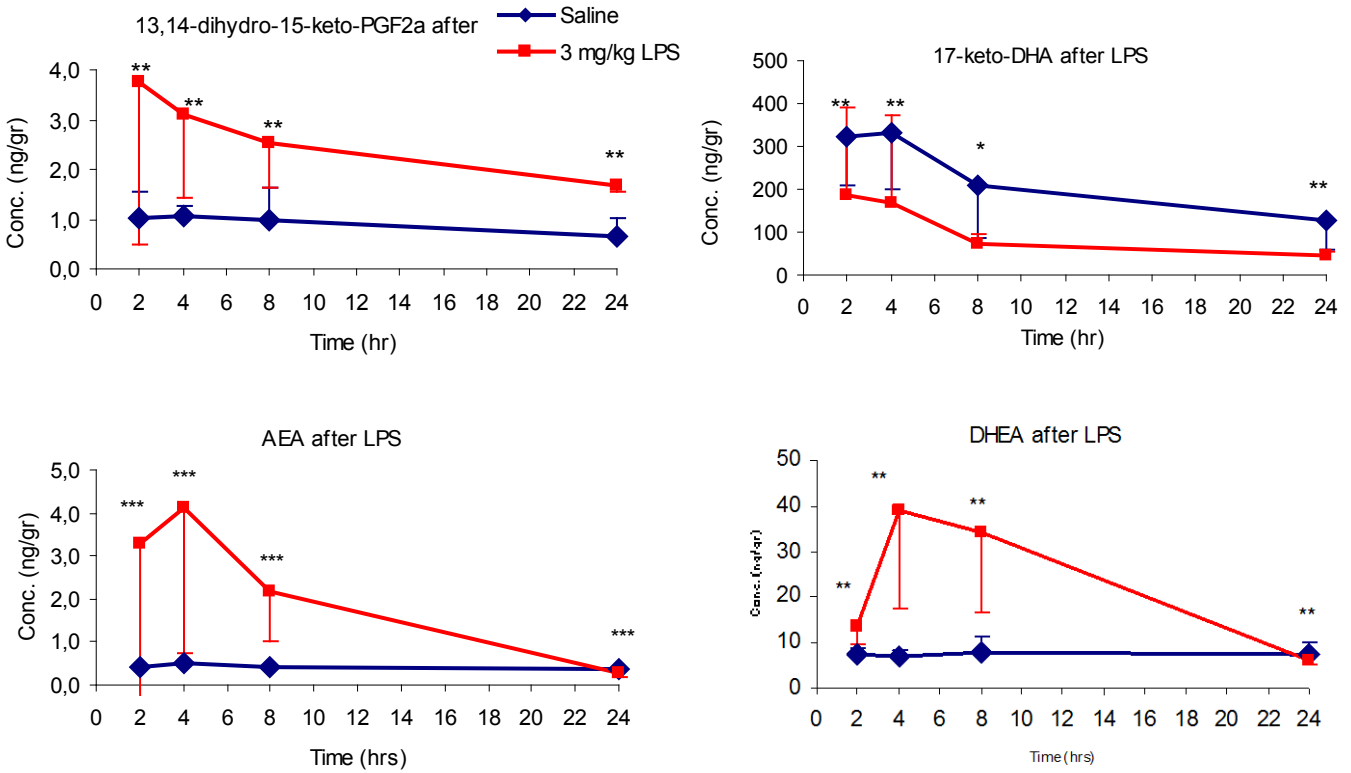
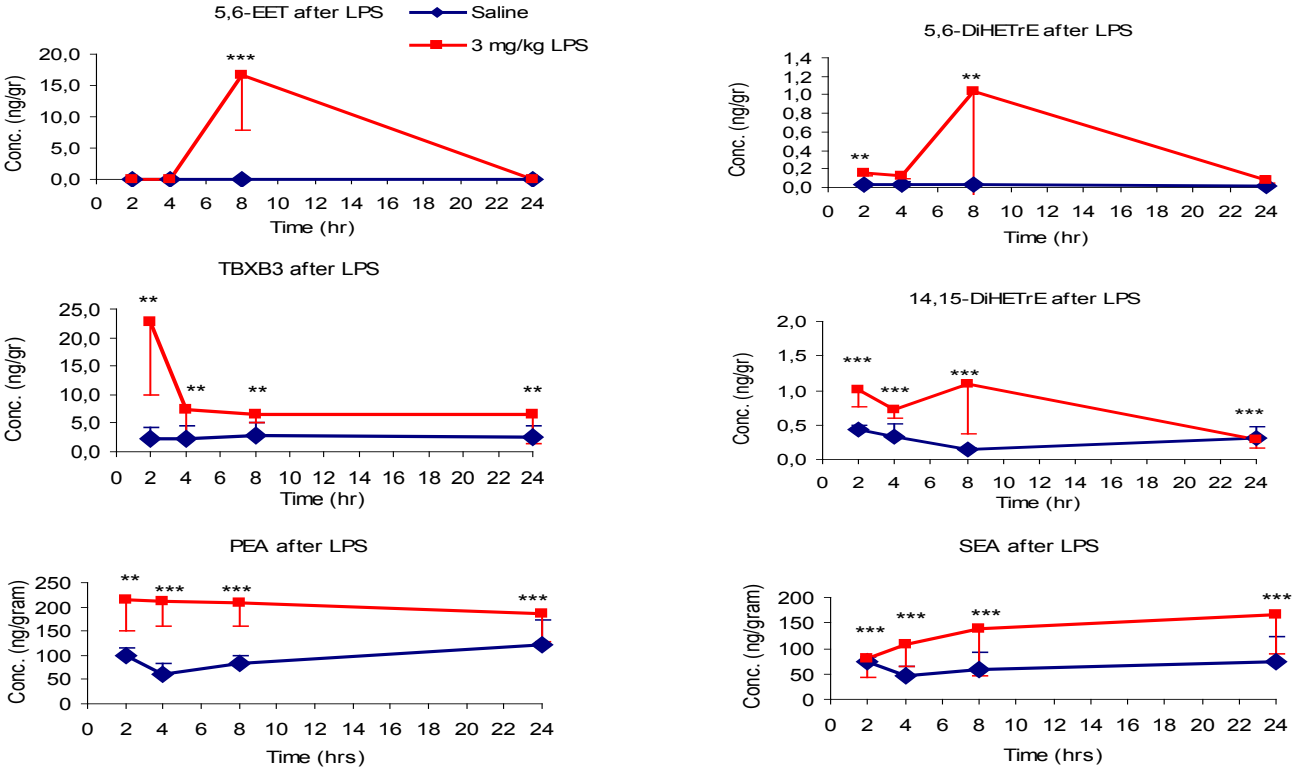


Figure 5.3: *Time curves of mean liver eicosanoid and endocannabinoid concentration levels in mice after treatment with either saline or LPS. Data indicate mean ± standard deviation (n=3 for 8 hr saline liver endocannabinoid analysis; n=3 for all 24 hr LPS analyses; n=4 for all other analyses). Time points with a significant LPS effect are marked (* P < 0.05; ** P < 0.01; *** P < 0.001).*

Figure 5.4: *Time curves of mean ileum eicosanoid and endocannabinoid concentration levels in mice after treatment with either saline or LPS. Data indicate mean ± standard deviation (n=3 for all 24 hr LPS analyses; n=4 for all other analyses). Time points with a significant LPS effect are marked (* P < 0.05; ** P < 0.01; *** P < 0.001).*



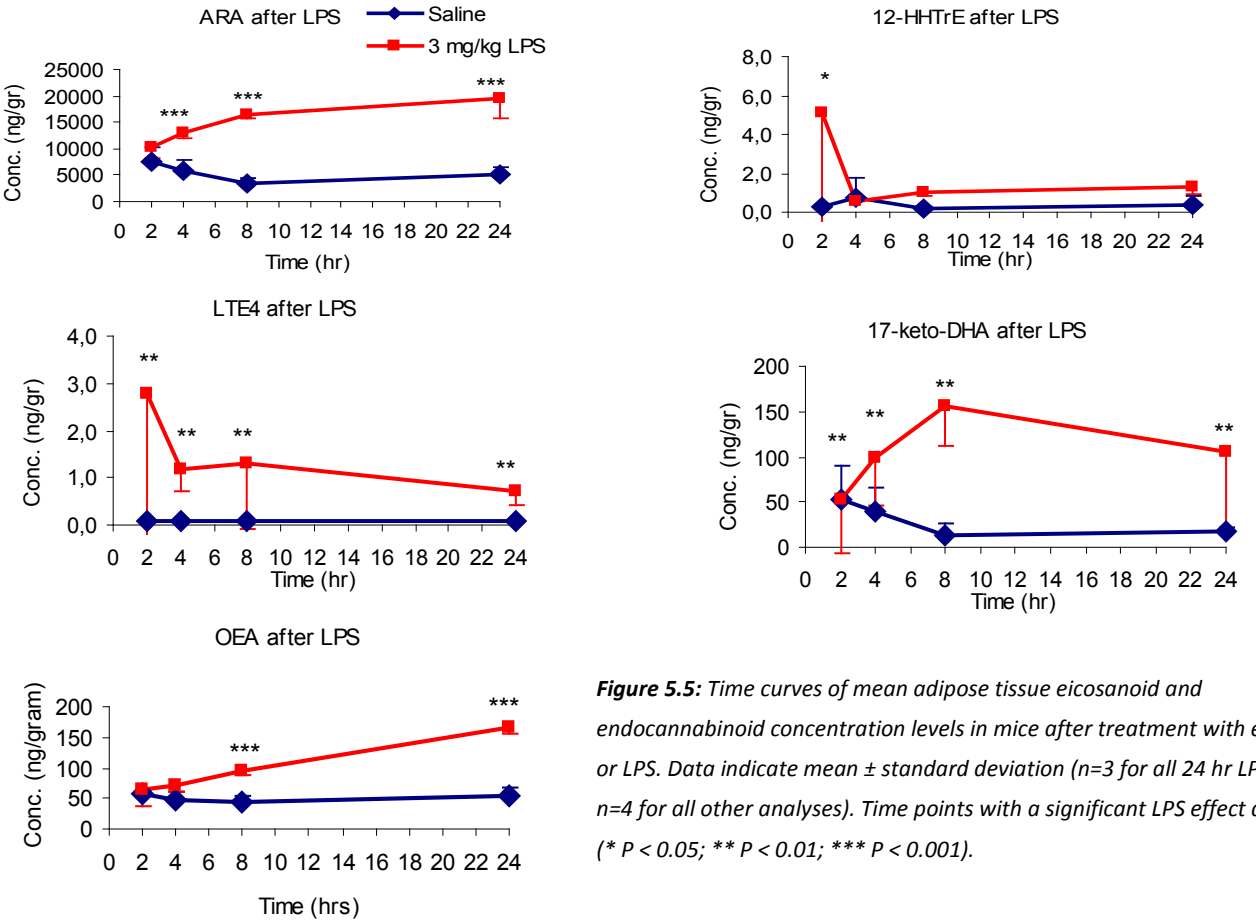


Figure 5.5: *Time curves of mean adipose tissue eicosanoid and endocannabinoid concentration levels in mice after treatment with either saline or LPS. Data indicate mean ± standard deviation (n=3 for all 24 hr LPS analyses; n=4 for all other analyses). Time points with a significant LPS effect are marked (* P < 0.05; ** P < 0.01; *** P < 0.001).*

Discussion

Eicosanoids and endocannabinoids are important regulators of inflammation, with highly dynamic concentrations. Considering the complexity of eicosanoid and endocannabinoid metabolism, it is likely that inflammation affects multiple compounds at the same time, and that differential effects between plasma and tissues exist. However, so far most studies have only focused on a limited number of (established) compounds in a restricted number of tissues or plasma instead of evaluating profiles of compounds. In addition, little is known about the effect of inflammation on (more recently discovered) n-3 fatty acid derived metabolites. The purpose of this study was to systematically evaluate the profiles of bioactive lipids using a multi-compartment targeted lipidomics approach in plasma and tissues following an inflammatory stimulus in mice fed a fish oil diet. The data show time- and tissue-dependent effects in all compartments, with the highest number of eicosanoids altered in ileum and adipose tissue.

Due to technical advances in the field of mass spectrometry, the capability to adequately measure more compounds at lower concentration levels in biological matrices has increased. Tandem MS methods capable of measuring > 80 compounds or more in a single analytical run are not unusual [23-25]. When considering all pathways and substrates in the fatty acid metabolome, this number of compounds is easily met. Their simultaneous quantification could potentially give new insights in associations between bioactive lipids after *e.g.* inflammatory stimulus or dietary intervention. Despite the advances in analytical chemistry, adequate bioactive lipid quantification is challenging due to their broad concentration range and different physiological characteristics. However , it is of high relevance to understand which bioactive lipids are formed and present at what time point after *e.g.* an inflammatory stimulus or dietary intervention, because regulation of their synthesis can depend on e.g. enzyme activity, time and availability of parent compounds.

Al already mentioned above, it can be concluded from our study that LPS treatment changed the profile of many bioactive lipids, which differed in time and were tissue specific, and with the most pronounced effects in ileum and adipose tissue. In the discussion, we will mainly focus on contrastive effects of LPS on eicosanoids across plasma and tissues, differential effects of LPS on eicosanoids derived from CYP enzymes, n-3 fatty acid derived eicosanoids, and finally effects on NAEs.

Interestingly, some contrastive effects of LPS on eicosanoids were seen. LPS treatment decreased the levels of 12-HHTrE, a PGH_2 metabolite [26] in plasma, whereas adipose tissue levels were increased. However, at the same time, plasma and adipose tissue levels of PGE_2, another PGH_2 metabolite [27], were increased by LPS. This could indicate selective synthesis towards PGE_2 at the expense of 12-HHTrE in plasma, or specific breakdown or uptake in *e.g.* adipose tissue of plasma 12-HHTrE. Similar opposite effects were observed for 9,10-DiHOME, which was increased in plasma, but decreased in liver after LPS. The significance and origin of this is not understood and requires further investigation.

LPS treatment affected eicosanoids derived from different enzymes, including COX- and LOX metabolites, and several differential effects were observed for CYP metabolites. Interestingly, we observed little effects in liver although it contains considerable levels of CYP. Theken and cowokers recently demonstrated that intrahepatic LPS reduced liver expression of several CYP enzymes after 24 hrs in mice, paralleled by decreased EET and HETE levels [28]. In our study, LPS treatment altered only few eicosanoids in liver, with only 13,14-dihydro-15-keto-$PGF_{2\alpha}$ increased and no effects on EETs or DiHETrEs were observed, but this discrepancy might relate to the fish oil diet which was used in the present study or the administration route of LPS. However, other work demonstrated that chemically induced colitis can reduce liver CYP enzyme expression [29], indicating that hepatic CYP expression can also be decreased when the site of inflammation is the intestine, such as the *i.p.* route in our work. Levels of 9,10-DiHOME, another compound related to the CYP pathway, was reduced in our work, which is in line with the effects on CYP enzymes published by Theken and coworkers.

In contrast to liver, we have observed numerous effects of LPS treatment on CYP metabolites in plasma and ileum. Levels of 11,12-EET, 5,6- DiHETrE, 11,12-DiHETrE and 14,15-DiHETrE in plasma, and 5,6-DiHETrE and 14,15-DiHETrE in ileum were increased by LPS. So far, most research on EETs focuses on effects on the vascular bed, cardiovascular health and ion channels, with few reports on intestinal effects. Their increase after LPS in the intestine might point to a new role for these compounds in gut inflammation, as these compounds are also known for their anti-inflammatory properties [3]. Ileum shows highest levels of 5,6-EET and 8,9-EET at 8 hrs after LPS, whereas in adipose tissue 5,6-EET levels peaked already after 2 hrs LPS, suggesting differential regulation of EET levels across different tissues.

The metabolism of n-3 fatty acids to *e.g.* eicosanoids and resolvins has received much attention due to their proposed anti-inflammatory properties. The effect of LPS treatment on n-3 fatty acid derived mediators could also be investigated as the mice received a fish oil diet for 6 weeks prior to the LPS treatment. Therefore, we hypothesized that these metabolites would be liberated by an inflammatory stimulus after consuming a fish oil diet. Levels of 10,17-DiHDoHE, 19,20-DiHoPE, and 17-keto-DHA were increased by LPS in certain instances. In contrast to our expectations, no resolvins were detected in any tissue at any time after LPS injection. The class of resolvins has attracted much attention over the last years due to their active inflammation resolving properties in relation to n-3 fatty acids [12, 30]. Possibly, resolvins are not released until 24 hrs after initiation of inflammation, or their levels remained below the detection limit of the LC-MS/MS method. Despite this, 10,17-DiHDoHE, also known as Protectin DX, and structurally related to 17-HDoHE, which is a marker for resolving synthesis [30, 31], was increased in adipose tissue after LPS challenge. The role of this compound in adipose tissue inflammation has not been explored yet, but is potentially relevant for metabolic diseases such as the metabolic syndrome which is characterized by adipose tissue inflammation. In contrast to effects on 10,17-DiHDoHE, no effects of LPS treatment on 17-HDoHE levels were found in the present study.

Next tot the resolvins and related compounds, n-3 fatty acids can also be converted to certain prostaglandins, such as PGE_3. LPS treatment triggered the increase of PGE_3 in plasma and $TBXB_3$ in ileum and adipose tissue. Ileal $TBXB_3$ levels peaked after 2 hrs, whereas in adipose tissue the increase was more gradual. These compounds have pro-inflammatory and pro-aggregatory properties, but are less potent than their ARA-derived counterparts and may thus be involved in the anti-inflammatory properties associated with dietary intake of n-3 fatty acids. PGE_2 and PGE_3 were not detected in plasma after saline, but were visible after LPS treatment. In plasma however, PGE_2 showed early peak levels and no significant increase after 24 hrs of LPS, whereas PGE_3 levels gradually increased over time with the strongest increase after 24 hrs. PGE_2 is a commonly studied plasma marker of an inflammatory response. Our data demonstrate again that studying markers like PGE_2 in isolation from other markers may lead to the wrong conclusion that the inflammatory response might have already turned down. Although it is accepted that both PGE_2 and PGE_3 are synthesized via the same

pathway, clearly different factors are involved in determining their ultimate levels. A comparable pattern is seen for the EETs/DiHETrEs in ileum, where 5,6-EET and its metabolite 5,6-DiHETrE were only increased after 8 hr, whereas other EETs and DiHETrEs were increased over a broader time range, although with peak levels after 8 hrs. It has been described that different CYP enzymes synthesize distinct proportions of the different EETs, with *e.g.* CYP2C8 and CYP2C9 producing specific ratios of 11,12-EET and 14,15-EET [32]. Inflammation is known to have different effects on CYP enzymes [33], thereby potentially affecting the EET profile. Specific regulation of CYP enzymes by LPS in the ileum might explain this difference, but further research should elucidate this.

