UNIVERSITÉ **LAVAL**

Régulation de l'inflammation par les lipides bioactifs : interactions biosynthétiques et fonctionnelles entre les endocannabinoïdes et les éicosanoïdes

Thèse

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Résumé

Les maladies inflammatoires chroniques sont un fardeau de santé important à travers le monde. Les traitements actuellement disponibles soulagent la douleur et l'inflammation, mais leurs effets secondaires rendent leur utilisation à long terme risquée. À la lumière de cette problématique, la communauté scientifique s'intéresse au potentiel d'anti-inflammatoires naturels comme les endocannabinoïdes. Les endocannabinoïdes sont des lipides endogènes qui activent les récepteurs cannabinoïdes (CB_1 et CB_2). Ils régulent ainsi divers processus physiologiques tels l'appétit, l'adipogénèse et la nociception. Les deux endocannabinoïdes les mieux caractérisés, le 2-AG et l'AEA, peuvent également moduler l'inflammation en activant le récepteur CB_2 à la surface des cellules immunitaires. Les souris déficientes pour le récepteur CB_2 présentent un phénotype inflammatoire exacerbé, suggérant que ce récepteur est anti-inflammatoire. Cependant, le rôle des endocannabinoïdes dans l'inflammation est beaucoup plus complexe puisqu'ils peuvent être métabolisés en une grande variété de médiateurs lipidiques de l'inflammation. Leur voie de dégradation principale est leur hydrolyse en acide arachidonique (AA), qui sert de précurseur à la biosynthèse d'éicosanoïdes pro-inflammatoires comme le leucotriène B_4 et la prostaglandine E_2. Ils peuvent également être métabolisés directement par certaines enzymes impliquées dans la synthèse d'éicosanoïdes, pour générer des médiateurs comme les prostaglandines-glycérol (PG-G). Par conséquent, les endocannabinoïdes peuvent générer un profil unique d'effets pro- et anti-inflammatoires. Des stratégies thérapeutiques visant à bloquer l'hydrolyse des endocannabinoïdes pour amplifier leurs effets anti-inflammatoires ont été étudiées, mais une très grande proportion de cette recherche a été effectuée sur des animaux. Les façons dont les endocannabinoïdes sont synthétisés et dégradés par les leucocytes humains, ainsi les effets de leurs métabolites sur les fonctions de ces cellules, sont mal définis. Bien que les résultats obtenus dans des modèles animaux soient prometteurs, ces mécanismes doivent être mieux caractérisés chez l'humain avant qu'il ne soit envisageable de les manipuler afin de traiter les maladies inflammatoires.

Le premier objectif de mon doctorat était de caractériser les voies de dégradation et de biosynthèse des endocannabinoïdes chez les leucocytes humains. Nous avons documenté l'expression de toutes les lipases hydrolysant le 2-AG, chez plusieurs types leucocytaires. Ces résultats ont souligné que chaque leucocyte exprime plusieurs 2-AG hydrolases et que les inhibiteurs sélectifs actuellement disponibles n'inhibent que partiellement l'hydrolyse du 2-AG chez ces cellules. Ces données ont également démontré que les leucocytes humains hydrolysent très efficacement le 2-AG, une découverte qui nous a permis de mettre en évidence une nouvelle voie de biosynthèse du 2-AG par les leucocytes. En présence d'inhibiteurs d'hydrolyse, les neutrophiles, éosinophiles et monocytes stimulés avec de l'AA ont produit des quantités de 2-AG environ 1000 fois supérieures aux niveaux rapportés dans la littérature. Ils ont également transformé d'autres acides gras polyinsaturés en leurs endocannabinoïdes-glycérol respectifs. Nous avons démontré que cette voie de

biosynthèse est dépendante de la réacylation des acides gras dans les phospholipides membranaires, et que leur métabolisme subséquent en endocannabinoïdes implique possiblement l'acide lysophosphatidique comme intermédiaire. Cette étude est la première à rapporter une biosynthèse significative d'endocannabinoïdes par les leucocytes humains, et à démontrer que cette biosynthèse est indépendante de la voie classique de biosynthèse du 2-AG.

Nous avions également pour objectif de caractériser l'impact du 2-AG et des PG-G sur les fonctions des leucocytes humains. Nous avons démontré qu'en présence d'IL-5, une cytokine impliquée dans l'inflammation éosinophilique présente dans l'asthme, le 2-AG induit une migration importante des éosinophiles. Cet effet du 2-AG requiert à la fois l'activation du récepteur CB_2 et l'hydrolyse du 2-AG en AA pour produire des métabolites de la 15-lipoxygénase. Ceci souligne que l'hydrolyse du 2-AG permet la production de médiateurs causant des effets pro-inflammatoires et qu'il serait souhaitable de bloquer cette hydrolyse in vivo. Finalement, nous avons étudié l'effet de la PGE_2-G sur les fonctions des neutrophiles et démontré qu'elle inhibe plusieurs fonctions effectrices de ces cellules. Cet effet inhibiteur nécessite l'hydrolyse de la PGE_2-G en PGE_2 par les neutrophiles, et l'activation du récepteur EP_2 à leur surface.

Nos travaux permettront de mieux comprendre la façon dont l'hydrolyse des endocannabinoïdes devrait être bloquée chez les humains, ainsi que tous les effets biologiques qui en découleront. Le but ultime est de développer de nouveaux traitements contre les maladies inflammatoires chroniques, qui maximiseront les effets analgésiques et anti-inflammatoires des endocannabinoïdes tout en limitant leurs effets néfastes.

Abstract

Chronic inflammatory diseases are an important health burden worldwide. The currently available treatments alleviate pain and inflammation, but their numerous adverse effects make their long term use difficult. Therefore, the scientific community is studying the anti-inflammatory potential of mediators such as endocannabinoids. Endocannabinoids are endogenous lipids that activate the cannabinoid receptors, namely CB_1 and CB_2. In doing so, they regulate various physiological functions and cognitive processes functions such as appetite, adipogenesis and nociception. The two best-characterized endocannabinoids, 2-AG and AEA, also exert effects on immune cell functions, leading to the modulation of immunity and inflammation. They do so by activating the CB_2 receptor, which is expressed in the periphery, notably on immune cells. Notably, it was shown that mice lacking the CB_2 receptor display an exacerbated inflammatory phenotype, suggesting that CB_2 activation by endocannabinoids is anti-inflammatory. However, the biological effects of endocannabinoids are far more complex, given that they can be metabolized into a wide variety of bioactive lipids. The main degradation pathway for 2-AG and AEA is their hydrolysis into arachidonic acid (AA), a fatty acid that acts a precursor for the biosynthesis of several pro-inflammatory eicosanoids such as leukotriene B_4 and prostaglandin E_2. They can also be directly metabolized by eicosanoid-biosynthetic enzymes, which generates mediators such as glyceryl-prostaglandins (PG-Gs). Therefore, endocannabinoids can generate an intriguing profile of pro- and anti-inflammatory effects, depending on the balance between their catabolic pathways and receptor activation. Therapeutic strategies aiming at blocking endocannabinoid hydrolysis to amplify their anti-inflammatory effects have been extensively studied. However, most of these studies were conducted in animals. Endocannabinoid metabolism by human leukocytes, as well as the effects of their metabolites on human leukocytes functions, are poorly defined. Although the data obtained from animal models is promising, these mechanisms must be characterized in humans before they can be manipulated to treat inflammatory diseases.

Our first aim was to characterize endocannabinoid biosynthetic and hydrolytic pathways in human leukocytes. We documented the expression of several 2-AG hydrolases in human neutrophils, eosinophils, monocytes, lymphocytes and alveolar macrophages. The data we obtained underscored that each cell type expresses several 2-AG hydrolases, and that the selective inhibitors that are currently available only partially block 2-AG degradation by leukocytes. Our results also show that human leukocytes are experts at hydrolyzing 2-AG, a finding that allowed us to establish a novel 2-AG biosynthetic pathway in human leukocytes. In the presence of 2-AG hydrolysis inhibitors, neutrophils, eosinophils and monocytes stimulated with AA produced 2-AG in amounts ~ 1000 times greater than those previously reported. They also converted other polyunsaturated fatty acids into their glycerol-containing endocannabinoid counterparts. We showed that this endocannabinoid

biosynthetic pathway depends on fatty acid reacylation into membrane phospholipids, and that their subsequent metabolism into endocannabinoid likely requires the production of a lysophosphatidic acid intermediate. This study is the first one to report a significant endocannabinoid synthesis by human leukocytes, and to show that this biosynthesis is independent from the classical 2-AG biosynthetic pathway.

We also aimed to characterize the impact of 2-AG and PG-Gs on human leukocyte functions. We showed that in the presence of IL-5, a cytokine involved in the eosinophilic inflammation found in asthma, 2-AG induces eosinophil migration. This requires the activation of the CB_2 receptor, as well as 2-AG hydrolysis into AA to produce 15-lipoxygenase metabolites. This underscores that 2-AG hydrolysis by eosinophils allows for the synthesis of mediators that have pro-inflammatory effects, and that blocking this hydrolysis in vivo may be beneficial. We also studied the biological effects of PGE_2-G and found that it inhibits several effector functions of human neutrophils. This inhibitory effect requires PGE_2-G hydrolysis into PGE_2 by neutrophils, and the activation of the EP_2 receptor on their surface.

Our work will allow a better understanding of how endocannabinoid hydrolysis should be blocked in humans, and of the biological effects that will result from this inhibition. The goal is to develop new treatments against chronic inflammatory diseases, which will enhance the analgesic and anti-inflammatory effects of endocannabinoids while limiting their deleterious effects.

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Abréviations

ABHD	*Alpha beta hydrolase domain*
ABPP	*Activity-based protein profiling*
AMP	Adénosine monophosphate
ARN	Acide ribonucléique
CES	Carboxylestérase
COX	Cyclooxygénase
CP55,940	(-)-cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol
DAG	Diacylglycérol
FAAH	*Fatty acid amide hydrolase*
fMLP	N-Formylméthionine-leucyl-phénylalanine
FP	Fluorophosphonate
GM-CSF	*Granulocyte-macrophage colony-stimulating factor*
GTP	Guanosine triphosphate
ICAM	*Intracellular adhesion molecule*
IL	Interleukine
JZL184	4-nitrophenyl-4-[bis(1,3-benzodioxol-5-yl)(hydroxy)methyl]piperidine-1-carboxylate
LPA	Acide lysophosphatidique
LT	Leucotriène
LYPLA	Lysophospholipase
MAFP	Méthyl arachidonoyl fluorophosphonate
MAG	Monoacylglycérol
MMP	*Matrix metallopeptidase*
NADP	Nicotinamide adenine dinucleotide phosphate
NDGA	Acide nordihydroguaiarétique
NET	*Neutrophil extracellular trap*
PAF	*Platelet activating factor*
PBMC	*Peripheral blood mononuclear cell*
PG	Prostaglandine
PLC	Phospholipase C
PPT	*Palmitoyl protein thioesterase*
PSGL	*P-selectin glycoprotein ligand*
ROS	Espèce oxygénée réactive
TAMRA	Tétraméthylrhodamine
THL	Tétrahydrolipstatine
UDP	Uridine diphosphate
WWL70	N-methyl-N-[[3-(4-pyridinyl)phenyl]methyl]-carbamic acid 4'-(aminocarbonyl)[1,1'-biphenyl]-4-yl ester
WWL113	4'-[[[Methyl[[3-(4-pyridinyl)phenyl]methyl]amino]carbonyl]oxy]-[1,1'-biphenyl]-4-carboxylic acid ethyl ester
VCAM	*Vascular cell adhesion protein*

Remerciements

Contrairement à bien des gens, j'ai entrepris mon doctorat sans hésiter et sans trop me questionner. Dans mon cas, le moment des doutes et des remises en question est arrivé plus tard, alors que j'étais rendue trop loin dans mon cheminement pour qu'abandonner soit une option. Malgré cela, je ne sais pas si j'aurais complété mon doctorat si je n'avais pas eu tout plein de gens fantastiques qui m'ont écoutée, épaulée et par moment, traînée vers la ligne d'arrivée.

Tout d'abord, je remercie mon directeur de recherche, le **Dr Nicolas Flamand**, d'avoir été mon mentor depuis mes débuts dans le monde de la recherche académique. Tu as su identifier mes forces et les mettre à profit, tout en travaillant fort pour que j'améliore mes faiblesses (et je sais que ça n'a pas été facile !). Je remercie également ma co-directrice, **Dre Marie-Renée Blanchet,** pour ses idées et son support tout au long des années. Merci au **Dr Michel Laviolette** d'avoir participé aussi activement à l'aspect clinique de mon projet de recherche et d'avoir été extrêmement généreux de son temps en apportant des révisions de qualité aux publications contenues dans cette thèse. Merci à tous les autres co-auteurs et collaborateurs impliqués dans les différentes études de mon projet de recherche, sans qui la réalisation de cette thèse aurait été impossible. Merci aux membres de mon jury, soit le **Dr Vincenzo Di Marzo,** le **Dr Cristoforo Silvestri** et le **Dr Patrick McDonald.**

Je veux remercier du fond du cœur les membres de l'équipe Flamand, avec qui j'ai travaillé pendant pas moins de 7 années. **Véro et Cyril**, je ne saurai jamais comment vous remercier pour tout le support technique et moral que vous m'avez apporté au fil des années. Véro, ta bonne humeur contagieuse et ton expertise inégalée ont été un modèle à suivre pour la bébé-scientifique que j'étais en débutant mes études. Cyril, tes journées passées devant le spectromètre de masse à mettre au point mes analyses (et à devoir inévitablement faire une courbe standard de plus, puis une autre, puis une autre...) étaient plus qu'appréciées. Presque autant que les bonbons que tu ramenais de France après tes vacances. Je vais m'ennuyer des niaiseries qu'on racontait dans le labo quand la porte était fermée ! Merci aux stagiaires qui ont été impliquées à différents stades de mon projet et m'ont donné un énorme coup de main : **Chloé, Mégane et Élizabeth**. Merci à **Francesco**, qui est débarqué au labo à la toute fin de mon doctorat mais avec qui j'ai beaucoup aimé travailler. Merci à tous mes **collègues de pneumo**, avec qui j'ai eu de fréquentes discussions de pertinence variable et qui ont contribué à rendre mes années de formation agréables. Merci à **Em**, grâce à qui j'ai survécu à des cours du lundi matin difficiles. Merci à **Ariane** d'avoir choisi notre labo et notre bureau comme quartiers généraux de procrastination et d'avoir donné lieu à bien des 5 à 7 (3 à 5) improvisés ! Et finalement, un immense merci à **Anne-So**, avec qui j'ai eu des milliers de fou rires et écouté des quantités impressionnantes de One Direction en travaillant dans le laboratoire. Je vais toujours me rappeler le moment où on a échoué

lamentablement à préparer un tampon phosphate (monobasique ou dibasique ?). Ton soutien, ainsi que ton acharnement légendaire à me rappeler mes gaffes de labo pour pouvoir rire de moi à l'infini, m'ont été indispensables et je suis très contente de pouvoir te compter parmi mes amis.

En dehors du laboratoire, j'ai été soutenue et encouragée par tellement de personnes que je ne sais pas par où commencer. Merci à mes parents, **Sylvain et Claire**, ainsi qu'à mon petit frère, **Christian**. Merci pour votre support, ainsi que pour votre compréhension face à la fréquence décroissante de mes voyages Québec-Edmundston à mesure que mon doctorat avançait. Merci à ma tante **Danielle**, qui a été mon inspiration depuis mon enfance et sans qui je n'aurais probablement jamais osé me diriger vers la recherche biomédicale. Merci à ma meilleure amie depuis toujours, **Pascale**. Si les gens voyaient tout le temps qu'on a passé à boire du vin, magasiner, aller voir des spectacles de musique et chanter dans l'auto en allant voir des spectacles de musique, ils auraient de la misère à croire qu'on a réussi à faire chacun doctorat à travers tout ça ! Merci à **Manue** et **Marilène** qui, en plus d'avoir été des amies formidables, m'ont endurée en tant que colocataire pendant mes études graduées. Merci à **Anne-Ju**, qui a toujours été une oreille attentive et qui était toujours partante pour sortir veiller tard (parfois même jusqu'à 22h30 un vendredi !).

Et finalement, le dernier mais non le moindre : MERCI à mon *partner in crime*, **Jo**. Tu as été extrêmement patient et compréhensif pendant la fin de mon doctorat et la rédaction de ma thèse, une période pendant laquelle j'ai malheureusement été une énorme boule de stress. J'ai apprécié chaque *pep talk*, chaque repas que tu as cuisiné quand je n'avais pas le temps, et surtout, ton grand sens de l'écoute. Maintenant que ce chapitre se termine, je vois dans notre avenir beaucoup d'aventures, de *geekiness* et de brassage de bière !

Avant-propos

Cette thèse contient les travaux effectués dans le cadre de mon doctorat, que ce soit des revues de la littérature ou de la recherche originale en laboratoire. Elle contient 6 articles, dont 5 sont publiés ou soumis pour publication. Les chapitres 1 et 2 sont des articles de revue de que j'ai écrits et publiés pendant mes études au doctorat. Ces chapitres sont complémentaires à l'introduction, et leur contenu et celui de mon chapitre d'introduction ne sont pas redondants. Ma contribution dans la préparation de ces articles, ainsi que celle de mes co-auteurs, se détaillent comme suit :

Chapitre 1

Caroline Turcotte, François Chouinard, Julie S Lefebvre and Nicolas Flamand. (2015) **Regulation of inflammation by cannabinoids, endocannabinoids and their metabolites.** *J Leukoc Biol.* 97(6):1049-70.

Je suis l'auteure principale de cet article de revue, publié en 2015 dans le *Journal of Leukocyte Biology*. La conception de l'article a été faite par moi, François Chouinard et Nicolas Flamand. L'ensemble des auteurs a participé à la recherche bibliographique, à la préparation des tableaux et à la rédaction du manuscrit. L'article a été inséré dans cette thèse sans aucune modification par rapport à la version publiée.

Chapitre 2

Caroline Turcotte, Marie-Renée Blanchet, Michel Laviolette and Nicolas Flamand. (2016) **The CB₂ receptor and its role as a regulator of inflammation.** *Cell Mol Life Sci.* 73(23):4449-4470.

Je suis l'auteure principale de cet article de revue, publié en 2016 dans le *Cellular and Molecular Life Sciences*. La conception de l'article a été faite par moi et les Dr Blanchet, Laviolette et Flamand. J'ai effectué la recherche bibliographique avec le Dr Flamand. J'ai rédigé la première version de l'article et préparé les tableaux y figurant. L'article a ensuite été révisé et bonifié par l'ensemble des auteurs. L'article a été inséré dans cette thèse sans aucune modification par rapport à la version publiée.

Quant au chapitre 3, il énonce mes hypothèses de recherche et les objectifs de mon doctorat. Les chapitres 4, 5, 6 et 7 contiennent les résultats des études reliées à mon programme de recherche au doctorat. Ma contribution à ces chapitres, ainsi que leur état de publication, se détaille comme suit :

Chapitre 4

Caroline Turcotte, Élizabeth Dumais, Anne-Sophie Archambault, Cyril Martin, Marie-Renée Blanchet, Élyse Bissonnette, Louis-Philippe Boulet, Michel Laviolette, Vincenzo Di Marzo, and Nicolas Flamand. **Human**

leukocytes differentially express endocannabinoid-glycerol lipases and hydrolyze 2-arachidonoyl-glycerol and its metabolites from the 15-lipoxygenase and cyclooxygenase pathways. *J Leukoc Biol.* 2019 Sep 26.

Je suis l'auteure principale de l'article, qui est sous presse dans le *Journal of Leukocyte Biology*. J'ai effectué la conception de l'étude et des expériences avec le Dr Flamand, le Dr Blanchet, le Dr Bissonnette, le Dr Boulet, le Dr Laviolette et le Dr Di Marzo. Les manipulations en laboratoire ont été effectuées par moi, Élizabeth Dumais, Anne-Sophie Archambault et Cyril Martin. Tous les auteurs ont participé à l'analyse et l'interprétation des données. J'ai rédigé avec le Dr Flamand la première version du manuscrit, que l'ensemble des auteurs a révisée et bonifiée.

Chapitre 5

Caroline Turcotte, Anne-Sophie Archambault, Élizabeth Dumais, Cyril Martin, Marie-Renée Blanchet, Vincenzo Di Marzo, Michel Laviolette and Nicolas Flamand. **Endocannabinoid hydrolysis inhibition unravels that unsaturated fatty acids induce a robust synthesis of glycerol-containing endocannabinoids in human myeloid leukocytes.**

Je suis l'auteure principale de l'article, qui sera prochainement soumis pour publication dans un journal scientifique avec révision par les pairs. J'ai effectué la conception de l'étude et des expériences avec le Dr Flamand, le Dr Blanchet, le Dr Laviolette et le Dr Di Marzo. Les manipulations en laboratoire ont été effectuées par moi, Anne-Sophie Archambault, Élizabeth Dumais et Cyril Martin. Tous les auteurs ont participé à l'analyse et à l'interprétation des données. J'ai écrit avec le Dr Flamand la première version du manuscrit, qui a ensuite été révisé et bonifié par l'ensemble des auteurs. Des différences pourraient figurer entre cette version et la version finale publiée, qui sera corrigée en fonction des commentaires des arbitres mandatés par le journal.

Chapitre 6

Marie-Chantal Larose*, Caroline Turcotte*, François Chouinard, Claudine Ferland, Cyril Martin, Véronique Provost, Michel Laviolette and Nicolas Flamand. (2014) **Mechanisms of human eosinophil migration induced by the combination of IL-5 and the endocannabinoid 2-arachidonoyl-glycerol.** *J Allergy Clin Immunol.* 133(5):1480-1482.

Je suis, avec Marie-Chantal Larose, co-première auteure de cet article. Marie-Chantal Larose, François Chouinard, Claudine Ferland, Cyril Martin, Véronique Provost et moi avons participé à la conception des

expériences, aux manipulations en laboratoire et à l'analyse des résultats. Le Dr Laviolette et le Dr Flamand ont conçu l'étude, participé à l'interprétation des résultats et écrit le manuscrit. Cet article a été publié en tant que lettre à l'éditeur dans le *Journal of Allergy and Clinical Immunology* en 2014. Puisque le format d'une lettre à l'éditeur ne suit pas le format classique d'un article scientifique, le texte de la version ci-insérée a été divisé et organisé en sections. De plus, puisque le nombre de figures de la lettre à l'éditeur était limité, les figures qui se trouvent dans le matériel supplémentaire ont été incorporées dans le chapitre. Aucune autre modification n'a été apportée au contenu.

Chapitre 7

Caroline Turcotte, Simona Zarini, Stéphanie Jean, Cyril Martin, Robert C. Murphy, David Marsolais, Michel Laviolette, Marie-Renée Blanchet and Nicolas Flamand. (2017) **The Endocannabinoid Metabolite Prostaglandin E$_2$ (PGE$_2$)-Glycerol Inhibits Human Neutrophil Functions: Involvement of Its Hydrolysis into PGE2 and EP Receptors.** *J Immunol.* 198(8):3255-3263.

Je suis l'auteure principale de cet article, qui a été publié *Journal of Immunology* en 2017. La conception de l'étude et des expériences a été faite par Stéphanie Jean, le Dr Laviolette, Le Dr Blanchet, le Dr Marsolais, le Dr Murphy, le Dr Flamand et moi. Les manipulations en laboratoire ont été effectuées par moi, Stéphanie Jean, Simona Zarini et Cyril Martin. Tous les auteurs ont participé à l'analyse et à l'interprétation des données. J'ai écrit avec le Dr Flamand la première version du manuscrit, qui a ensuite été révisé et bonifié par l'ensemble des auteurs. L'article a été inséré dans cette thèse sans aucune modification par rapport à la version publiée.

Pendant mes études graduées, j'ai aussi participé, à titre de co-auteure, à des études qui ne figurent pas dans cette thèse mais qui portaient aussi sur le rôle des lipides bioactifs dans l'inflammation. En voici la liste :

Anne-Sophie Archambault, Samuel Poirier, Julie S. Lefebvre, Marie-Chantal Larose, Caroline Turcotte, Cyril Martin, Véronique Provost, Luc H. Boudreau, Patrick P. McDonald, Michel Laviolette, Marc Surette and Nicolas Flamand. (2019) **20-Hydroxy- and 20-carboxy-leukotriene (LT) B$_4$ downregulate LTB$_4$ -mediated responses of human neutrophils and eosinophils.** *J Leukoc Biol*. doi: 10.1002/JLB.MA0718-306R. [Epub ahead of print]

Caroline Turcotte*, Anne-Sophie Archambault*, Cyril Martin, Véronique Provost, Marie-Chantal Larose, Catherine Laprise, Jamila Chakir, Élyse Bissonnette, Michel Laviolette, Ynuk Bossé and Nicolas Flamand. (2018) **Comparison of eight 15-lipoxygenase (LO) inhibitors on the biosynthesis of 15-LO metabolites by human neutrophils and eosinophils.** *PLoS One*. 17;13(8):e0202424. *Co-premières auteures.

Anne-Sophie Archambault, <u>Caroline Turcotte</u>, Cyril Martin, Julie S Lefebvre, Véronique Provost, Michel Laviolette, Nicolas Flamand. (2017) **Leukotriene B$_4$ Metabolism and p70S6 Kinase 1 Inhibitors: PF-4708671 but Not LY2584702 Inhibits CYP4F3A and the ωOxidation of Leukotriene B$_4$ In Vitro and In Cellulo.** *PLoS One.* 9;12(1):e0169804.

<u>Caroline Turcotte</u>, Marie-Renée Blanchet, Michel Laviolette, Nicolas Flamand. (2016) **Impact of Cannabis, Cannabinoids, and Endocannabinoids in the Lungs.** *Front Pharmacol.* 15;7:317.

François Chouinard, <u>Caroline Turcotte</u>, Xiaochun Guan, Marie-Chantal Larose, Samuel Poirier, Bouchard L, Provost V, Flamand L, Grandvaux N, Flamand N. (2013) **2-Arachidonoyl-glycerol- and arachidonic acid-stimulated neutrophils release antimicrobial effectors against E. coli, S. aureus, HSV-1, and RSV.** *J Leukoc Biol.* 93(2):267-76.

Introduction

Introduction générale

L'inflammation est une réponse critique de l'organisme en réponse à un danger ou une blessure. Orchestrée par les cellules immunitaires, la réaction inflammatoire est essentielle à l'élimination d'une menace et au retour à l'homéostasie. Elle peut prendre deux formes : aiguë ou chronique. L'inflammation aiguë sert à protéger l'organisme, par exemple lors d'une infection. Par contre, lorsque la réaction inflammatoire devient exagérée et/ou ne se résout pas, elle cause des dommages aux tissus affectés. On parle alors d'inflammation chronique, une condition observée dans plusieurs maladies dont l'arthrite rhumatoïde, le psoriasis, l'asthme et les maladies inflammatoires chroniques de l'intestin. Bien que plusieurs traitements existent afin de soulager temporairement les symptômes des personnes atteintes, aucun de ces médicaments ne permet de résoudre l'inflammation associée à ces maladies sans causer d'effets néfastes significatifs, soulignant un important besoin de nouvelles stratégies thérapeutiques.

La réaction inflammatoire : un survol

L'inflammation est un enchainement de réactions et d'évènements cellulaires menant au rétablissement d'un tissu infecté ou endommagé (1, 2). Elle se manifeste habituellement par un ou plusieurs des signes appelés les cinq signes cardinaux de l'inflammation : la rougeur (érythème), le gonflement (œdème), la douleur, la sensation de chaleur et l'altération de la fonction du tissu affecté. Ces signes sont causés par le recrutement et l'activation des cellules du système immunitaire, qui infiltrent rapidement le tissu affecté. La réaction inflammatoire se déroule en plusieurs étapes distinctes : l'initiation, la phase aiguë et la phase de résolution.

Suite à une agression, l'inflammation est initiée localement par les cellules structurales et les leucocytes résidents, qui y répondent en libérant des signaux de danger. Ceci, en combinaison avec la présence d'antigènes microbiens dans le cas d'une infection, entraîne la production par les macrophages de chimiokines et autres médiateurs solubles, dont le rôle est de recruter des leucocytes au tissu. Il y a alors une augmentation de la circulation et de la perméabilité vasculaire, ce qui contribue à l'infiltration du tissu par des leucocytes présents dans la circulation. Le neutrophile est habituellement le premier leucocyte circulant à être recruté et sa présence dans un tissu constitue un signe clinique d'inflammation. Les neutrophiles peuvent exercer une multitude de fonctions associées à la défense de l'hôte : la phagocytose de bactéries, la libération du contenu de leurs granules (dont des peptides antimicrobiens), la production d'espèces oxygénées réactives et la formation de trappes extracellulaires. Lors de ce stade d'activation des neutrophiles, ces derniers libèrent

des médiateurs qui promeuvent le recrutement des monocytes inflammatoires circulants. Les neutrophiles, les monocytes et les macrophages participent ensemble à la clairance du pathogène.

L'arrêt du recrutement des neutrophiles est un élément crucial de la phase de résolution de l'inflammation, qui est hautement régulée afin de protéger l'hôte des dommages causés par une présence prolongée de neutrophiles activés dans un tissu. Une fois le pathogène éliminé, les neutrophiles présents entrent en apoptose et participent ainsi à freiner l'influx de neutrophiles, en plus d'induire la migration de monocytes. Les monocytes nouvellement recrutés, sous l'influence des médiateurs présents, se différencient en macrophages possédant un phénotype pro-résolution. Ces macrophages, en plus de produire des lipides diminuant le recrutement de neutrophiles, phagocytent les neutrophiles apoptotiques par un processus appelé efferocytose. Les macrophages ont également pour tâche de promouvoir le retour à l'homéostasie en produisant des cytokines anti-inflammatoires et des facteurs de croissance favorisant la réparation tissulaire (3).

Figure 1.1 : Les phases de la réaction inflammatoire et les principaux acteurs impliqués.
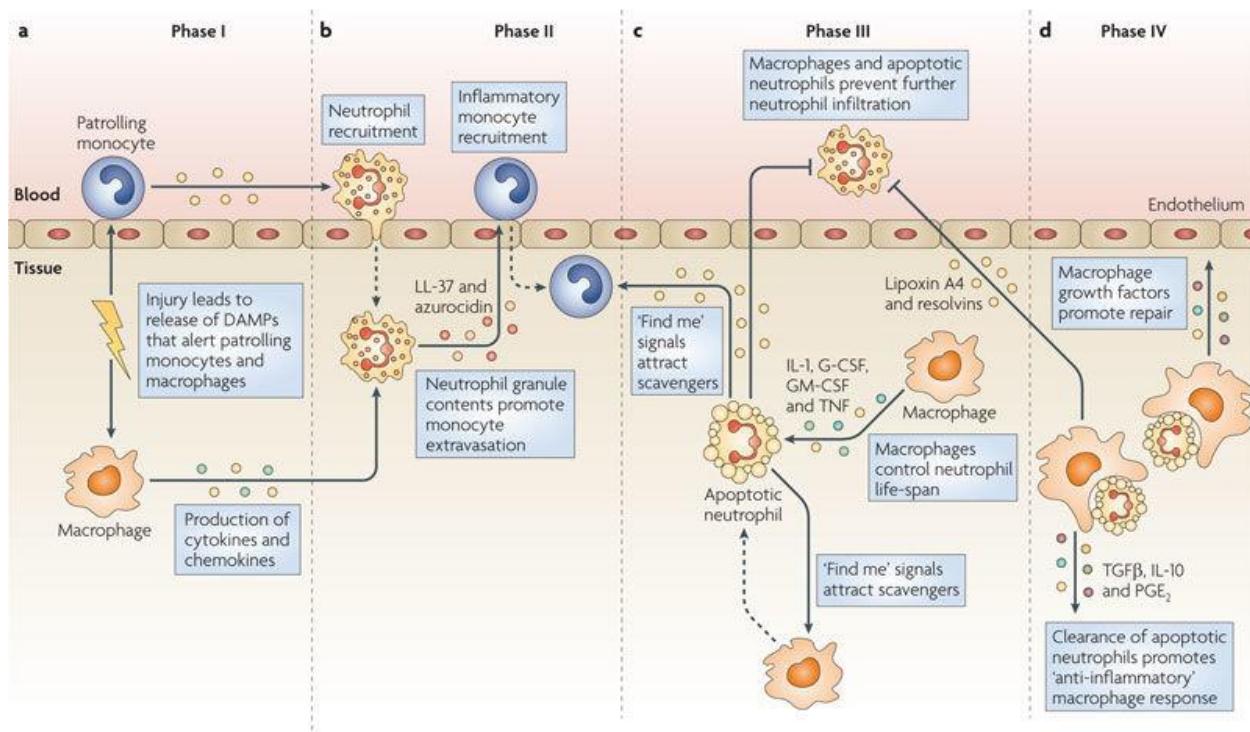
Utilisé avec l'autorisation de Springer Nature: Nature Reviews Immunology. Phagocyte partnership during the onset and resolution of inflammation. Oliver Soehnlein et al., 2010.

Le neutrophile et son rôle dans l'inflammation

Le neutrophile est un leucocyte appartenant à la famille des granulocytes. Il constitue 40-60 % des leucocytes sanguins, ce qui en fait le leucocyte le plus abondant chez les mammifères (4, 5). On le distingue de l'éosinophile et du basophile par son noyau multilobé et ses granules qui restent incolores lorsqu'il est coloré à l'hématoxyline et/ou à l'éosine. Le neutrophile joue un rôle si crucial dans la réaction inflammatoire qu'il est le premier leucocyte circulant à être recruté au tissu inflammé. Une fois arrivé au tissu, il exerce diverses fonctions visant à éliminer la menace, qui seront détaillées dans les paragraphes à venir. Dans tous les cas, l'activation de ces fonctions survient rapidement, orchestre la phase aiguë de l'inflammation et se termine avec mort du neutrophile. En effet, le neutrophile est une cellule possédant une durée de vie très courte, avec une demi-vie inférieure à 8 heures dans la circulation (4). Chez l'humain, une baisse du compte de neutrophiles sanguins (appelée neutropénie) peut mener à une immunodéficience importante (6). Par conséquent, le nombre de neutrophiles présents, ainsi que leur niveau d'activation, doivent être équilibrés afin de permettre une immunité innée efficace sans causer d'inflammation chronique. Ces processus sont hautement régulés à tous les niveaux, en commençant avec le recrutement du neutrophile au foyer inflammatoire.

Recrutement du neutrophile

Le recrutement du neutrophile au site d'inflammation se fait par un phénomène appelé chimiotaxie. Le neutrophile est attiré vers le site d'infection grâce à diverses molécules, appelées agents chimiotactiques, qui ont été libérées lorsque les cellules y ont détecté un danger. Les agents chimiotactiques du neutrophile incluent les peptides N-formylés (7), l'IL-8 (8-10), le fragment 5a du complément (11), et le LTB_4 (12). Suite à son activation par un agent chimiotactique, le neutrophile doit migrer à travers l'endothélium afin de passer de la circulation sanguine au tissu. Ce processus implique des molécules d'adhésion exprimées à la surface des cellules endothéliales, qui en reconnaissant leur ligand à la surface du neutrophile permettront l'attachement de ce dernier et sa transmigration à travers l'endothélium. Cela se produit en une série d'étapes ordonnées : le roulement, l'adhésion à l'endothélium et la transmigration.

L'élément déclencheur de la migration des neutrophiles circulants est l'expression de molécules d'adhérence à la surface des cellules endothéliales. L'expression de ces molécules est induite par des médiateurs pro-inflammatoires libérés par les cellules présentes au tissu inflammé. Les E- et P-sélectines nouvellement exprimées à la surface de l'endothélium reconnaissent leurs ligands à la surface des neutrophiles et ces adhésions faibles et transitoires provoquent la capture des neutrophiles circulants, puis leur roulement sur l'endothélium. Tout d'abord, les P-sélectines lient le PSGL-1 (*P-Selectin Glycoprotein Ligand-1*) qui se trouve à la surface des leucocytes. Les E-sélectines sont ensuite induites et interagissent également avec PSGL-1 à la surface du neutrophile ainsi qu'avec un autre ligand, ESL-1 (*E-Selectin Ligand-1*). L'intégrine $\alpha_4\beta$ (VLA-4)

sur le neutrophile interagit avec VCAM-1 sur l'endothélium, ce qui augmente l'adhésion des intégrines du neutrophile $\alpha_M\beta$ (MAC-1) et $\alpha_L\beta$ (LFA-1) à leurs ligands ICAM sur l'endothélium. L'adhésion du neutrophile est maintenant ferme, et ce dernier peut procéder au passage à travers l'endothélium par remodelage de son cytosquelette (5, 13).

Fonctions du neutrophile associées à la défense de l'hôte

Explosion oxydative

L'activation du neutrophile par des médiateurs pro-inflammatoires ou la présence de microorganismes entraîne un phénomène appelé explosion oxydative. La cellule consomme alors de grandes quantités d'oxygène afin de produire des espèces oxygénées réactives (ROS) comme l'anion superoxyde ($O_2^{\bullet-}$), dont la synthèse est catalysée par le complexe NADPH oxydase (14, 15). L'assemblage et l'activité de ce complexe protéique sont hautement régulés par la cellule étant donné la nature de ses produits, qui sont toxiques tant pour l'organisme que pour les bactéries. Lorsque le neutrophile est au repos, les composantes du complexe sont dispersées : les protéines p40^{phox}, p47^{phox} et p67^{phox} sont dans le cytoplasme alors que gp91^{phox} et p22^{phox} sont localisés aux membranes cellulaires pour former le cytochrome b_{558}. Lorsque la cellule est activée, l'assemblage du complexe est déclenché par deux principaux évènements : la phosphorylation de p47^{phox} et l'activation de la GTPase Rac2 (16, 17). Les composantes du complexe se rassemblent alors dans la membrane pour former une NADPH oxydase active, qui assure le transfert d'électrons du cytoplasme aux molécules d'oxygène pour générer l'anion $O_2^{\bullet-}$ (18). Une fois produit, l'anion superoxyde est dismuté en peroxyde d'hydrogène (H_2O_2). Le neutrophile peut utiliser la myéloperoxydase (MPO) pour transformer le peroxyde d'hydrogène en HOCl, un autre produit très nocif pour les bactéries (19).

Un exemple soulignant l'importance de la production de ROS comme mécanisme de défense de l'hôte est la maladie granulomateuse chronique, qui cause un défaut de certaines composantes du complexe NADPH oxydase. Les individus atteints présentent une déficience à générer des ROS se manifestant par une susceptibilité à des infections par de nombreuses bactéries (20, 21). L'importance de la synthèse de ROS comme fonction antimicrobienne du neutrophile n'est pas uniquement due à leur capacité à endommager directement les bactéries (18). En effet, leur formation entraîne également la libération du contenu des granules et la formation de NETs, des fonctions qui sont décrites dans les prochaines sous-sections.

Dégranulation

Les granules des neutrophiles sont de petites organelles pouvant contenir environ 300 différentes protéines, dont une grande proportion possède des propriétés antimicrobiennes (22). Les granules sont formés au cours du développement du neutrophile et sont nommés selon le stade de développement au cours duquel ils

apparaissent, puisque cela influence grandement leur contenu. Les neutrophiles circulants sont exempts d'ARN messager codant pour la majorité des protéines granulaires, indiquant que les granules sont formés avant que le neutrophile ne quitte la moelle osseuse (23). Les granules peuvent libérer leur contenu dans le milieu extracellulaire par exocytose ou encore fusionner avec le phagolysosome ou autres vésicules intracellulaires. De plus, la membrane des granules possède des molécules de surface qui se retrouvent dans la membrane plasmique lors de l'exocytose et qui peuvent jouer un rôle, par exemple, dans l'adhésion du neutrophile à l'endothélium (23).

Les premiers granules à être formés sont les granules **azurophiles**. Ils se distinguent par leur contenu riche en myéloperoxydase (MPO) et varient beaucoup en terme de taille et de forme (24). Les granules azurophiles ne subissent que très peu d'exocytose et semblent plutôt avoir un rôle dans la dégradation de microorganismes présents dans le phagosome. Ils sont très riches en défensines et contiennent également l'azurocidine, la cathepsine G, la protéinase-3 et l'élastase (25). Les granules **spécifique**s, quant à eux, se distinguent par la présence de lactoferrine. Leur contenu peut être libéré pour éliminer les pathogènes tant à l'extérieur de la cellule que dans le phagolysosome. Finalement, les granules de **gélatinase** contiennent beaucoup moins de substances antibiotiques que les granules spécifiques. Ils sont plus facilement exocytosés et sont notamment d'importantes sources de molécules de surfaces nécessaires à la transmigration du neutrophile (26, 27). Il est à noter que ces trois grandes classes de granules sont hétérogènes et pourraient être divisées en sous-classes, un sujet qui ne sera pas abordé ici.

Formation de trappes extracellulaires (NETs)

Les NETs sont des structures extracellulaires composées de l'ADN du neutrophile, d'histones citrullinées et d'une variété de protéases actives (28). Ils servent de pièges ayant pour utilité la capture et la destruction de bactéries dans le milieu extracellulaire (29). L'éjection d'une telle structure à base d'ADN dans l'environnement entraine la mort du neutrophile dans la plupart des cas. Ce mécanisme de mort cellulaire, distinct de l'apoptose et de la nécrose, a été nommé NETose (30).

La formation de NETs débute lorsque l'activation du neutrophile entraîne la production de ROS par le complexe NADPH oxydase. Cependant, bien que tous les neutrophiles répondent à divers stimuli pro-inflammatoires par une explosion oxydative, seulement ~20% d'entre eux produisent des NETs (31). La première étape est le bris de la membrane nucléaire en de multiples vésicules, libérant la chromatine décondensée dans la cellule. Ce contenu se mélange avec celui des granules, qui ont perdu leur intégrité. Finalement, la cellule se contracte et libère les NETs, perforant du coup sa membrane (32). Cependant, la NETose n'est pas toujours une forme de suicide pour le neutrophile. En effet, les NETs peuvent être libérés sans bris de la membrane plasmique et dans ces cas, le neutrophile demeure vivant et conserve sa capacité à

5

migrer et à phagocyter (33-35). Par contre, des différences importantes ont été observées entre la production de NETs *in vitro* et *in vivo*, soulignant que notre compréhension de l'importance des NETs dans la défense de l'hôte est incomplète (36).

Phagocytose

Un autre outil dont dispose le neutrophile afin de combattre l'infection est la phagocytose, un phénomène par lequel une cellule capture et ingère des particules solides, par exemple des bactéries. Pour qu'un neutrophile puisse reconnaître une bactérie et enclencher le processus de la phagocytose, il est préférable que la bactérie soit recouverte d'opsonines, le plus souvent des immunoglobulines ou des fragments du complément. Les neutrophiles reconnaissent ces opsonines par le biais des récepteurs Fcγ ou de intégrines β_2 exprimés à leur surface (37, 38). Une fois la bactérie reconnue, les neutrophiles créent des pseudopodes afin de l'entourer, et l'internalisent en formant une structure appelée phagosome. Une fois le phagosome formé, celui-ci doit subir une maturation afin d'être en mesure de tuer et de disposer des bactéries internalisées. Cette maturation du phagosome implique son acidification et l'acquisition de composantes comme des enzymes protéolytiques et le complexe NADPH oxydase. Chez le neutrophile, une augmentation des niveaux de calcium intracellulaire peut entraîner une fusion du phagosome avec les granules, lui permettant d'acquérir leur contenu hautement antimicrobien (39). Une fois cette étape complétée, le pathogène peut être digéré par le neutrophile (40).

L'éosinophile et son rôle dans l'inflammation

Dans des conditions comme les infections parasitaires ou certains types d'asthme, c'est l'éosinophile qui est abondamment recruté et qui mène la phase aiguë. L'éosinophile est un granulocyte décrit pour la première fois en 1879 par Paul Ehrlich. Il tient son nom d'une de ses propriétés uniques, c'est à dire la capacité de ses granules à retenir des colorants acides comme l'éosine. Il est retrouvé en faibles quantités dans la circulation d'individus sains, soit moins de 400 cellules par µl de sang ou moins de 5% des leucocytes totaux (41). Il s'agit, comme le neutrophile, d'une cellule ayant une durée de vie courte, avec une demi-vie d'environ 18h dans la circulation (42). Le rôle de l'éosinophile dans le combat contre les infections parasitaires lui a d'abord été attribué en raison d'images histologiques montrant son association à des helminthes, et a été consolidé plus tard par des études montrant que des protéases contenues dans ses granules ont des propriétés antiparasitaires (43). Outre ses fonctions dans la défense de l'hôte, l'éosinophile est impliqué dans de nombreuses pathologies dans lesquelles il promeut l'inflammation et donc, est considéré comme étant nocif (44). Parmi ces pathologies, nous retrouvons notamment l'asthme, la dermatite atopique et l'œsophagite à éosinophiles.

Le recrutement de l'éosinophile

Les niveaux d'éosinophiles circulants sont augmentés dans plusieurs maladies inflammatoires comme l'asthme, et la migration de ces éosinophiles vers les tissus est souvent délétère. Cette migration des éosinophiles se fait en plusieurs étapes, la plus cruciale étant la formation d'un gradient chimiotactique servant à attirer l'éosinophile vers le site inflammatoire. Ce gradient peut être formé d'un grand éventail de molécules ayant des propriétés chimiotactiques pour les éosinophiles, comme les chimiokines et les lipides bioactifs. Le processus de transmigration de l'éosinophile est pratiquement identique à celui décrit plus haut pour le neutrophile, c'est à dire qu'il subit des étapes de roulement, d'adhésion et de migration à travers l'endothélium qui sont régulées par des molécules de surface comme les sélectines et les intégrines.

Parmi les médiateurs lipidiques qui agissent en tant qu'agents chimiotactiques envers l'éosinophile, le plus puissant est le 5-oxo-ETE (45, 46). En plus de son rôle de chimioattractant, ce dernier facilite la migration des éosinophiles à travers l'endothélium en augmentant son expression et sa production de protéases comme la MMP-9, qui dégradent les composantes de la matrice extracellulaire (47). Chez l'humain, l'injection intradermale du 5-oxo-ETE mène à une infiltration d'éosinophiles au tissu chez des sujets sains ou asthmatiques (48). Cependant, cette réponse est plus robuste chez les sujets asthmatiques, soulignant l'importance du 5-oxo-ETE dans l'éosinophilie observée dans l'asthme allergique. La prostaglandine D_2 (PGD_2) est un autre exemple de médiateur lipidique induisant la migration des éosinophiles (49). Il a été démontré que ses niveaux augmentent après l'exposition à un allergène, et qu'un antagoniste de son récepteur améliore la fonction pulmonaire des asthmatiques (50-53).

Il existe un groupe de chimiokines, les éotaxines, qui sont sélectives pour l'éosinophile. Trois chimiokines font partie de ce groupe, soit la CCL11 (éotaxine-1), la CCL24 (éotaxine-2) et la CCL26 (éotaxine-3). Elles peuvent recruter d'autres leucocytes, mais beaucoup moins efficacement. Chez l'humain, ces chimiokines sont sécrétées tant par les cellules structurales comme celles de l'épithélium bronchique, que par des cellules immunitaires comme les macrophages alvéolaires et les éosinophiles (54-57). Leurs niveaux sont augmentés dans divers tissus de sujets asthmatiques comme le sang, les expectorations induites et le lavage broncho-alvéolaire (58-64). Les éotaxines recrutent les éosinophiles via l'activation du récepteur CCR3 à leur surface. Ce récepteur présente lui aussi une expression augmentée dans les voies respiratoires de sujets asthmatiques par rapport aux sujets sains (65). À cet égard, le rôle des éotaxines a été étudié dans des modèles murins à l'aide de souris déficientes pour le récepteur CCR3. Cependant, le potentiel de transposer ces résultats à la santé humaine sont limitées par les différences importantes entre l'éosinophile humain et murin, et par le fait que la souris n'exprime pas la CCL26 (66). Dans le passé, notre groupe de recherche a démontré que la CCL26 induit plus efficacement la migration des éosinophiles que la CCL11 ou la CCL24, et

que la CCL26, et que l'IL-13 augmentait sélectivement l'expression de la CCL26 par les cellules épithéliales bronchiques de sujets asthmatiques sévères (67, 68). Nous avons également démontré dans cette étude qu'il y a une corrélation entre le nombre d'éosinophiles et le niveau de CCL26 dans les expectorations induites de sujets asthmatiques. Ces résultats suggèrent que cette éotaxine en particulier joue un rôle clé dans le recrutement des éosinophiles chez les asthmatiques sévères, un rôle dont l'étude chez la souris n'est pas possible.