TBXB2, the inactive metabolite from TBXA2, was decreased in plasma after LPS at all time points. TBXA2 is normally involved in platelet aggregation and vasoconstriction. Previous work demonstrated that during endotoxemic shock TBXA2 levels would first increase, followed by a subsequent decrease [34]. Interestingly, in the present study, 24 hrs after LPS administration, the mice showed severe signs of systemic inflammation, including shock, which might relate to the decrease in TBXA2 levels, but this remains speculative at this point. Moreover, it is likely that the shock symptoms were caused by alterations in the eicosanoid and endocannabinoid metabolomes as a whole, rather than by individual compounds.

LPS clearly increased NAE levels in ileal tissue, resulting in increased levels of most NAEs from 2 hrs after LPS treatment, with enhanced levels still present after 8 or 24 hrs. Increases in plasma or adipose tissue NAE levels were usually at later time points, if present. These findings are in line with a previously hypothesized role of the endocannabinoid system in protecting against inflammatory stress in the gut [17, 20] or elsewhere [18, 21]. Interestingly, although levels of EPEA were affected by LPS in both plasma and ileum, DHEA levels were barely affected. In addition, in contrast to previous reports suggesting increased levels of systemic AEA during inflammation [19], we did not find significant effects on plasma AEA levels after LPS treatment. This might relate to the differences in diets between the studies, as dietary n-3 fatty acids are known to decrease levels of AEA in at least peripheral tissues [35-37]. The dietary fish oil might have decreased baseline plasma AEA levels, and this might translate into a poor response of plasma AEA to inflammatory stress.

In contrast to limited effects on hepatic eicosanoid levels, hepatic levels of the endocannabinoids AEA, DHEA, EPEA and OEA were affected by LPS. Other work, investigating effects of hepatic reperfusion injury on liver endocannabinoids, also observed increased levels of AEA and OEA [38]. Interestingly, the latter study also showed that AEA and 2-AG levels correlated with markers of inflammation and tissue damage, and that targeting CB2 receptors resulted in decreased inflammatory responses after reperfusion stress. Considering the anti-inflammatory properties of DHEA and EPEA [15, 16] and binding to CB2 [39], their levels might also be involved in limiting hepatic tissue damage during inflammatory or reperfusion stress.

Despite pronounced effect on eicosanoids in adipose tissue, only OEA levels were affected by LPS here. Obesity, also characterized by induction of inflammation in the adipose tissue compartment [40], is known to result in changes in endocannabinoid levels. Epididymal fat of diet-induced obese mice or visceral fat from obese humans contained more 2-AG than their controls, but also effects of obesity on AEA were found [41]. Unfortunately, the authors did not report any effects on OEA or other NAEs in these studies. Interestingly, in subcutaneous adipose tissue, a high fat diet reduced AEA and 2-AG levels [42]. This shows that different adipose tissue compartments can react differently to different types of inflammatory stress, and more work is needed to understand this.

In summary, we demonstrated that treatment of mice with LPS when fed a fish oil diet changed the profile of eicosanoids and endocannabinoids in plasma, liver, ileum and adipose tissue. Effects were observed during a 24 hr time course, with tissue and compound specific differences. Feeding mice with a fish oil diet yielded detectable levels of n-3 derived eicosanoids and endocannabinoids. Markers of resolvin synthesis were detected, but the resolvins themselves were not detectable 24 hrs after induction of inflammation. The data indicate that changes in plasma profiles do not reflect effects in tissues, thus meaning that extrapolating effects in plasma to biological effects in tissues is not valid without further investigation. Nevertheless, it is clear that during *in vivo* inflammation the levels of eicosanoids and endocannabinoids are highly dynamic. Therefore, if we want to better understand the role of eicosanoids in endocannabinoids in (patho)-physiology, it is important to determine the profile of these metabolomes rather than quantifying individual compounds.

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Chapter 6

Fish oil and inflammatory status alter the n-3 to n-6 balance of the endocannabinoid and eicosanoid metabolomes in mouse plasma and tissues

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Abstract

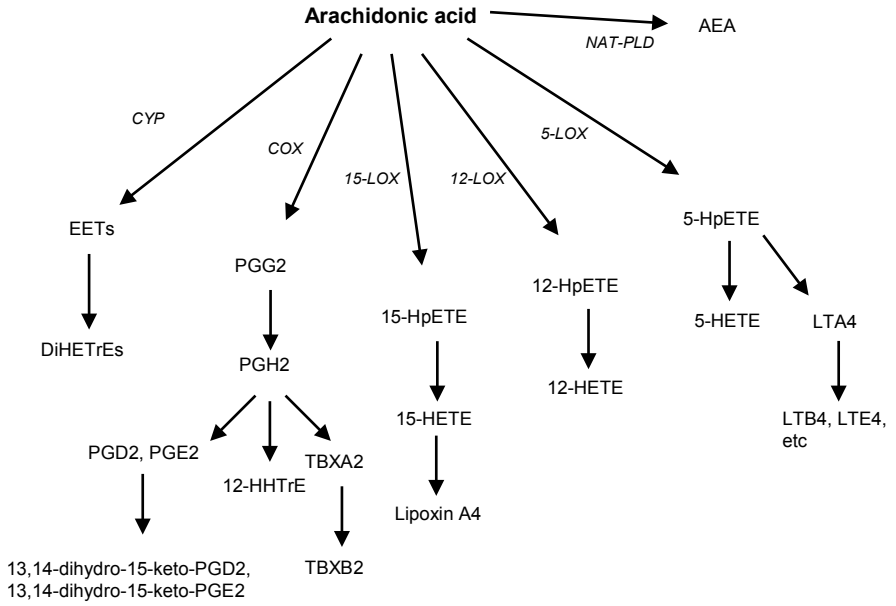
It is well established that dietary intake of n-3 fatty acids is associated with anti-inflammatory effects, and this has been linked to modulation of the eicosanoid and endocannabinoid metabolomes. However, the amount of data on specific tissue effects is limited, and it is not known how inflammation affects this relation. In the present study we systematically explored the combined effects of n-3 fatty acid diets and inflammation on the *in vivo* endocannabinoid and eicosanoid metabolomes using a multicompartment, detailed targeted lipidomics approach. Male C57BL/6 mice received diets containing 0%, 1%, or 3% w/w fish oil (FO) for 6 weeks, after which 2 mg/kg LPS or saline was administered *i.p.* Levels of endocannabinoids/*N*-acylethanolamines (NAEs) and eicosanoids, covering n-3 and n-6 fatty acid derived compounds, were determined in plasma, liver, ileum and adipose tissue using LC-MS/MS. FO generally increased 'n-3' NAEs and eicosanoids at the expense of compounds derived from other fatty acids, affecting all branches of the eicosanoid metabolome. LPS generally increased levels of endocannabinoids/NAEs and eicosanoids, with opposing effects across plasma and tissues. Multivariate data analysis revealed that separation between diet groups in the saline treated groups was primarily explained by decreases in other than n-3 derived compounds. In the LPS treated groups, the separation was primarily explained by increases in n-3 derived compounds. In conclusion, FO caused marked changes in the n-3 to n-6 balance of the endocannabinoid and eicosanoid metabolomes, with specific effects depending on inflammatory status.

Introduction

Dietary intake of long-chain n-3 polyunsaturated fatty acids (PUFAs), like docosahexaenoic acid (DHA; 22:6 n-3) and eicosapentaenoic acid (EPA; 20:5 n-3), is known to have beneficial health effects in both humans and animals, which are partly explained by a reduction of inflammatory processes [1-4]. The mechanisms behind this are not completely understood, but involve binding of n-3 PUFAs to GPR120 [5], their conversion to resolvins [6], and the alteration of the eicosanoid balance [1]. Increased dietary intake of n-3 PUFAs leads to enhanced incorporation of DHA and EPA in cell membranes, at the expense of incorporation of the n-6 PUFA arachidonic acid (ARA; 20:4 n-6). This results in decreased synthesis of ARA-derived eicosanoids, for example prostaglandin E_2 (PGE_2), after *e.g.* an inflammatory stimulus [7]. At the same time, increased levels of n-3 fatty acid derived eicosanoids are observed. These n-3 fatty acid derived metabolites are often referred to as '3-series' or '5-series' eicosanoids, comprising structures like prostaglandin D_3 (PGD_3), PGE_3, thromboxane B_3 ($TBXB_3$), and 5-hydroxyeicosapentaenoic acid (5-HEPE), or leukotriene B_5 (LTB_5), respectively (see Figure 6.1a and 6.1b for an overview of eicosanoids and their origin). These compounds are in general also pro-inflammatory, but considered less potent than the ARA-derived metabolites under certain circumstances, thereby contributing to a reduction of the general inflammatory status and specific inflammatory processes associated with fish oil consumption [1, 3, 4].

Over the last decades, several endocannabinoids and related *N*-acyl ethanolamines (NAEs) have emerged as important regulators of metabolism and inflammation [8-11]. Like the eicosanoids, these compounds are also derived from fatty acids following incorporation in cell membranes [12, 13]. Arachidonoyl ethanolamide (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG) are two endocannabinoids which are derived from arachidonic acid, but combinations derived with other fatty acids also exist, such as palmitoyl ethanolamide (PEA) and the n-3 fatty acid derived NAEs docosahexaenoyl ethanolamide (DHEA), eicosapentaenoyl ethanolamide (EPEA). Both AEA and PEA are known for their anti-inflammatory properties [14, 15].

Several *in vitro* and animal studies have demonstrated a link between availability of specific fatty acids in the diet and the presence of endocannabinoids and related NAEs. Berger and coworkers reported enhanced levels of anandamide and 2-AG in piglet brain after feeding milk supplemented with ARA, with a diet rich in



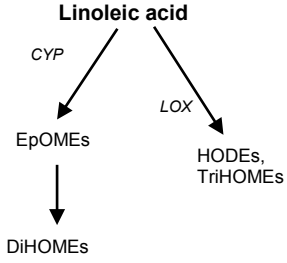


Figure 6.1a: *Overview of enzymatic pathways involved in eicosanoid and NAE synthesis. Arachinoic acid is the best studied precursor for eicosanoid synthesis, but linoleic acid can also serve as substrates for different enzymes, including COX, CYP and the LOX isoenzymes, yielding distinct eicosanoids and intermediates.*

DHA showing even higher levels of its NAE metabolite, DHEA [16]. Wood and coworkers showed that a two-week diet rich in DHA elevated plasma and brain levels of DHEA in mice, while decreasing plasma 2-AG [17]. Artmann and coworkers demonstrated that feeding rats a fish oil (FO) diet, by nature rich in n-3 PUFAs, decreased jejunal levels of AEA and PEA, but increased the levels of n-3 NAEs DHEA and EPEA [18]. Fish oil also decreased adipose tissue levels of AEA and 2-AG in a rat model of obesity [19]. It thus seems that the profile of NAEs

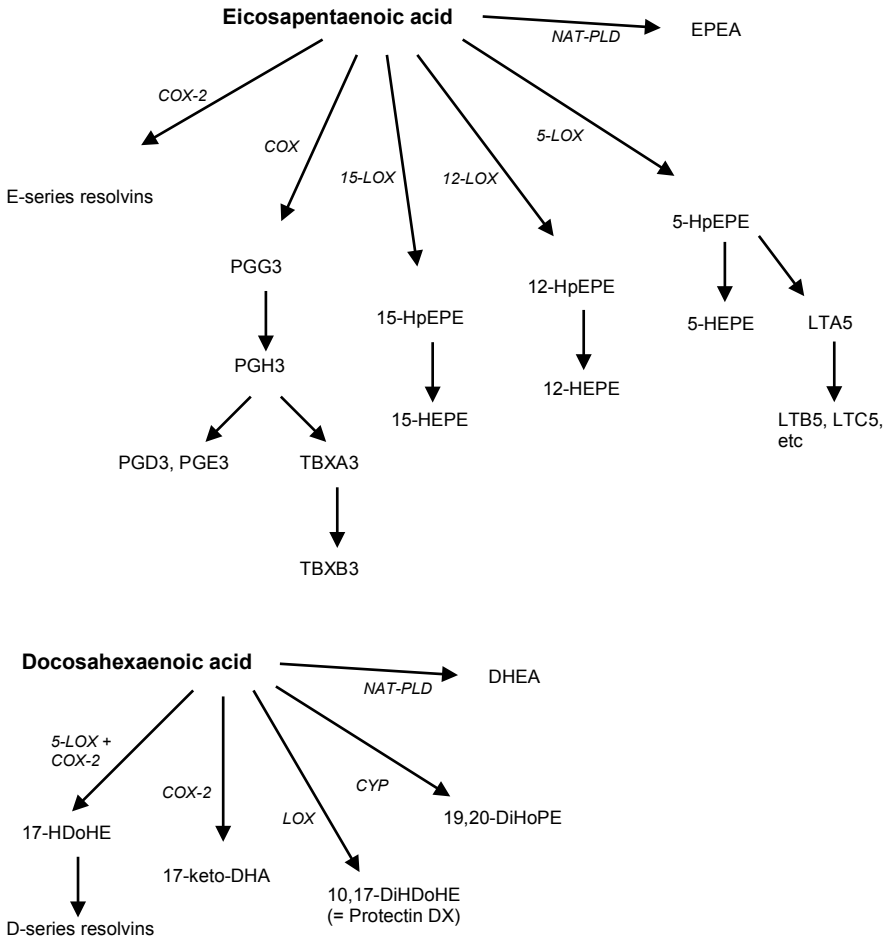


Figure 6.1b: *Overview of enzymatic pathways involved in eicosanoid and NAE synthesis. EPA and DHA can also serve as substrates for different enzymes, including COX, CYP and the LOX isoenzymes, yielding distinct eicosanoids, intermediates, and NAEs.*

represents the relative abundance of fatty acids in the diet. Recently, is was shown that DHEA and EPEA display anti-inflammatory properties in macrophages and adipocytes [20, 21], indicating that these compounds might be involved in the anti-inflammatory effects which are related to dietary n-3 PUFA intake.

In addition to diet, inflammation is also known to affect the synthesis and/or release of both eicosanoids and NAEs [22], but it is not known how inflammation itself affects *e.g.* DHEA and EPEA tissue levels *in vivo*. Moreover, it is not known if changes induced by dietary fatty acids also persist under inflammatory conditions, or if the effect of diet is different under inflammatory conditions.
In the present study, we systematically explored in detail the (combined) effect of dietary fish oil and inflammation on levels of endocannabinoids/NAEs and eicosanoids in plasma, liver, ileum and adipose tissue of wild type C57BL/6 mice using a targeted lipidomic approach. In total, levels on 61 compounds were analyzed, including levels of PGE_3, PGD_3, $TBX\text{-}B_3$, 5-HEPE, resolvin D_1, DHEA and EPEA. Both univariate and multivariate data analysis tools were used to assess differences in metabolite patterns between the intervention groups. Our data show in detail that dietary intake of fish oil shifted the n-3 to n-6 balance in the endocannabinoid and eicosanoid metabolomes in all tissues examined. In addition, the direction of this shift appeared to be affected by inflammation, and was different between the examined tissues.

Materials and methods

Chemicals and reagents

Lipopolysaccharide (0111:B4; LPS), indomethacin, paraoxon and butylated hydroxytoluene (BHT) were from Sigma (Steinheim, Germany). Phenylmethylsulfonyl fluoride (PMSF) was from Fluka (Steinheim, Germany). 12-[(tricyclo[3.3.1.13,7]dec-1-ylamino)carbonyl]amino]-dodecanoic acid (AUDA) and URB602 was purchased from Cayman (Ann Arbor, MI, USA). Milli-Q water (Milli-Q Advantage unit, Millipore, Amsterdam, The Netherlands) was used in all analyses. ULC-grade acetonitrile (ACN), formic acid (FA) and trifluoro acetic acid (TFA) were obtained from Biosolve (Valkenswaard, The Netherlands). LC-MS grade methanol was from Riedel-de-Häen (Steinheim, Germany). Isopropanol and ethanol were from JT Baker (Deventer, The Netherlands). All analytical and internal standards, except EPEA, were purchased from Cayman. EPEA was synthesized as described earlier [23]. For eicosanoids, stock solutions were prepared in ethanol, aliquoted and stored at -80 °C until analysis. For endocannabinoids/NAEs, stocks were prepared in ACN, aliquoted and stored at -80 °C until analysis. C8 SPE columns (Bond Elut; 200 mg, 3 mL) were from Varian Inc (Lake Forest, CA, USA). HLB SPE

columns (Oasis, 60 mg, 3 mL) were from Waters (Etten-Leur, The Netherlands). ELISA kits were from R&D Systems (Minneapolis, MN, USA).

Animal experiment

Wild type male C57BL/6 mice were obtained from Harlan (Horst, The Netherlands) and housed two or three per cage in a temperature controlled environment with a 12 hour light-dark cycle (light at 6.00-18.00). The mice, 4 weeks old at arrival, had free access to a standard run-in diet (AIN93-M, with a 4% w/w fat content whereof 1% soy bean oil and 3% high-oleic acid sunflower oil (HOSF)) for two weeks. At the age of 6 weeks the mice were divided in to three groups of 16 mice; group 1 was kept on the standard diet, group 2 received a diet containing AIN93-M with 1% fish oil (1% FO) (Marinol®), 2% HOSF, and 1 % soy bean oil. The third group had access to a diet containing AIN93-M with 1% soy bean oil and 3% fish oil (3% FO). The diets and water were available *ad libitum*. Diets were prepared by Research Diet Services (Wijk bij Duurstede, The Netherlands) and the Marinol® was a kind gift from Lipid Nutrition (Wormerveer, The Netherlands). Diets were stored in air-tight bags at -20 °C until just before feeding, and fresh food was provided two times per week to minimize oxidation of the fatty acids in the diet. GC-MS based analysis of the diets confirmed that the correct amounts of DHA and EPA were present, and re-analysis after 4 weeks revealed that its amounts were stable under the described conditions (data not shown). Food consumption and animal weight were measured two times per week, revealing no differences in food intake or body weight between the diet groups.