Les médiateurs lipidiques de l'inflammation

Les fonctions des leucocytes présentés dans les sections précédentes sont régulées par plusieurs classes de médiateurs. Parmi ces médiateurs, les médiateurs lipidiques de l'inflammation jouent un rôle particulièrement important. Ils sont synthétisés rapidement par une grande variété de cellules et de tissus, et sont essentiels tant au recrutement et l'activation des leucocytes dans la phase aiguë de l'inflammation qu'à sa résolution. Les travaux de ma thèse portent sur les endocannabinoïdes, des lipides mimant certains effets du cannabis et modulant l'immunité et l'inflammation. Cependant, les endocannabinoïdes exercent une partie de leurs effets par le biais de leurs nombreux métabolites, notamment les dérivés de l'acide arachidonique. Les principaux lipides faisant partie de ce système complexe, qui sont schématisés à la figure 1.2 afin d'en donner une vue d'ensemble, sont décrits dans la présente section.
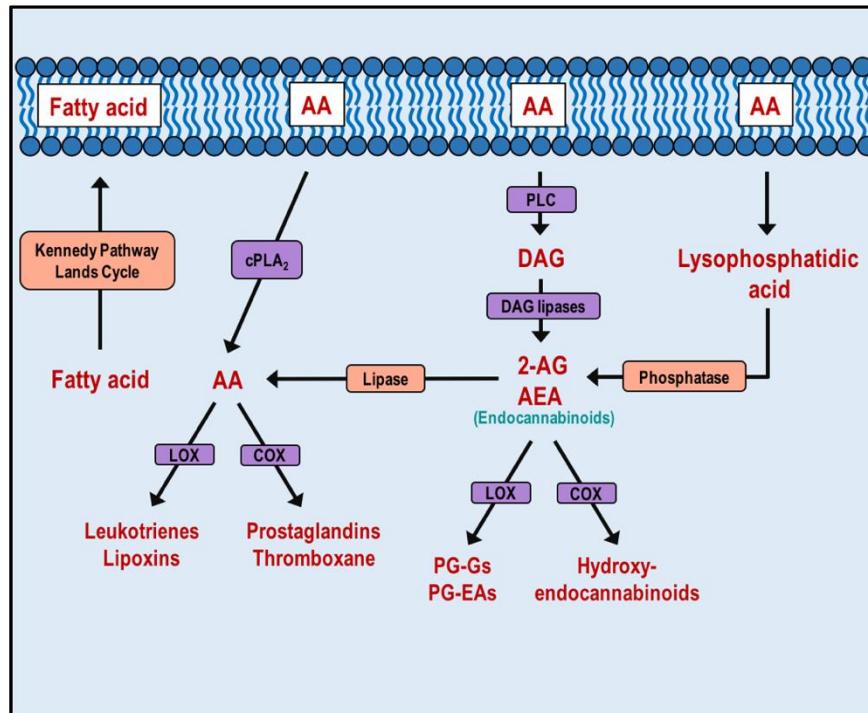
Figure 1.2 : Les médiateurs lipidiques de l'inflammation.

Les acides gras

Les acides gras sont indispensables à la régulation de l'inflammation puisqu'ils sont les précurseurs de la grande majorité des médiateurs lipidiques de l'inflammation, qu'ils soient pro- ou anti-inflammatoires (figure 1.3). On définit les acides gras comme étant des acides carboxyliques comportant une chaîne de carbones. Ils peuvent être nommés et classés de plusieurs façons, selon la longueur de la chaîne de carbones, le nombre d'insaturations (doubles liaisons) et la position de ces dernières. Par exemple, l'acide arachidonique est un acide gras 20 :4, puisqu'il possède 20 carbones et 4 insaturations. Il s'agit également d'un acide gras ω-6, puisque la dernière insaturation est positionnée au 6e carbone à partir de l'extrémité ω.

Les acides gras peuvent être obtenus par les cellules de deux façons, soit par le métabolisme des phospholipides de la membrane cellulaire ou par le biais de la diète. À cet égard, un acide gras peut être
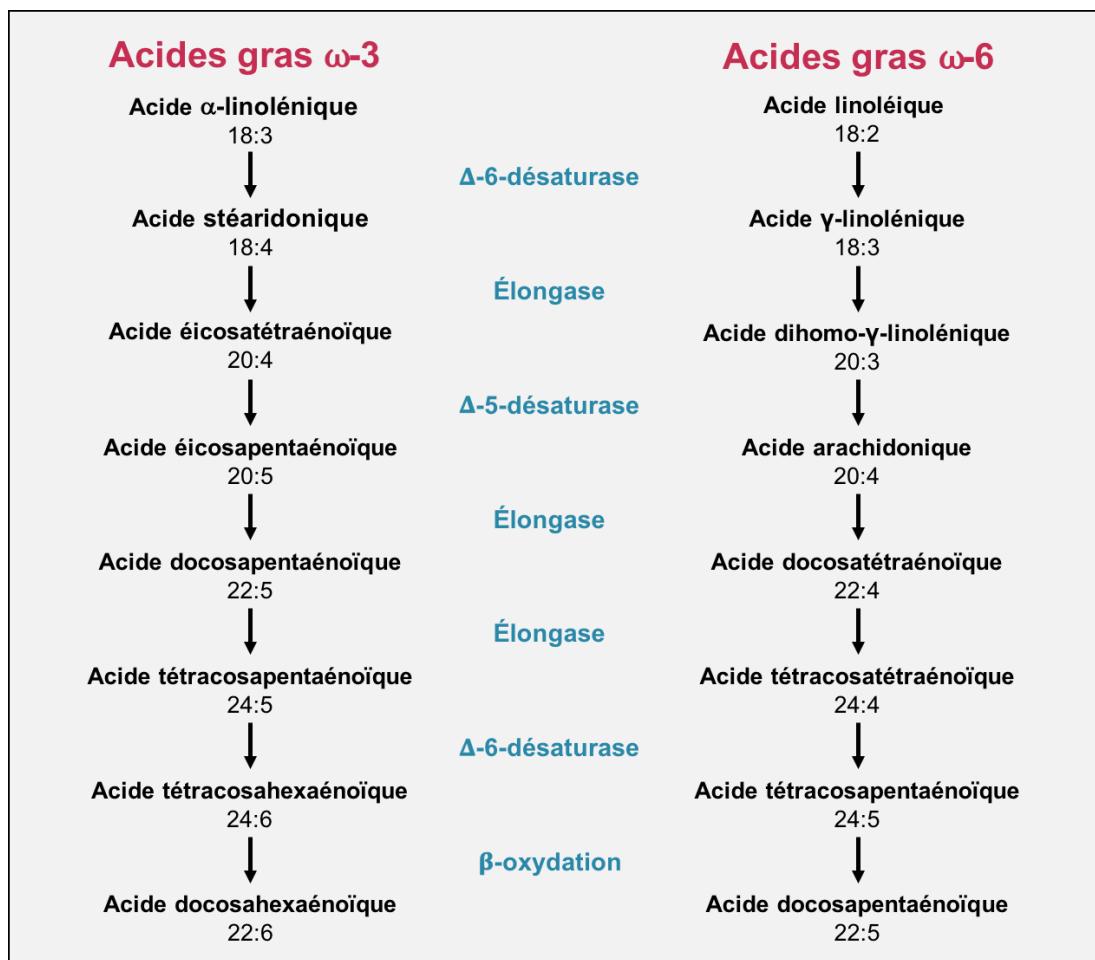
Figure 1.3 : Voies de biosynthèse des acides gras polyinsaturés ω-3 et ω-6 par les cellules humaines

Traduit avec l'autorisation de John Wiley and Sons: Wiley Books. Fatty Acids: Structures and Properties. Rustan A et al., 2005.

désigné comme étant essentiel ou non-essentiel, selon la nécessité de sa présence dans la diète d'un organisme donné. Un acide gras non-essentiel peut être synthétisé par les cellules de l'organisme à partir de substrats déjà présents. Un acide gras essentiel est nécessaire à l'organisme mais ne peut être synthétisé *de novo* et donc, doit être présent dans l'alimentation (69).

Chez l'humain, les acides gras monoinsaturés sont obtenus dans certains aliments comme l'huile d'olive, ou synthétisés à partir de précurseurs saturés retrouvés naturellement dans les cellules, le plus souvent l'acide stéarique (18 :0) ou l'acide palmitique (16 :0). Quant aux acides gras polyinsaturés, l'humain est capable d'effectuer certaines conversions pour les synthétiser à partir d'autres qui ont été obtenus dans l'alimentation. Par conséquent, seulement deux acides gras sont essentiels pour un humain en bonne santé : l'acide linoléique (18 :2) et l'acide α-linolénique (18 :3). Les cellules possèdent les enzymes nécessaires afin d'effectuer la transformation de ces deux acides gras en une panoplie d'autres acides gras polyinsaturés, tel qu'illustré à la figure 1.3 (70).

Lorsqu'un acide gras entre dans une cellule, il n'y demeure pas à l'état libre très longtemps. S'il n'est pas rapidement métabolisé, il est réincorporé dans les phospholipides membranaires, où il fera partie de la structure de la membrane jusqu'à ce qu'il soit utilisé comme précurseur pour la synthèse de médiateurs lipidiques. Ce phénomène est d'une grande importance puisqu'il régule la disponibilité des acides gras libres à des fins de biosynthèse de médiateurs lipidiques, en plus de déterminer la composition en acides gras des membranes cellulaires.

Le remplacement des acides gras présents dans les phospholipides par de nouveaux acides gras est nommé le cycle de Lands et est illustré en figure 1.4 (71). Une telle réacylation des acides gras libres dans une cellule requiert d'abord qu'un phospholipide perde un de ses deux acides gras par une réaction de déacylation, formant un lysophospholipide pouvant accueillir un nouvel acide gras. Puis, l'acylation de l'acide gras libre peut se faire par plusieurs différents mécanismes, CoA-dépendants ou –indépendants. L'acylation Co-A dépendante, dont l'importance physiologique est mieux définie, s'effectue en deux étapes : 1) la liaison de l'acide gras libre avec un coenzyme A (CoA) par une acyl-CoA synthase; et 2) le transfert de cet acyl-CoA à un lysophospholipide par une acyltransférase (72). En contraste, le processus consistant à synthétiser des phospholipides *de novo* se nomme la voie de Kennedy (73). Cette voie vise à former de l'acide phosphatidique (PA), afin de le transformer en phospholipide et de l'incorporer à la membrane cellulaire. Une fois les acides gras incorporés dans des phospholipides, ils peuvent être transférés dans des phospholipides d'une espèce différente par des transacylases, un phénomène appelé le remodelage des acides gras (72).
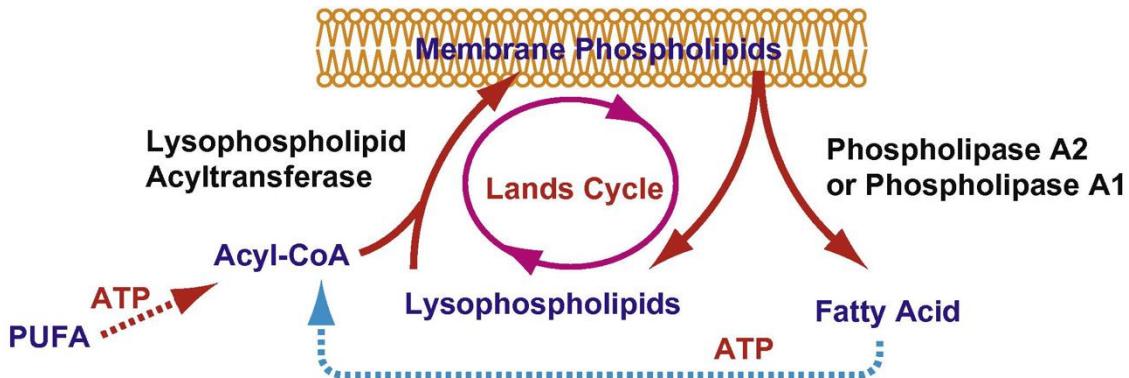
Figure 1.4. Le cycle de Lands.

Adapté avec l'autorisation de Elsevier: Progress in Lipid Research. Acyltransferases and transacylases that determine the fatty acid composition of glycerolipids and the metabolism of bioactive lipid mediators in mammalian cells and model organisms. Yamashita A et al., 2014.

L'acide arachidonique

L'acide arachidonique (AA) est au centre d'une cascade menant à la biosynthèse de nombreux lipides pro- et anti-inflammatoires. Puisque les dérivés de l'acide arachidonique possèdent 20 carbones, ils font partie d'une famille de médiateurs lipidiques appelés les éicosanoïdes. Le destin de l'acide arachidonique dépend à la fois du type cellulaire et du tissu, qui influencent la disponibilité des enzymes requises à la formation des divers métabolites, et de l'emplacement auquel l'AA est formé dans la cellule.

L'AA est libéré à partir des phospholipides membranaires par l'action d'enzymes appelées les phospholipases A_2 (PLA$_2$) (74, 75). Celles-ci sont divisées en quatre classes : les PLA$_2$ cytosoliques (cPLA$_2$), les PLA$_2$ sécrétées (sPLA$_2$), les PLA$_2$ indépendantes du calcium (iPLA$_2$) et les PLA$_2$ associées à des lipoprotéines (Lp-PLA$_2$). Dans un contexte inflammatoire, c'est la PLA$_2$ cytosolique du groupe IVA (cPLA$_2\alpha$) qui est responsable de la majeure partie de la production d'acide arachidonique et de ses dérivés (76). Celle-ci clive l'acide arachidonique en position *sn-2* des phospholipides et son activation est hautement régulée à plusieurs niveaux. Sa forme inactive se retrouve dans le cytosol et son activation est dépendante d'une augmentation des taux intracellulaires de calcium (Ca^{2+}) de l'ordre des bas micromolaires (77). Les ions Ca^{2+} lient la sous-unité C2 de l'enzyme, entraînant un changement de conformation et sa translocation vers les membranes comme celle du Golgi, du réticulum endoplasmique et du noyau (78-80). Plusieurs enzymes métabolisant l'AA en éicosanoïdes se trouvent également à ces endroits ou vont y transloquer une fois la cellule activée, favorisant le métabolisme de l'AA formé (81). Il est toutefois possible que l'enzyme soit activée sans élévation significative des taux de Ca^{2+} dans la cellule (82, 83).

Les leucotriènes

Les leucotriènes sont formés à partir de l'acide arachidonique par la voie de la 5-LO. La première étape est la conversion de l'AA en LTA_4, qui se fait en deux étapes. Deux éléments sont nécessaires à cette conversion, soit la 5-lipoxygénase (5-LO) et sa protéine associée, la *5-lipoxygenase-activating protein* (FLAP), qui présente l'AA à la 5-LO (84, 85). L'AA est d'abord converti en 5-HpETE, qui est instable et spontanément transformé en 5-HETE. La 5-LO/FLAP agit à nouveau en transformant le 5-HETE en LTA_4. Le LTA_4 étant très instable, il n'est pas bioactif et est transformé en divers leucotriènes en fonction des enzymes présentes. La figure 1.4 illustre les voies de biosynthèse des leucotriènes qui sont présentés plus en détails dans les paragraphes suivants.

Le leucotriène B_4

Le LTB_4 a été décrit pour la première fois en 1979 par Samuelsson et ses collaborateurs, qui étudiaient le métabolisme de l'AA par la voie de la 5-LO (86, 87). Peu de temps après la découverte du LTB_4, son activité chimiotactique envers les neutrophiles a été rapportée (88), ce qui a grandement contribué à sa réputation de médiateur pro-inflammatoire et nocif (89). De plus, le LTB_4 stimule diverses fonctions des leucocytes associées à la défense de l'hôte, dont la libération de peptides antimicrobiens et la phagocytose (90-93). Bien que ses effets associés à la défense de l'hôte fassent du LTB_4 un lipide essentiel à l'homéostasie, il est crucial qu'il ne soit pas synthétisé excessivement puisqu'il devient alors un médiateur pro-inflammatoire et potentiellement délétère.

Le LTB_4 est synthétisé à partir du LTA_4 par les cellules exprimant la LTA_4 hydrolase, comme le neutrophile, le monocyte et le macrophage (94). Par contre, il a été démontré que plus de 50% du LTA_4 synthétisé par ces cellules via la 5-LO est libéré dans le milieu extracellulaire plutôt que d'être métabolisé par la cellule l'ayant produit (95). Ce phénomène s'insère dans un concept voulant que les leucotriènes puissent être formés de façon transcellulaire. De cette façon, les cellules exprimant la 5-LO libèrent une partie du LTA_4 qu'ils synthétisent afin de le fournir aux cellules n'exprimant pas la 5-LO et leur permettre de synthétiser des leucotriènes. Parmi les cellules ayant besoin d'accepter de LTA_4 extracellulaire afin de synthétiser des leucotriènes, on compte les érythrocytes, les cellules endothéliales, les neutrophiles et les plaquettes (96-99).

Le LTB_4 possède deux récepteurs, soit le BLT_1 et le BLT_2. Le BLT_1 est le récepteur pour lequel le LTB_4 possède la plus grande affinité, avec une constante de dissociation (K_d) de 1.5 nM (100). Quant au récepteur BLT_2, il a initialement été décrit comme un récepteur du LTB_4 à faible affinité avant que son ligand naturel, le 12-HHT, ne soit identifié. Par conséquent, les effets biologiques engendrés par l'activation du BLT_2 sont

majoritairement attribuables au 12-HHT plutôt qu'au LTB4. Contrairement au BLT1 qui est surtout retrouvé chez les leucocytes, le récepteur BLT2 est exprimé de façon relativement ubiquitaire parmi les tissus humains (101).
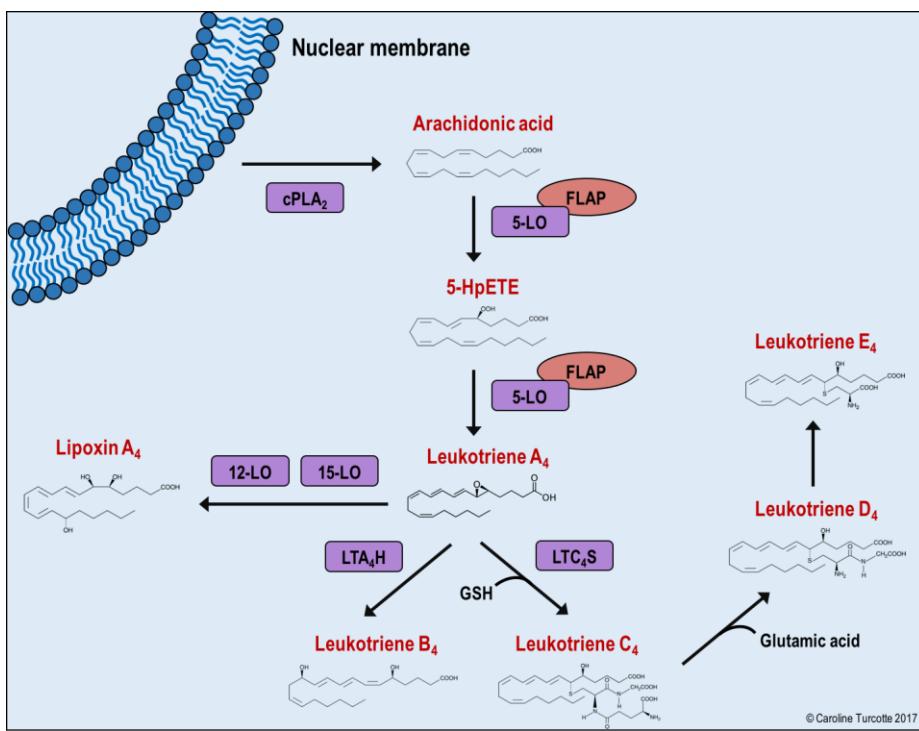
Figure 1.5. Voies de biosynthèse des leucotriènes.

Le LTB4 a une courte demi-vie, ce qui limite grandement sa bioactivité. Chez les neutrophiles, la principale voie de dégradation du LTB4 est son ω-oxydation par une enzyme de la classe des cytochromes P450, le CYP4F3A (102, 103). Cette réaction mène à la formation de deux métabolites inactifs, soit le 20-OH-LTB4 et le 20-COOH-LTB4 (désignés ω-LTB4). Ce dernier est formé par l'action d'une déshydrogénase sur le 20-OH-LTB4 (104). Ces métabolites sont capables de lier le BLT1 avec une affinité inférieure à celle du LTB4, mais suffisante pour qu'ils puissent agir en tant qu'antagonistes et limiter l'activation du récepteur par le LTB4 (105, 106). D'ailleurs, les ω-LTB4 inhibent les fonctions des neutrophiles humains induites par le LTB4, mais pas celles induites par des agonistes BLT1-indépendants (107).

Malgré l'importance du LTB4 dans la défense de l'hôte, il est impliqué dans la physiopathologie de plusieurs conditions dans lesquelles il est synthétisé excessivement et participe au maintien de l'inflammation. Parmi ces conditions, on compte la MPOC, l'arthrite rhumatoïde, le cancer, l'allergie et les maladies cardiovasculaires (108-112). La régulation de la synthèse et de l'activité du LTB4 est donc une approche thérapeutique séduisante pour ces maladies, et plusieurs stratégies sont possibles afin d'y arriver. Il est possible de bloquer la voie de biosynthèse du LTB4, que ce soit par l'inhibition de la 5-LO, de la LTA4H ou de

la FLAP. Ces trois avenues ont été étudiées par la génération de souris déficientes pour chacune des cibles, qui ont démontré une diminution de l'infiltration de neutrophiles et par conséquent, une inflammation moins sévère que les souris sauvages (113-117). Il est important de noter que certains modèles, notamment les souris 5-LO$^{-/-}$ infectées à *Klebsiella pneumoniae*, sont plus susceptibles à l'infection que les souris sauvages, ce qui soulève des questionnements quant à l'utilisation d'inhibiteurs de la 5-LO chez les humains (118). Néanmoins, des inhibiteurs de 5-LO ont été développés et l'un d'entre eux, le Zileuton, est disponible sur le marché pour traiter l'asthme et montre également un potentiel dans le traitement d'autres conditions comme l'arthrite rhumatoïde et les maladies inflammatoires de l'intestin (119). Quant au développement d'inhibiteurs de FLAP, la première vague de composés à entrer en essais cliniques dans les années 1990 n'a mené à aucune mise en marché. Par contre, l'élucidation de la structure 3D de la FLAP, qui a eu lieu entre-temps, a permis la synthèse de nouveaux composés qui sont présentement étudiés pour le traitement de diverses maladies inflammatoires (120, 121).

Les cystéinyl-leucotriènes

Une fois le LTA$_4$ synthétisé par la 5-LO, les cellules exprimant la LTC$_4$ synthase (comme l'éosinophile) peuvent le transformer en LTC$_4$ en le conjuguant avec le glutathion. Ce dernier pourra ensuite être transformé en LTD$_4$ et LTE$_4$ (122, 123). Le LTC$_4$, LTD$_4$ et LTE$_4$ sont appelés cystéinyl-leucotriènes (CysLT) en raison de la présence de l'acide aminé cystéine dans leur structure. Ils ont été observés pour la première fois dans les années 1930, dans les expectorations induites de sujets asthmatiques et dans les poumons de cobayes sensibilisés à un allergène. Étant donné qu'ils causaient des contractions du muscle lisse dans un modèle de choc anaphylactique, l'ensemble des trois leucotriènes avait alors été nommé *slow-reacting substance of anaphylaxis* (124).

Comme l'indique bien ce nom, les CysLT sont surtout connus pour leur rôle central dans la réaction allergique. Ils sont principalement synthétisés par les éosinophiles, les basophiles, les mastocytes et les monocytes/macrophages, qui sont tous des acteurs de l'allergie. Les CysLT induisent des contractions du muscle lisse bronchique, une augmentation de la perméabilité vasculaire et une augmentation de la sécrétion de mucus (125). Ils exercent ces effets par l'activation de deux récepteurs couplés à des protéines G, soit CysLT$_1$ et CysLT$_2$. CysLT$_1$ est un récepteur du LTD$_4$ à haute affinité, avec une affinité plus faible pour le LTC$_4$. Quant à CysLT$_2$, il lie le LTD$_4$ et le LTC$_4$ avec des affinités semblables mais plus faibles que le récepteur CysLT$_1$ (126). Chez l'humain, les récepteurs CysLT sont exprimé chez les éosinophiles, les mastocytes, les neutrophiles, les macrophages et les cellules endothéliales et épithéliales des voies respiratoires (127). Leur activation par les CysLT stimule une variété de réponses pro-inflammatoires chez les leucocytes, comme la production de ROS, de cytokines pro-inflammatoires, de chimiokines (128). Les CysLT induisent aussi le recrutement des éosinophiles et leur adhésion à la paroi endothéliale et à l'épithélium bronchique, contribuant

ainsi au maintien du phénotype inflammatoire observé dans l'asthme (129, 130). Le récepteur CysLT$_1$ est d'ailleurs la cible du Montelukast, du Pranlukast et du Zafirlukast, des antagonistes couramment utilisés en combinaison avec d'autres thérapies pour le traitement des symptômes de l'asthme et de la rhinite allergique (131).

Les prostanoïdes

Le terme « prostanoïde » désigne l'ensemble des médiateurs formés par le métabolisme des acides gras par la voie des cyclooxygénases. Ils sont classés selon l'acide gras à partir duquel ils sont formés; les prostanoïdes de la série 1 sont formés à partir de l'acide γ-linolénique (GLA), la série 2 à partir de l'AA et la série 3 à partir de l'acide éicosapentaénoïque (EPA) (132). Les prostanoïdes de série 2, puisqu'ils proviennent de l'acide gras abondant chez les mammifères qu'est l'AA, sont les prostanoïdes qui seront discutés dans cette section et étudiés dans cette thèse (figure 1.6).
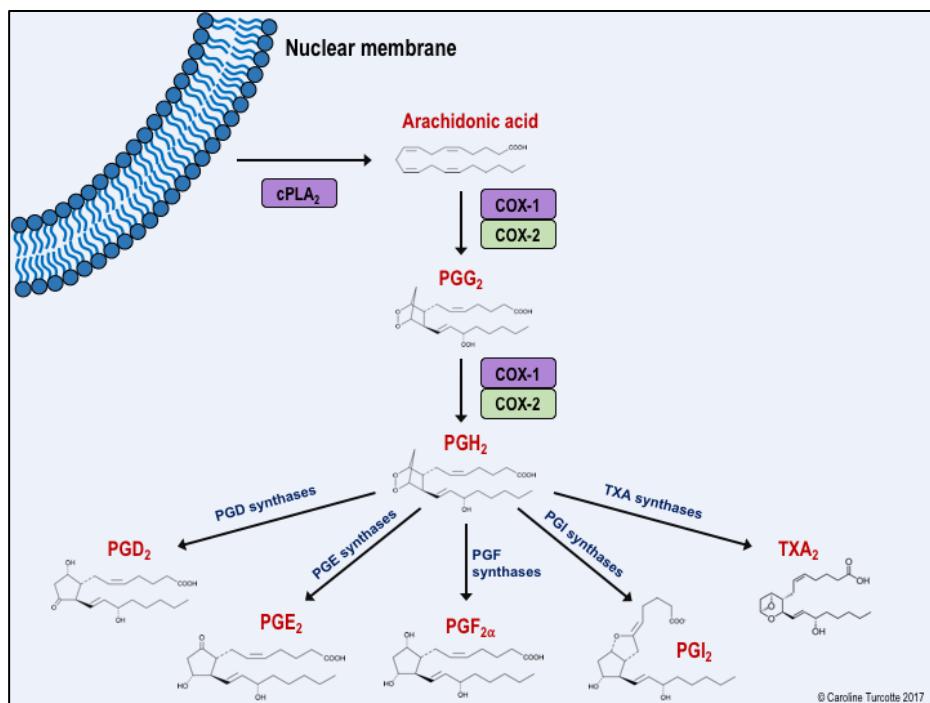
Figure 1.6. Biosynthèse des prostanoïdes dérivée de l'acide arachidonique.

Le métabolisme de l'AA par la voie des cyclooxygénases (COX) peut mener à la formation de prostaglandines et du thromboxane. Deux isoformes, la COX-1 et la COX-2, sont capables d'effectuer cette conversion de l'AA. Ces isoformes diffèrent surtout par leur patron d'expression. La COX-1 est exprimée constitutivement dans une grande variété de tissus et cellules. Quant à la COX-2, son expression est induite par la présence de

médiateurs pro-inflammatoires. Cette différence fondamentale a mené à une théorie voulant que la COX-1 soit responsable du maintien des niveaux de base des prostaglandines possédant un rôle protecteur, alors que la COX-2 causerait l'élévation des niveaux de prostaglandines dans un contexte d'inflammation.

La conversion de l'AA en prostanoïdes nécessite d'abord la synthèse de la PGH_2, un précurseur commun. Cette PGH_2 est obtenue en deux étapes successives, soit l'oxygénation et la peroxydation, toutes deux effectuées par les COX. Ensuite, la transformation de la PGH_2 en divers prostanoïdes est effectuée par des isomérases dont l'expression varie entre les tissus, déterminant ainsi le destin de la PGH_2 (133). Les prostanoïdes pouvant être ainsi synthétisées sont le thromboxane A_2 (TXA_2), la prostaglandine D_2 (PGD_2), la prostaglandine E_2 (PGE_2), la prostaglandine $F_{2\alpha}$ ($PGF_{2\alpha}$) et la prostacycline (PGI_2).

Les effets biologiques des prostanoïdes

Les prostanoïdes activent divers récepteurs dont les patrons d'expression parmi les tissus, ainsi que les voies de signalisations sous-jacentes, varient grandement (134-136). Aussi tôt que dans les années 1960, des études ont rapporté que les prostanoïdes étaient associés à des changements dans les niveaux de seconds messagers, notamment l'AMP cyclique (AMPc) et le calcium intracellulaire (Ca^{2+}) (132). Il a ensuite été découvert que plusieurs tissus possèdent des sites de liaison de haute affinité pour les prostanoïdes. Cependant, les récepteurs des prostanoïdes ont seulement été clonés dans les années 1990, en commençant avec le récepteur du thromboxane (137). Les études sur ce récepteur ont révélé que les récepteurs des prostanoïdes sont des récepteurs à sept domaines transmembranaires couplés à des protéines G. Ces récepteurs, leurs ligands et les évènements de signalisation leur étant associés sont illustrés à la figure 1.7.
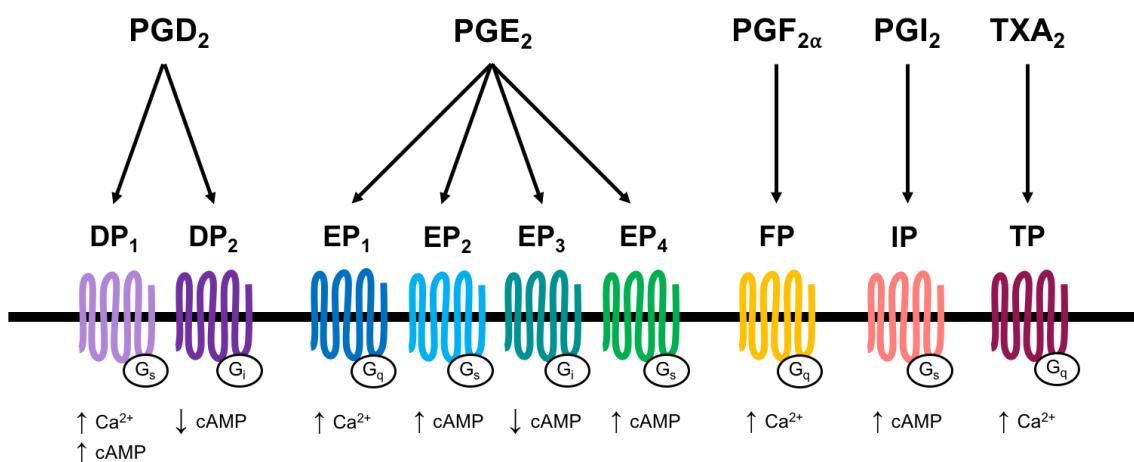
Figure 1.7 : Les récepteurs des prostanoïdes et les principaux évènements de signalisation intracellulaire qu'ils induisent.

L'activation de ces récepteurs mènent à des effets biologiques extrêmement variés selon le récepteur et le tissu, et les prostaglandines sont notoires pour leurs rôles complexes dans l'inflammation (133).

Les prostaglandines, cibles des anti-inflammatoires non-stéroïdiens

Les anti-inflammatoires non-stéroïdiens (NSAIDs) sont des inhibiteurs des COX visant à réduire la production de prostaglandines. Ils ont notamment été développés en réponse aux inquiétudes concernant l'utilisation fréquente de stéroïdes, parfois à long terme, pour traiter l'inflammation (138). Vu le rôle central de la PGE_2 dans la manifestation de plusieurs signes cardinaux de l'inflammation, il est logique qu'une diminution de sa synthèse apporte un soulagement de la douleur associée à l'inflammation (136). L'utilisation des NSAIDs est extrêmement courante et demeure une option de choix pour soulager l'inflammation et la douleur à court terme. Par contre, les inhibiteurs non-sélectifs bloquant les deux isoformes de COX ne discriminent pas entre les prostaglandines pro-inflammatoires et les prostaglandines protégeant la muqueuse gastro-intestinale. Par conséquent, l'utilisation des NSAIDs à long terme augmente drastiquement le risque de lésions de la muqueuse gastro-intestinale (139-141).

Ce problème a initialement été adressé par le développement d'inhibiteurs sélectifs pour la COX-2, les COXIBs. Cette stratégie visait à préserver la production de prostaglandines impliquées dans l'homéostasie, principalement synthétisées par la COX-1, et bloquer celles qui sont produites en conditions inflammatoires lorsque la COX-2 est induite. Les COXIBs ont été mis sur le marché et après quelques années, des rapports d'incidents cardiovasculaires reliés à leur utilisation ont vu le jour. Ces incidents s'expliquent par le fait que l'inhibition d'un seul isoforme de COX détruit l'équilibre entre la production de prostaglandines pro- et anti-thrombotiques, favorisant un phénotype pro-thrombotique. Depuis, des études ont confirmé les risques associés à leur utilisation chez les patients atteints de maladies cardio-vasculaires (142), alors que d'autres études ont conclu que les risques n'étaient pas nécessairement plus élevés que ceux associés aux NSAIDs (143, 144).

Les effets secondaires ne sont pas la seule raison pour laquelle les NSAIDs sont déconseillés pour le traitement de l'inflammation à long terme. Bien qu'ils soulagent la douleur associée à l'inflammation, leur mécanisme d'action ne permet pas d'induire sa résolution. À cet égard, plusieurs études dans des modèles murins ont démontré que la COX-2 est essentielle au déclenchement de la phase de résolution de l'inflammation (145-150). Donc, l'inhibition pharmacologique de la COX-2 par les NSAIDs pourrait empêcher la résolution de l'inflammation. Il est important de noter que ce phénomène n'implique potentiellement pas uniquement les prostaglandines. Les COX peuvent également métaboliser les endocannabinoïdes et certains

acides gras omega-3 pour produire des médiateurs potentiellement anti-inflammatoires et pro-résolution (151-153).

Les endocannabinoïdes et leurs métabolites

Les endocannabinoïdes sont des lipides bioactifs activant les mêmes récepteurs que les cannabinoïdes contenus dans le cannabis, soit les récepteurs CB_1 et CB_2. Le récepteur CB_1 est fortement exprimé dans le système nerveux central et son activation est responsable des effets psychotropes associés avec la consommation de cannabis. Quant au récepteur CB_2, il s'agit du récepteur cannabinoïde périphérique et on le retrouve à la surface de la plupart des leucocytes. Plus de deux décennies de recherche sur le système endocannabinoïde ont permis d'établir que des souris déficientes en récepteur CB_2 ont généralement un phénotype inflammatoire exacerbé, suggérant qu'il s'agit d'un récepteur anti-inflammatoire. Par contre, le rôle des endocannabinoïdes dans l'inflammation est beaucoup plus complexe que leurs effets médiés par le récepteur CB_2 étant donné qu'ils peuvent être métabolisés en une grande variété de lipides bioactifs. Le 2-arachidonoyl-glycérol (2-AG) et l'arachidonoyl-éthanolamide (AEA), les deux endocannabinoïdes les mieux caractérisés, sont hautement susceptibles à être hydrolysés en acide arachidonique, ce qui en fait une source importante d'éicosanoïdes pour la cellule (figure 1.3). De plus, les endocannabinoïdes peuvent être métabolisés directement par plusieurs enzymes de la voie des COX et des LOX, générant ainsi des métabolites potentiellement bioactifs dont les effets ne sont pas encore bien définis. Par conséquent, le système endocannabinoïde offre un grand nombre de possibilités en ce qui a trait au traitement de maladies inflammatoires.

Les chapitres 1 et 2 sont constitués d'articles de revue de la littérature, rédigés et publiés au cours de mes études doctorales. Ils complètent la présente introduction en offrant une vue d'ensemble sur les voies de biosynthèse et de métabolisme des endocannabinoïdes, leurs effets sur les cellules immunitaires (dépendants et indépendants du récepteur CB_2) et leur rôle dans une multitude de modèles de maladies inflammatoires.

Chapitre 1. Regulation of inflammation by cannabinoids, endocannabinoids and their metabolites

Caroline Turcotte, François Chouinard, Julie S Lefebvre et Nicolas Flamand.

Résumé

Le 2-arachidonoyl-glycérol (2-AG) et l'anandamide (AEA) sont des endocannabinoïdes impliqués dans une grande variété de troubles physiologiques incluant l'obésité, le syndrome métabolique, la douleur, les troubles neurologiques et l'inflammation. Leurs effets immunomodulateurs sont nombreux et n'impliquent pas toujours les récepteurs cannabinoïdes. Ce phénomène reflète la présence d'une molécule d'acide arachidonique dans leur structure, qui sert de précurseur à la biosynthèse de nombreux lipides pro- ou anti-inflammatoires. Le 2-AG et l'AEA peuvent donc servir de source d'acide arachidonique ou être métabolisés par la plupart des enzymes impliquées dans la biosynthèse d'éicosanoïdes, générant ainsi des médiateurs lipidiques dont les effets sont mal définis. À cet égard, l'augmentation des niveaux d'endocannabinoïdes par l'utilisation d'inhibiteurs de leur hydrolyse est susceptible d'augmenter également les niveaux de ces lipides, qui pourraient eux aussi exercer des effets biologiques. Cet article de revue présente les voies métaboliques impliquées dans la synthèse et la dégradation du 2-AG et de l'AEA, ainsi que le rôle de ces endocannabinoïdes et de leur lipidome dans la régulation de l'inflammation.

Abstract

2-arachidonoyl-glycerol (2-AG) and arachidonyl-ethanolamide (AEA) are endocannabinoids implicated in many physiological disorders including obesity, metabolic syndromes, hepatic diseases, pain, neurological disorders and inflammation. Their immunomodulatory effects are numerous and are not always mediated by cannabinoid receptors. This reflects the presence of an arachidonic acid molecule in their structure, the latter being the precursor of numerous bioactive lipids that are pro- or anti-inflammatory. 2-AG and AEA can thus serve as a source of arachidonic acid but can also be metabolized by most eicosanoid biosynthetic enzymes, yielding additional lipids. In this regard, enhancing endocannabinoid levels using endocannabinoid hydrolysis inhibitors will likely augment the levels of these lipids that could regulate inflammatory cell functions. This review summarizes the metabolic pathways involved in the biosynthesis and metabolism of AEA and 2-AG, as well as the biological effects of the 2-AG and AEA lipidomes in the regulation of inflammation.

Introduction

Historical notes

The use of cannabis for recreational purposes is widely known among the population. Indeed, ingesting the plant or its extracts leads to important psychotropic effects, as documented by Baudelaire in the beginning of the nineteenth century (1). In addition, cannabis has been used as a medicine for millennia (2). With the colossal technological increment we have experienced over the past decades, key cellular and molecular mechanisms by which cannabinoids and their endogenous counterparts exert their effects have been established. In this regard, the cannabinoid system has been involved in several disorders, notably obesity, metabolic syndrome, pain, and multiple sclerosis (3-5).

The structure elucidation of the main psychoactive substance found in *Cannabis sativa*, (-)-Δ9-tetrahydrocannabinol (THC) back in 1964, and the chemical synthesis of the THC analog CP 55,940 in the mid-eighties, paved the way for cannabinoid research (6-9). These compounds were key chemical probes for the pharmacological characterization of a rat receptor localized in the brain, and its cloning from rats and humans. Importantly, this brain receptor, now referred to as CB_1, is primarily found in the brain and testes (10-12). Another receptor, now referred to as CB_2, was rapidly cloned from the human promyelocytic cell line HL-60 (13). In sharp contrast to CB_1, CB_2 is mainly expressed in the periphery, notably in lymphoid tissues and by myeloid cells (10-13). Both the CB_1 and the CB_2 receptors belong to the G-protein coupled receptors (GPCR) superfamily and their activation triggers $G\alpha_{i/o}$ signaling events such as adenylyl cyclase inhibition and mitogen activated protein kinases (MAPK) activation (reviewed in (14)). GPR55 is another GPCR that was shown to bind THC and CP 55,940 (15). However, subsequent studies indicated that GPR55 better responds to lysophosphatidylinositol than to classical cannabinoid receptor agonists (16;17).

Following the cloning of the CB_1 and CB_2 receptors in 1990 and 1993 respectively, their endogenous ligands, endocannabinoids, were rapidly identified. Using porcine brain extracts and the radiolabeled synthetic cannabinoid canbisol (HU-243), Devane and colleagues defined arachidonyl-ethanolamide (AEA; Figure 2.1) as a high affinity CB_1 receptor agonist (Ki value of 39 ± 5 nM). The authors originally termed AEA anandamide, which is made up from the Sanskrit term ananda (meaning bliss and referring to its psychoactive effects) and the amide nature of the molecule (18). The cloning of the CB_2 receptor by Munro and colleagues further established AEA as a potent endocannabinoid (13) and led to the identification of other endogenous ligands. In this respect, 2-arachidonoyl-glycerol (2-AG; Figure 2.2) was subsequently identified by Mechoulam and colleagues in 1995. They isolated 2-AG from the canine gut, characterized its binding properties to CB receptors using CB_1- and CB_2-transfected COS-7 cells, and demonstrated that intravenously injected AEA or

2-AG mimicked the effect of THC (decreased nociception, spontaneous activity and rectal temperature) in mice (19;20). In parallel, Sugiura and colleagues isolated 2-AG from rat brain extracts and showed that it was competing against CP 55,940 in binding assays using rat synaptosomal membranes (19). Interestingly, the identification of 2-AG as a CB_1 and CB_2 ligand took place more than ten years after its biosynthesis was observed by Prescott and Majeurus in thrombin-stimulated platelets (21).

Several other endocannabinoids such as noladin ether (22), O-arachidonyl-ethanolamine (23) and N-arachidonyl-dopamine (24) also activate the CB_1 and CB_2 receptors. As of today, numerous endogenous molecules have been documented as CB receptor ligands. Their biosynthesis and biological effects have been recently reviewed and will not be documented in this manuscript (25-27).

Endocannabinoid biosynthesis

AEA biosynthesis

Although AEA can be obtained from the reverse reaction of the fatty acid amide hydrolase (FAAH) (28), its levels are increased in FAAH-deficient mice, indicating that FAAH is mostly involved in its hydrolysis and that other enzymes are responsible for AEA biosynthesis (29). In fact, the main precursor of AEA is N-acyl-phosphatidyl-ethanolamine (NAPE), which is obtained from the acylation of the ethanolamine portion of phosphatidyl-ethanolamines by Ca^{2+}-dependent and -independent N-acyl-transferases (30;31). Four pathways have been described for the metabolism of NAPE into AEA (figure 2.1): **1)** a one-step hydrolysis of NAPE by at least two distinct phospholipases D (PLD) that are specific for NAPE (32-35); **2)** a two-step process involving the hydrolysis of NAPE by a PLC, generating a phospho-AEA intermediate that is dephosphorylated by phosphatases such as PTPN22 (36); **3)** the generation of lyso-NAPE by a PLA_2 activity followed by its hydrolysis by a lyso-PLD (37); or **4**) the generation of a lyso-NAPE by a PLA_2 activity, its hydrolysis into glycerophospho-N-arachidonyl-ethanolamine by the serine esterase ABHD4, followed by the action of glycerophosphodiesterase 1 (38).

2-AG biosynthesis

Several pathways have been documented for the biosynthesis of 2-AG and the generation of diacylglycerol (DAG) is common to most of them. DAG can be produced by the hydrolysis of glyceryl-phosphatidyl-inositol by phospholipases C (PLC) (21;39) or by the combined action of PLD and phosphatidic acid phosphatase on glyceryl-phosphatidyl-choline (40). 2-AG is then obtained when arachidonic acid (AA)-containing DAG species are hydrolyzed by DAG lipase α or DAG lipase β (41;42). 2-AG can also be obtained from the

dephosphorylation of arachidonoyl-lysophosphatidic acid by a lysophosphatidic acid phosphatase (43) (figure 2.2).

Endocannabinoid metabolism

Endocannabinoids released into the extracellular space are rapidly cleared by cellular uptake and/or by hydrolysis. Four distinct mechanisms by which endocannabinoids are transported from the extracellular space to the cytoplasm have been documented: **1)** facilitated transport by a carrier protein; **2)** gradient-driven passive diffusion coupled with intracellular metabolism by enzymes such as monoacylglycerol (MAG) lipase and FAAH; **3)** gradient-driven passive diffusion coupled with endocannabinoid sequestration by fatty acid binding proteins; and **4)** caveolae-mediated transport. These mechanisms and recent data regarding endocannabinoid clearance from the extracellular space have been reviewed by Yates and Barker (44). Following their uptake, 2-AG and AEA can be hydrolyzed by serine esterases into AA and glycerol/ethanolamine, or metabolized by a variety of enzymes involved in eicosanoid metabolism including lipoxygenases (LOX), cyclooxygenases (COX) and epoxygenases.

Hydrolysis of AEA

The membrane-associated FAAH-1 and -2 are the main enzymes responsible for the hydrolysis of AEA into AA and ethanolamine. While FAAH-1 is expressed in rodents and humans, FAAH-2 is expressed in humans but not rodents. The tissue distribution profiles of FAAH-1 and -2 are slightly different: FAAH-1 is mainly found in the brain, kidney, liver, lung, prostate, testis, small intestine while FAAH-2 is mostly found in the heart, kidney, liver, lung, prostate and ovary. Interestingly, very little FAAH-1 or -2 was detected by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of cDNA obtained from leukocytes (45-47). The pharmacological or genomic deletion of FAAH-1 increases basal levels of AEA and abrogates the hydrolysis of exogenously added AEA (29;48-50). This underscores the key role of FAAH-1, and possibly FAAH-2, in AEA hydrolysis and establishes FAAH as a promising avenue to treat pain, anxiety and possibly inflammatory diseases. Importantly, FAAH inhibition reduces nociception, anxiety and depression while other effects associated to cannabinoids such as hypothermia and decreased coordination are not observed (29;51).

Hydrolysis of 2-AG

With the help of a biotinylated fluorophosphonate derivative, which covalently binds to and inactivates serine hydrolases, Blankman and colleagues unravelled several enzymes capable of hydrolyzing 2-AG into arachidonic acid and glycerol in the mouse brain. Indeed, they found that although MAG lipase accounted for 85% of the 2-AG hydrolase activity, other enzymes such as α/βhydrolase domain (ABHD) 6, ABHD12 and FAAH-1 were responsible for the remaining 15% (52). Importantly, ABHD6 plays a key role in 2-AG hydrolysis

(and CB$_2$ receptor activation) in the microglial cell line BV-2, which does not express MAG lipase. Moreover, the dual inhibition of FAAH and ABHD6 significantly increases 2-AG and AEA levels in neurons (53-55). The importance of each 2-AG-hydrolyzing enzyme has yet to be determined in humans and might be different to what has been described in rodents. In that regard, other enzymes such as carboxylesterases 1/2 are involved in 2-AG hydrolysis in cultured human THP-1 cells (56). This also suggests that the profiles of enzymes that hydrolyze 2-AG in the periphery and in the brain may be different.