The diets were continued for six weeks, after which the animals received either *i.p.* saline (8 mice per diet group) or 2 mg/kg LPS (8 mice per diet group). After 24 hrs, the animals were anesthetized, blood was collected from the orbital sinus and captured in 1.3 mL EDTA coated tubes (Sarstedt; Etten-Leur, The Netherlands) and put on ice until centrifugation (10', 10,000 rpm at 4 °C). After centrifugation, plasma was aliquoted. For eicosanoid analysis, 200 µL plasma was stored in 1 mL methanol containing paraoxon, BHT, AUDA, indomethacin, and PMSF to prevent eicosanoid oxidation and breakdown. For endocannabinoid/NAE analysis, 100 µL plasma was stored in the presence of PMSF and URB602. Subsequently, the animals were sacrificed by cervical dislocation after which liver, ileum and adipose tissue were collected and immediately snap-frozen in liquid nitrogen. All plasma and tissue samples were stored at -80 °C until further analysis. Analysis of plasma

interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) levels confirmed that LPS had triggered an inflammatory response by showing strongly increased IL-6 and MCP-1 levels in LPS-treated mice (data not shown).
The study was conducted according to the Netherlands Law on Animal Experiments, and approved by the local Animal Experiments Committee of Wageningen University.

Extraction of endocannabinoids/NAEs from plasma
Plasma (100 µL) was thawed and 400 µL extraction mixture containing 100 µM PMSF and internal standards (AEA-d8, 2-AG-d8 and OEA-d4) in ACN was added while the sample was gently vortexed. After subsequent centrifugation (5' at 13,000 rpm and RT), the supernatant was transferred to a clean eppendorf tube and evaporated to dryness in a vacuum concentrator (Scanvac; Lynge, Denmark). The dried extracts were reconstituted in 100 µL ACN containing 0.1% TFA and used for LC-MS/MS analysis.

Extraction of endocannabinoids/NAEs from tissues
Endocannabinoid/NAE were extracted from freeze-dried liver and ileum using a method adapted from a previously published protocol for plasma [24]. Approximately 50 mg freeze-dried liver or 10 mg freeze-dried ileum were extracted by adding 1 mL extraction mixture (ACN) and sonication. The samples were centrifuged (5' at 14,000 rpm), the supernatant was transferred to a clean 15 mL tube, and this was repeated once. The pooled ACN fractions were diluted with MQ water containing 0.13% TFA until the final ACN concentration was 20% prior to SPE clean-up as described before [24]. In short, columns were washed with 20% v/v ACN in MQ water containing 0.1% TFA, eluted with 80% v/v ACN in MQ water containing 0.1% TFA and evaporated to dryness using vacuum centrifugation. The dried extracts were reconstituted in 100 µL ACN containing 0.1% TFA and used for LC-MS/MS analysis.
For adipose tissue, approximately 100 mg 'wet' tissue was extracted with 1 mL extraction solution (ACN) by sonication. The samples were centrifuged for 5' at 14,000 rpm and RT, the supernatant was transferred to a clean 2.0 mL eppendorf tube, and the ACN extraction was repeated once. The 2 mL ACN extract was subsequently evaporated to dryness, reconstituted in 100 µL ACN containing 0.1% TFA and used for LC-MS/MS analysis.

Compounds	*Parent (m/z)*	*Product (m/z)*
AEA	348	62
2-AG (NH_4 adduct)	379 (396)	287
DHEA	372	62
EPEA	346	62
DLE	350	62
OEA	326	62
PEA	300	62
SEA	328	62
Internal standards		
AEA-d8	356	63
OEA-d4	330	66
2-AG-d8 (NH_4 adduct)	387 (404)	294

Table 6.1a: *m/z Values of parent and product ions of endocannabinoids/NAEs used in the LC-MS/MS method. For tissue, 2-AG analyses (performed on TSQ Quantum MS), the ammonium adduct was the most dominant parent ion. For plasma 2-AG analyses (performed on Xevo TQ-S MS), the molecular ion (M+H) was the most dominant parent.*

LC-MS/MS analysis of endocannabinoids/NAEs

Two LC-MS/MS systems were used for endocannabinoid/NAE analysis. Plasma extracts were analyzed by UPLC coupled to a Xevo TQ-S mass spectrometer (Waters; Etten-Leur, The Netherlands) because high sensitivity was essential for adequate quantification in extracts obtained from 100 µL plasma samples. Liver, ileum and adipose tissue were analyzed on a Surveyor HPLC coupled to a TSQ Quantum Discovery mass spectrometer (Thermo Finnigan; Breda, The Netherlands).

For the UPLC-Xevo system, 3 µL plasma extract was injected on a Acquity C8 BEH UPLC column (2.1 x 100 mm, 1.7 µm) and was separated using gradient elution with a stable flow of 500 µL/min. The gradient started with 100% A (40:40:20 v/v/v of MQ water : methanol : ACN with 0,1% FA) which was maintained until 0.35 minutes, followed by a linear increase to 100% B (7:3 v/v methanol : ACN with 0.1% FA) which was achieved at 7.0 minutes and was maintained until 9.0 minutes. Finally, the column equilibrated for 3 minutes at 100% A. The column was maintained at 60 °C during analysis, and the samples were kept at 10 °C. The MS was operating in selective reaction mode using electrospray ionization in

Compounds	*Parent (m/z)*	*Product (m/z)*
12-HHTrE	279.0	179.3
13-HODE	295.1	195.0
9-HODE	295.1	171.0
EPA	301.1	257.2
ARA	303.1	259.2
12,13-DiHOME	313.2	183.0
9,10-DiHOME	313.2	201.0
15-deoxy-d-12,14-PGJ_2	315.0	271.1
12-HEPE	317.2	179.0
5-HEPE	317.2	115.0
11,12-EET	319.1	167.0
12-HETE	319.1	179.2
5-HETE	319.1	203.1
14,15-EET	319.1	219.2
5,6-EET	319.1	191.3
11-HETE	319.1	167.0
8,9-EET	319.1	167.0
15-HETE	319.2	219.1
20-HETE	319.2	275.3
2,3-dinor-8-iso-$PGF_{2\alpha}$	325.1	237.2
DHA	327.1	283.1
9,10,13-TriHOME	329.2	171.1
9,12,13-TriHOME	329.2	211.1
PGB_2	333.2	174.9
LTB_4	335.1	194.8
14,15-DiHETrE	337.1	207.0
11,12-DiHETrE	337.1	166.9
5,6-DiHETrE	337.1	144.8
8,9-DiHETrE	337.1	127.0
17-keto-4(z),7(z),10(z),13(z),15(e),19(z) DHA	341.5	111.0
17-HDoHE	343.1	281.4
PGE_3	349.0	269.1
PGD_3	349.1	269.1
PGD_2	351.1	271.1
Lipoxin A_4	351.1	114.9
13,14-dihydro-15-keto-PGD_2	351.1	175.0
13,14-dihydro-15-keto-PGE_2	351.1	175.1
PGE_2	351.1	271.2
13,14-dihydro-15-keto-$PGF_{2\alpha}$	353.1	113.1
8-iso-$PGF_{2\alpha}$	353.1	193.0
11β-$PGF_{2\alpha}$	353.1	193.0
$PGF_{2\alpha}$	353.1	193.0
$PGF_{2\beta}$	353.2	193.1
10,17-DiHDoHE	359.1	152.9
Maresin	359.6	177.1
19,20-DiHDoPE	361.1	272.7
$TBXB_3$	367.1	168.9
$TBXB_2$	369.1	169.0
Resolvin D_2	375.1	175.0
Resolvin D_1	375.1	140.8
LTE_4	438.0	351.0
n-acetyl LTE_4	480.0	351.1
LTD_4	495.1	142.9

Table 6.1b: *m/z Values of parent and product ions of eicosanoids used in the LC-MS/MS analysis. Compounds are ranked based on their parent m/z value.*

Internal standards	*Parent (m/z)*	*Product (m/z)*
13-HODE-d4	299.2	198.2
ARA-d8	311.2	267.2
20-HETE-d6	325.1	281.3
15-HETE-d8	327.2	226.1
14,15-EET-d11	330.2	268.3
PGB_2-d4	337.1	178.9
CUDA	339.1	214.1
LTB_4-d4	339.1	197.1
8,9-DiHETrE-d11	348.2	127.0
PGE_2-d4	355.1	275.1
13-14-dihydro-15-keto-$PGF2_\alpha$-d4	357.1	187.0
8-iso-$PGF_{2\alpha}$-d4	357.1	196.9
11β-$PGF_{2\alpha}$-d4	357.1	313.4
$PGF_{2\alpha}$-d4	357.1	313.4
PGD_2-d9	360.3	280.1
$TBXB_2$-d4	373.1	173.0
LTD_4-d5	500.0	142.9

Table 6.1b (cont.): *m/z Values of parent and product ions of eicosanoids used in the LC-MS/MS analysis. Compounds are ranked based on their parent m/z value.*

positive ion mode, with a capillary voltage of 1.5 kV, a source temperature of 150 °C and a desolvation temperature of 500 °C. Cone voltage and collision energy were optimized for each compound individually (see Table 6.1a for parent and product *m/z* values). Peak identification and quantification was performed using MassLynx software version 4.1. Calibration curves were run in duplicate from which one regression equation was generated.

For the analysis of liver, ileum and adipose tissue, a TSQ Quantum Discovery was used as described before [24]. Five µL extract was separated on an Xterra C8 MS column (2.1 x 150 mm, 3.5 µm) using gradient elution with a constant flow of 150 µl/min. The same solutions were used as in the Xevo system, but now 1 g/L ammonium acetate was added (the most dominant parent for 2-AG in this MS is the ammonium adduct). The gradient started with 100% A which was maintained until 2.0 minutes, followed by a linear increase to 100% B which was achieved at 8.00 minutes and maintained until 16.0 minutes, and the column was left to equilibrate for 5 minutes at 100% A. The column was maintained at 40 °C during analysis and the samples were cooled at 4 °C. The MS was operating in selective reaction mode using electrospray ionization in positive ion mode, with a capillary voltage of 4.5 kV and a capillary temperature of 350 °C. Cone voltage and collision energy were optimized for each compound individually. Peak identification and

quantification was performed using LCquan software version 2.5.5. Calibration curves were run in duplicate from which one regression equation was generated.

Extraction of eicosanoids from plasma

Internal standards were added to the plasma samples which were already precipitated with methanol (see section 'animal experiment'), and the samples were put on ice for 30 minutes. Samples were subsequently centrifuged (5' at 3000 x *g* and 4 °C) and the supernatant was transferred to a glass tube. Just before loading on activated HLB columns, 4.75 mL MQ water containing 0.1% v/v FA was added to the methanol extract, diluting the extract to 20% methanol. After loading, the columns were washed with 2 mL 20% methanol in MQ water containing 0.1% FA, and the columns were allowed to dry for 15 minutes. The SPE columns were eluted with 2 mL methanol and the samples were captured in tubes already containing 20 µL of 10% glycerol and 500 µM BHT in ethanol. The tubes were placed in a water bath at 40 °C and the methanol was evaporated under a gentle stream of nitrogen, after which the samples were reconstituted in 100 µL ethanol containing another internal standard (CUDA) and immediately used for LC-MS/MS analysis.

Extraction of eicosanoids from tissues

The extraction of eicosanoids from liver, ileum and adipose tissue was similar to plasma eicosanoid extraction. Approximately 100 mg liver and adipose tissue, and 50 mg ileum was extracted with 1 mL methanol containing internal standards and sonication. After centrifugation (5' at 3000 x *g* and 4°C), the supernatants were transferred to clean tubes and the methanol extraction was repeated once. Just before loading on HLB SPE columns, 8 mL MQ water containing 0.1% formic acid was added to the methanol extracts. For the SPE procedure and further, see section 'Plasma extraction of eicosanoids'.

LC-MS/MS analysis of eicosanoids

All eicosanoid analyses were performed on a UPLC coupled to a Xevo TQ-S mass spectrometer (Waters). Five µL extract was injected on a Acquity C18 BEH UPLC column (2.1 x 100 mm, 1.7 µm) and was separated using gradient elution with a stable flow of 600 µL/min. The gradient started with 95% A (MQ water with 0.1% FA) and 5% B (ACN with 0.1% FA) followed by a linear increase to 70% A and 30%

B which was achieved at 5.0 minutes. This was followed by a linear increase towards 50% B which was achieved at 11.25 minutes and maintained until 13.25 minutes. The system was subsequently switched to 100% B, which was achieved at 15.75 minutes and maintained until 16.75 minutes, after which the column was left to equilibrate at 5% B for approximately 3 minutes. The column was maintained at 50 °C during analysis, and the samples were kept at 10 °C. The MS was operating in selective reaction mode using electrospray ionization in negative ion mode, with a capillary voltage of 3.3 kV, a source temperature of 150 °C and a desolvation temperature of 600 °C. Cone voltage and collision energy were optimized for each compound individually (see Table 6.1b for parent and product *m/z* values). Peak identification and quantification was performed using MassLynx software version 4.1. Calibration curves were run in duplicate from which one regression equation was generated. During data analysis, 5 peaks of unknown identity were found to be influenced by diet or LPS treatment, and these compounds are listed UK1 to UK5. These peaks were visible in the transitions *m/z* 295.2 > 195.2 and *m/z* 295.2 > 171.1. ARA, DHA and EPA were also determined using this method.

Data analysis

Univariate analysis was performed with SAS version 9.1 (2002-2003 by SAS Institute Inc, Cary, NC, USA). ANOVA assumptions were checked for each variable. If these assumptions were not met, rank transformation was applied for that particular variable. Partial tests were performed using Tukey-Kramer multiple comparison correction. Benjamini and Hochberg false discovery rate correction was applied to correct for false positives. In all statistical tests that were performed, the null hypothesis (no effect) was rejected at the 0.05 level of probability ($\alpha = 5\%$).

The added value of multivariate data analysis in addition to univariate statistics is that correlations between variables are taken into account, so combinations of variables which are associated with differences between treatment groups. Multivariate data analysis summarizes all the variables into one variable by means of a linear combination, now called the 'principal component', which accounts for a significant amount of variance in the data. Using principal component analysis (PCA), we screened for group separation, outliers, (undesired) patterns and this was further analyzed with principal component discriminant analysis (PCDA).

PCDA includes the original group designation of the animals in the model and is therefore called a supervised classification technique. PCA and PCDA were performed in the Matlab environment (R2008b, 1984-2008, The Mathworks Inc, Natick, MA, USA) using the PLS toolbox for Matlab version 5.0.3 (r 6466, 1995-2008, Eigenvector Research Inc, Wenatchee, WA, USA). PCA and PCDA are described in more detail elsewhere [25, 26]. For all multivariate models data were autoscaled to mean zero and variance 1 for each variable. For PCDA, stability of the model was evaluated by 10-fold cross-validation, revealing correct classification rates of typically 80-100%. PCA and PCDA were performed on the combined data ('fused data'), containing data on both endocannabinoids/NAEs and eicosanoids from plasma, liver ileum and adipose tissue combined in one data set.

Results

Fish oil diet and inflammation alter the endocannabinoid/NAE balance

To investigate the effect of dietary n-3 fatty acids and inflammation on endocannabinoid/NAE and eicosanoid levels, wild-type male C57BL/6 mice received a diet containing either no, 1% w/w or 3% w/w FO followed by either saline or 2 mg/kg LPS *i.p.* injection. Endocannabinoid/NAE levels were determined in plasma, liver, ileum and adipose tissue.