The effects of a pharmacological or a genetic disruption of MAG lipase underscored its crucial role in 2-AG hydrolysis in mice. Indeed, the administration of the MAG lipase inhibitor JZL184 results in a 10-fold increase in 2-AG levels while decreasing the levels of unesterified AA in the brain by ~50%, similarly to what is observed in MAG lipase deficient animals (57;58). As opposed to FAAH disruption, the chronic inhibition of MAG lipase with JZL184 induces a loss of analgesic effects, development of physical dependence, impaired endocannabinoid-dependent synaptic plasticity, and CB$_1$ desensitization in the brain (58;59).

Oxygenation of AEA and 2-AG

The arachidonoyl moiety of AEA and 2-AG makes them susceptible to metabolism by eicosanoid biosynthetic enzymes, notably through the COX, LOX and epoxygenase pathways. These are often seen as endocannabinoid inactivation pathways, considering most of the obtained metabolites have limited or no affinity towards cannabinoid receptors. However, given the importance of eicosanoids in inflammation, endocannabinoid oxygenation should be seen as a unique process leading to the production of numerous bioactive lipids that will modulate the inflammatory response.

Cyclooxygenation

AEA was initially described as a good substrate for COX-2 by Yu and colleagues in 1997. The authors documented that AEA was metabolized into PGH$_2$-EA by human recombinant COX-2, but not COX-1; and into PGE$_2$-EA by a human foreskin fibroblast cell line expressing COX-2. Interestingly, AEA metabolism by COX-2 was almost as efficient as that of AA (60). The biosynthesis of PGE$_2$-EA was later confirmed in RAW 264.7 cells (61). Additional studies further demonstrated COX-2 metabolism of AEA leads to the AEA-derived prostaglandins PGG$_2$-EA, PGH$_2$-EA, PGD$_2$-EA, PGE$_2$-EA, PGF$_{2\alpha}$-EA and PGI$_2$-EA (62-64). The first evidence that PG-EAs might play important roles in the regulation of multiple pathways *in vivo* came from two studies: one that showed the pharmacological profile of PGF$_{2\alpha}$-EA was identical to the PGF$_{2\alpha}$ analog bimatoprost, currently used for the treatment of glaucoma (65); and the other demonstrated that PGD$_2$-EA, PGE$_2$-EA and PGF$_{2\alpha}$-EA were produced *in vivo* in FAAH-deficient mice (66). PG-EA are poor activators of CB$_1$ and CB$_2$ receptors (67) and are less potent at activating PG receptors than PGs (65;68). Although PG-EA can directly

activate PPAR-γ (69) and EP receptors (70) (figure 2.3), several studies highlight the existence of a specific PG-EA receptor (reviewed in (71)).

2-AG can also be oxygenated by COX-2 as efficiently as AA but, similarly to AEA, the oxygenation of 2-AG by COX-1 is ineffective (72). 2-AG can be metabolized into PGG$_2$-glycerol (G), PGH$_2$-G, PGD$_2$-G, PGE$_2$-G, PGF$_{2\alpha}$-G, PGI$_2$-G, and HHT-G. Although thromboxane (TX) B$_2$-G could be detected, the conversion of 2-AG into TXA$_2$-G by the TX synthase is minimal, compared to AA and PGH$_2$ (62). Interestingly, PGE$_2$-G activates RAW 264.7 cells at pM concentrations with a distinct pharmacological profile than PGE$_2$, with which it shares affinity for EP$_1$ and EP$_3$ receptor (73). This indicates that PG-G potently activates cells by interacting with PG-G-specific receptors (73).

Lipoxygenation

Soon after the identification of AEA and 2-AG as endocannabinoids, the metabolism of AEA by leukocyte-type 12-LOX and 15-LOX was documented (74;75). Three main lipoxygenase (LOX) activities are observed in mammals. Indeed, AA can be metabolized by 5-, 12-, and 15-LOX. Interestingly, AEA and 2-AG are good substrates for the leukocyte-type 12- and 15-LOX enzymes but are not oxidized by platelet-type 12-LOX nor 5-LOX (74-77).

The oxidation of AEA by 12- and 15-LOX leads to the biosynthesis of 12(S)- and 15(S)-hydroperoxyeicosatetraenoyl-EA (12-HpETE-EA and 15-HpETE-EA), respectively. They are then reduced to their hydroxyl derivative (e.g. 15-HETE-EA) (74;75). Even though 12-HETE-EA displays minimal binding to the CB$_1$ receptor (74;78) these mediators seem to exert their biological effects by activating vanilloid receptors (79-81). 2-AG oxidation by 12- and 15-LOX results in the formation of 12- and 15-HETE-G (76;77). Very little is known regarding the biological effects of 12- and 15-HETE-G and whether they activate cell surface receptors. That being said, 15-HETE-G was reported to be a PPARα agonist (77). The biosynthesis of 12- and 15-HETE-EA was observed in human platelets and granulocytes (78) incubated with AEA while the biosynthesis of 12- and 15-HETE-G was detected in human keratinocytes (76;77).

Epoxygenation

2-AG and AEA are also substrates for P450 enzymes. The metabolism of AEA into 20-HETE-EA, 5,6-, 8,9-, 11,12- and 14,15-epoxyeicosatrienoic (EET)-EA was observed in human liver, kidney and brain (82;83). Similarly, 2-AG can be metabolized into 11,12-, and 14,15-EET-GE (84) which promote the vasodilatation of rat mesenteric arteries (85). While no specific GPCR has been characterized for these P450-derived metabolites of AEA and 2-AG, they bind to CB$_1$ and CB$_2$ with the same affinity as AEA and 2-AG (84-86).

Cannabinoid receptors and immune cells

Following the cloning of CB_2 from HL-60 cells (13), numerous studies have investigated cannabinoid receptor expression by leukocytes and whether endocannabinoids and cannabinoids would modulate their functions. Leukocytes were initially shown to express the CB_2 receptor and, to a lesser extent, the CB_1 receptor (87;88). However, the level of expression of each CB receptor in immune cell subsets should be considered with caution, most importantly when only mRNA data were provided. Contamination by other cell types during isolation of a specific subset of leukocytes is frequent and difficult to avoid. Considering mRNA can be over amplified very effectively by PCR, a minor cell contamination can produce false positives. As an example, the removal of contaminating eosinophils in human neutrophil suspensions considerably diminishes the amplification of the CB_2 mRNA, thereby questioning whether neutrophils express that receptor or not (89-92). Furthermore, initial results obtained with commercially available CB receptor antibodies can be misleading since they have shown many differences in expression patterns (93;94). Table 2 summarizes most studies that have documented the presence or absence of CB receptors in human immune cells at the mRNA and protein levels.

Role of the cannabinoid system in inflammation

Endocannabinoids levels fluctuate in many inflammatory conditions, notably brain injury (95), cerebral ischemia (96), hepatic ischemia-reperfusion injuries (97), Huntington disease (98), multiple sclerosis (99), rheumatoid arthritis (100), atherosclerosis (101), sepsis (102), ulcerative colitis (103), contact dermatitis (104), and inflammatory pain (105). In this regard, three main strategies were used to define the immunomodulatory effects of endocannabinoids *in vivo*: 1) increasing their levels using 2-AG or AEA hydrolysis inhibitors; 2) the administration of exogenous endocannabinoids or cannabinoids; and 3) the genetic or pharmacological disruption of CB receptors.

Anti-inflammatory effects of endocannabinoids and cannabinoids in vivo

Endocannabinoids were shown downregulate inflammation in numerous experimental models such as experimental hepatitis (106), hepatic ischemia reperfusion injuries (107;108), lipopolysaccharides (LPS)-induced pulmonary inflammation (109), inflammatory pain (48;110-112), trinitrobenzene sulfonic acid-induced colitis (103;113), polymicrobial sepsis (114), contact hypersensitivity (104) and multiple sclerosis (115). In these studies, activation of the cannabinoid system was linked to decreased inflammatory cell recruitment and enhanced anti-inflammatory cytokine production. Tables 3, 4 and 5 respectively summarize the anti-inflammatory effects of CB receptors, AEA and 2-AG *in vivo.*

CB receptor agonists also downregulate the immune response *in vivo* (116-124) but can worsen *Legionella pneumophila* (125)*, Herpes simplex virus-2* (126) and *Listeria monocytogenes* infections (127) as well as increasing tumor progression (128). In these particular cases, the immunosuppressive effects induced by CB receptor activation on immune cells promote the proliferation of pathogens and cancer cells. Importantly, high concentrations of CB receptor agonists can modulate inflammation independently of CB receptor activation. Peroxisome proliferator-activated receptors, which are known to modulate immune cell functions, are indeed activated by high concentrations of the cannabinoid receptor agonists THC, WIN55212-2, CP 55,940 and HU-210 (129-131)**.** Table 6 summarizes the anti-inflammatory actions of CB receptor agonists described to date.

Other cannabis constituents such as cannabidiol have anti-inflammatory properties even though they do not activate CB receptors. Indeed, cannabidiol activates GPR55 (15) and inhibits the nucleoside transporter 1 (132). The latter results in increased extracellular adenosine concentrations, which can putatively downregulate inflammatory cells by activating the adenosine A_{2A} receptor (133-135).

Anti-inflammatory effects of endocannabinoids and cannabinoids ex vivo

In agreement with the anti-inflammatory effects promoted by 2-AG and AEA *in vivo*, endocannabinoids can downregulate immune cell functions such as leukocyte migration (92;136;137), the production of reactive oxygen species (138-140), the release of pro-inflammatory cytokine (69;138;139;141-143) and the increased of anti-inflammatory cytokine (IL-10) release (144). However, some of the data is conflicting. For example, while it was published that AEA was a potent inhibitor of human neutrophil migration (137), others reported it as effectless (89;103;145) and these discrepancies remain unexplained. Cannabinoid receptor activation by synthetic agonists mimics most of the anti-inflammatory effects of endocannabinoids including inhibition of leukocyte migration (136;146-150) and of the release of pro-inflammatory mediators (115;151-153). Furthermore, some studies suggest that cannabinoid receptor activation induces a shift from the production of cell-mediated immunity (Th1) cytokines to humoral immunity (Th2) cytokines (115;151;152;154). Tables 7 and 8 summarize the documented anti-inflammatory effects of endocannabinoids (2-AG and AEA) and cannabinoids on immune cells.

Pro-inflammatory effects of 2-AG, AEA, and cannabinoids in vivo

Although a large body of evidence supports that endocannabinoids are anti-inflammatory mediators, several studies have reported a pro-inflammatory role of endocannabinoids. Indeed, endocannabinoids contribute to the development of inflammation in hypersensitivity (155;156), neuropathy (157), cardiomyopathy (158), and experimental dermatitis (159;160). These pro-inflammatory effects of endocannabinoids were associated with

enhanced leukocyte recruitment, the production of reactive oxygen species and the release of pro-inflammatory cytokines (Tables 9 and 10). In agreement with the above-mentioned pro-inflammatory effects, endocannabinoids can also activate leukocyte functions and participate to the development of the inflammatory process. 2-AG participates to the recruitment of leukocytes (89;91;161-165) and to the release of pro-inflammatory mediators. Endocannabinoids can also increase the release of pro-inflammatory cytokines and autacoids (101;166-169) as well as enhancing phagocytosis, suggesting that they might play a role in host defense (162;170). Interestingly, the vast majority of the pro-inflammatory effects attributed to endocannabinoids involve 2-AG and but not AEA (table 10). In contrast to endocannabinoids, cannabinoids do not appear to stimulate leukocyte functions in experimental models of inflammation and a limited number of studies show that they stimulate immune cells [159, 160]. This suggests that endocannabinoids, 2-AG in particular, might modulate inflammation independently of CB receptor activation and through their numerous metabolites.

Involvement of endocannabinoid-derived metabolites in the regulation of inflammation

Given that both AEA and 2-AG are susceptible to metabolism by eicosanoid biosynthetic enzymes, the immunomodulatory role of endocannabinoids is very likely the combination of the effects of endocannabinoids and their metabolites. The hydrolysis of AEA and 2-AG will result in increased AA levels, putatively promoting eicosanoid biosynthesis. However, FAAH and MAGL inhibitors will not only increase the effects of endocannabinoids by preventing their hydrolysis. They will also increase their likelihood to undergo oxidation by COX-2, LOX and p450 enzymes. A good example for this is the detection of PG-EA in FAAH knockout mice (66). Moreover, pro-inflammatory mediators such as LPS reduce the expression of FAAH and promote COX-2 expression, which results in enhanced PG-EA and/or PG-G production (72;171;172).

COX-2 derivatives of AEA and 2-AG are the most studied endocannabinoid metabolites. Their biosynthesis was observed *in vivo* in rat (173); and inflammatory mediators like LPS and zymosan increase their production *in vitro* (72;174). This is not surprising considering that PG biosynthesis is a hallmark of inflammation. Among their biological effects, PGD_2-EA and PGE_2-EA induce cell death in cancer cell lines (175;176). Additionally, $PGF_{2\alpha}$-EA induces the contraction of the cat iris sphincter (177) and PGE_2-G induces Ca^{2+} mobilization, activation of PKC, hyperalgesia and allodynia, and enhances glutamatergic synaptic transmission (73;173;178). The reported biological effects of COX-2-derived 2-AG and AEA metabolites are detailed in table 11. Further studies will be required in order to establish a role for these mediators in inflammatory diseases.

Noteworthy, 2-AG-derived COX-2 metabolites have a short half-life *in vivo.* This half life is 14 seconds in rat plasma and 10 minutes in human plasma (179). Indeed, PGE_2-G is hydrolyzed into PGE_2 and glycerol by

several hydrolases including MAG lipase, carboxylesterase 1, lysophospholipase 2 and palmitoyl protein thioesterase 1 (180;56;181;182). Thus, PG-Gs likely act at the vicinity of their biosynthesis and are then rapidly cleared from the tissues. Moreover, some biological effects attributed to PG-Gs may in fact be the consequence of their hydrolysis products. PGE_2-EA, in contrast, is not hydrolyzed but slowly dehydrated into its isomer PGB_2-EA. It is far more stable in human and rat plasma, with a half-life greater than 5 hours (179).

The 12- and 15-LOX also oxygenate endocannabinoids. In humans, 15-LO is constitutively expressed in reticulocytes, eosinophils, and airway epithelial cells (183;184). Th2 cytokines like IL-4/13 induce the expression of 15-LO in monocytes, macrophages, and neutrophils. 12-LO is expressed in human platelets (reviewed in (185)). Although the biosynthesis of 12/15-HETE-AE and 12/15-HETE-G is observed *ex vivo*, their levels have never been reported *in vivo*. However, LOX inhibitors abrogate some of the effects of endocannabinoids, suggesting that the transformation by 12/15 LOX occurs and that these products have biological activity. These effects are summarized in table 12. The detection and modulation of 12/15-HETE-AE and 12/15-HETE-G biosynthesis in humans or animals could determine the biological relevance of these metabolites. While AEA and 2-AG are not oxidized by 5-LO, the hydrolysis of AEA and 2-AG can lead to substantial levels of AA, potentially leading to significant eicosanoid biosynthesis. In this respect, our group recently documented a functional link between 2-AG and leukotriene B_4 biosynthesis. We found that 2-AG activated neutrophil function. This required its hydrolysis into AA, which was rapidly metabolized into LTB_4, which then activated human neutrophils through the BLT_1 receptor (89). Other studies also reported that eicosanoids were mediating, at least in part, the effects of endocannabinoids (186-188).

Concluding remarks

A growing body of evidence supports the immunomodulatory roles of 2-AG and AEA. First, CB receptors are expressed by human leukocytes. Second, mice in which CB receptors are deficient display an altered, usually worsened, inflammatory phenotype. Third, mice in which endocannabinoid levels are increased, either by a genetic or a pharmacological blockade, also display an altered inflammatory phenotype. When 2-AG and AEA were identified as CB receptor ligands, the story was relatively simple: they modulated cell functions by activating CB receptors. However, the large metabolome derived from 2-AG and AEA, which includes eicosanoids, significantly increases the complexity of endocannabinoid research in inflammation.

It will be important to pursue research in this field having in mind that perhaps one or more 2-AG or AEA metabolites participate to inflammation in a coordinated and timely fashion and that additional receptors, aside from CB_1 and CB_2, are involved (figure 2.3). This is important given that eicosanoid biosynthetic enzyme expression is constantly changing during the course of an inflammatory response. In this regard, 2-AG can be

the source of pro-inflammatory leukotrienes in human neutrophils (89) and prostaglandins in murine macrophages (189), which are both usually involved in the acute phase of inflammation. Further, the 2-AG metabolite PGD$_2$-G was shown to promote the resolution of inflammation in the lungs of mice (190), indicating that the biosynthesis of the latter may occur during the resolution phase and may participate in the anti-inflammatory effects of COX-2 previously described (191).

Finally, the blockade of AEA and 2-AG hydrolysis not only increases endocannabinoid levels, but also their availability to undergo oxidation by COX and LOX enzymes. Therefore, endocannabinoid hydrolysis inhibitors may have a more complex impact than the intended increase in cannabinoid receptor signalling. This should be taken into consideration in future studies, especially when assessing the potential of AEA and 2-AG hydrolysis inhibitors in the treatment of inflammatory diseases. It will also be important to establish, in humans, which enzymes hydrolyze 2-AG and AEA as well as their expression profile in order to develop pharmacological tools that could limit inflammation and possibly promote its resolution.

Abbreviations

2-AG, 2-arachidonoyl-glycerol; **5,8,11-ETI,** 5,8,11-eicosatriynoic acid; **AA**, arachidonic acid; **AEA**; arachidonyl-ethanolamide; **ABHD**, α/β hydrolase domain; **AM251**, *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole 3-carboxamide; **AM281**, 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide; **AM630**, 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)-methanone; **Baicalein**, 5,6,7-trihydroxyflavone; **COX**, cyclooxygenase; **CP55,940**, (-)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)-cyclohexanol; **DAG**, diacyl-glycerol; **DFU**, [5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-*2*(5H)-furanone]; **EET**, epoxyeicosateraenoyl; **FAAH**, fatty acid amid hydrolase; **GPCR**, G-protein coupled receptor; **GW627368X**, 4-(4,9-diethoxy-1,3-dihydro-1-oxo-2H-benz[f]isoindol-2-yl)-N-(phenylsulfonyl)-benzeneacetamide; **GW6471**, [(2*S*)-2-[[(1*Z*)-1-Methyl-3-oxo-3-[4-(trifluoromethyl)phenyl]-1-propenyl]amino]-3-[4-[2-(5-methyl-2-phenyl-4-oxazolyl)ethoxy]phenyl]propyl]-carbamic acid ethyl ester; **GW9662**, 2-Chloro-5-nitro-*N*-phenylbenzamide; **HpETE**, hydroperoxyeicosatetraenoyl; **HU-201**, (6a*R*)-*trans*-3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[*b,d*]pyran-9-methanol; **HU-243,** [(3H)HU 243,3-dimethylheptyl-11-hydroxyhexahydrocannabinol; **IL**, interleukin; **JWH-015**, (2-Methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone; **JWH-133**, (6a*R*,10a*R*)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b,d*]pyran; **LT**, leukotriene; **LOX**, lipoxygenase; **LPS**, lipopolysaccharide; **MAFP**, (5*Z*,8*Z*,11*Z*,14*Z*)-5,8,11,14-eicosatetraenyl-methyl ester phosphonofluoridic acid; **MAG**, monoacyl-glycerol; **MAPK**, mitogen-activated protein kinases; **NAPE**, N-acyl-phosphatidyl-ethanolamine; NS398, *N*-[2-Cyclohexyloxy-4-nitrophenyl]methanesulfonamide; **OMDM1-2**, (9*Z*)-*N*-[1-((*R*)-4-Hydroxbenzyl)-2-hydroxyethyl]-9-octadecenamide; **OTFP**, 3-octylthio-1,1,1-trifluoropropan-2-one; **PF3845**, *N*-3-Pyridinyl-4-[[3-[[5-