Significant differences between diet groups and LPS treatment were obtained with the ANOVA test and are summarized in tables 6.2a-b for endocannabinoids/NAEs and 6.3a-b for eicosanoids. A *diet effect* is here defined as an effect of the diet which (in magnitude and direction) was the same for saline and LPS-treated mice. The term *LPS effect* refers to situations in which LPS induced a change in a concentration of a compound, which was similar for all diet groups. An *interaction effect* indicates that only certain (combinations of) diets with saline or LPS resulted in significant differences, and therefore separate comparisons ('partial tests') should be interpreted rather than main effects. Table 2a shows diet effects on NAEs/endocannabinoids, and 2b LPS effects. Compounds with an interaction effect are highlighted with * in the tables.

			plasma	liver	ileum	adi. tiss.
Ctrl vs 1% FO	n-3 derived	EPEA	↑	*	↑	↑
		DHEA	↑	↑	↑	↑
	other	AEA	*	↓	↓	↓
		2-AG	↓	↓	↓	↓
		DLE	↓	-	↓	↓
		OEA	↓	↓	-	*
Ctrl vs 3% FO	n-3 derived	EPEA	↑	*	↑	↑
		DHEA	↑	↑	↑	↑
	other	AEA	*	-	↓	↓
		2-AG	↓	↓	↓	↓
		DLE	↓	-	↓	↓
		OEA	↓	↓	-	*
		SEA	↓	-	-	↑
1% FO vs 3% FO	n-3 derived	EPEA	-	*	↑	↑
		DHEA	-	↑	↑	↑
	other	2-AG	-	↓	-	↑
		OEA	↓	-	-	*
		SEA	↓	-	-	↑

Table 6.2a: *Effect of the fish oil diets on endocannabinoid/NAE levels in plasma, liver, ileum and adipose tissue (diet effect). Only statistically significant effects are listed, with ↑ representing an increase by the FO diet, and ↓ a decrease. ND indicates that the compound was not detected in the particular matrix, and '-' indicates that no statistical significant differences were observed. * indicates an interaction effect. Adi. tiss.= adipose tissue.*

The fish oil diets altered endocannabinoid levels with different effects in plasma, liver, ileum and adipose tissue (Table 6.2a). DHEA was increased by both fish oil diets in all compartments compared to control diet. For EPEA, an interaction effect was observed in liver, but the compound was increased by the fish oil diets in plasma, ileum and adipose tissue. The endocannabinoids/NAEs derived from other fatty acids, such as AEA and 2-AG, were in general decreased by the fish oil diets, but some deviations were observed. For instance, 2-AG levels in adipose tissue and liver were decreased in both fish oil groups compared to the control diet. When comparing the 1% versus the 3% FO group, liver 2-AG was lower in the 3% group, but higher in adipose tissue. DLE in liver was not influenced by the diets, but was decreased in plasma, ileum and adipose tissue in the fish oil groups. OEA was decreased in liver and plasma, but not in ileum. When comparing the

control diet group with the 3% FO group, SEA displayed opposite effects in adipose tissue and plasma; fish oil was found to decrease plasma levels, but increased adipose tissue levels of SEA. This was also observed when comparing the 1% versus the 3% FO groups.

			plasma	liver	ileum	adi.tiss.
Saline vs LPS	n-3 derived	EPEA	↑	*	-	↑
		DHEA	↑	↑	↑	↑
	other	AEA	*	↑	↑	-
		2-AG	↓	-	-	↑
		DLE	↑	↑	↑	-
		PEA	*	↓	↑	*
		OEA	↑	↑	↑	*
		SEA	↑	↓	↑	↓

Table 6.2b: *Effect of LPS on endocannabinoid/NAE levels in plasma, liver, ileum and adipose tissue (LPS effect). Only statistically significant effects are listed, with ↑ representing an increase by LPS and ↓ a decrease. ND indicates that the compound was not detected in the particular matrix, and '-' indicates that no statistical significant differences were observed. * indicates an interaction effect. Adi. tiss.= adipose tissue.*

The effect of LPS on endocannabinoids appeared to be both compound and tissue specific (Table 6.2b). LPS increased DHEA levels in all compartments, but for some compounds tissue-specific effects were seen. LPS decreased plasma 2-AG, whereas it increased adipose tissue 2-AG. A similar divergence is seen for SEA and PEA. LPS increased plasma and ileum SEA levels, but decreases liver and adipose tissue SEA levels. PEA levels were decreased in liver by LPS, but increased in ileum. In summary, both the fish oil diets and the LPS treatment affected plasma and tissue endocannabinoid/NAE levels. In general, DHEA and EPEA were increased by the fish oil diets, and compounds derived from other fatty acids were decreased, with different effects for 1% and 3% FO diets. LPS raised endocannabinoid/NAE levels in general, but opposing effects were seen for 2-AG, PEA and SEA across the tissues investigated.

			plasma	liver	ileum	adi. ti.
Ctrl vs 1% FO	fatty acids	ARA	*	↓	-	↓
		DHA	↑	-	↑	-
		EPA	*	↑	↑	↑
	n-3 eicosanoids	5-HEPE	↑	↑	↑	↑
		12-HEPE	*	↑	↑	↑
		PGD_3	ND	ND	↑	↑
		PGE_3	-	ND	↑	↑
		17-HDoHE	-	-	↑	↑
		10-17-DiHDoHE	ND	ND	↑	↑
		19,20-DiHoPE	↑	↑	↑	↑
		$TBXB_3$	*	ND	↑	↑
	n-6 eicosanoids	5,6 EET	-	↓	-	-
		11,12 EET	-	↓	↓	↓
		14,15 EET	↓	↓	↓	↓
		LTB_4	ND	↑	↓	*
		LTD_4	ND	ND	↓	-
		5,6-DiHETrE	-	↓	↓	↓
		8,9-DiHETrE	↓	↓	↓	*
		11,12-DiHETrE	↓	↓	↓	↓
		14,15-DiHETrE	↓	↓	↓	*
		PGE_2	-	↓	-	-
		$PGF_{2\alpha}$	-	↓	-	-
		8-iso-$PGF_{2\alpha}$	-	-	-	↓
		13,14-dihydro-15-keto-PGD_2	ND	ND	↓	ND
		13,14-dihydro-15-keto-PGE_2	↓	↓	↓	↓
		13,14-dihydro-15-keto-$PGF_{2\alpha}$	ND	↓	↓	↓
		12-HHTrE	-	↓	-	↓
		5-HETE	↓	↓	-	↓
		11-HETE	-	↓	-	↓
		12-HETE	-	↓	-	-
		15-HETE	-	↓	-	↓
		20-HETE	ND	↓	-	ND
		$TBXB_2$	-	↓	-	-
		13-HODE	-	↓	-	-
		9,10,13-TriHOME	-	*	-	↓

Table 6.3a (1/3): *Effect of the fish oil diets on eicosanoid levels in plasma, liver, ileum and adipose tissue (diet effect). Only statistically significant effects are listed, with ↑ representing an increase by the FO diet, and ↓ a decrease. ND indicates that the compound was not detected in the particular matrix, and '-' indicates that no statistical significant differences were observed. * indicates an interaction effect. Adi. tiss.= adipose tissue.*

Fish oil diet and inflammation alter the eicosanoid balance

The results of the eicosanoid analyses in plasma, liver, ileum and adipose tissue are presented in Tables 6.3a-b, with 6.3a showing diet effects and 6.3b LPS effects. Compounds with an interaction effects are highlighted with an * in the tables. The fish oil diets decreased levels of ARA and increased DHA and EPA, confirming that the increased dietary intake of n-3 fatty acids was reflected in tissue fatty acid levels (Table 6.3a). Furthermore, n-3 derived eicosanoid levels were increased by the fish oil diets, with the most pronounced effects observed in ileum and adipose tissue. The eicosanoids derived from other fatty acids were in general decreased by the fish oil diets, with some exceptions, and effects were not always consistent over all tissues tested. Ileum levels of LTB_4 were decreased in ileum and adipose tissue by the fish oil diets, but liver levels were increased. Lipoxin A_4 levels were increased in the 3% FO group compared to the control and 1% FO diet in liver, ileum and adipose tissue. When comparing the 1% and 3% FO diets, ileal 5-HETE levels were decreased in the 3% FO group, but its level was increased in adipose tissue. The fish oil diets decreased eicosanoids belonging to different branches of the fatty acid eicosanoid cascade, including the cyclooxygenase pathway (COX; PGD_2, PGE2 and their metabolites 13,14-dihydro-15-keto-PGD_2 and –PGE_2, $PGF_{2\alpha}$, $TBXB_2$), the 15-lipo-oxygenase pathway (15-LOX; 15-HETE), 12-LOX (11-HETE and 12-HETE), 5-LOX (5-HETE, LTB_4 and LTD_4) and the cytochrome P450 pathways (EETs and DiHETrEs) (see Figure 6.1 for an overview).

Treatment with LPS generally resulted in increased levels of fatty acids, n-3 derived eicosanoids and other eicosanoids, with the most compounds affected in plasma and adipose tissue, and the least number of compounds altered in ileum (Table 6.3b). Again, opposing effects were observed between compartments for some components. LPS decreased plasma levels of 11-HETE, but increased liver levels. TBXB2 was decreased by LPS in plasma, but increased in ileum and adipose tissue.

			plasma	liver	ileum	adi. tiss.
Ctrl vs 3% FO	fatty acids	ARA	*	↓	-	-
		DHA	↑	-	↑	-
		EPA	*	↑	↑	↑
		17-keto DHA	ND	-	↑	*
	n-3 derived eicosanoids	5-HEPE	↑	↑	↑	↑
		12-HEPE	*	↑	↑	↑
		PGD_3	ND	ND	↑	↑
		PGE_3	-	ND	↑	↑
		17-HDoHE	-	↑	↑	↑
		10-17-DiHDoHE	ND	ND	↑	↑
		19,20-DiHoPE	↑	↑	↑	↑
		$TBXB_3$	*	ND	↑	↑
	other eicosanoids	5,6 EET	-	↓	-	-
		8,9 EET	ND	ND	↓	-
		11,12 EET	↓	↓	↓	↓
		14,15 EET	↓	↓	↓	-
		LTB_4	ND	↑	↓	*
		LTD_4	ND	ND	↓	-
		n-acetyl-leukotriene E_4	ND	↓	*	ND
		5,6-DiHETrE	↓	-	↓	↓
		8,9-DiHETrE	↓	↓	↓	*
		11,12-DiHETrE	↓	↓	↓	↓
		14,15-DiHETrE	↓	↓	↓	*
		PGD_2	↓	*	-	-
		PGE_2	↓	↓	-	-
		$PGF_{2\alpha}$	-	↓	-	-
		8-iso-$PGF_{2\alpha}$	-	-	-	↓
		13,14-dihydro-15-keto-PGD_2	ND	ND	↓	ND
		13,14-dihydro-15-keto-PGE_2	↓	↓	↓	↓
		13,14-dihydro-15-keto-$PGF_{2\alpha}$	ND	↓	↓	↓
		12-HHTrE	↓	↓	-	↓
		5-HETE	↓	-	-	-
		11-HETE	↓	↓	-	-
		12-HETE	↓	↓	-	↓
		15-HETE	↓	↓	-	↓
		20-HETE	ND	↓	-	ND
		$TBXB_2$	↓	↓	-	-
		9-HODE	-	-	-	↓
		13-HODE	↓	↓	-	↓
		lipoxin A_4	ND	↑	↑	↑

Table 6.3a (2/3): *Effect of the fish oil diets on eicosanoid levels in plasma, liver, ileum and adipose tissue (diet effect). Adi. tiss.= adipose tissue.*

			plasma	liver	ileum	adi. tiss.
1% FO vs 3% FO	fatty acids	DHA	-	-	↑	-
		EPA	*	-	↑	↑
		17 keto-DHA	ND	-	↑	*
	n-3 derived eicosanoids	5-HEPE	-	↑	↑	↑
		12-HEPE	*	-	↑	↑
		PGD_3	ND	ND	-	↑
		PGE_3	-	ND	-	↑
		17-HDoHE	-	↑	-	-
		19,20-DiHoPE	-	↑	-	↑
	other eicosanoids	5-HETE	-	-	↓	↑
		11-HETE	↓	-	-	-
		13-HODE	↓	-	-	-
		15-HETE	↓	-	-	-
		PGE_2	↓	-	-	-
		13,14-dihydro-15-keto-PGE_2	-	-	↓	-
		13,14-dihydro-15-keto-$PGF_{2\alpha}$	ND	↓	-	-
		lipoxin A_4	ND	↑	↑	↑
		$TBXB_2$	↓	-	-	-

Table 6.3a (3/3): *Effect of the fish oil diets on eicosanoid levels in plasma, liver, ileum and adipose tissue (diet effect). Adi. tiss.= adipose tissue.*

Multivariate data analysis shows separation between diet groups and LPS treatment

The univariate data analysis approach revealed that both fish oil and LPS altered endocannabinoid/NAE and eicosanoid levels, and effects were seen in plasma, liver, ileum and adipose tissue. In total, 244 variables obtained in four compartments were evaluated, which were, due to complexity, further analyzed with multivariate data analysis to evaluate differences between treatment groups. Two methods were used, the unbiased Principal component analysis (PCA) and Principal component discriminant analysis (PCDA). In the PCA plot (Figure 6.2), a good separation of the 6 intervention groups can be seen. The first principal component (PC) explains the separation between the diet groups, and the second PC distinguishes between saline or LPS treatment.

			plasma	liver	ileum	adi. tiss.
Saline vs LPS	fatty acids	ARA	*	↑	-	↑
		DHA	↑	↑	-	-
		EPA	*	-	-	↑
	n-3 derived eicosanoids	5-HEPE	↑	-	-	↑
		PGD_3	ND	ND	-	↑
		PGE_3	-	ND	↑	↑
		10(S)-17(S)-DiHDoHE	ND	ND	-	↑
		19,20-DiHoPE	↑	↑	↑	↑
		$TBXB_3$	*	ND	↑	↑
	other eicosanoids	5,6 EET	-	-	-	↑
		11,12 EET	-	-	-	↑
		14,15 EET	-	↑	-	-
		LTB_4	ND	↓	-	*
		LTD_4	ND	ND	-	↑
		n-acetyl leukotriene E_4	ND	↑	*	ND
		5,6 DiHETrE	-	-	-	↑
		8,9-DiHETrE	↑	-	-	*
		11,12-DiHETrE	↑	-	-	-
		14,15-DiHETrE	↑	-	-	*
		PGE_2	↑	↑	-	↑
		$PGF_{2□}$	-	-	-	↑
		8-iso-$PGF_{2□}$	-	↑	-	-
		13,14-dihydro-15-keto-PGE_2	↑	-	-	↑
		13,14-dihydro-15-keto-$PGF_{2\alpha}$	ND	↑	-	-
		12-HHTrE	↓	-	-	↑
		5-HETE	↑	-	-	↑
		11-HETE	↓	↑	-	↑
		12-HETE	-	↓	-	-
		15-HETE	↓	-	-	-
		20-HETE	ND	↑	-	ND
		$TBXB_2$	↓	-	↑	↑
		9-HODE	↑	-	↑	↑
		13-HODE	-	-	↑	↑
		9,10,13-TriHOME	↑	*	-	-
		lipoxin A_4	ND	-	-	↑

Table 6.3b: *Effect of LPS on eicosanoid levels in plasma, liver, ileum and adipose tissue (LPS effect). Only statistically significant effects are listed, with ↑ representing an increase by LPS and ↓ a decrease. ND indicates that the compound was not detected in the particular matrix, and '-' indicates that no statistical significant differences were observed. * indicates an interaction effect. Adi. tiss.= adipose tissue.*

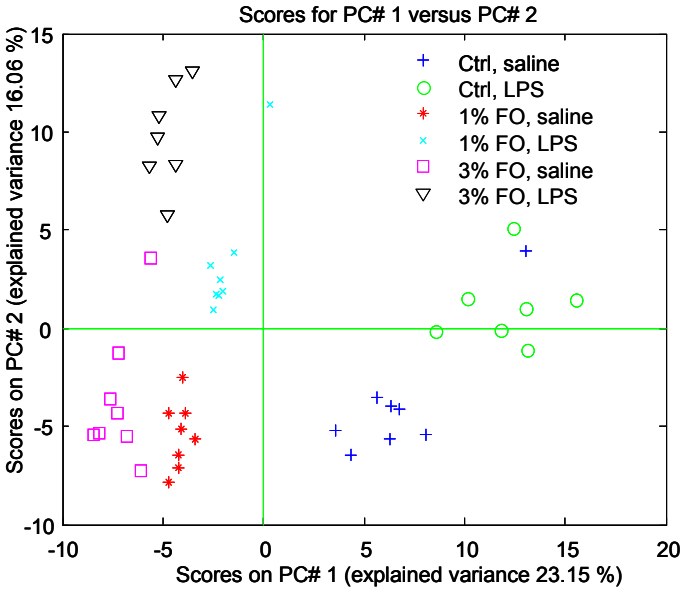


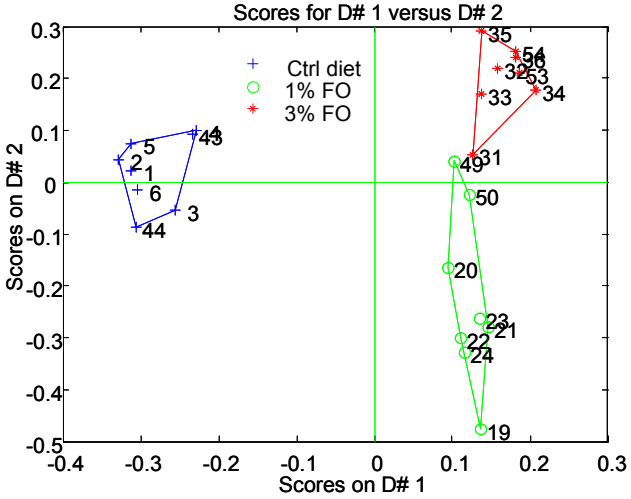
***Figure 6.2**: PCA analysis on fused data. The PCA plot shows good separation of the three diet groups. PC1 describes mainly the diet effect, and PC2 mainly the LPS effect.*

The diet effect in the saline treated mice is explained by other variables than the diet effect in the LPS-treated mice

To further explore differences between diet groups, PCDA was performed. The data was split for saline and LPS-treated mice, thus resulting in two separate PCDA plots. PCDA analyses showed that there is separation based on diet for both the saline and LPS-treated mice (Figure 6.3). The contribution of a variable in the PCDA model is expressed as its D-score, with a positive score meaning an increase by the FO diets, and a negative score indicating a decrease.

Analysis of D-scores revealed that the diet groups are separated by increased levels of n-3 derived compounds in the fish oil groups, and compounds derived from other fatty acids were generally decreased by the fish oil diets (Table 6.4). In addition to this, both endocannabinoids/NAEs and eicosanoids show up in the top of the rank lists, indicating that both classes of compounds are important to describe the diet effect. The ranking, number and origin of n-3 derived metabolites in the models is different between the saline and LPS treated animals. Out of the 50 compounds ranking highest for the saline treated mice, only 12

Saline



LPS

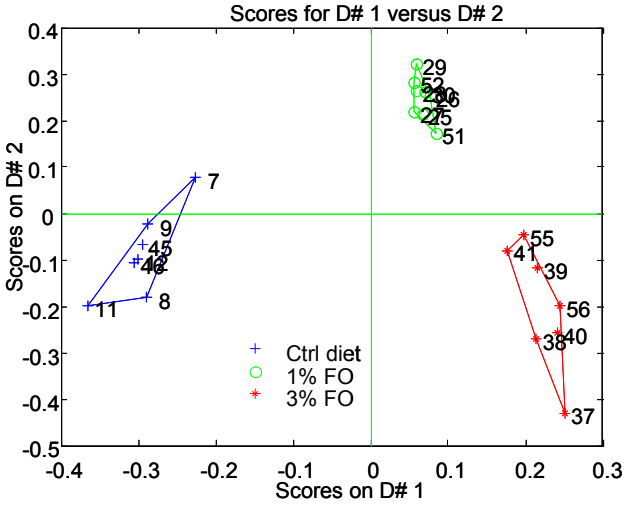


***Figure 6.3**: PCDA analysis, split for saline (upper panel) and LPS treated (lower panel) mice. A separation of diet groups is observed in both saline and LPS treated mice, with a more prominent separation in the LPS treated mice.*

compounds are n-3 fatty acid derived metabolites, while for the LPS treated mice, the top-50 list contains 25 n-3 fatty acid derived metabolites. In addition to this,

the majority of n-3 derived compounds in the LPS treated mice from this list originated from adipose tissue.
From these results, it can be concluded that the diet effect of fish oil in the saline treated animals is mainly explained by a decrease of compounds derived from other than n-3 fatty acids, and to a lesser extent by an increase of n-3 derived metabolites. However, for the LPS treated mice, the diet effect is principally explained by an increase of n-3 derived metabolites, and to a lesser extent by a decrease of metabolites derived from other than n-3 fatty acids.