(trifluoromethyl)-2-pyridinyl]oxy]phenyl]methyl]-1-piperidine carboxamide; **PG**, prostaglandin; **PLA₂**, phospholipase A_2; **PLC**, phospholipase C; **PLD**, phospholipase D; **PPAR** proliferating peroxisome activating receptor; **RT-PCR**, reverse transcriptase-polymerase chain reaction; **SC-19920**, 8-chloro-dibenz[b,f][1, 4]oxazepine-10(11H)-carboxy-(2-acetyl)hydrazide; **SR144528**, 5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-N-[(1S, 2S,4R)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1H-pyrazole-3- carboxamide; **SR144716**, 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide; **T0070907**, 2-chloro-5-nitro-N-4-pyridinyl-benzamide; **THC**, (-)-Δ9-tetrahydrocannabinol. **UCM707**, (5*Z*, 8Z, 11Z, 14*Z*)-*N*-(3-Furanylmethyl)-5,8,11,14-eicosatetraenamide; **URB597**, (3'-(aminocarbonyl)[1,1'-biphenyl]-3-yl)-cyclohexylcarbamate; **URB602**, [1,1'-biphenyl]-3-yl-carbamic acid, cyclohexyl ester; **VDM11**, (5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(4-Hydroxy-2-methylphenyl)-5,8,11,14-eicosatetraenamide; **WIN55-212**, (*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone
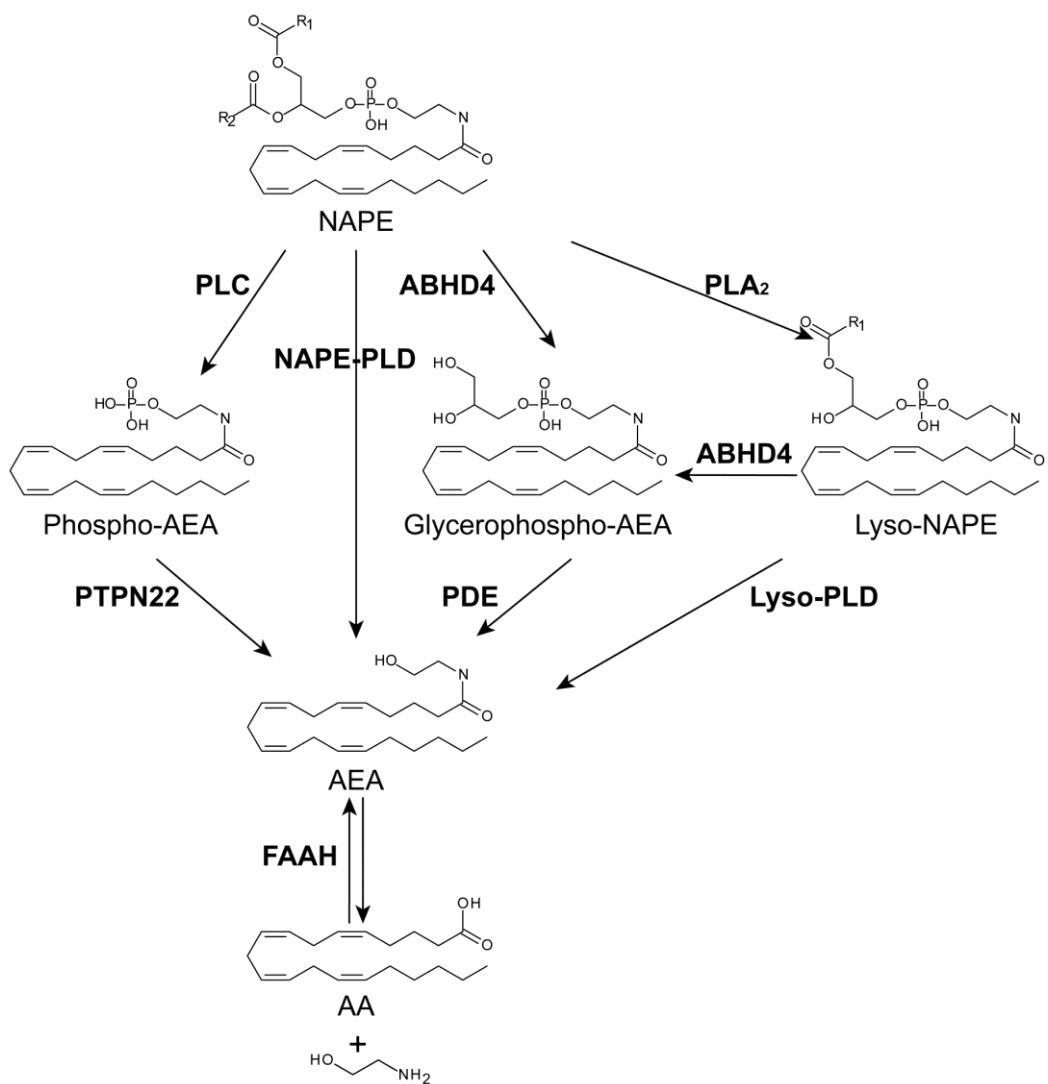
Figure 2.1. Biosynthetic pathways of AEA. AEA can be obtained by conjugation of ethanolamine and AA from the reverse reaction of FAAH. Otherwise AEA is obtained from its precursor NAPE which is produced from acylation of phosphatidyl-ethanolamines by N-acyl-transferases. NAPE can be metabolized into AEA by four pathways: 1) hydrolysis of NAPE by two distinct PLD; 2) hydrolysis of NAPE by a PLC to generate a phospho-AEA, which is dephosphorylated by the phosphatase PTPN22; 3) Cleavage of NAPE by PLA_2 and the hydrolysis of the lyso-NAPE by a lyso-PLD; 4) the generation of a lyso-NAPE by a PLA_2 activity, its hydrolysis by the serine esterase ABHD4, followed by a phosphodiesterase activity (glycerophosphodiesterase-1).
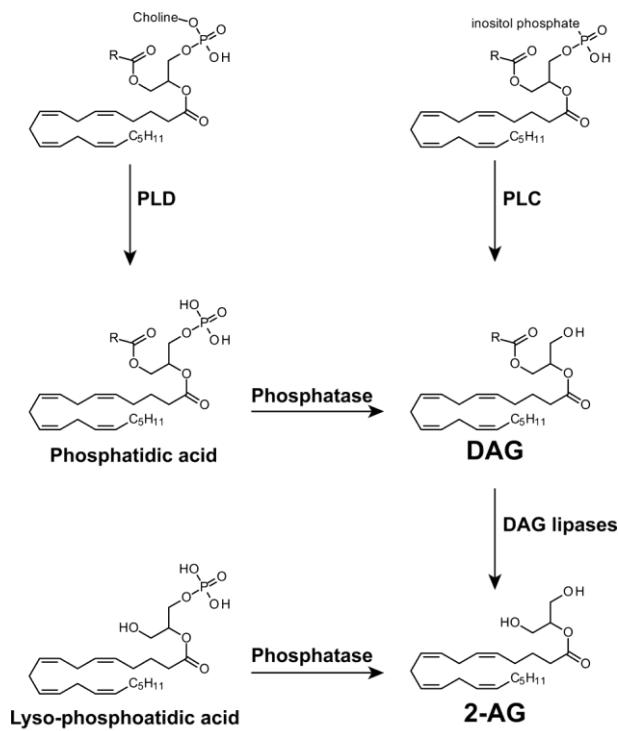
Figure 2.2. Biosynthetic pathways of 2-AG. 2-AG is obtained from DAG that can be produced by the hydrolysis of glyceryl-phosphatidyl-inositol by PLC or by or the combined action of PLD and phosphatidic acid phosphatase on glyceryl-phosphatidyl-choline. DAG-containing arachidonic acid is then hydrolyzed by DAG lipase α or DAG lipase βto generate 2-AG. The latter can also be obtained from the dephosphorylation of arachidonoyl-lysophosphatidic acid by a lysophosphatidic acid phosphatase.
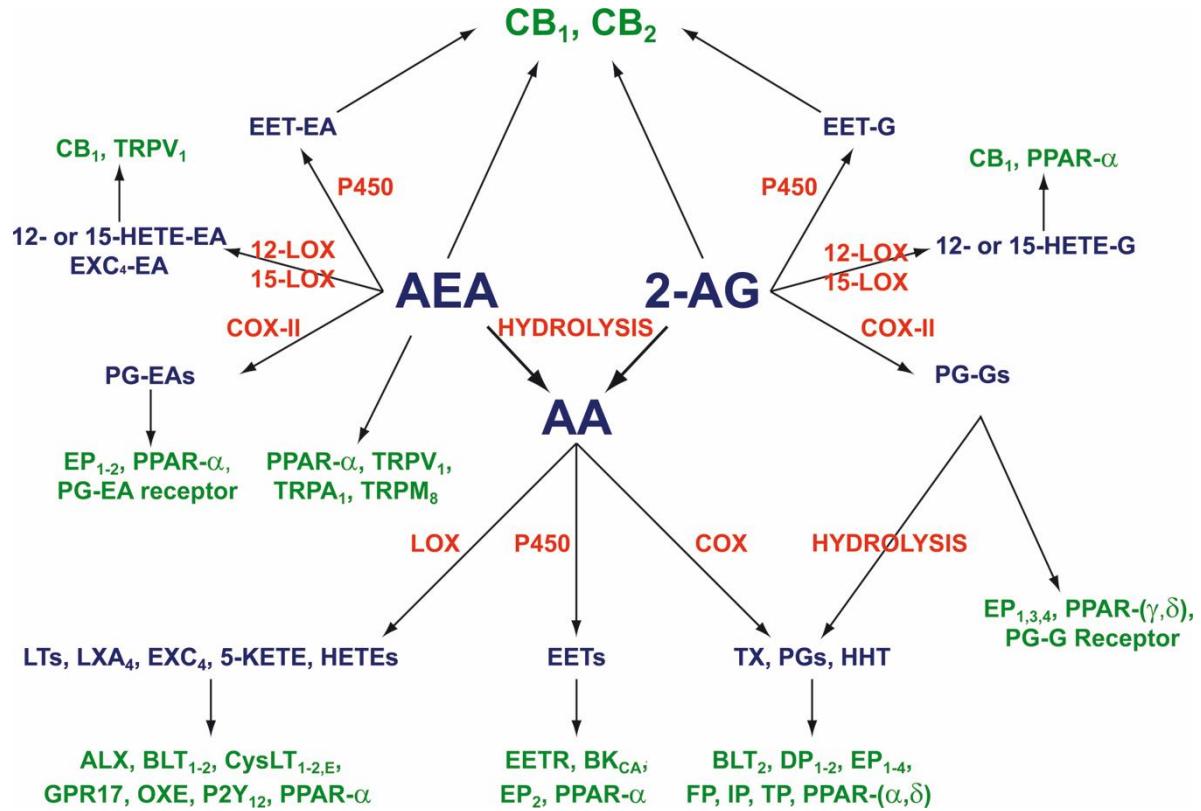
Figure 2.3. The complex metabolome of AEA and 2-AG is likely involved in their ability to regulate inflammation. The endocannabinoids AEA and 2-AG can modulate inflammatory cell functions by activating the CB_1 and CB_2 receptors. However, their hydrolysis into AA or their metabolism by eicosanoid biosynthetic enzymes (red) results in a plethora of bioactive lipids that activate additional receptors (green). Thus, the final outcome of 2-AG and AEA as regulators of inflammation will likely results in the combination of effects involving cannabinoid receptors, eicosanoid receptors and endocannabinoid metabolites receptors.

Table 2.1. 2-AG and/or AEA biosynthesis by inflammatory cells.

Cell type	Origin (cell line)	Endocannabinoid	Ref.
Astrocytes	Mouse	AEA	(192)
		2-AG	(193)
Dendritic cells	Human (monocyte-derived)	2-AG	(194)
Lymphocytes	Human	AEA	(171)
Mast cells	Rat (RBL-2H3)	AEA	(195)
Microglial cells	Mouse	2-AG	(196)
	Rat (RTMGL1)	2-AG, AEA	(197)
Macrophages	Mouse (peritoneal)	2-AG	(174) (198)
	Mouse (J774)	AEA	(199;200)
		2-AG	(200)
	Mouse (RAW 264.7)	AEA	(200;201) (202)
	Mouse (P388D1)	AEA	(203)
		2-AG	(203;204)
	Rat	2-AG, AEA	(200)
Platelets	Human	2-AG	(21;204)
	Rat	2-AG	(201)

Table 2.2. Expression of CB receptors by human leukocytes

Leukocytes	Receptor	mRNA	Protein	Ref.
Astrocytes	CB_1	+		(205)
	CB_2	-		
Basophils	CB_1	+		(206)
	CB_2	+		
B cells	CB_1	+	+ (FACS)	(207)
	CB_2	+		(87;207;208)
$CD4^+$	CB_1	-	+ (FACS)	(207)
	CB_2	+		(87;207)
$CD8^+$	CB_1	-	+ (FACS)	(207)
	CB_2	+		(87;207)
Dendritic cells	CB_1	+	+ (WB)	(194)
	CB_2	+	+ (WB)	
Eosinophils	CB_1	+		(206)
	CB_2	+	+ (WB)	(89-91)
Mast cells	CB_1	+	+ (FACS), + (IHC)	(209;209-211)
	CB_2	+	+ (FACS)	(209;209)
Macrophages (monocyte-derived)	CB_1	+	+ (FACS)	(139)
	CB_2	+	+ (FACS)	(212)
Microglia	CB_1	-	+ (IHC)	(213)
	CB_2	+		(213)
Monocytes	CB_1	+	+ (IHC)	(87;139)
	CB_2	+	+ (FACS)	(87;147;208)
Neutrophils	CB_1	+		(87)
	CB_2	+, -	+ (FACS), - (WB)	(87;89-92)
NK cells	CB_1	+		(87)
	CB_2	+	+ (FACS)	(87;207)
Platelets	CB_1		+ (WB), + (CM, WB)	(214)
	CB_2		+ (WB), - (CM, WB)	(215)
T lymphocytes	CB_1	+		(208;216)
	CB_2	+	+ (FACS)	

CM, Confocal microscopy; **FACS,** Fluorescence-activated cell sorting**; IHC**, immunohistochemistry; **WB**, western blot; **+**, detected; **-**, not detected.

Table 2.3. Anti-inflammatory effects observed in CB receptor deficient mice *in vivo*

Model	Species	Intervention	Effects	Ref.
TNBS-induced colitis	Mouse	CB_1, CB_2 and $CB_{1/2}$ K.O	↑ Colitis ↑ TNF-α and IL-1β	(113)
Sepsis caused by cecal ligation	Mouse	CB_2 knockout	↓ Sepsis induced mortality ↓ IL-10, IL-6 and MIP-2	(114)
DNFB-induced contact hypersensitivity	Mouse	CB_1, CB_2 and $CB_{1/2}$ K.O	↑ Neutrophil recruitment ↑ Ear swelling	(104)
Hepatic IR injury	Mouse	CB_2 K.O	↑ Liver damage ↑ Neutrophil recruitment ↑ Inflammatory cytokines	(107)
Influenza infection	Mouse	$CB_{1/2}$ K.O.	↑ APC function ↑ Pulmonary damage ↑ Inflammatory cell infiltration	(217)

APC, Antigen-presenting cell; **DNBS**, 2,4,4-trinitrobenzenesulphonic acid; **DNFB**, 2,4-Dinitro-1-Fluorobenzene; **IR**, ischemia reperfusion; **K.O** knock-out **MHC**, major histocompatibility complex; **MIP**, macrophage inflammatory proteins; **MPO**, myeloperoxidase; **FAAH-NS**, express FAAH exclusively in nervous tissue; **TNBS**, 2,4,6-trinitrobenzene sulfonic acid, **TNF**, tumor necrosis factor.

Table 2.4. Anti-inflammatory effects linked to enhanced AEA levels *in vivo*

Model	Species	Intervention	Effects	Ref.
ConA-induced hepatitis	Mouse	AEA	↓ Liver damage ↓ Inflammatory cytokines	(106)
Collagen-induced arthritis	Mouse	FAAH K.O FAAH-NS FAAH inhibitor URB597	↓ Severity of arthritis (Pannus, inflammation, bone damage, cartilage damage)	(50)
Carrageenan-induced acute inflammation	Mouse	FAAH inhibitor URB937	↓ Paw oedema ↓ Hyperalgesia ↓ Indomethacin-induced gastric lesions	(218)
Peripheral nerve injury	Mouse	FAAH inhibitor URB937	↓ Hyperalgesia and allodynia ↓ Indomethacin-induced gastric lesions	(218)
Antigen-induced arthritis	Mouse	FAAH inhibitor URB937	↓ Hyperalgesia	(218)
LPS-induced pulmonary inflammation	Mouse	AEA	↓ TNF-α ↓ Neutrophil recruitment	(109)
LPS-induced inflammatory pain	Mouse	FAAH K.O	↓ Oedema ↓ Hyperalgesia ↓ TNF-α and IL-1β	(112)
			↓ Allodynia	(219)
		FAAH inhibitors PF-3845, URB597 and OL-135	↓ Allodynia	(219)
DNBS-induced colon inflammation	Mouse	AEA reuptake inhibitor VDM11	↓ Colon inflammation ↓ MPO ↑ AEA	(103)
Theiler's encephalo-myelitis disease	Mouse	AEA reuptake inhibitor OMDM1-2	↓ Motor symptoms ↓ Microglial cell activation ↓ MHC class II	(115)
Osteoarthritis	Rat Guinea Pig	FAAH inhibitor URB597	↓ Nociception	(220)
Periodontitis	Rat	AEA	↓ TNF-α and IL-1β	(221)
Age-related long term potentiation	Rat	FAAH inhibitor URB597	↓Age-related microglial activation ↓ Age-related increase in TNF-α and IL-1β	(222)
Periodontitis	Rat	AEA	↓ TNF-α and IL-1β	(221)
LPS-induced brain inflammation	Rat	FAAH inhibitor URB597	↓ TNF-α and IL-1β	(223)
Hypoxic-ischemic injury	Rat	AEA	↓ Apoptosis ↓ ROS production	(224)
Neuropathic pain	Mouse	FAAH inhibitor	↓ Nociception	(225)

(3 models)	Rat	ST4070		
Cyclophosphamide-induced cystitis	Mouse	FAAH K.O.	↓ NGF, COX-2, iNOS	(226)
Kaolin-carrageenan induced osteoarthritis	Mouse	FAAH inhibitor URB597	↓ Leukocyte rolling and adhesion ↓ Inflammation-induced hyperaemia	(227)
Traumatic brain injury	Mouse	FAAH inhibitor PF-3845	↓ COX-2 expression ↓ iNOS and ↑ Arg-1 in microglial cells ↓ Cortex lesion volume	(228)
mBSA-induced delayed type hypersensitivity	Mouse	AEA	↓ Footpad swelling ↓ IL-17 and IFN-γ ↓ RORγT expression ↑ IL-10	(229)
TNBS-induced colitis	Mouse	FAAH inhibitor PF-3845	↓ Colon inflammation ↓ PGE_2	(230)
TMEV infection	Mouse	AEA	↓ IL-1β and IL-6 ↑ IL-10	(231)

TMEV, Theiler's encephalomyelitis virus.

Table 2.5. Anti-inflammatory effects of enhanced 2-AG levels *in vivo*

Model	Species	Intervention	Effects	Ref.
Acute experimental autoimmune encephalomyelitis	Mouse	2-AG	⬆ Activation and ramification of microglia ⬆ M2 macrophages	(232)
Acute inflammation	Mouse	MAGL inhibitor URB-602	⬇ Nociception ⬇ Oedema	(110)
Traumatic brain injury	Mouse	ABHD6 inhibitor WWL70	⬇ Cortex lesion volume ⬇ Neurodegeneration ⬇ iNOS and COX-2	(233)
Formalin-induced inflammatory pain	Rat	MAGL inhibitor URB-602	⬇ Nociception	(111)
Hypoxic-ischemic injury	Rat	2-AG	⬇ Apoptosis ⬇ ROS production	(224)
LPS-induced expression of cytokines in frontal cortex and plasma	Rat	MAGL inhibitor JZL-184	⬇ TNF- α, IL-1β, IL-6, IL-10 ⬇ Arachidonic acid levels	(234)
Alzheimer's disease	Mouse	MAGL K.O.	⬇ Gliosis and neuroinflammation ⬇ TNF- α, IL-1β IL-6, arachidonic acid, PGE2, PGD2, TXB2 ⬇ Amyloid plaques	(235)
Hepatic injury	Mouse	MAGL inhibitor JZL-184 MAGL K.O.	⬇ Cell death ⬇ Neutrophil infiltration ⬇ TNF-α and IL-1β	(236)
Carrageenan-induced paw oedema	Mouse	MAGL inhibitor KML29	⬇ Paw oedema ⬇ Mechanical allodynia	(237)
Sciatic nerve injury	Mouse	MAGL inhibitor KML29	⬇ Mechanical and cold allodynia	(237)
Diclofenac-induced gastric haemorrhage	Mouse	MAGL inhibitor KML29	⬇ Development of gastric haemorrhages	(237)
Chronic constriction injury	Mouse	JZL184 (MAGL) and Diclofenac (COX)	⬇ Mechanical and cold allodynia	(238)
LPS-induced lung injury	Mouse	MAGL inhibitor JZL184	⬇ Leukocyte recruitment ⬇ Adhesion molecule expression ⬇ TNF-α, IL-6, MCP-1	(239)

Table 2.6. Anti-inflammatory effects of CB receptor agonists *in vivo*

Model	Species	Intervention	Effects	Receptor	Ref.
Theiler's encephalo-myelitis disease	*Mouse*	WIN55-212	⬇ ICAM-1 and VCAM-1 ⬆ Motor coordination ⬇ CD4$^+$ recruitment	PPARγ (GW9662)	(116)
Infection with *L. pneumophila*	Mouse	THC	⬇ INF-γ and IL-12 ⬆ IL-4	CB$_1$ and CB$_2$ K.O	(240)
Infection with influenza viruses	Mouse	THC	⬇ Lymphocyte and monocyte recruitment ⬆ viral hemagglutinin	CB$_1$ and CB$_2$ K.O	(241)
Experimental autoimmune uveoretinitis	Mouse	JWH-133	⬇ Lymphocytes recruitment ⬇ INF-γ, TNF-α and IL-10	-	(117)
Experimental autoimmune encephalomyelitis	Mouse	THC JWH-133	⬇ Mononuclear cells recruitment ⬇ INF-γ and IL-2 ⬇ T cells proliferation	CB$_1$ and CB$_2$ K.O	(118)
Atherosclerosis ApoE$^{-/-}$ KO	Mouse	THC	⬇ Atherosclerotic lesions ⬇ Macrophage infiltration ⬇ Leukocyte adhesion	CB$_2$ (SR144528)	(119)
		WIN55-212	⬇ Atherosclerotic lesions ⬇ Macrophage infiltration ⬇ MCP-1, IL-6 and TNF-α	CB$_2$ (AM630)	(120)
Breast cancer cells injection	Mouse	THC	⬇ Splenocyte proliferation ⬆ Tumor mass ⬆ Metastasis	CB$_2$ (SR144528)	(128)
Ovalbumine induced-asthma	Mouse	THC	⬇ IL-2, IL-4 and, IgE	-	(121)
Streptozotocin-induced diabetes	Mouse	THC	⬇ Serum glucose ⬆ Pancreatic insulin ⬇ INF-γ, TNF-α, IL-12	-	(122)
Thioglycollate-induced peritoneal inflammation	Mouse	HU-210 WIN55-212	⬇ Neutrophil recruitment ⬇ MCP-1	CB$_1$ (SR141716)	(124)
Myocardial I/R	Mouse	WIN55-212	⬇ MPO activity ⬇ IL-1β and IL-8	CB$_2$ (AM-630)	(123)
Allergen-induced airway inflammation	Mouse	THC	⬇ Total cell count in bronchoalveolar lavage	-	(242)
Ovalbumine-induced asthma	Guinea Pig	CP55940	⬇ MPO activity ⬇ Mast cell degranulation ⬇ TNF-α and PGD$_2$	CB$_1$ (AM-251) CB$_2$ (SR144528)	(243)
Amyloid -induced experimental Alzheimer	Rat	WIN55-212	⬇ Microglia cell activation ⬆ Cognitive functions ⬇ Toxic effects	CB$_1$ (SR141528)	(244)
CCI-induced allodynia	Rat	AM1241	⬇ Allodynia	CB$_2$	(245)
Brain ischemia	Mouse	JWH-133	⬇ Microglia/macrophages ⬇ Middle cerebral artery occlusion-induced gene	CB$_2$ (SR144528 and CB$_2$ K.O.)	(246)

			expression of IL-6, TNF-α, MCP-1, MIP-1α, RANTES, iNOS		
Traumatic brain injury	Mouse	O-1966	↓Microglia/macrophage infiltration ↓ Blood-brain barrier disruption ↓ Neurodegeneration	CB$_2$	(247)
Periodontitis	Rat	Meth-AEA	↓ LPS-induced TNF-α and iNOS ↓ Alveolar bone loss	CB$_1$	(248)
Sepsis	Mouse	HU308	↓ Adherent leukocytes in submucosal venules	CB$_2$ (AM630)	(249)
LPS-induced interstitial cystitis	Mouse	JWH015	↓ Leukocyte infiltration ↓ Myeloperoxidase activity ↓ IL-1α, IL-1β and TNF-α	CB$_2$	(250)
Stress-induced neuro-inflammation	Mouse	JWH-133	↓ TNF-α and MCP-1 ↓ NF-κB, COX-2 and NOS-2 expression	CB$_2$	(251)

ICAM, Inter-Cellular Adhesion Molecule; **INF**, interferon; **Ig**, immunoglobulin; **MCP**, monocyte chemotactic protein; **VCAM**, vascular cell adhesion molecule; **-** not determined

Table 2.7. Anti-inflammatory effects of 2-AG and AEA *in vitro*

Leukocyte	Origin	Intervention	Effects	Receptor	Ref.
Astrocytes	Rat	AEA uptake inhibitor UCM707	↓ TNF-α, IL-1β and NO	CB$_1$ (SR141716) CB$_2$ (SR144528)	(138)
CD4+	Human	AEA	↓ INF-γ, TNF-α and IL-17	CB$_2$ (SR144528) (JWH-015)	(142)
CD8+	Human	AEA	↓ INF-γ and TNF-α	CB$_2$ (SR144528)	(142)
		AEA	↓ Migration induced by SDF-1	CB$_2$ (JWH-015)	(145)
Dendritic cells	Human	AEA	↓ R848-induced IL-6, IL-12 and IFN- α production	CB$_2$ (SR144528)	(252)
Macrophages	Mouse (RAW264.7)	AEA	↓ ROS, TNF-α and MCP-1	CB$_1$ (SR141716)	(139)
	Mouse (Peritoneal)	2-AG	↓ TNF-α	-	(253)
		AEA	↓ NO and IL-6	-	(253)
	Mouse (Peritoneal)	AEA	↓ Killing of TNF-sensitive cells	-	(254)
Mast cells	Human	AEA	↓ Mast cell maturation and degranulation	CB$_1$ (CB$_1$ K.O)	(210)
Microglia	Mouse	AEA	↑ IL-10	CB$_2$ (SR144528)	(144)
	Mouse (microglia cell line BV-2)	AEA	↓ NO	CB$_2$ (AM630)	(140)
		2-AG and AEA	↓ TNF-α release	-	(255)
	Mouse	AEA	↓ IL-12p70 and IL-23 ↑ IL-10	CB$_2$ (SR144528)	(256)
Mononuclear cells	Human	AEA	↓ IL-8, TNF-α and IL-6	-	(143)
Muller glia	Human	AEA and 2-AG	↓ Tat induced retinal cell death ↓ TNF- α and IL-6 ↑ IL-10 and TGF-β	-	(257) (258)
Neutrophils	Human	AEA	↓ Migration induced by fMLP and LTB$_4$	-	(137)
		2-AG	↓ Migration induced by fMLP	CB$_2$ (SR144528)	(92)
Splenocytes	Human	2-AG and AEA	↓ IL-2	PPARγ	(141)
		AEA	↓ Plaque forming cell assays	CB$_2$ (SR144528)	(259)

T cells		AEA	↓ Cell proliferation	CB$_2$ (SR144528)	(142)
	Human	AEA	↓ Cell proliferation ↑ Apoptosis	-	(260)
		2-AG	↓ Migration induced by SDF-1	CB$_2$ (JWH-015)	(136)
	Human (Jurkat)	2-AG	↓ IL-2	PPARγ	(261)
U87MG (malignant glioma)	Human	2-AG	↓ NF-κB activation ↓ Cell growth	CB$_1$ (AM281)	(262)
Platelets	Human	AEA	↓ Platelet aggregation	-	(263)

fMLP, formylMethionyl-Leucyl-Phenylalanine; **NO**, nitric oxide; **ROS**, reactive oxygen species; **SDF**-1, stromal cell derived factor-1.