Discussion

Our results support the general idea that increasing dietary n-3 fatty intake results in increased levels of n-3 derived -endocannabinoids/NAEs and eicosanoids. However, to the best of our knowledge, our study is the first one describing effects of dietary fish oil on the balance between the "endocannabinoid" and eicosanoid pathways in such detail, in different compartments simultaneously, and in relation with inflammation. In addition, our study illustrates the risk of obtained potentially premature conclusions when only a few mediators are analysed in a limited number of matrices. Several studies, focusing on for example AEA (anandamide) and 2-AG only, have concluded that dietary fish oil leads to an overall down regulation of the endocannabinoid system [19, 27]. However, as we show other (n-3 derived-) endocannabinoids might be affected in an opposite direction following fish oil intake. Although there are still several questions regarding their biological role, there are reports showing that n-3 derived ethanolamides have affinity for CB1 and CB2 receptors [28, 29], and have anti-inflammatory properties [20, 21].
LPS was found to produce a general increase of *in vivo* endocannabinoid/NAE and eicosanoid levels, although there were some exceptions (see below). Multivariate data analysis showed that the diet effect was also present during inflammatory conditions. Without LPS, the effect of a fish oil diet was mainly explained by a reduction of mediators other than those derived from n-3 fatty acids, and to a lesser degree by increased levels of n-3 derived metabolites. However, after LPS, the balance was shifted in favor of an increase of n-3 derived mediators while lower associations were found with reductions of non n-3 derived metabolites.

	Saline	d-score			LPS	d-score
	P_AEA	-4,6879			L_2-AG	-4,3994
	L_2-AG	-4,5742			P_AEA	-4,2737
	P_DLE	-4,5412		1	**F_EPA**	**4,2374**
	F_AEA	-4,4914		2	**F_12-HEPE**	**4,1634**
	P_ARA	-4,4486		3	**P_EPA**	**4,1441**
	P_11,12-DiHETrE	-4,4257			P_2-AG	-4,1053
1	**F_DHEA**	**4,3978**			P_AA	-4,0854
	P_14,15-DiHETrE	-4,3961		4	**I_PGE_3**	**4,0450**
	L_ARA	-4,3915		5	**F_$TBXB_3$**	**4,0374**
	L_13,14-dihydro-15-keto-PGF_{2a}	-4,3025		6	**F_PGE_3**	**3,9827**
	L_12-HETE	-4,2590			P_DLE	-3,9498
	F_8,9-DiHETrE	-4,2553			L_14,15-DiHETrE	-3,8977
	L_15-HETE	-4,2343		7	**F_DHEA**	**3,8946**
2	**L_5-HEPE**	**4,2162**		8	**I_EPA**	**3,8541**
	L_11-HETE	-4,1548		9	**L_EPEA**	**3,7505**
3	**L_EPA**	**4,1323**			L_11,12-DiHETrE	-3,7410
4	**P_EPA**	**4,1269**		10	**L_DHEA**	**3,7222**
	F_DLE	-4,1166			L_ARA	-3,6999
	P_8,9-DiHETrE	-4,0892		11	**L_EPA**	**3,6953**
	I_13,14-dihydro-15-keto-PGE_2	-4,0783		12	**I_DHA**	**3,6912**
	P_OEA	-4,0592		13	**I_12-HEPE**	**3,6874**
	F_11,12 EET	-4,0186			L_8,9-DiHETrE	-3,6672
	F_OEA	-4,0148		14	**F_EPEA**	**3,6327**
	L_8,9-DiHETrE	-4,0079			L_13,14-dihydro-15-keto-$PGF_{2\alpha}$	-3,6261
	I_2-AG	-3,9897			I_2-AG	-3,6231
	F_9,10-DiHOME	-3,9147		15	**P_DHA**	**3,5903**
	P_9,10-DiHOME	-3,8645			F_AEA	-3,5678
	L_LTB4	3,8175			L_PGD_2	-3,5455
	F_11,12-DiHETrE	-3,7600		16	**F_PGD_3**	**3,5197**
	F_14,15-DiHETrE	-3,7376		17	**F_19,20-DiHoPE**	**3,5168**
	L_5,6 EET	-3,7094			L_AEA	-3,5158
	I_13,14-dihydro-15-keto-$PGF_{2\alpha}$	-3,7008		18	**I_19,20-DiHoPE**	**3,4924**
	P_12,13-DiHOME	-3,6962			L_14,15 EET	-3,4871
	F_12,13-DiHOME	-3,6718		19	**I_EPEA**	**3,4515**
5	**P_DHEA**	**3,6677**		20	**I_5-HEPE**	**3,4391**
	P_UK4	-3,6605			I_AEA	-3,4310
	L_14,15 EET	-3,6511			F_SEA	3,4187
6	**F_EPA**	**3,6069**			L_20-HETE	-3,4104
7	**I_19,20-DiHoPE**	**3,5938**			I_13,14-dihydro-15-keto-PGE_2	-3,3956
	F_5,6-DiHETrE	-3,5357		21	**P_5-HEPE**	**3,3787**
	P_2-AG	-3,5155		22	**L_5-HEPE**	**3,3617**
8	**I_12-HEPE**	**3,4899**			L_$TBXB_2$	-3,3200
	L_11,12 EET	-3,4781			L_12-HHTrE	-3,3155
9	**F_EPEA**	**3,4723**			P_5,6-DiHETrE	-3,3080
10	**L_19,20-DiHoPE**	**3,4679**		23	**F_5-HEPE**	**3,3048**
	L_12-HHTrE	-3,4525		24	**P_DHEA**	**3,2978**
	P_UK2	-3,4144			P_11,12-DiHETrE	-3,2966
	L_PGE_2	-3,4143		25	**F_17 keto-DHA**	**3,2931**
11	**I_EPA**	**3,3758**			L_11-HETE	-3,2792
12	**P_5-HEPE**	**3,3581**			P_15-HETE	-3,2618

Table 6.4: *Top lists of endocannabinoids/NAEs and eicosanoids changed in saline and LPS treated mice. Negative D-scores indicate that the compound is decreased in the fish oil groups; positive D-scores mean that it is increased by fish oil. P = plasma; L=liver; I=ileum; F=adipose tissue. Decrease of other than n-3 derived compounds ranks relatively high in the saline diet effect, whereas an increase in n-3 derived compounds (printed in* ***bold****) ranks high for the diet effect in the LPS treated mice.*

The relation between dietary fatty acid intake and the presence of endocannabinoids/NAEs and eicosanoids in plasma and tissues has been established before [30, 31], but not under conditions of inflammation. Previous work with rats demonstrated that patterns of organ levels of NAEs follow the relative abundance of fatty acids in the diet [18]. Other work, investigating the effect of DHA on murine levels of endocannabinoids/NAEs in brain and plasma, showed strongest changes in plasma [17]. Interestingly, plasma AEA levels were not significantly affected by DHA alone, whereas other NAEs were decreased by DHA. Other work, supplementing krill oil or menhaden oil to human subjects also did not show an effect on plasma AEA levels [27]. Our work shows that six weeks of a fish oil diet is capable of reducing plasma AEA and 2-AG levels. This discrepancy might originate from differences in n-3 fatty acids sources, or length of the period in which the n-3 fatty acids were supplemented.

Many studies analyze plasma levels of endocannabinoids/NAEs or eicosanoids. The present work shows that plasma levels do not always reflect effects in liver, ileum or adipose tissue. For example, plasma 2-AG levels decreased after LPS, but were increased in adipose tissue, and similar divergences were also observed for PEA, SEA, several HETEs, and $TBXB_2$. The origin and significance of these findings are not known yet, but this could be related to synthesis, release, uptake or breakdown which might be differentially regulated by LPS or other factors across different organs. Nevertheless, based on our results, extrapolating effects found in plasma to effects on peripheral tissues is not always appropriate.

The LPS treated mice had a lower food intake combined with a small loss of body weight (data not shown), whereas the saline treated animals displayed normal food consumption and stable body weight. Previous work showed that levels of endocannabinoids and related NAEs depend on fasting status [32-34]; their tissue levels being high during fasting, followed by a rapid postprandial decrease. Possibly, the effect of LPS on endocannabinoid levels might in part be mediated through such a 'fasting' effect. In addition, inflammation reduces FAAH expression, and inhibition of FAAH or monoacyl glycerol lipase (MGL) has been shown to reduce disease symptoms in several models of inflammation [22, 35, 36]. Similarly, studies using CB2 knock-out models under induced inflammatory conditions showed that increased levels of NAEs likely contribute to suppress inflammation [37]. Together, this suggests that increased levels of endocannabinoids/NAEs are part of a normal response protecting against

inflammatory stress. Previous work identified DHEA and EPEA as having anti-inflammatory properties in macrophages and adipocytes [20, 21], and these compounds could be another link between fish oil and its anti-inflammatory effects as n-3 derived NAEs were more effective than AEA in suppressing nitric oxide release from macrophages [21].

The fish oil diets also influenced levels of eicosanoids, including metabolites from the COX, CYP450, and 5-LOX, 12-LOX and 15-LOX pathways (figure 1), and these effects were in general also seen during inflammatory conditions. Another strength of the present study is that we analyzed both n-6 and n-3 related eicosanoids simultaneously in different compartments. In general, levels of n-3 fatty acid derived eicosanoids (e.g. PGD_3, PGE_3, 5-HEPE, 12-HEPE and $TBXB_3$) were increased with fish oil at the expense of eicosanoids derived from other fatty acids (e.g. PGD_2, PGE_2, PGF_2 ,$TBXB_2$ and members of the EET and HETE subclasses). A functional role in inflammation has been described for several of these compounds, and it is likely that the changes in profiles which are found in this study (and before) are causally related to the anti-inflammatory effects which are associated with n-3 fatty acid intake. For example, PGE_3 is less potent than PGE_2 in inducing COX-2 expression and IL-6 release [38]. A similar principle applies to the thromboxanes [39, 40] and for 5-HETE/5-HEPE [41], which were also altered by the fish oil diets.

Interestingly, the fish oil diets increased liver LTB_4 levels, whereas ileum LTB_4 levels were decreased. LTB_4 has multiple pro-inflammatory functions in the immune system [42], but the different effect of fish oil on organ levels of LTB_4 is not understood. Lipoxin A_4, a compound with anti-inflammatory properties [43], was increased by the 3% FO diet. This indicates that at least for this compound, which is synthesized from ARA, its level is not directly related to dietary supply of precursor, but that other presently unknown factors are involved.

Levels of several EETs were reduced by the FO diets, especially in the liver. EETs play regulatory roles in heart and vascular physiology with effects on blood pressure regulation, but also have anti-inflammatory effects [44]. It thus seems that EETs do not play a role in the anti-inflammatory properties of n-3 fatty acids. Another line of evidence suggests that EETs very specifically alter the release of either insulin or glucagon [45, 46], pointing to a potential link between n-3 fatty acids and glucose metabolism. EETs might therefore also be part in mediating

effects of dietary fatty acids on metabolism, but this relation has not been given much attention yet.

In the present study we did not detect resolvins in any of the samples. It might be that these compounds are not formed in quantities high enough to be detected with our method during the first 24 hrs after the initiation of the inflammatory response, or the detection limit of the analytical method was not sufficient to detect these compounds. However, the presence of 17-HDoHE (also known as 17-HDHA), a marker for resolvin synthesis [47] with anti-inflammatory properties [48], was increased by the fish oil diets. The fish oil diets as well as LPS increased levels of 10,17-DiHDoHE, (also known as protectin DX) which was previously shown to reduce inflammation and accelerate its resolution [49]. Altogether, the fish oil diets altered all branches in the eicosanoid metabolome in a way that is largely associated with suppression of inflammation.

A major finding of this work is that the effects of fish oil were also persistent under inflammatory conditions. Multivariate data analysis revealed that both endocannabinoids and eicosanoids are responsible for separation between diet groups. Under non-inflammatory conditions, the diet groups could be primarily separated based on the reduction of other than n-3 derived endocannabinoids and eicosanoids. In contrast, with LPS treatment, the diet groups were primarily separated by increases in levels of n-3 fatty acid derived endocannabinoids and eicosanoids. The combined approach of comparing normal versus inflammatory conditions was thus useful in demonstrating that effects of diet on eicosanoids and endocannabinoids are depending on inflammatory status.

Recent evidence suggested that relatively high intakes of fish oil impairs the host's resistance to microbial infection [50-52]. In our study, the mice that had received 3% FO showed relatively more severe signs of shock after LPS, and one mouse from the 3% FO + LPS group died shortly before the end of the experiment. This would be in line with the notion that high fish oil intake might impair the host's resistance to inflammatory stress, or to suppress the capability to overcome the inflammatory stimulus. We observed that plasma $TBXB_2$ levels, a compound related to $TBXA_2$ which is involved in vasoconstriction [53], was decreased in the 3% FO group compared to the 1% FO group, but also by LPS. The combination 3% FO and LPS treatment might have caused a decrease in $TBXA_2$ levels below its physiological range, potentially increasing the risk of inducing excessive vasodilatation and shock. Alternatively, pre-treatment of rats with a CB1 blocker

was effective in reducing hypotension after LPS administration [54], suggesting that increases in endocannabinoids after LPS might also contribute to the shock observed for the 3% FO + LPS group. Future work should point out which (combination of) metabolites account for the impaired resistance in the 3% FO + LPS group. Furthermore, future work should clarify which intake levels of n-3 fatty acids are beneficial to reduce symptoms of inflammatory diseases and where the inhibition of inflammation starts to interfere with an efficient response to an inflammatory stimulus.

In conclusion, dietary fish oil caused marked changes in the n-3 to n-6 balance of the endocannabinoid and eicosanoid metabolomes, with specific effects depending on inflammatory status. The effects on metabolites are in line with the anti-inflammatory effects associated with n-3 fatty acid intake.

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Chapter 7

Free and esterified fatty acid *N*-acyl ethanolamines in plasma and blood cells: evidence for previously ignored pools of esterified NAEs in plasma and blood cells

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Abstract

The origin of plasma NAEs is not exactly known, and it is assumed that plasma levels are a reflection of system levels as plasma may act as a 'spill-over' sink. However, plasma contains numerous classes of lipids which could contain esterified NAEs, and blood cells might also synthesize these compounds. In the present study, we i) compared free to esterified plasma NAE levels in mice fed diets with different amount of n-3 fatty acids, and ii) investigated the presence of NAE in blood cells. For this purpose a LC-MS/MS method was developed and validated for the quantification of AEA, 2-AG, DHEA, DLE, EPEA, OEA, PEA and SEA in 100 µL plasma using a simple acetonitrile extraction step. Plasma extracts contained 20-60 fold higher levels of esterified NAEs than free NAEs. Moreover, the effect of dietary n-3 fatty acids on free plasma NAE profiles was similar for esterified NAEs. Finally, esterified NAEs were also present in blood cells, and their pattern followed the same diet effect as observed for free and esterified plasma NAEs. Together, these data point to the presence of previously ignored pools of esterified NAEs in plasma and blood cells, which correlated with free plasma NAE levels.

Introduction

Over the last decades, the fatty acid *N*-acyl ethanolamines (NAEs) have emerged as important regulators of diverse biological processes, including food intake, energy metabolism, and inflammation [1-4]. The best studied NAE is the endocannabinoid arachidonoyl ethanolamide (AEA; anandamide), but NAEs synthesized from other fatty acids also exist, such as docosahexaenoyl ethanolamide (DHEA), dihomo-γ-linolenoyl ethanolamide (DLE), eicosapentaenoyl ethanolamide (EPEA), oleoyl ethanolamide (OEA), palmitoyl ethanolamide (PEA), and stearoyl ethanolamide (SEA). These compounds are widely present throughout the body and several publications have reported on their levels in tissues, plasma, and other body fluids [5-12]. Several papers have described analytical methods for NAE quantification, typically using solvent extraction and/or solid phase extraction (SPE) protocols combined with liquid-chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis [13-21]. These procedures extract and quantify NAEs which are present in "free form", meaning that these NAEs were not-covalently bound *e.g.* esterified to for example phospholipids. Usually large amounts (*e.g.* 1 mL) of plasma are needed, making such methods not practical for the use in *e.g.* mouse studies. NAEs are often quantified in (human) plasma, but it should be kept in mind that the plasma compartment may act as a 'spill-over' sink for *e.g.* liver or adipose tissue synthesis, and the exact origin of plasma NAEs is thus not known. Peripheral blood also contains cells which could potentially synthesize NAEs, and could thereby contribute to (changes in) plasma NAE levels. Unfortunately, this has not received much attention yet. In the present study, we i) compared plasma levels of free NAEs to esterified NAE levels, and ii) investigated whether blood cells, obtained from the same blood sample, contained esterified NAE levels. To this end, mice were fed with diets containing different fatty acid compositions and subsequently 100 µl plasma was extracted with or without a saponification (hydrolyzation) step. The NAEs in red blood cells were measured after saponification. NAEs were quantified using a new and more sensitive developed LC-MS/MS method. Higher levels of NAEs were found in plasma extracts after saponification, and NAEs were also detected in red blood cells. Together, these results indicate that both plasma and blood cells contain a pool of esterified NAEs which is only revealed after hydrolyzation of samples.

Materials and methods

Chemicals and reagents

Lipopolysaccharide (0111:B4; LPS) was from Sigma (Steinheim, Germany). Phenylmethylsulfonyl fluoride (PMSF) was from Fluka (Steinheim, Germany). URB602 was from Cayman (Ann Arbor, MI, USA). Milli-Q water (Milli-Q Advantage unit, Millipore, Amsterdam, The Netherlands) was used in all analysis. ULC-grade acetonitrile (ACN), isopropanol (IPA) formic acid (FA) and trifluoro acetic acid (TFA) were obtained from Biosolve (Valkenswaard, The Netherlands). LC-MS grade methanol was from Riedel-de-Häen (Steinheim, Germany). Analytical grade IPA was from JT Baker (Deventer, The Netherlands). Potassium hydroxide (KOH) was from Merck (Darmstadt, Germany). All analytical and internal standards, except EPEA, were from Cayman. EPEA was synthesized as described earlier [22]. NAE stocks were prepared in ACN, aliquoted and stored at -80 °C until analysis. C8 SPE columns (Bond Elut; 200 mg, 3 mL) were from Varian Inc (Lake Forest, CA, USA).

Animal experiment

The plasma and blood cells were collected from an animal experiment described in more detail elsewhere [23]. In short, wild-type C57BL/6 mice were fed for six weeks with a control diet (ctrl), 1% fish oil (1% FO) or 3% fish oil (3% FO). After six weeks, blood was collected from the orbital sinus and captured in 1.3 mL EDTA coated tubes (Sarstedt; Etten-Leur, The Netherlands) and put on ice until centrifugation (10', 10,000 rpm at 4 °C). After centrifugation, 100 µL plasma was stored in the presence of 100 µM PMSF and 100 µM URB602. The blood cells were reconstituted in phosphate-buffered saline to a 1:1 ratio. Both plasma and blood cells were stored at -80 °C until analysis, thereby ensuring blood cell lysis.

Plasma extraction of free NAEs

Plasma (100 µL) was thawed and 400 µL ACN containing 100 µM PMSF and AEA-d8, 2-AG-d8 and OEA-d4 was added while the sample was gently vortexed. After subsequent centrifugation (5' at 13,000 rpm, RT), the supernatant was transferred to a clean eppendorf tube and evaporated to dryness in a vacuum concentrator (Scanvac; Lynge, Denmark). Prior to LC-MS/MS analysis, the dried extracts were reconstituted in 100 µL ACN containing 0.1% TFA.

Hydrolyzation and extraction of NAEs from plasma or blood cells

Plasma or blood cell lysate (100 µL) was thawed and 400 µL ULC-grade IPA containing 100 µM PMSF and AEA-d8, 2-AG-d8 and OEA-d4 was added while the sample was gently vortexed. IPA was used to ensure extraction of lipophilic structures, including phospholipids and lipoprotein complexes. After centrifugation (5' at 13,000 rpm, RT), the supernatant was transferred to a clean glass tube. Hydrolysis was performed through saponification by adding 200 µL 0.5 M KOH in methanol and placing the tube at 60 °C for 45'. Hereafter, 4 mL MQ water containing 0.13% v/v TFA was added, and the extract was loaded on an activated C8 SPE column. The column was washed with 2 mL of 20% ACN containing 0.1% TFA, and subsequently eluted with 2 mL of 80% ACN containing 0.1% TFA. The extract was evaporated to dryness in a vacuum concentrator and finally reconstituted in 100 µL ACN containing 0.1% TFA prior to LC-MS/MS analysis. The saponification step should hydrolyze all ester bonds, and the absence of 2-AG-d8 in the final extract was used as an indicator of complete sample hydrolysis.

LC-MS/MS analysis of NAEs

Extracts were analyzed on a Waters UPLC-XEVO TQ-S system. Three µL extract was injected on a Acquity C8 BEH UPLC column (2.1 x 100 mm, 1.7 µm) and was separated using gradient elution with a stable flow of 500 µL/min. The gradient started with 100% A (40:40:20 v/v/v of MQ water : methanol : ACN with 0.1% FA) which was maintained until 0.35 minutes, followed by a linear increase to 100% B (7:3 v/v methanol : ACN with 0.1% FA) which was achieved at 7.0 minutes. This was maintained until 9.0 minutes, and at 9.10 minutes the gradient was returned to 100% A, which was maintained until 12.0 minutes. The column was kept at 60 °C during analysis, and the samples at 10 °C. The MS was operated in selective reaction mode using electrospray ionization in positive ion mode, with a capillary voltage of 1.5 kV, a source temperature of 150 °C and a desolvation temperature of 500 °C. Cone voltage and collision energy were optimized for each compound individually. Peak identification and quantification was performed using MassLynx software version 4.1. Calibration curves were run in duplicate from which one regression equation was generated.

Validation of the analytical method

The accuracy and precision of the analytical method was evaluated using quality control samples (QCs), which were prepared by spiking NAE and 2-arachidonoyl glycerol (2-AG) stock solutions to 100 µL human plasma obtained from an apparently healthy volunteer. The spike levels correspond to one, three, and six times the basal plasma concentrations.

Statistical analysis

Data are presented as mean ± standard deviation where appropriate. Comparisons between groups were made with one-way analysis of variance (ANOVA) followed by Dunnett's *t* test using SPSS Statistics 17.0 software. $P < 0.05$ was considered as significant level of difference.

Results

Accuracy and precision of the analytical method

A new analytical method was developed which allowed quantification of NAEs and 2-AG in 100 µL plasma. The accuracy of the method was evaluated by spiking NAEs and 2-AG at three concentration levels to human plasma prior to sample work-up (see Table 7.1 for accuracies and related information). In most cases, good values for accuracy (70-120%) and precision (< 20%) were obtained, with usually higher precision values at the lowest spike level. It must be noted that 2-AG was not detected in the particular batch of human plasma used for the validation, but this compound has been quantified in 100 µL murine plasma (unpublished results). The new ULPC-based chromatography allowed separation of 2-AG from its isomer 1-AG (see Figure 7.1 for chromatograms). Matrix effect was determined using internal standard areas, showing ion suppression for AEA-d8 (60.5%), 2-AG-d8 (52.8%) and OEA-d4 (80.9%), and ion enhancement for PEA-d4 (178.4%).

Hydrolyzed plasma extracts contain higher levels of NAEs than non-hydrolyzed extracts

To investigate whether plasma contains a pool of esterified NAEs, murine plasma samples were hydrolyzed using KOH saponification and concentrations were compared to the same plasma sample without saponification.

Compound	Concentration spiked	Accuracy	Precision (intra-day)	LLOQ	Blanc human plasma levels	Linear range
	ng/ml	%	% RSD	ng/mL	ng/mL	ng/mL
AEA	0.21	107.3	18.2	0.056	0.14	0.056-406.6
	0.62	106.3	4.9			
	1.23	117.7	6.9			
2-AG	6.13	72.0	16.8	0.771	-	0.771-5617.2
	18.39	72.6	6.5			
	36.77	79.2	6.1			
DHEA	0.15	101.1	31.5	0.064	0.18	0.064-140.4
	0.45	103.8	8.4			
	0.89	117.5	7.9			
DLE	0.02	110.8	47.5	0.019	0.04	0.019-140.4
	0.07	110.8	18.1			
	0.13	133.8	12.6			
EPEA	1.58	75.3	8.6	0.069	-	0.069-499.9
	4.74	80.3	6.8			
	9.47	82.1	5.9			
OEA	1.18	95.1	4.0	0.064	0.96	0.064-140.4
	3.53	95.4	1.8			
	7.05	96.4	2.8			
PEA	1.03	97.4	18.8	0.064	0.94	0.064-140.4
	3.08	101.3	3.3			
	6.16	110.9	4.7			
SEA	0.38	68.9	26.5	0.095	0.41	0.095-69.6
	1.14	75.1	7.1			
	2.28	82.3	6.4			

Table 7.1: *Validation results for the quantification of NAEs and 2-AG from 100 µL human plasma.*

Figure 7.2 shows the esterified and free plasma NAE levels from murine samples. From Figure 7.2 can be concluded that NAE values after hydrolysis are higher (20 to 60 fold) compared to free plasma values. EPEA, an n-3 fatty acid derived NAE, is not detectable in free plasma from mice fed the control diet, but was quantifiable after hydrolysis.

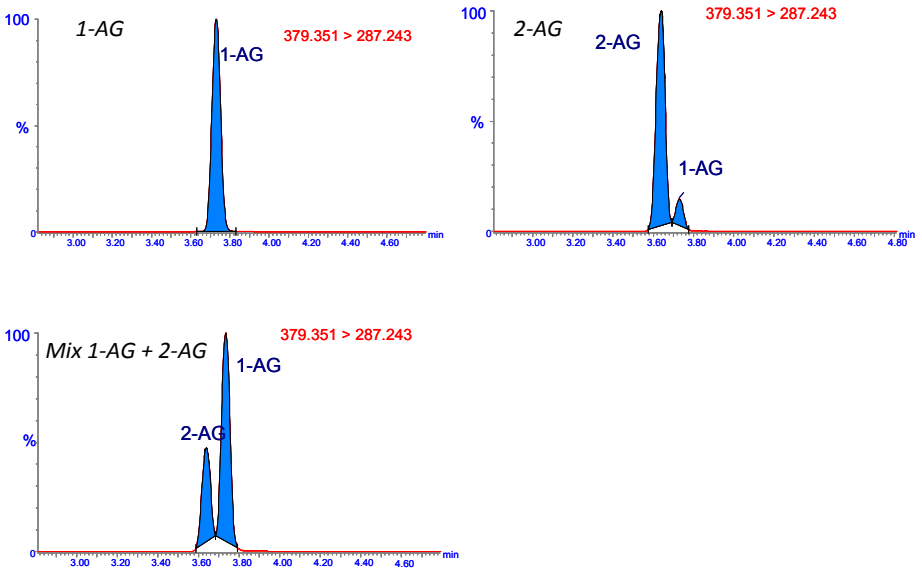

Figure 7.1: *Chromatograms from the injection of 1-AG (upper left), 2-AG (upper right) or a 1:1 mix of 1-AG and 2-AG (lower left), showing that 2-AG and 1-AG are separated. As 2-AG is prone to isomerization to 1-AG, some 1-AG is present in the chromatogram from the 2-AG injection.*

In addition, the effects of dietary FO on free plasma NAE levels are also observed in saponificated samples; *e.g.* the fish oil diets decreased free plasma AEA, and the same decrease is observed in the hydrolyzed samples. In general, the concentrations in the hydrolyzed samples were 20-60 fold higher compared to the free NAE levels. Hydrolyzed samples contained no detectable 2-AG-d8 levels, indicating that the hydrolysis was complete. AEA-d8 and OEA-d4 levels were not affected by the hydrolyzation (data not shown).

To confirm whether human plasma also contains esterified NAEs, plasma from 10 healthy volunteers was hydrolyzed and the NAE levels were compared to free NAE levels from the same samples. As shown in Table 7.2, the NAE levels in hydrolyzed extracts are higher compared to the free NAE levels, indicating that human plasma also contains pools of esterified NAEs.

Blood cells contain quantifiable NAE levels

As can be seen from Figure 7.3, hydrolyzed blood cell extracts contain quantifiable levels of all NAEs. In addition, the effect of the diets which was observed in free plasma levels is also evident in blood cell NAE levels; the fish oil diets increased levels of DHEA and EPEA and in general reduced levels of other NAEs. However, some differences between free plasma levels and levels in blood cells were obvious (*e.g.* PEA and SEA). Non-hydrolyzed blood cell extract also contained NAEs, but their levels were lower compared to hydrolyzed extracts (data not shown).

Discussion

There are numerous reports in literature presenting plasma NAE levels, but the site of synthesis of these compounds remains speculative as the plasma compartment may act as a 'spill-over' sink for organs with established NAE biosynthesis, such as liver or adipose tissue. The possibility that plasma or blood cells contain pools of esterified NAEs has not been investigated before. The purpose of this study was to compare free plasma NAE levels to esterified plasma NAEs in groups of mice fed with diets containing different fatty acid compositions, and to investigate whether blood cells also contain esterified NAEs.

NAEs are in general present at low concentrations in plasma, and their accurate quantification requires adequate sample volumes, *e.g.* 1 mL of plasma, depending on the sensitivity of the instruments used. We have developed and validated a new analytical method for the quantification of NAEs from 100 µL plasma, extending its applicability to animal studies. Another advantage of the present method is the relatively simple sample work-up procedure which does not require a solid phase extraction (SPE) step, thus speeding up analysis time and reducing costs. The plasma NAE concentrations found in the human control sample are in agreement with previously published values [13, 15, 24, 25]. As the present method does not use sample clean up, special attention was paid to ion suppression caused by matrix effect. No differences with respect to the '1 mL' method were observed [13]. Although 2-AG was not detected in the human control sample, it was possible to quantify this compound in 100 µL murine plasma samples (unpublished data). The levels of NAEs in hydrolyzed plasma extracts were in general 20-to-60 fold higher compared to non-hydrolyzed extracts, indicating that plasma contains significant pool of esterified NAEs. In

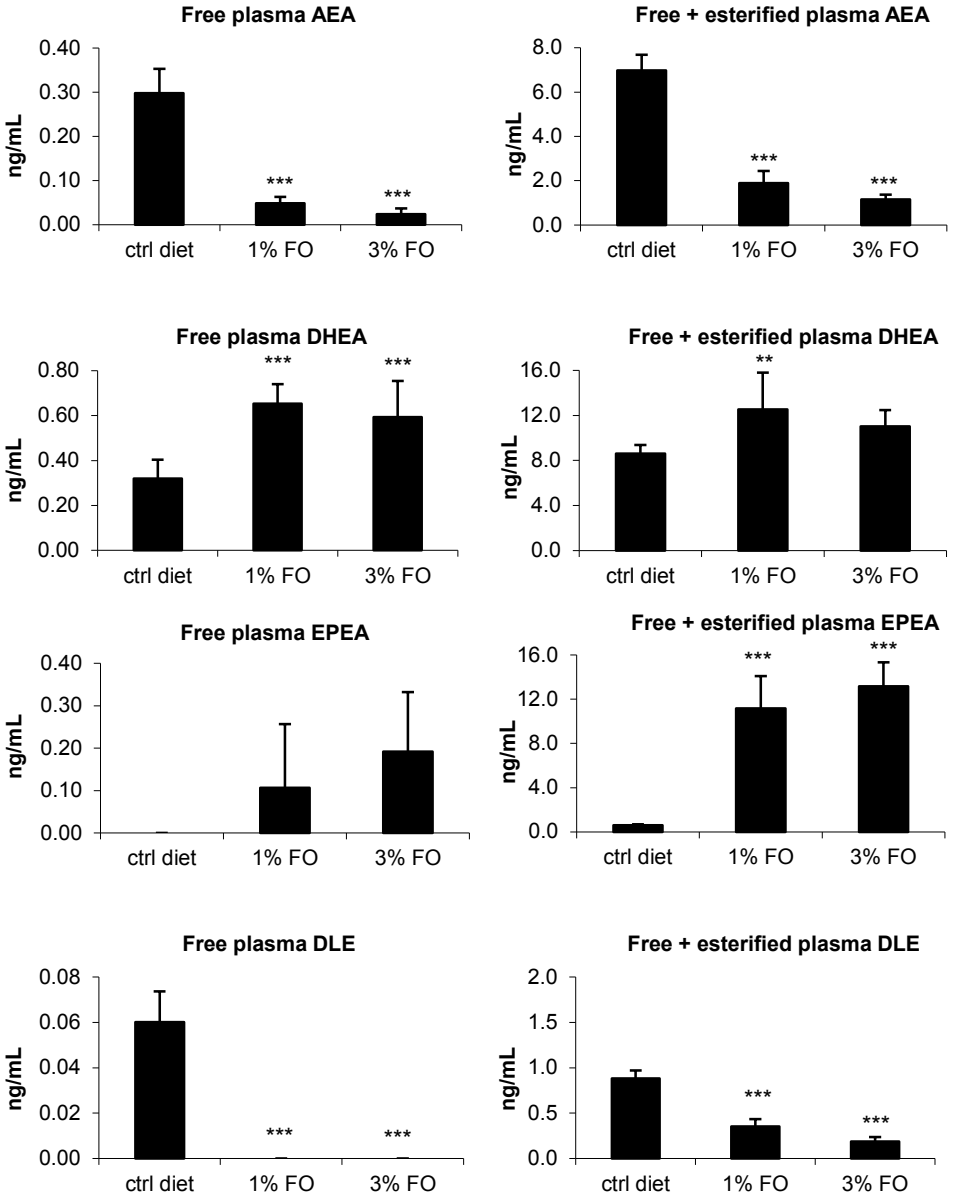

Figure 7.2: *Free plasma NAE levels (left) and free + esterified NAE levels (right) in mice fed a control diet, 1% FO or 3% FO diet. The effects of the FO diets are comparable for free plasma and esterified NAE levels, but the saponificated extracts contain higher NAE levels. * P<0.05; ** P<0.01; *** P<0.001 compared to ctrl diet.*