Table 2.8. Anti-inflammatory effects of CB receptor agonists *in vitro*

Leukocytes	Origin	Intervention	Effects	Receptor	Ref.
Astrocytes	Human	WIN55-212	↓ NO, TNF-α, IP-10, MCP-1 and RANTES	CB$_1$ (SR141716) CB$_2$ (SR144528)	(152)
CD8$^+$	Human	JWH-015	↓ Migration induced by SDF-1	CB$_2$ (AM630)	(146)
Dendritic cells	Mouse	THC	↑ NF-κB-dependant apoptosis	CB$_1$ (SR141716A) and CB$_2$ (SR144528)	(264)
Macrophages	Mouse (RAW264.7)	WIN55-212	↓ ROS	CB$_2$ (AM630)	(153)
	Mouse (RAW264.7)	WIN55-212	↓ NO production	CB$_2$ (SR144528)	(265)
	Mouse (Peritoneal)	CP55, 940	↓ Migration induced by fMLP	CB$_1$ (SR141716)	(149)
		THC	↓ Migration induced by RANTES	CB$_2$ (SR144528)	(150)
			↓ Yeast phagocytosis	-	(266)
		JWH-133	↓ IL-12p40 ↑ IL-10	CB$_2$ (SR144528)	(267)
	Mouse (J774)	THC	↓ NO, IL-6 and PGE$_2$	-	(166)
	Mouse (Clone 63)	THC	↓ Activation of CD4$^+$	CB$_2$ (SR144528)	(268)
Mast cells	Rat (RBL-2H3)	WIN55-212 CP55, 940	↓ β-Hexosaminidase release	CB$_2$ (AM630)	(269)
Microglia	Mouse (Microglia cell line BV-2)	THC	↓ IL-1β, INF-β and IL-6 ↓ Phosphorylation of STAT-1	-	(151)
		CD-101	↓ LPS-induced production of NO, COX-2, TNF-α, IL-1β and IL-6	-	(270)
Neutrophils	Human	JWH-133	↓ Migration induced by TNF-α	-	(271)
	Mice	JWH-133	↓ Migration induced by MIP-2α	CB$_2$ K.O	(148)
Splenocytes	Mice	JWH-015	↑ Apoptosis	-	(272)
T cells	Human	JWH-133	↓ Migration induced by SDF-1	-	(136)
		THC	↓ Cytokine proliferation switch to Th2	CB$_2$ (SR144528)	(154)
	Leukemia (Jurkat)	CP55, 940 WIN55-212 JWH-015	↓ Migration induced by SDF-1	CB$_2$ (AM630)	(146)
Thymocytes	Mice	JWH-015	↑ Apoptosis	CB$_2$ (SR144528)	(272)

IP, Interferon gamma-induced protein; **RANTES,** regulated upon activation, normal T-cell expressed, and secreted; **STAT**, signal transducer and activator of *Transcription.*

Table 2.9. Pro-inflammatory effects of endocannabinoids *in vivo*

Model	Species	Intervention	Effects	Receptor	Ref.
Ovalbumin	Mouse	AEA	↑ Delayed type hypersensitivity ↑ Cell proliferation ↑ INF-γ	-	(156)
Immunization	Mouse	2-AG and Peptidoglycan *keyhole limpet* hemocyanin	↑ Delayed type hypersensitivity ↓ IL-4	CB_2 (SR144528)	(155)
Cisplatin-induced neuropathy induced	Mouse	CB_1 K.O	↓ Renal dysfunction ↓ Inflammatory response	CB_1	(157)
Doxorubicin-induced cardiomyopathy	Mouse	CB_1 K.O	↓ Cell death ↓ Fibrosis ↓ Oxidative stress	CB_1	(158)
Oxazolone-induced contact-dermatitis	Mouse	CB_2 (SR144528)	↓ Swelling ↓ MCP-1, MIP-1 and TNF-α ↓ Eosinophil recruitment	CB_2	(159)
TPA-induced ear inflammation	Mouse	CB_2 (SR144528)	↓ Swelling ↓ LTB_4 ↓ Eosinophil recruitment	CB_2	(160)
Endotoxin-induced uveitis	Rabbit	AEA	↑ Ocular inflammation	CB_1 (AM251)	(273)
Atherosclerosis (ApoE (-/-))	Mouse	FAAH K.O.	↑ Neutrophil recruitment in aortas ↑ IFN-γand TNF-α	-	(274)

TPA, 12-O-tetradecanoylphorbol-13-acetate.

Table 2.10. Pro-inflammatory effects of endocannabinoids *in vitro*

Leukocyte	Origin	Intervention	Effects	Receptors	Ref.
B cells	Human lymphoblast	2-AG	↑ Migration	CB$_2$ (SR144528)	(275)
	Mouse	2-AG	↑ Migration	CB$_2$ (SR144528)	(276) (277)
Dendritic cells	Human	2-AG	↑ Migration	CB$_2$ (SR144528)	(155)
Eosinophils	Human	2-AG	↑ Migration	CB$_2$ (SR144528)	(91) (278)
	Human	2-AG	↑ Migration	CB$_2$ (SR144528 and AM630)	(279)
Endothelial cells	HUVECs and Jurkat cells	2-AG	↑ P- and E-selectins on HUVECs ↑ PGSL1 on Jurkat cells	-	(280)
Macrophages	Human	LPS	↓ IL-1β IL-6, IL-8, TNF-α and MMP-9	CB$_1$ (SR141716A)	(101)
	Mouse (peritoneal)	CB$_2$ K.O	↓ Apoptosis	-	(281)
	Mouse (Peritoneal)	2-AG	↑ Zymosan phagocytosis	CB$_2$ (SR144528)	(170)
	Human (HL-60 macrophages)	2-AG	↑ Actin polymerization ↑ Adhesion to fibronectin	CB$_2$ (SR144528)	(163)
		2-AG	↑ IL-8 and MCP-1 production	CB$_2$ (SR144528)	(282)
	Mouse (J774)	2-AG	↑ NO	-	(283)
			↓ IL-6	-	(166)
Mast cells	Rat (Peritoneal)	AEA	↑ Histamine release	-	(168)
	Human (RBL-2H3 cell line)	2-AG	↑ TNF-α ↑ βHexosaminidase	-	(284)
Microglia	Mouse (Microglial cell line BV-2)	2-AG	↑ Migration	CB$_2$ (SR144528)	(165)
Monocytes	Human	2-AG	↑ Adhesion to fibronectin	CB$_2$ (SR144528)	(283)
			↑ Migration	CB$_2$ (SR144528)	(163)
			↑ NO release	CB$_1$ (SR141716A)	(167)
Neutrophils	Human	2-AG	↑ LTB$_4$ biosynthesis ↑ MPO release	-	(89)
NK cells	Human	2-AG	↑ Migration	CB$_2$ (SR144528)	(164)
Platelets	Human	2-AG	↑ Platelet aggregation	-	(285) (286)

Macrophages	Mouse (Peritoneal)	DAG lipase β blockade	↓ PGE_2 ↓ PGD_2 ↓ LPS-induced TNF-α	-	(198)

MMP, matrix metalloproteinase.

Table 2.11. Effects of COX-2-derived metabolites of AEA and 2-AG on inflammation

Cell type	Effects	Metabolite	Ref.
Aortic rings (Rat)	Vasorelaxation induced by AEA	COX-2 products Blocked by FAAH inhibitor URB597 Blocked by COX-2 inhibitor DFU Blocked by EP_4 antagonist GW627368X	(287)
Carcinoma (colorectal HT29)	Cell death induced by AEA	COX-2 products Prevented by COX-2 inhibitor NS398 Mimicked by PGD_2-EA and PGE_2-EA	(175)
Carcinoma (cell line JWF2)	Cell death induced by AEA	COX products Mimicked by PGD_2 and PGD_2-EA	(176)
Cat Iris	Contraction of iris sphincter	$PGF_{2\alpha}$-EA > PGD_2-AEA ≈ PGE_2-EA	(177)
Hippocampus	Enhance glutamatergic synaptic transmission Enhance neuronal injury/death	PGE_2-G	(178)
Human Umbilical Vein Endothelial Cells	Tissue factor release and PPAR-δ activation induced by 2-AG	PGI_2-G Prevent by COX-2 inhibitor NS398 Inhibited by PGI_2S siRNA	(288)
Lung (Rabbit)	Pulmonary arterial pressure induced by 2-AG and AEA	COX-2 products Blocked by FAAH inhibitor MAFP Blocked by COX-2 inhibitor nimesulide Blocked by EP_1 antagonist SC-19220	(289)
Monocytes (human and THP-1 cell line)	↓ LPS-induced TNF-α production	PGE_2-EA	(290)
Mouse macrophages (RAW 264.7)	Transcriptional activity of the IL-12p40 genes induced by AEA	COX-2 products Prevented by COX-2 inhibitor NS398 Mimicked by PGE_2-EA Blocked by EP_2 antagonist AH6809	(291)
	Ca^{2+} accumulation Activation of PKC	PGE_2-G	(73)
Mouse splenocytes	Inhibition of IL-2 releases induced by AEA	COX-2 products Unaffected by FAAH inhibitor MAFP Blocked by COX-2 inhibitor NS398 Blocked by PPAR-γ antagonist T0070907	(69)
Platelets	Aggregation induced by 2-AG	COX products Prevented by COX inhibitor aspirin	(292)
Rat	Hyperalgesia and allodynia	PGE_2-G	(173)
Carrageenan-induced knee inflammation (Rat)	↑ Nociception ↓ Paw withdrawal latency	$PGF_{2\alpha}$EA	(293)
Ex vivo human	↓ Cytokine-evoked	PGE_2-EA	(294)

mucosal explant colitis	epithelial damage	PGF$_{2\alpha}$-EA	
Renal medulla (Mouse)	↓ Mean arterial pressure ↑ Renal blood flow	PGE$_2$-EA	(295)
Peritoneal macrophages (Mouse)	↓ LPS-induced inflammation	PGD$_2$-G	(190)
T cells (Jurkat)	IL-2 release induced by 2-AG	COX-2 products Prevented by COX-2 inhibitor NS398	(141)
	↓ IL-2	15d-PGJ$_2$-G	(296)

Table 2.12. Involvement of lipoxygenases in the effects of 2-AG and AEA.

Cell type	Effects	Metabolite	Ref.
Brain (Mouse)	↑ NAPE-PLD activity ↓ AEA uptake ↓ DAG lipase activity	15-HETE-EA	(297)
Brain (Rat)	Decrease oedema induced by ouabain	12-HETE-EA	(298)
Bronchus (Guinea Pig)	Contraction induced by AEA	LO products Blocked by LO inhibitor 5,8,11-ETI	(80)
Carcinoma (PC-3)	Cell invasion induced by 2-AG	12-LO products Mimicked by AA and 12(S)-HETE Blocked by 2-AG hydrolysis inhibitor OTFP Blocked by 12-LO inhibitor baicalein	(186)
Distal colon (Guinea Pig)	Contraction of longitudinal muscle induced by 2-AG	LO products Blocked by LO inhibitor NDGA	(299)
Neuroblastoma (Human)	FAAH inhibition induced by AEA and 2-AG	LO products Mimicked by AA Blocked by 5-LO inhibitor MK886	(187)
Neutrophil (Human)	2-AG promotes migration, kinase activation, Ca^{2+} mobilization, and antimicrobial peptide release	LTB_4 Mimicked by AA Blocked by 2-AG hydrolysis inhibitor MAFP Blocked by FLAP antagonist MK-0591 Blocked by LTB_4 receptor antagonist CP105, 696	(89;90)
Vagus nerve (Guinea Pig)	Depolarization induced by AEA	LO products Blocked by LO inhibitor 5,8,11-ETI	(81)
Sciatic nerve injury (Rat)	FAAH inhibition reduced nociception	LO products Blocked by 12/15-LO inhibitor baicalein	(300)

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Chapitre 2.   The CB₂ receptor and its role as a regulator of inflammation

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Résumé

Le récepteur CB_2 est un récepteur cannabinoïde exprimé principalement en périphérie. Il est fortement exprimé dans les tissus immunitaires, expliquant le rôle immunomodulateur du système endocannabinoïde. À cet égard, il a été démontré que le récepteur CB_2 module les fonctions des leucocytes, tant *in cellulo* que dans des modèles animaux de maladies inflammatoires. Plusieurs études ont d'ailleurs rapporté que des souris déficientes en récepteur CB_2 présentent un phénotype inflammatoire exacerbé. Ceci suggère que des stratégies thérapeutiques visant à augmenter la signalisation au récepteur CB_2 seraient prometteuses pour le traitement de diverses maladies inflammatoires. Dans cet article de revue, nous présentons la pharmacologie du récepteur CB_2, son patron d'expression et les voies de signalisation induites par son activation. Nous examinons également la régulation des fonctions des cellules immunitaires par le récepteur CB_2 et les données générées à partir de leucocytes humains primaires, de lignées cellulaires immortalisées et de modèles animaux d'inflammation. Finalement, nous discutons des possibilités de thérapies visant le récepteur CB_2 et des questions qui doivent être étudiées davantage afin de déterminer si ce récepteur sera utile pour le traitement des maladies inflammatoires.

Abstract

The CB$_2$ receptor is the peripheral receptor for cannabinoids. It is mainly expressed in immune tissues, highlighting the possibility that the endocannabinoid system has an immunomodulatory role. In this respect, the CB$_2$ receptor was shown to modulate immune cell functions, both *in cellulo* and in animal models of inflammatory diseases. In this regard, numerous studies have reported that mice lacking the CB$_2$ receptor have an exacerbated inflammatory phenotype. This suggests that therapeutic strategies aiming at modulating CB$_2$ signaling could be promising for the treatment of various inflammatory conditions. Herein, we review the pharmacology of the CB$_2$ receptor, its expression pattern and the signaling pathways induced by its activation. We next examine the regulation of immune cell functions by the CB$_2$ receptor and the evidence obtained from primary human cells, immortalized cell lines and animal models of inflammation. Finally, we discuss the possible therapies targeting the CB$_2$ receptor and the questions that remain to be addressed in order to determine whether this receptor could be a potential target to treat inflammatory disease.

Introduction

The psychotropic effects induced by cannabis promoted its widespread use among the population. These effects are mediated by a cannabinoid receptor that is mainly expressed in the central nervous system, namely CB_1. The identification of a receptor that is selectively activated by cannabinoids suggested that the human body synthesizes at least one natural ligand for this receptor. This hypothesis was confirmed by the discovery of two high-affinity ligands for the CB_1 receptor: arachidonoyl-ethanolamide (AEA) [1] and 2-arachidonoyl-glycerol (2-AG) [2]. As these novel lipid mediators were uncovered, a second cannabinoid receptor (CB_2) was being cloned and characterized. Its expression profile among tissues was found to be distinct from that of CB_1. It was primarily found in immune cells and was initially not detected in the brain, although this was later proven incorrect by several studies. In light of these findings, the CB_2 receptor was postulated to be responsible for the immunomodulatory effects of cannabinoids and endocannabinoids. In the past two decades, this hypothesis was tested in a wide array of cellular and animal models. This article offers a comprehensive review of the evidence that was gathered in these studies, with a focus on peripheral inflammation. The CB_2 receptor's potential as a therapeutic target in inflammatory disease is also discussed.

Cloning of the CB_2 receptor

The non-psychoactive effects of cannabinoids were initially believed to be mediated either centrally or through their interaction with non-receptor proteins. Although there are phytocannabinoids that exert non-psychoactive effects without binding to the CB_2 receptor (e.g. cannabidiol (CBD), cannabigerol (CBG)), discovering the latter explained many of the peripheral effects of cannabinoids. Munro et al. cloned the human CB_2 receptor in 1993 from the promyelocytic leukaemic cell line HL-60 [3]. To achieve this, cells were treated with dimethylformamide to induce granulocyte differentiation, a cDNA library was prepared, polymerase chain reaction (PCR) was performed using degenerated primers and the amplification products were cloned and sequenced. One of the clones showed homology to the G-protein-coupled-receptor (GPCR) family and was related to the CB_1 receptor. The protein encoded by this sequence was found to have 44% homology with the CB_1 receptor. This homology increased to 68% when only the transmembrane portion was considered. Binding assays showed that this receptor had high affinity for the cannabinoid receptor ligands WIN 55,212-2 and CP 55,940, as well as the endocannabinoid AEA and the phytocannabinoid Δ^9-THC. The authors suggested that the previously described central receptor be named CB_1 and that this novel, peripheral receptor be named CB_2.

A few years later, Shire et al [4] cloned the murine CB_2 receptor from a mouse splenocyte cDNA library. They found it to be 82% homologous to the human CB_2 receptor and to have similar affinity for the ligands AEA, CP55,940 and Δ^9-THC. WIN 55,212-2, however, bound the mouse CB_2 receptor with an affinity 6-fold lower

than that documented for human CB_2. This was followed by the cloning of the rat CB_2 receptor by Brown et al. [5]. The authors also compared the sequence of their clone with those of the mouse and human CB_2 receptor and found significant differences in protein length, although these were mainly the consequence of disparities in carboxyl termini. Amino acid conservation was highest in the transmembrane regions of the three receptors.

In addition to binding the endocannabinoids AEA and 2-AG, the CB_2 receptor binds many phytocannabinoids. The pharmacology of endocannabinoids and of the CB_2 receptor were rigorously reviewed in the past [6,7]. Table 3.1 provides a summary of the various endocannabinoids and phytocannabinoids and their affinity for the human CB_2 receptor.

Available tools to study CB_2 receptor functions

Pharmacological compounds

Synthetic cannabinoids such as CP 55,940 and WIN 55,212-2 were already available when the CB_2 receptor was cloned. They were subsequently shown to be potent CB_2 ligands, but to also lack selectivity as they activate CB_1 with comparable efficiency. In this respect, several agonists and antagonists were rapidly developed and made available to the scientific community. The most widely used compounds are the agonist JWH 133, and the antagonists SR144528 and AM630. Still, many compounds display good potency and selectivity towards CB_2. Table 3.2 contains a comprehensive list of those compounds, as well as their binding potency towards human CB_2 and in some cases, the other receptors they target.

Knockout mice

The first CB_2 receptor-deficient mouse was generated by Buckley et al in 2000 [32]. The *CNR2* gene was inactivated by homologous recombination, by replacing a 341 bp fragment of its coding sequence with the neomycin gene. This mutation eliminated part of intracellular loop 3, transmembrane domains 6 and 7, and the carboxyl extremity of the receptor. Autoradiography experiments confirmed the absence of specific binding of [^{3}H]CP 55,940 in the spleen of $CB_2^{-/-}$ mice. No significant difference in the binding of [^{3}H]CP 55,940 between wild-type and knockout animals was found in the brain, supporting that CB_1 receptor expression was not altered in $CB_2^{-/-}$ animals. The authors confirmed this by demonstrating that knockout mice were as responsive to the psychotropic effects of Δ^9-tetrahydrocannabinol as wild-type animals.

$CB_2^{-/-}$ mice display no morphological differences when compared to their wild-type counterparts. They are a normal size and weight, are fertile, have normal litter sizes and care for their young. However, subsequent studies by other groups show that $CB_2^{-/-}$ mice develop differences at the cellular level. In this regard, Ofeck et al have demonstrated that $CB_2^{-/-}$ mice have lower counts of osteoblast precursors and increased numbers and

activity of osteoclasts [33]. In consequence, these mice have a low bone mass phenotype that worsens with age. They also present abnormalities in the development of several T and B cell subsets [34]. While this might impair immune homeostasis, $CB_2^{-/-}$ mice fail to spontaneously develop any observable immune disease. Therefore, they are suitable to study CB_2 function and have since become invaluable tools in cannabinoid research. In this respect, they have been used to define the impact of CB_2 deficiency in a variety of inflammatory disease models, and the results of these studies will be discussed in section 7.1.

Antibodies

As it is the case with numerous GPCRs, CB_2 protein detection is difficult due to the lack of specificity of primary antibodies. This concept was underscored in a recent study by Marchalant et al. [35], who showed that a commercially available and widely used CB_2 polyclonal antibody is heavily cross-reactive towards other proteins. Noteworthy, they demonstrated that some of the proteins detected by the antibody were not membrane-bound, ruling out the previously suggested hypothesis that the additional bands represent glycosylation variants of the CB_2 receptor. Moreover, Graham et al. [36] compared several CB_2 primary antibodies in flow cytometry experiments on human primary leukocytes. The antibodies they compared generated different expression patterns between cell types. Therefore, data regarding CB_2 protein detection must be interpreted with caution.

The detection of the CB_2 receptor using antibodies can be substituted, to some extent, by alternate methods. For example, Schmöle et al. [37] recently generated a Bacterial Artificial Chromosome (BAC) transgenic mouse model that expresses a green fluorescent protein (GFP) under the CB_2 promoter. This mouse can be used to determine CB_2 expression in mouse tissues *in vitro* and *in situ*, by several techniques including RT-PCR, qPCR, immunoblot, flow cytometry and immunofluorescence. This system, based on GFP detection, is an alternative to the use of CB_2 antibodies on mouse tissues. It is more reliable in the sense that most antibodies directed against GFP are specific and yield reproducible data. However, this kind of approach cannot be used for CB_2 detection in human primary cells and tissues, which remain problematic. A different strategy that was evaluated by Petrov et al. involves the synthesis of fluorescent CB_2 agonists [38]. The synthesized compound showed marked selectivity for CB_2 over the CB_1, $5-HT_{2A}$ and $5-HT_{2C}$ receptors. This agonist was validated as a flow cytometry probe to detect the CB_2 receptor in cells, and also to evaluate CB_2 receptor binding using fluorescence microscopy. Other methods of detection could also be added to CB_2 ligands in order to use them as probes, such as biotinylation [39].

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CB$_2$ expression profiles in human and animal tissues

Expression profile of CB$_2$ among tissues

Upon cloning the human CB$_2$ receptor from HL-60 cells, Munro et al. isolated a portion of a rat homologue by PCR [3]. They used this homologue to probe various rat tissues and detected high CB$_2$ receptor mRNA levels in the spleen, but not in the liver, nasal epithelium, thymus, brain, lung or kidney. Cell sorting allowed the authors to associate CB$_2$ receptor expression to the monocyte/macrophage population of the spleen rather than T cells. Two years later, Galiègue et al. published the first study describing CB$_2$ receptor expression in various human tissues and isolated leukocyte populations [40]. The authors found high CB$_2$ mRNA levels in tonsils, spleen, PBMC and thymus, and were able to detect the CB$_2$ protein in tonsils by immunohistochemistry using an anti-CB$_2$ polyclonal antibody. They also evaluated CB$_2$ receptor mRNA expression in numerous human organs and found it to be absent from most non-immune tissues, with the exception of pancreas, lung and uterus, which had relatively low mRNA levels. Several reports have since shown that the CB$_2$ receptor is expressed in both male [41] and female [42,43] reproductive tissues. In this regard, the CB$_2$ receptor exerts an important role in the fertility of both sexes, which has already been extensively reviewed [44-47].

The pattern of CB$_2$ receptor expression among human tissues is consistent between studies. More groups have reported the presence of the CB$_2$ receptor mRNA and protein in the human spleen [48] and tonsils [49]. Moreover, the high level of CB$_2$ expression in human immune tissues was also reported in murine and rodent spleen [50,37,51-56] and thymus [37,54].

The presence and role of the CB$_2$ receptor in the central nervous system has yet to be fully elucidated, and the issue was discussed in a review article recently published by Atwood and Mackie [57]. It was initially believed that it was not expressed in non-immune cells of the central nervous system, because Munro et al. did not detect CB$_2$ receptor mRNA in any brain part when they cloned the receptor [3], which is supported by many studies [40,58,59,54]. However, we now know that the CB$_2$ receptor is not completely absent from the brain since it is expressed in microglia [60]. Still, the concept of the CB$_2$ receptor being a second central cannabinoid receptor is up for debate for three main reasons: 1) A study showed that the CB$_2$ receptor agonists JWH-015 and JWH-133 modulate peripheral neuron functions [61] and 2) the CB$_2$ receptor was detected in the uninjured brain by immunochemistry on numerous occasions [62-64], and 3) A recent study found that hippocampal principal neurons express CB$_2$ mRNA, and that CB$_2$-selective agonist HU-308 modulated the activity of these cells [65]. Conversely, a study that relied on GFP detection to determine the expression of the CB$_2$ receptor in the murine brain showed that the signal is located in microglia [37]. Therefore, the lack of reliability of the antibodies that were used in immunochemistry experiments stresses the need for more research in order to

expand our knowledge on the involvement of the CB_2 receptor in the central nervous system and neuroinflammation.