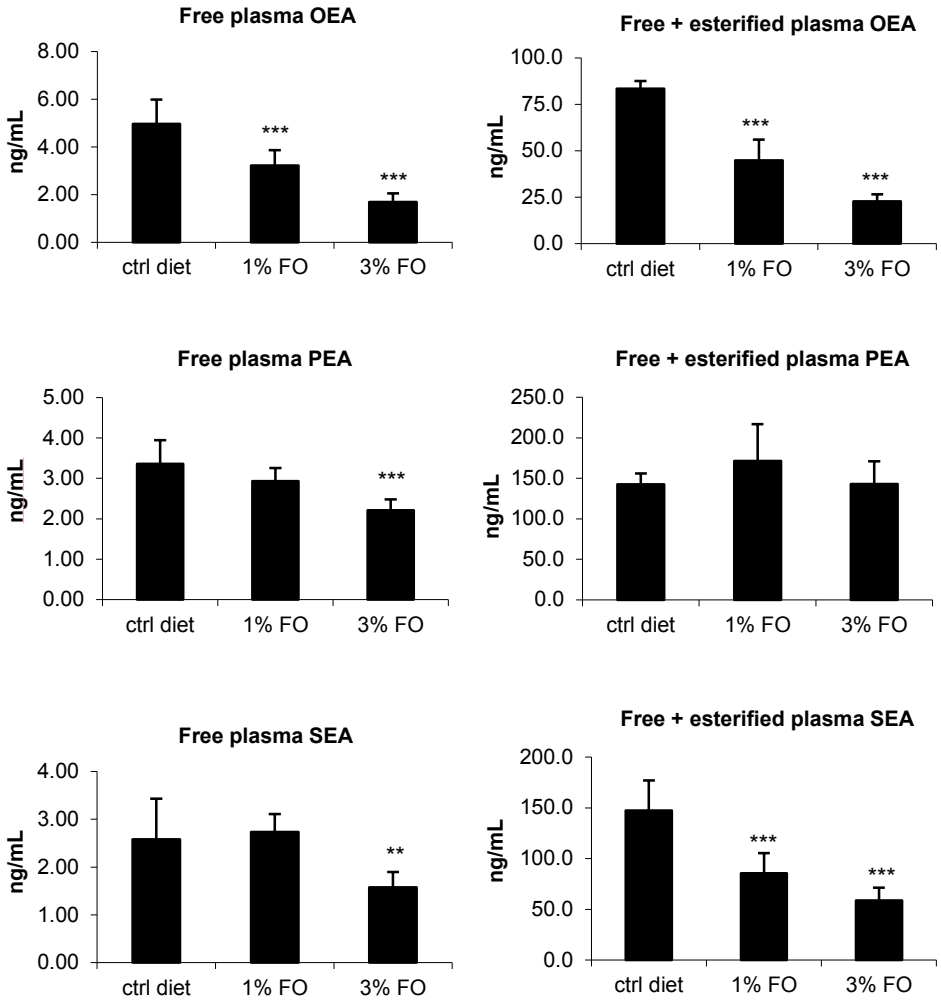

Figure 7.2 (cont.): *Free plasma NAE levels (left) and free + esterified NAE levels (right) in mice fed a control diet, 1% FO or 3% FO diet. The effects of the FO diets are comparable for free plasma and esterified NAE levels, but the saponificated extracts contain higher NAE levels. * P<0.05; ** P<0.01; *** P<0.001 compared to ctrl diet.*

addition, dietary fatty acids had the same effect on both free and esterified NAE profiles, increasing DHEA and EPEA levels and in general decreasing other NAEs. A

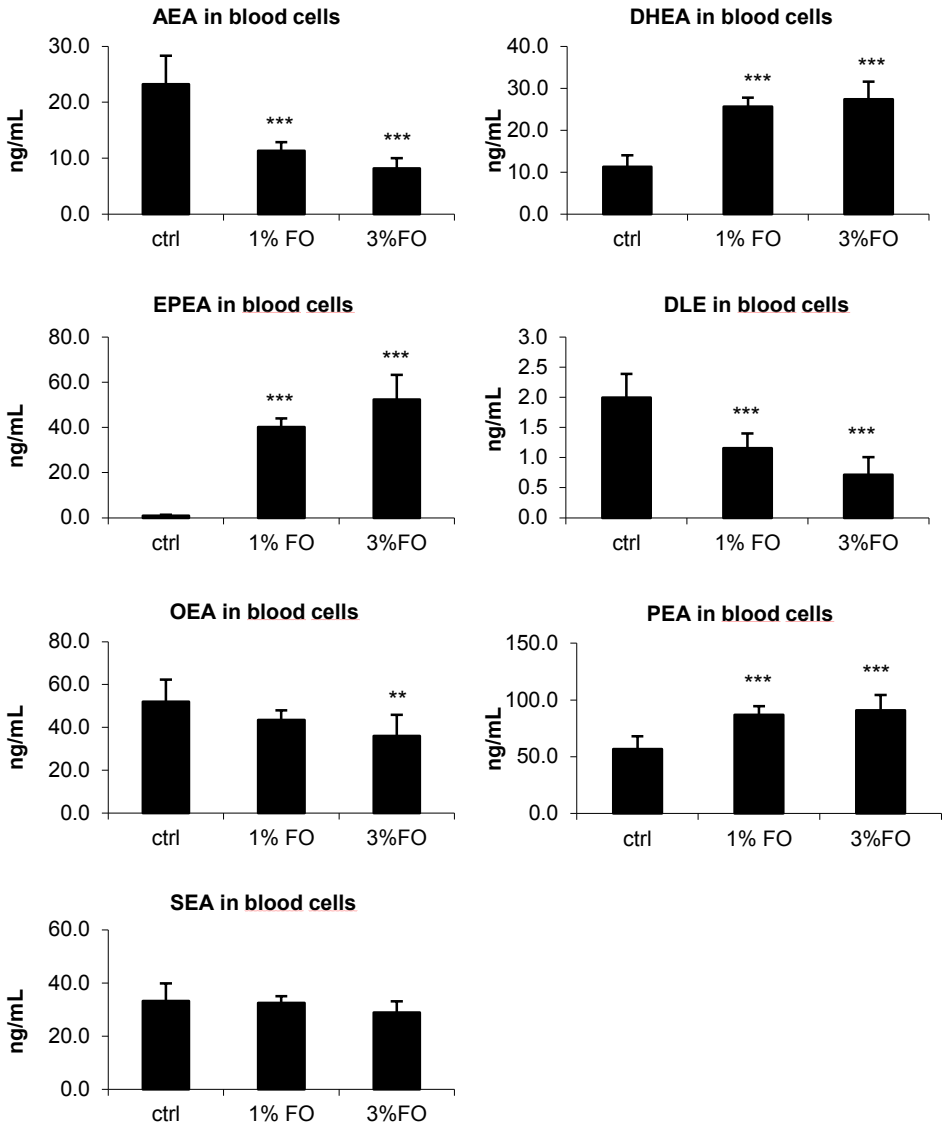

Figure 7.3: *NAEs in hydrolyzed blood cell extracts. * P<0.05; ** P<0.01; *** P<0.001 compared to ctrl diet.*

similar relation between dietary fatty acids and *in vivo* levels of NAEs has been described before [5, 26-29]. This shows that esterified NAEs are an appropriate

marker for free NAE levels, and it could be hypothesized that free plasma NAE levels are directly released from the esterified plasma NAE pool. However, the biochemical relation between free and esterified NAEs is not clear yet, and several considerations should be kept in mind. First, the compounds to which the NAEs are coupled to are not known, but the nature of the saponification hydrolysis combined with the structure formulas of NAEs rule out triglycerides as the source. Therefore, esterification to phospholipids is more likely. Plasma is known to contain *N*-acylphosphatidylethanolamines (NAPEs) [30]. NAPEs are cell membrane-associated intermediates in NAE biosynthesis and contain NAEs in esterified form [31, 32], but their contribution to the effects observed in the present chapter remains speculative. Secondly, it is not known whether the free plasma NAEs are directly derived from the as yet unidentified esterified plasma pool, from blood cells, liver or adipose tissue, or a combination of these. The solid relation between free and esterified NAE profiles in plasma does not necessarily rule out involvement of peripheral organs, as potential sources for esterified NAEs might be ultimately synthesized in *e.g.* liver. In addition to this, it is not clear what stimulus would result in the liberation of esterified NAEs in plasma, if this would be the case.

	Free NAE (ng/mL)	Free + esterified NAE (ng/mL)
AEA	0.37 ± 0.12	8.07 ± 1.45
DHEA	0.41 ± 0.24	3.99 ± 1.73
DLE	0.05 ± 0.03	0.73 ± 0.24
OEA	1.98 ± 0.70	22.66 ± 4.21
PEA	1.46 ± 0.38	50.92 ± 11.68
SEA	0.62 ± 0.22	34.31 ± 8.90

Table 7.2: *Free NAE levels and free + esterified NAE levels in human plasma (n=10).*

Blood cells were also shown to demonstrate significant amounts of esterified NAEs. In general, NAEs are synthesized from cell membrane phospholipids, involving calcium ion signaling and the action of multiple enzymes [31, 32], but these pathways have not been extensively investigated in blood cells. Blood contains different cell types, like erythrocytes and lymphocytes, and platelets. The contribution of these different cell types to the total esterified blood cell NAE

levels is not known, nor the relation to free plasma NAEs, but it is clear that blood cells contain significant amounts of NAEs. This might be of biological relevance as different studies have described effects of NAEs on immune cells [33, 34].
To summarize, we successfully developed and validated a new analytical method for the quantification of NAEs from 100 µL plasma samples, which was used to demonstrate that plasma contains levels of esterified NAEs which are approximately 20-to-60 fold higher than free NAE levels. In addition, blood cells also contain significant levels of esterified NAEs. Together, these data show that blood contains a previously ignored pool of NAEs which are esterified to both plasma and blood cell structures. The physiological relevance of these pools remains to be demonstrated.

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Chapter 8

General discussion

It is well established that dietary n-3 fatty acid intake is associated with anti-inflammatory effects. The mechanisms behind these effects have been shown to include binding to nuclear and G-protein coupled receptors, interactions with eicosanoid synthesis, and conversion to resolvins which display anti-inflammatory properties. Fatty acids are also precursors for *N*-acylethanolamines (NAEs)/endocannabinoids, but the anti-inflammatory effects of n-3 fatty acids have not been linked to the endocannabinoid system before. This thesis describes the link between (dietary) fatty acids, NAEs/endocannabinoids, and inflammation.

Nature's combinatorial chemistry: endocannabinoid levels depend on (dietary) supply of fatty acids

According to the most common view, endocannabinoids and other NAEs are locally released and rapidly broken down by enzymes including fatty acid amide hydrolase (FAAH) and monoacyl glycerol lipase (MAGL). The patterns of molecules which are released are considered to reflect the local availability of their precursor molecules. This can be regarded as a form of *combinatorial chemistry*: different end products are formed depending on the supply of precursors to a system. Using *in vitro* and *in vivo* studies, we demonstrated that the principle of combinatorial chemistry also applies for fish oil-derived endocannabinoids. The work showed that dietary fatty acids determine the profile of endocannabinoids rather than just individual compounds.

Adipocytes are known for their capacity to synthesize endocannabinoids and were previously used to study conversion of dietary fatty acids to NAEs [1]. In chapter 3, we demonstrated that adipocytes can convert the n-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) to their respective NAEs, namely docosahexaenoyl ethanolamide (DHEA) and eicosapentaenoyl ethanolamide (EPEA). When DHA and EPA were supplied to the adipocytes via the in medium, levels of other NAEs were barely affected. In contrast, when DHA and EPA were supplied in dietary fish oil to mice as described in chapter 6, the endocannabinoid and eicosanoid metabolomes were altered, with increased levels of DHEA, EPEA and n-3 derived eicosanoids such as the prostaglandins (PGs) PGD_3, PGE_3, thromboxane B3 ($TBXB_3$), and 5-hydroxyeicosapentaenoic acid (5-HEPE). This was paralleled by decreased levels of metabolites not derived from n-3 fatty acids. This demonstrated that fish oil alters metabolite profiles rather than

individual compounds, and that the principle of combinatorial chemistry also applies for *in vivo* condition after dietary fish oil. In general, these n-3 derived compounds are known to be less potent inducers of inflammation than its arachidonic acid-derived analogues [2-7]. As such, increased levels of n-3 derived compounds are thus in line with anti-inflammatory properties associated with n-3 fatty acid intake. Moreover, inflammation triggered the release of NAEs, including DHEA and EPEA.

The authors of previous papers have concluded that fish oil leads to a 'reduction' or 'inhibition' of the endocannabinoid system [8, 9], but such statements seem to be incorrect as our studies show that dietary fish oil changes the endocannabinoid profile towards increased levels of DHEA and EPEA, and reduced levels of other endocannabinoids.

Taken together, the *in vitro* and *in vivo* experiments described in this thesis demonstrate that the principle of combinatorial chemistry also applies to the relation between n-3 fatty acids and endocannabinoids.

Increases of DHEA and EPEA following a fish oil diet: what are the consequences?

Changes in metabolite patterns following a fish oil diet are descriptive, and it thus remains relevant to establish biological effects of n-3 fatty acid metabolites and, more particularly, patterns of n-3 and other fatty acid-derived metabolites. To the best of our knowledge, we were the first to report biological effects of n-3 derived NAEs. Using adipocytes, we demonstrated in chapter 3 that DHEA and EPEA have anti-inflammatory properties, thus suggesting that DHEA and EPEA play a role in the anti-inflammatory properties associated with fish oil intake [10]. As adipocytes play a key role in the pathophysiology of obesity, these findings might have implications for obesity research [11-14]. As for DHEA and EPEA, only anti-proliferative [15] and synaptogenic [16] properties are described in recent literature besides the anti-inflammatory effects we have reported, and clearly more work is needed to understand the biological effects of these fish oil derived endocannabinoids, especially in *in vivo* situations.

It should not be ignored that anti-inflammatory properties have been described for the other NAEs which were decreased by the fish oil diet. For instance, previous work from Cencioni *et al.* pointed to anti-inflammatory properties for arachidonoyl ethanolamide (AEA) [17]. This raises questions concerning the

relative efficacy of NAEs. When DHEA, EPEA, and AEA were compared in their effects on nitric oxide (NO) release from stimulated macrophages, DHEA and EPEA proved to inhibit NO release at lower doses compared to AEA [18]. Thus, fish oil replaces AEA for DHEA and EPEA, being more potent anti-inflammatory compounds compared to AEA in at least *in vitro* models of inflammation, and this might be one of the explanations for the anti-inflammatory effects of n-3 fatty acids *in vivo*.

Anti-inflammatory properties have also been described for oleoyl ethanolamide (OEA), palmitoyl ethanolamide (PEA) and stearoyl ethanolamide (SEA), and it has been hypothesized that the endocannabinoid system is part of a protective response against inflammatory stress [17, 19-26]. The effects observed for DHEA and EPEA are in line with this general finding, and the superior efficacy of these molecules suggests that dietary n-3 fatty acids exert their anti-inflammatory properties at least partly through fine-tuning the NAE profile towards increased proportions of DHEA and EPEA. In view of the effect of diet and inflammation on the levels of DHEA and EPEA, combined with their anti-inflammatory properties, it seems that these endocannabinoids are linking dietary n-3 fatty acids to their anti-inflammatory effects. Therefore, endocannabinoids represent a new branch to the tree which summarizes the pathways linking dietary n-3 fatty acid intake to anti-inflammatory effects (see Figure 8.1).

Fish oil fatty acids and their metabolites: other classes of metabolites besides NAEs

Repeating the experiments with the adipocytes using deuterated fatty acids revealed that only a small fraction of the n-3 fatty acids is converted to NAEs (chapter 3). The question emerges about the metabolic fate of the majority of the n-3 fatty acids. They could be left unchanged and dissolved in the medium, incorporated in cell membranes, or metabolized to other mediators which were not included in the MS platform used at that time. For instance, cyclo-oxygenase (COX) was shown to metabolize AEA to prostaglandin ethanolamides, and it has been speculated that cytochrome P450 (CYP) could convert AEA to 20-HETE-ethanolamide [27, 28]. In parallel to this, it could be that DHEA and EPEA are also substrates for COX or CYP, potentially yielding other metabolites with interesting biological properties. Another publication reported effects of a fish oil diet on 2-docosahexaenoyl glycerol and 2-eicosapentaenoyl glycerol levels [29], pointing to

the notion that n-3 fatty acids are also converted to other classes of metabolites besides eicosanoids and NAEs. In a recent paper we demonstrated the presence, formation, and putative effects of serotonin derivates of DHA and EPA in the intestinal tract [30]. Together these data show that fatty acids are converted to a (still expanding) variety of compounds, which can subsequently be converted to other metabolites with currently largely unknown biological effects. Thus, focusing on DHEA and EPEA will not be sufficient to understand the anti-inflammatory effects of n-3 fatty acids, and clearly more research is needed to identify and characterize other n-3 derived metabolites.

Targeted lipidomics combined with a multi-compartment approach revealed tissue- and compound specific effects of dietary fatty acids and inflammation

In order to investigate the effects of diet and inflammation on endocannabinoid profiles in more detail, an analytical method capable of quantifying a broad range of compounds was needed. At the start of the project, such methods were scarcely available. Therefore, we developed and validated an LC-MS/MS based analytical method for the quantification of a broad range of endocannabinoids and related structures, including several NAEs, 2-arachidonoyl glycerol (2-AG), fatty acid dopamines, and *N*-arachidonoyl glycine (NAGly) (chapter 2). Although similar in structure to a certain degree, the structural differences between these molecules made that the recoveries for these compounds were strongly dependent on the type of solid phase extraction (SPE) column used. As discussed in chapter 2, also the evaporation technique proved to be critical for recovery, with the dopamines and NAGly being the most difficult to recover. Nevertheless, by combining C8 SPE columns with vacuum concentration, we were able to quantify endocannabinoids and related compounds in plasma, and also from cell culture media and tissues. The versatility of the developed method was demonstrated with the addition of DHEA and EPEA in a later stage, since these compounds were not available at the time of initial method development and validation. The broad range of compounds which are included in this method proved to be critical to demonstrate that dietary fatty acid alter profiles of endocannabinoids rather than levels of individual compounds.

The combination of the LC-MS/MS methodology with a multi-compartment approach revealed additional information which would have been overlooked

when only plasma was investigated. In chapters 5 and 6, we describe that effects of lipopolysaccharide (LPS) and fish oil can not be generalized for certain metabolites, but can be opposite and site-specific. This holds important implications for research in this field, because effects on plasma levels of endocannabinoids or eicosanoids clearly do not reflect effects in peripheral tissues, and extrapolation from *e.g.* plasma values to tissue levels is thus not justified. Studying the plasma and multiple tissues endocannabinoid levels provides additional information and should be considered for future studies in this field.

Using multivariate data analysis we were able to show that adipose tissue contained a large number of metabolites which could separate the different diet groups under inflammatory conditions, clearly demonstrating increased levels of n-3 derived eicosanoids and endocannabinoids. This suggests that adipose tissue endocannabinoid and eicosanoid levels are reactive to changes in dietary patterns, especially during inflammatory conditions. As with adipokines, adipocyte-derived lipid mediators might be released to the bloodstream to act systemically, which might also be relevant for inflammatory processes elsewhere in the body. Perhaps, these mediators might also be relevant during normal conditions for maintenance of health. The data show that adipose tissue is an important and reactive site of synthesis for both endocannabinoids and eicosanoids, underlining the need to use multi-compartment approaches when investigating effects of diet on metabolism.

High n-3 fatty acid intake and acute inflammation

To study the effects of nutrition on health and disease, clear and practical definitions of health and disease are required. In many cases, the boundaries between health and disease are not well defined. Increased knowledge on how diseases develop and improved diagnostics have demonstrated that there is often a continuum between a healthy state and disease. Diseases are often diagnosed or measured based on a relatively small number of variables which are considered to be deviant or outside the normal range. At the same time, “health” refers a multi-dimensional, dynamic and time-dependent situation which is difficult to quantify, in particular when only a limited number of biomarkers are measured. Based on the classical principles of homeostasis and biological evolution, it is proposed that the term ‘health’ be defined as ‘the ability to adapt’ to internal and

external stimuli [31]. New methods and models are currently being developed that better take into account the complexity and balance of homeostatic

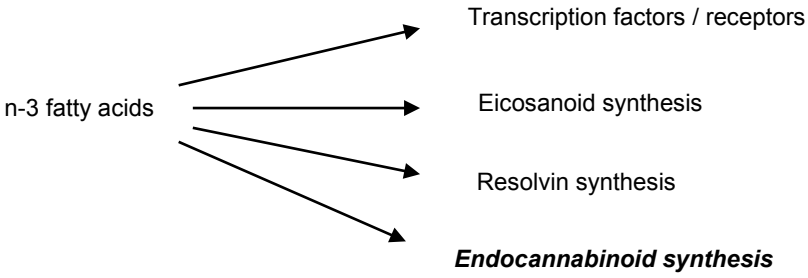

Figure 8.1: *Revised representation of the mechanisms through which n-3 fatty acids modulate inflammatory processes. In addition to the established mechanisms, the data in this thesis point to a role for endocannabinoids as mediators of the anti-inflammatory effects of n-3 fatty acids.*

mechanisms. These models are based on dynamic processes instead of single endpoints. Recent advances in genomics and systems biology enable researchers to measure and model biomarker profiles and to translate these into dynamic processes. According to these concepts, healthy individuals are better able to cope efficiently with stressors they encounter, meaning that they recover quickly from any disturbance of homeostasis. Strategies to measure health include "challenge" or "stress" tests in which individuals are subjected to a stressor and their reaction (biochemical, physiological, immunological) is monitored [32]. Several models to 'stress' individuals have been developed, subjecting individuals to nutritional stress (*e.g.* high glucose or fat intake in a short period of time) or functional stress (*e.g.* vigorous exercise or induction of inflammation by LPS) [32]. In the animal experiment described in chapter 6 we exposed animals to LPS and observed that the animals who received 3% fish oil (FO) showed more severe signs of illness when challenged with LPS compared to the control and 1% FO groups. Clearly, the 3% fish oil intake suppressed the animal's capability to overcome the LPS challenge. We were not the first to report that relatively high intakes of fish oil impair the hosts' immunological response to *e.g.* infection [33-

35]. Dietary n-3 fatty acid intake has been shown to reduce inflammation under conditions of chronic low-grade inflammation, such as rheumatoid arthritis or chronic inflammatory bowel disease [3, 4]. However, there are now several indications that high n-3 fatty acid intakes impair the resilience towards acute inflammatory stress. This should have implications on the way we look at beneficial effects of n-3 fatty acids as these might have adverse effects in certain situations, at least under acute inflammatory stress. It must be noted here that the 3% fish oil diet represents a rather high intake: allometric scaling from mouse (30 gr mouse; consuming 2 gr/day of a diet containing 3% fish oil of which 36% DHA + EPA) to a 75 kg human and a metabolic conversion factor of 6 would result in a daily dose of 9 gr DHA + EPA. This dose strongly exceeds the recommendation from the National Health Council, currently advising a daily intake of 450 mg. This also relates to the effects on the endocannabinoid and eicosanoid metabolomes, and it would be interesting to repeat this study using lower, more representative intakes of fish oil, and to make an effort to relate alterations in metabolomes to biomarkers of health or disease. Nevertheless, our data support previous observations that (very) high fish oil intakes can disturb the capability to recover from an acute inflammatory stimulus.

Endocannabinoid levels are personal and depend at least on diet

EPEA is normally not detected in plasma from mice fed a standard diet, but is present at quantifiable levels after the mice were fed a fish oil diet (chapter 6), indicating that endocannabinoid levels can depend on dietary patterns. In chapter 4, we described a positive correlation between serum free fatty acid levels and plasma NAE levels in women. The differences in NAEs and fatty acids between the subjects might be explained by differences in dietary habits, but this was not investigated here. Nevertheless, this shows that endocannabinoid levels are quite ‘personal’ as their levels are influenced by diet and postprandial status, demanding study designs which preferably do not compare different intervention groups in parallel, but rather use cross-over designs because this takes inter-subject variation more into account. As a consequence, it is difficult to define ‘normal’ values of endocannabinoid levels and it is better to refrain from using or defining such values.

Directions for future research

Advances in the field of analytical chemistry

As discussed above, it is clear that at present (n-3) fatty acid derived metabolites are still being identified, such as the fatty acid serotonin conjugates and further COX or CYP metabolites of NAEs. This suggests that there are still numerous compounds waiting to be discovered. The scientific community will not be able to fully understand the biology n-3 fatty acids unless a complete overview of their metabolism is available. Thus, the field of analytical chemistry should focus on the identification and characterization of novel (n-3) fatty acid derived metabolites, methods that enable to measure several metabolites in one sample, and statistical models to interpret these complex lipidomic data sets.

Advances in the field of biology

It is clear from our data that dietary fatty acids alter the profile of endocannabinoids, but it is unclear how this would affect health and disease. A better understanding of the relation between dietary fatty acids and health requires more knowledge on the biological effects of fatty acid metabolites, and special attention should be given to biological effects of endocannabinoid profiles rather than individual compounds. As for the endocannabinoids, AEA and 2-AG are the best studied compounds, and far less is known about *e.g.* DLE, DHEA, and EPEA. This gap needs to be closed, thus requiring more functional characterization of at least DHEA and EPEA, but also other n-3 derived metabolites. In line with the notion that diet affects endocannabinoid profiles, it would be interesting to test the effect of combinations of *e.g.* endocannabinoids on inflammation instead of testing only single compounds. Exposure to multiple compounds at the same time is also a more relevant representation of the *in vivo* situation as endocannabinoids are co-existing. Related to this, the individual endocannabinoids and NAEs have a distinct pharmacodynamic signature, targeting receptors like CB1, CB2, TRPV-1, and the PPARs with different affinities [36-41]. Thus, as these endocannabinoids are present at the same time, their concentration profile determines the biological outcome as different receptors might be more or less stimulated. Diet has an effect on the balance of the endocannabinoid metabolome, increasing levels of certain compounds at the

expense of others, supporting the importance of testing combinations or patterns of endocannabinoids rather than individual compounds.

Towards a role for nutrition-based modulation of endocannabinoids in health and disease in humans using n-3 fatty acids

To the best of our knowledge, no data has been published that points to a role for nutrition-based modulation of endocannabinoids in health and disease using n-3 fatty acids. Considering the regulatory role of endocannabinoids in metabolism and inflammation, and their dependence on at least nutritional habits and inflammatory status, a role as a biomarker is at hand. There is a limited amount of literature available on this topic, pointing to such a relation with cardiometabolic risk factors and obesity [42-44]. Increased plasma AEA and 2-AG levels are observed in obesity [45, 46], and one report demonstrated that life style intervention in obesity improves cardiometabolic risk factors in parallel to decreases in plasma AEA and 2-AG levels [43]. For most risk factors, correlations with 2-AG were established, but this might be explained by the fact that DHEA and EPEA are often not reported. An exception to this is the publication from Sipe *et al.*, pointing to decreased DHEA levels in obese persons compared to normal-weight counterparts [44]. Considering the above-mentioned correlations in the obese state, it would be interesting to investigate whether endocannabinoid levels have a predictive capacity regarding the future development of disease in apparently healthy individuals, *e.g.* whether the development of disease is preceded by (early) alterations in endocannabinoid profiles. In addition to this and considering the findings presented in this thesis, it is very interesting to explore whether dietary n-3 fatty acids modulate the endocannabinoid system in humans, and to investigate how a possible alteration correlates with the maintenance of health and the development of disease. Of course, such a (descriptive) correlation would only point to a role as a biomarker, and a causal relation between altered endocannabinoid levels and effects on health still remain to be established. The relation between dietary n-3 fatty acid intake and *in vivo* endocannabinoid levels in obese subjects was published by Banni and coworkers in 2010 [8], but no data on DHEA or EPEA were included in that report, and associations with cardiometabolic risk factors were not investigated. Nevertheless, the established endocannabinoids are interesting biomarkers, and DHEA and EPEA might also

have potential use as biomarkers of health and disease and might causally link dietary n-3 fatty acids to health effects.

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Samenvatting

Gezonde voeding is een van de belangrijkste factoren die van invloed zijn op onze gezondheid. Van veel voedingsstoffen is bekend dat ze een negatieve invloed hebben op onze gezondheid, maar tegelijkertijd zijn er ook voedingsstoffen die een gunstig effect kunnen hebben. Een voorbeeld hiervan is visolie, waarvan gebleken is dat het ontstekingsremmende eigenschappen heeft. De mechanismen hierachter zijn nog niet geheel doorgrond, maar het is duidelijk dat de zogenaamde omega-3 (ook wel genoteerd als n-3 of ω-3) vetzuren die in visolie zitten van invloed zijn op de vorming van eicosanoïden, een groep vetzuur-afgeleide verbindingen die ontstekingsprocessen reguleren. Vetzuren kunnen ook omgezet worden tot de zogenaamde *N*-acyl ethanolamines (NAEs), waarvan de endocannabinoïd anandamide (AEA) een bekend voorbeeld is. Ook NAEs hebben invloed op ontstekingsprocessen, maar een verband tussen inname van visolie, vorming van NAEs, en ontsteking was nog niet eerder onderzocht. Dit proefschrift beschrijft een reeks studies die zijn uitgevoerd om een verband tussen vetzuren uit het dieet, de vorming van endocannabinoïden/NAEs, en ontsteking te onderzoeken.

Om de invloed van dieet op de vorming van endocannabinoïden/NAEs te bestuderen, is het nodig om een analysemethode te hebben waarmee een breed aantal verbindingen gemeten kan worden. Hiervoor is een analysemethode op basis van vloeistofchromatografie gekoppeld aan massaspectrometrie (LC-MS/MS) ontwikkeld. Met behulp van deze methode is aangetoond dat n-3 vetzuren door vetcellen (adipocyten) *in vitro* kunnen worden omgezet naar hun endocannabinoïde analogen. Deze n-3 afgeleide NAEs, genaamd docosahexaenoyl ethanolamide (DHEA) en eicosapentaenoyl ethanolamide (EPEA), bleken tevens ontstekingsremmende eigenschappen te hebben in lipopolysaccharide (LPS)-gestimuleerde adipocyten, waarbij de secretie van de cytokines interleukine-6 (IL-6) en momocyte chemotactic protein (MCP-1) verminderd werd. Vervolgstudies toonden aan dat er een correlatie was tussen serum vetzuurconcentraties en plasma NAE concentraties, en ook bleek dat plasma AEA en oleoyl ethanolamide (OEA) concentraties correleerden met de 'body mass index' (BMI).
Gezien de complexiteit van het metabolisme van endocannabinoïden en eicosanoïden is het waarschijnlijk dat de concentraties ervan dynamisch en weefsel-specifiek zijn gedurende ontsteking. De meerderheid van eerder gepubliceerde studies waren echter gericht op slechts enkele verbindingen en in

een beperkt aantal weefsels. Daarnaast was nog niet bekend wat het effect van ontsteking op DHEA en EPEA zelf was. Om beter inzicht hierin te krijgen, werd in een dierstudie het tijdsafhankelijke effect van ontsteking op endocannabinoïden en eicosanoïden in plasma, lever, ileum en vetweefsel in detail bestudeerd ('multi-compartment targeted lipidomics approach'). De resultaten lieten zien dat de concentraties DHEA en EPEA werden verhoogd door LPS, maar er werden ook tijds- en weefselafhankelijke effecten gevonden. Op basis van deze data werd een tweede dierstudie uitgevoerd, waarin het gecombineerde effect van verschillende visoliediëten én ontsteking op de profielen van endocannabinoïden en eicosanoïden werd bestudeerd met behulp van dezelfde 'targeted lipidomics approach'. Uit deze studie bleek dat n-3 vetzuren en ontsteking de profielen van endocannabinoïden en eicosanoïden beïnvloedden waarbij er hogere concentraties van n-3 afgeleide metabolieten aanwezig waren, hetgeen ten koste ging van metabolieten die afkomstig zijn van andere vetzuren. Multivariate data analyse liet zien dat er scheiding was van de verschillende dieetgroepen bij zowel normale condities en tijdens ontsteking. Tijdens normale condities werd de scheiding tussen de dieetgroepen voornamelijk verklaart door een afname van anders dan n-3 afgeleide metabolieten. Echter, tijdens ontsteking werd scheiding tussen de dieetgroepen voornamelijk verklaart door een toename van n-3 vetzuur afgeleide metabolieten. Tot slot bleek uit aanvullende analyses dat plasma en bloedcellen aanzienlijke hoeveelheden veresterde NAEs bevatten. De hoeveelheden veresterde NAEs waren ongeveer 20 tot 60 keer hoger dan de vrije NAE waarden, en de profielen ervan komen overeen met die van vrije NAEs.

Samenvattend verhogen n-3 vetzuren uit het dieet de concentraties van DHEA en EPEA, en deze verbindingen hebben ontstekingsremmende eigenschappen. Hoewel de n-3 vetzuren waarschijnlijk naar nog meer klassen van metabolieten omgezet kunnen worden, suggereert het werk in dit proefschrift dat 'visolie-afgeleide' endocannabinoïden een nieuwe verbinding vormen tussen visolie en ontstekingsremmende eigenschappen. Meer onderzoek is nodig om een verband aan te tonen tussen de verandering van profielen van endocannaboïden door middel van dieet en effecten hiervan op ziekte en gezondheid.

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About the author

Curriculum vitae

Michiel Gerard Juliaan Balvers was born on June 28, 1981 in ‘s-Hertogenbosch, the Netherlands. After completing secondary school at the Jeroen Bosch College in ‘s-Hertogenbosch in 2000, he started studying Biomedical Sciences at the Radboud University Nijmegen and specialized in pathobiology and toxicology. He completed traineeships at the department of Pathology of the Radboud University Nijmegen Medical Centre, and department of Physiological Sciences at TNO Quality of Life (Zeist, the Netherlands), where he gained experience in molecular biology, *in vitro* experiments, and analytical chemistry. After obtaining his MSc degree in 2005, he first completed a one-year teacher training course before being appointed as a PhD candidate at the division of Human Nutrition of Wageningen University and TNO to work on the so-called ‘Balance project’. This research project investigated the relation between dietary fatty acids, endogenous fatty acid metabolites, and inflammation as described in this thesis. The experimental work was largely performed at TNO and involved the development and implementation of lipidomic analyses, *in vitro* experiments, and dietary studies in animals.
Michiel currently works in the group of Prof. dr. Renger Witkamp (division of Human Nutrition, Wageningen University) where he investigates the effects of newly discovered fatty acid metabolites on intestinal physiology and inflammatory processes.

List of publications

Balvers MGJ, Verhoeckx KCM, Meijerink J, Bijlsma S, Rubingh CM, Wortelboer HM, and Witkamp RF. *Fish oil and inflammatory status alter the n-3 to n-6 balance of the endocannabinoid and oxylipin metabolomes in mouse plasma and tissues*. Accepted for publication, Metabolomics

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Verhoeckx KCM, Voortman T, **Balvers MGJ**, Hendriks HF, Wortelboer HM, and Witkamp RF. *Presence, formation and putative biological activities of N-acyl serotonins, a novel class of fatty-acid derived mediators, in the intestinal tract.* Biochimica Biophysica Acta - Molecular and Cell Biology of Lipids (2011)

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Balvers MGJ, Verhoeckx KCM, and Witkamp RF. *Development and validation of a quantitative method for the determination of 12 endocannabinoids and related compounds in human plasma using liquid chromatography-tandem mass spectrometry.* Journal of Chromatography B (2009)

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van Herpen CM, van der Laak JA, de Vries IJ, van Krieken JH, de Wilde PC, **Balvers MGJ**, Adema GJ, and de Mulder PH. *Intratumoral recombinant human interleukin-12 administration in head and neck squamous cell carcinoma patients modifies locoregional lymph node architecture and induces natural killer cell infiltration in the primary tumor.* Clinical Cancer Research (2005)

Overview of completed educational activities

Discipline specific activities

Dutch Society for Massaspectometry (NVMS) annual meetings (2007 & 2011)
Avans Plus LCMS training course (2007)
NUTRIM course Regulation of food intake and satiety (2008)
Thermo Scientific LCMS user meeting (2008)
ECO 2008 conference (2008)
NWO Nutrition annual meetings (2008-2011)
Federation Innovative Medicine Research (FIGON) annual meetings (2008-2010)
International Cannabinoid Research Society (ICRS) annual conferences (2009-2011)
ISSFAL conference (2010)
Pharma-Nutrition conference (2011)

General activities

TNO Good Laboratory Practice (GLP) introduction course & SOP writing (2007-2008)
VLAG PhD week (2008)
TNO Time Management workshop (2011)
TNO Reporting and communicating with clients (2010-2011)
TNO Technician training & technical support (2011)

Optionals

Preparation Research proposals
Human Nutrition PhD tour (2009)
TNO Scientific colloquia (2007-2011)

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