In 2009, Liu et al showed that two distinct isoforms of the CB_2 receptor exist [66]. The novel CB_2 isoform was a splicing variant of the earlier cloned receptor, and was identified from a human neuroblastoma cDNA library. Splicing variants were also discovered in mice and rats, although their genomic structures and transcripts were different from those found in humans. Furthermore, the two human variants were found to display tissue-specific expression patterns. While the classical CB_2 isoform was predominantly found in spleen and other immune tissues, the novel isoform was detected in higher levels in testis and brain regions of the reward system. The identification of this new CB_2 variant could shed some light on the confusing expression patterns that were previously reported. Finally, it underscores the possibility of a role for CB_2 in reproductive and central nervous systems that is distinct from the immunomodulatory role of the classical CB_2 isoform.

CB_2 expression in immune cells

It is well known that the CB_2 receptor is widespread among cells of the immune system. Table 3.3 provides the literature associated with the expression of the CB_2 receptor in human leukocytes. Every cell type that has been investigated was found to express both mRNA and protein in at least one report. However, there is conflicting data associated with a few cell types. For example, there is no consensus in the literature regarding the presence of the CB_2 receptor in human neutrophils. Of note, not every study was conducted on purified, eosinophil-depleted neutrophils. Given that eosinophils have very abundant amounts of CB_2 receptor mRNA, a small number of eosinophils among the neutrophil sample could result in a false positive. This is consistent with the observation that CB_2 levels are lower in neutrophils than in eosinophils.

As discussed in the previous section, the scientific community should always be critical when interpreting protein data, especially of GPCRs. A large number of researchers have now reported expression data obtained with commercially available antibodies, and most of them relied on a positive control to validate their results. It was later underscored that in the case of the CB_2 receptor, a reliable negative control is absolutely necessary in order to confirm that the signal is not generated by non-specific binding of the antibody [35,67].

CB_2 receptor signaling

The CB_2 receptor was associated to the GPCR family when it was cloned. However, the signal transduction pathways induced by CB_2 receptor activation are far less characterized than those of CB_1. CB_1 is known to inhibit adenylyl cyclase, to modulate ion channels, and to activate numerous downstream signaling events, including p38 and p42/44 MAPK (ERK-1/2), PI3K, calcium mobilization (phospholipase C/IP$_3$), the arachidonic acid cascade, and nitric oxide production (reviewed in [83]). A few studies have aimed to compare the signaling events of CB_1 and CB_2 in a given cell system and found some divergences between the two

receptors. This section recapitulates the evidence regarding the signaling events downstream of the CB_2 receptor.

$G_{i/o}$ protein coupling and adenylyl cyclase inhibition

Like the CB_1, the CB_2 receptor couples with $G_{i/o}$ proteins. This was established by Slipetz et al., who found that in CB_2-transfected Chinese Hamster Ovary (CHO) cells, pretreatment with pertussis toxin (PTX) abolished the effect of cannabinoids on forskolin-induced cAMP production [84]. Other groups using CB_2-transfected cell models found signaling events to be PTX-sensitive, supporting the involvement of $G_{i/o}$ proteins [85,86]. This interaction was later confirmed in murine microglial cells [87], the murine macrophage cell line J774-1 [88], the human promyelocytic cell line HL-60 [89-91] and human bronchial epithelial cells [92]. Since it has proven to couple to $G_{i/o}$ proteins, the impact of CB_2 activation on adenylyl cyclase activity was also investigated. As expected, adenylyl cyclase was inhibited upon treatment of cells with CB_2 receptor agonists and/or synthetic cannabinoids, resulting in a decrease in intracellular cAMP levels [84,85,93,94].

Potassium channels

As opposed to the CB_1 receptor, the CB_2 receptor does not appear to couple to potassium channels. A study by Felder et al [9] investigated the possible modulation of inwardly rectifying potassium current (K_{ir}) channels in CB_2-transfected AtT-20 cells. In these cells, activation of the CB_2 receptor with WIN 55,212-2 failed to have an impact on K_{ir}. Another study showed that in *Xenopus laevis* oocytes co-expressing the CB_2 receptor and G protein-gated inwardly rectifying potassium (GIRK) channels, WIN 55,212-2 failed to induce consistent coupling of the CB_2 receptor to GIRK channels [95]. Of note, the CB_1 receptor was able to couple with GIRK channels and to modulate agonist-induced currents in the same cellular model. This important difference between CB_1 and CB_2 receptors established CB_2 as a functionally distinct receptor.

Mitogen-activated protein kinases (MAPK)

Signal transduction pathways induced by CB_2 receptor activation were first investigated in CB_2-CHO cells by Bouaboula et al. [86]. They found that upon CP 55,940 addition, adenylyl cyclase inhibition was followed by ERK-1/2 phosphorylation. This effect was significantly diminished by the protein kinase C (PKC) inhibitor GF 109203X, suggesting that PKC was involved in MAPK activation. Moreover, they were able to confirm their findings in HL-60 cells, which express the CB_2 receptor. Another group investigated MAPK activation by various CB_2 ligands in HL-60 cells and found that CP 55,940, 2-AG and AEA increased ERK-1/2 phosphorylation [89]. This effect was blocked by the CB_2 receptor antagonist SR144528 and was stronger in cells stimulated by 2-AG and CP 55,940 than in those treated with AEA. MAPK activation downstream of CB_2 activation was also demonstrated *in vitro* in murine osteoblasts [96], in DAUDI leukemia cells [94], murine microglia [97] and human primary monocytes [78]. Finally, this pathway was showed to be activated *in vivo*, in

a mouse model of acute experimental pancreatitis. In this model, a CB_2 receptor agonist reduced inflammation through the p38-MK2 pathway [98].

Intracellular calcium concentrations and phospholipase C activity

A study conducted in calf pulmonary endothelial cells showed that CB_2 activation modulates intracellular calcium concentrations [99]. In this model, AEA initiated phospholipase C (PLC) activation and inositol 1,4,5-triphosphate (IP_3) production, which led to intracellular Ca^{2+} release from the endoplasmic reticulum, as well as an increase in mitochondrial Ca^{2+}. This effect of AEA was not mimicked by arachidonic acid (AA), was blocked by SR144528 and was unchanged by treatment with SR141716A, confirming the involvement of the CB_2 but not the CB_1 receptor. Another group later confirmed this in HEK-293 cells co-expressing the CB_2 receptor with chimeric G_i and G_o proteins [100]. In this model, treatment with CP 55,940 or other CB receptor agonists was found to increase intracellular Ca^{2+} levels. The phospholipase C inhibitor U73122 abrogated the effect of CP 55,940 on calcium mobilization, as did thapsigargin. This evidence shows that in these cells, CB_2 receptor activation induces calcium mobilization via the PLC-IP_3 signaling pathway.

In vitro studies of CB_2 receptor functions

CB_2 activation by endocannabinoids in vitro

The endocannabinoids 2-AG and AEA both act on various immune cell types through CB_2 receptor activation (summarized in table 3.4). Interestingly, there is a sharp contrast between the anti-inflammatory effects that are triggered by the two lipids. 2-AG was most often found to modulate functions related to leukocyte recruitment such as chemokine release, adhesion to fibronectin and migration. This positive regulation of immune cell recruitment by 2-AG is the main pro-inflammatory effect of endocannabinoids or cannabinoids *in vitro* that has been reported. AEA, on the other hand, was found to downregulate leukocyte functions such as pro-inflammatory cytokine release and nitric oxide production. A few reports also show increased production of the anti-inflammatory cytokine IL-10 by cells treated with AEA. In all cases, the involvement of the CB_2 receptor was confirmed by the use of a selective antagonist. However, it is still possible that endocannabinoid metabolites are involved in the reported effects. Noteworthy, this hypothesis was tested in human eosinophils, which were shown to migrate in response to 2-AG [101]. In this model, the effect of 2-AG on eosinophil transmigration was blocked by the pre-incubation of cells with a CB_2 receptor antagonist. However, a CB_2-selective agonist failed to mimic the impact of 2-AG, and its 15-LO-derived metabolites were suggested to be necessary for eosinophils to migrate. Therefore, the successful blockade of endocannabinoid-induced effects with a CB_2 antagonist does not always rule out the possibility that other mediators, notably endocannabinoid metabolites, are involved as well [102]. This concept could explain why endocannabinoids can induce both pro- and anti-inflammatory effects.

CB$_2$ activation by exogenous agonists in vitro

In contrast to endocannabinoids, CB$_2$ receptor agonists have only been shown to exert anti-inflammatory effects on leukocytes, which are detailed in table 3.5. Some of the studies were performed using a nonselective cannabinoid, but the involvement of the CB$_2$ receptor was always confirmed with an antagonist. In addition to downregulating leukocyte functions such as cytokine release, reactive oxygen species production and migration, CB$_2$ agonists limited HIV-1 expression and replication in human macrophages and microglia [75,125].

In vivo studies of CB$_2$ receptor functions

Impact of CB$_2$ knockout in inflammation models

Transgenic mice have greatly contributed to our understanding of this receptor's role in human disease, including inflammatory conditions. In this regard, several models have shown that mice that are lacking the CB$_2$ receptor have exacerbated inflammation (summarized in table 3.6). The effects that were usually observed in *CB$_2^{-/-}$* animals included increased leukocyte recruitment (often neutrophils) and pro-inflammatory cytokine production, which often caused tissue damage. Conversely, one study found CB$_2$-deficient mice to be in better condition than the wild-type group [142]. However, the model was cecal ligation-induced sepsis, a condition in which efficient bacterial clearance by the immune system is vital. The authors' observations that the *CB$_2^{-/-}$* group had less mortality and less bacterial invasion were explained by the lower levels of IL-10 in these mice, which might have led to a better phagocytic response. Overall, these findings are consistent with the other reports of increased immune cell functions in the absence of the CB$_2$ receptor.

CB$_2$ activation by exogenous agonists in vivo

The potential of activating CB$_2$ *in vivo* to treat inflammation has been investigated in numerous studies. Two main strategies are employed: 1) the administration of a CB$_2$ receptor agonist; 2) the administration of an endocannabinoid hydrolysis inhibitor to augment endocannabinoid signaling.

The administration of CB$_2$ receptor agonists has been performed in several inflammation models. Table 3.7 summarizes the data that were generated with this approach. In many instances, the chosen agonist was not CB$_2$-selective and targeted both cannabinoid receptors, in which case the involvement of CB$_2$ was confirmed by showing that the treatment of animals with a CB$_2$ antagonist abrogated the effects of the cannabinoid receptor agonist. Altogether, the results of those studies point to the conclusion that CB$_2$ activation improves inflammation in mice. The recruitment of leukocytes to tissues and the production of pro-inflammatory cytokines and reactive oxygen species were downregulated in various inflammation models. In the case of atherosclerosis, two studies showed not only a decrease in inflammatory cells and mediators upon

cannabinoid treatment, but also a slower progression of the disease [148,149]. Indeed, oral Δ^9-THC administration, at doses that are suboptimal for inducing psychotropic effects, resulted in reduced atherosclerotic lesion development. Since these effects of Δ^9-THC were shown to be mediated by the CB_2 receptor, this supports that a selective CB_2 receptor agonist might be a valuable tool for the treatment of atherosclerosis.

CB_2 activation by endocannabinoids in vivo

The most widely used approach to investigate the impact of endocannabinoids *in vivo* is the blockade of their hydrolysis, as it is an efficient way to increase their levels in tissues. Despite the numerous studies that have used this method in animal models, it is still unclear whether the effects of endocannabinoids are pro- or anti-inflammatory. This is due, in part, to the presence of numerous enzymes that can metabolize them into other bioactive lipids. The main pathway is hydrolysis into AA by lipases such as MAG lipase for 2-AG [164] and FAAH for AEA [165]. AA is a precursor for the biosynthesis of leukotrienes, prostaglandins, and other lipid mediators of inflammation. Alternatively, endocannabinoids can undergo oxidation and the biological effects of the metabolites that originate from these pathways are not very well characterized [166]. Therefore, it is not possible to conclude that endocannabinoids exert their effects through CB_2 in an inflammation model unless this is confirmed by the genetic or pharmacological blockade of the receptor. In this respect, table 3.8 only presents studies that have thoroughly confirmed the involvement of the CB_2 receptor in the effects they observed.

A limited number of studies reported pro-inflammatory effects of endocannabinoids *in vivo*, and only three of those (listed in table 3.9) were confirmed to involve the CB_2 receptor. In two models of dermatitis in mice, treatment with the CB_2 antagonist SR144528 improved inflammation by inhibiting granulocyte recruitment and pro-inflammatory mediator production [174,175]. In both cases, this translated in a measurable decrease in swelling. As presented above in table 3.6, 2-AG has been implicated in the recruitment and migration of B and T cells, dendritic cells, eosinophils, monocytes and natural killer cells in a CB_2-dependent manner, which could very well translate to *in vivo* studies. However, to this day, there is no published data demonstrating that exogenous cannabinoids and selective CB_2 receptor agonists have pro-inflammatory effects. Therefore, it is possible that the pro-inflammatory effects of endocannabinoids that are presented in table 3.9 are a result of CB_2 activation and the action of one or more endocannabinoid metabolites [102].

Of note, many disorders cause a change in CB_2 receptor protein levels, due to pre-existing pro-inflammatory conditions. In multiple sclerosis and amyotrophic lateral sclerosis, for instance, the expression of CB_2 in microglia is increased, both in human tissues and mouse models [176,177]. A similar effect was reported in a rodent model of neuropathic pain [178]. This certainly facilitates the impact of CB_2 receptor activation by exogenous agonists of endocannabinoids in these inflammation models.

The CB$_2$ receptor as a potential therapeutic target

While there is a large body of evidence supporting that CB$_2$ receptor activation has anti-inflammatory effects, it has yet to be targeted to treat human disease. In sections 2.5 and 2.6, we presented *in vitro* and *in vivo* studies that suggested a role for the CB$_2$ receptor in numerous inflammatory conditions. In this section, we discuss the potential of the CB$_2$ receptor as a target in the treatment of chronic inflammatory diseases such as rheumatoid arthritis, atherosclerosis and inflammatory bowel disease.

Potential in rheumatoid arthritis

Rheumatoid arthritis (RA) is an inflammatory disease that affects approximately 1% of the adult population worldwide. RA is characterized by chronic inflammation of the synovium, cartilage destruction and bone loss. Patients with RA exhibit an influx of innate (neutrophils, macrophages) and adaptive (lymphocytes) immune cells in the synovial cavity. These cells promote inflammation and connective tissue damage by producing cytokines (TNF-α, IL-6, IL-1β, pro-inflammatory lipids and metalloproteinases (MMPs). The synovial lining becomes hyperplastic and an invasive structure (the pannus) is formed. Osteoclasts become exaggeratedly activated and cause bone resorption [180].

2-AG and AEA are present in the synovial fluid of patients with RA but not healthy volunteers, suggesting an involvement of the endocannabinoid system in the disease. CB$_1$ and CB$_2$ mRNA and proteins were also found in the synovial tissues of RA patients [181]. CB$_2$ activation can inhibit the production of pro-inflammatory cytokines and MMP release from fibroblast-like synoviocytes (FLSs) [182,183]. It can also promote osteoblast differentiation *in vitro* [33,184] and inhibit FLS proliferation [182]. These observations indicate that CB$_2$ receptor activation in RA joints could improve multiple aspects of the disease, including inflammation, FLS hyperplasia and bone loss.

In vivo, CB$_2$ agonists have proven to be beneficial in a murine model of rheumatoid arthritis, collagen-induced arthritis (CIA). One study showed treatment with the CB$_2$ receptor agonist JWH 133 to improve arthritis severity and to reduce bone destruction and leukocyte infiltration in the joints [183]. Another group investigated the impact of a different CB$_2$-selective agonist, HU-308. They found that the agonist decreased swelling, synovial inflammation and joint destruction, in addition to lowering circulating antibodies against collagen II [185]. Finally, the agonist HU-320 ameliorated established CIA [186]. Of note, CB$_2$ agonists did not prevent the onset of RA in any of those reports, as there were no differences in disease incidence between groups.

This growing body of evidence establishes the CB$_2$ receptor as a promising target for the treatment of RA. In all three of the abovementioned studies, the CIA model was used to test CB$_2$ agonists. Given that there is no animal model of RA that perfectly duplicates all aspects the human condition, these findings should be confirmed in different models.

Potential in atherosclerosis

Atherosclerosis is an inflammatory disease that is characterized by the presence of arterial plaques. These lesions contain immune cells, lipid-laden macrophages (foam cells), cholesterol, smooth muscle cells and collagen fibres [187]. The physical rupture of the plaques causes the occlusion of arteries, which can lead to tissue infarction. Plaque development is influenced by inflammatory mediators such as cytokines and chemokines, which are crucial to the recruitment of immune cells to the intima. In this respect, therapies that would downregulate the production of these mediators could reduce the progression of atherosclerotic lesion development. Since the CB_2 receptor is known to decrease the production of numerous chemokines and to inhibit leukocyte migration *in vitro* and *in vivo*, it emerged as a potential target to treat atherosclerosis.

A recent study specifically aimed to characterize the endocannabinoid system in human foam cells [188]. The authors found that the CB_2 agonist JHW-015 significantly decreased oxLDL accumulation in these macrophages. Moreover, it reduced the production of TNF-α, IL-6 and IL-10 and the expression of CD36, a scavenger receptor that is responsible for the uptake of modified lipoproteins by macrophages and the induction of foam cell formation. The endocannabinoids 2-AG and AEA mimicked these effects, which were block by the CB_2 antagonist SR144528. These findings are in accordance with a previous study that showed that CB_2 activation by WIN 55,212-2 reduces the oxLDL-induced inflammatory response in rat macrophages [131].

As briefly discussed in section 2.6, the role of the CB_2 receptor was investigated in mouse models of atherosclerosis. The first study to demonstrate the benefits of CB_2 activation in atherosclerosis was performed in *ApoE*$^{-/-}$ mice using low doses of the cannabinoid Δ^9-THC, which diminished inflammation and blocked the progression of the disease [149]. These effects were prevented by SR144528, confirming the involvement of the CB_2 receptor. The anti-atherosclerotic effects of CB_2 in the *ApoE*$^{-/-}$ model was later confirmed with WIN 55,212-2 as an agonist, and the antagonist AM630 confirmed the mechanism to be CB_2-dependent [189,148]. In *Ldlr*$^{-/-}$*CB_2*$^{-/-}$ double knockout mice, lesional macrophage and smooth muscle cell contents was higher than in *Ldlr*$^{-/-}$*CB_2*$^{+/+}$ animals [190]. In *Ldlr*$^{-/-}$ mice deficient for CB_2 in hematopoietic cells only, plaque area after 12 weeks on an atherogenic diet was larger than in mice with no CB_2 deficiency [191].

In summary, a large body of evidence strongly suggests that CB_2 receptor activation is an appropriate target for atherosclerosis treatment. CB_2 agonists have the potential to be beneficial on many levels as they were shown to improve inflammatory cell recruitment and activation, lipid uptake by macrophages and the size of atherosclerotic plaques. However, a few reports show conflicting data, especially in the *Ldlr*$^{-/-}$ model. A report shows unaltered lesion size following WIN 55,212-2 treatment in this model, although CB_2 receptor activation did decrease lesional macrophage accumulation [192]. Another group treated *Ldlr*$^{-/-}$ mice with JWH-133 and found no significant effect on lesion size or on their content in macrophages, lipids, smooth muscle cells,

collagen and T cells [193]. More investigation is required to determine the causes of these discrepancies before moving forward in the development of therapies targeting CB_2 for atherosclerosis.

Potential in inflammatory bowel disease

Inflammatory bowel disease (IBD) includes two main conditions: ulcerative colitis and Crohn's disease. They are caused by an excessive immune response and can affect any part of the gastrointestinal tract [194]. The endocannabinoid system first gained interest in IBD pathophysiology in light of a study that described a protective effect of CB_1 in DNBS-induced colitis [195]. Cannabinoids were then shown to enhance epithelial wound healing in a CB_1-dependent fashion [76]. The authors of the latter study also evaluated the expression of cannabinoid receptors in human IBD tissue by immunochemistry. They found that the CB_1 receptor was expressed in the normal human colon, but that CB_2 expression was higher in IBD tissues and that its presence was concentrated in plasma cells and macrophages. These findings raised the hypothesis that the CB_2 receptor was also involved in the inflammatory component of IBD.

A subsequent study reported that a FAAH inhibitor decreased inflammation in the TNBS-induced colitis model, and that the deletion of either CB_1 or CB_2 abrogated this effect [196]. In the same colitis model, the use of the MAG lipase inhibitor JZL184 to increase 2-AG levels also inhibited the development of colitis [173]. Mice treated with JZL184 had less colon alteration and lower expression of pro-inflammatory cytokines, and these effects were abolished by the antagonists AM251 (CB_1) and AM630 (CB_2).

Several groups tested the impact of a CB_2 receptor agonist in IBD models. The CB_2-selective agonists JWH-133 and AM1241 both protected against TNBS-induced colitis, whereas AM630 worsened it [197]. The non-psychotropic cannabinoid cannabigerol (CBG) was tested in DNBS-induced colitis and was found to reduce the colon weight/colon length ratio (an indirect marker of inflammation), MPO activity and iNOS expression by a CB_2-dependent mechanism [198]. Finally, the plant metabolite and unconventional CB_2 agonist (E)-β caryophyllene (BCP) was also evaluated in a model of DSS-induced colitis. Oral administration of BCP decreased micro- and macroscopic colon damage, MPO activity, NF-κB activation and pro-inflammatory cytokine production [199].

This wide array of CB_2 receptor agonists being able to improve IBD in animal models prompted the development of highly selective compounds that could be used to treat the disease in humans. In this regard, a research group synthesized a series of CB_2-selective agonists and tested the resulting lead compounds in models of experimental colitis [200,201]. Intra-peritoneal injection of the agonists was effective at protecting mice against colitis. Of note, a selective compound that is orally effective in experimental colitis was later synthesized [202].

Conclusion

In light of the evidence that was generated over the past two decades by the scientific community, we can draw a few general conclusions regarding the role of the CB_2 receptor. First, it is mainly found in immune tissues and is expressed in most immune cell types. Second, its deletion in animals causes an exacerbated inflammatory phenotype in several models, due to an upregulation of immune cell functions. Third, CB_2 activation by cannabinoids, either *in vitro* or *in vivo*, usually decreases inflammatory cell activation. Finally, the administration of CB_2 agonists in animal models of inflammatory disease can slow the progression of some diseases, in addition to reducing inflammation.

Several questions still need to be investigated. For example, there is no consensus regarding the expression of the CB_2 receptor in non-immune brain cells, and the role that CB_2 might play in brain functions is unknown. **Moreover, the impact of endocannabinoids on immune cells is still unclear. While most animal studies show that the blockade of endocannabinoid hydrolysis results in less inflammation, it is not possible to tell whether these effects are caused only by CB_2 activation and whether the opposite would occur in humans**. In this respect, endocannabinoids can induce human leukocyte migration (section 2.5.1). However, the impact of endocannabinoid metabolites on leukocyte functions is not well defined, and this should be addressed before endocannabinoid hydrolysis inhibitors can be considered as a valid strategy to enhance CB_2 receptor signaling [102]. Finally, the few CB_2 agonists that are currently being developed aim at treating inflammatory pain [203-205]. Perhaps these novel compounds are worthy of sparking new studies in order to define their putative beneficial role in inflammatory diseases.

Abbreviations

2-AG = 2-arachidonoyl-glycerol; **AA =** Arachidonic acid; **AEA =** *N*-arachidonoyl-ethanolamide; **AM1241 =** (2-iodo-5-nitrophenyl)-(1-(1-methylpiperidin-2-ylmethyl)-1H-indol-3-yl)methanone; **AM630 =** 6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone; **CB65 =** *N*-Cyclohexyl-7-chloro-1-[2-(4-morpholinyl)ethyl]quinolin-4(1*H*)-one-3-carboxamide; **cAMP =** Cyclic adenosine monophosphate; **CBD =** Cannabidiol; **CBG =** Cannabigerol; **CBN =** Cannabinol; **COX =** Cyclooxygenase; **CP 55,940 =** (-)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; **Δ⁹-THC =** (-)-Δ^9-tetrahydrocannabinol; **ERK-1/2 =** Extracellular signal-regulated kinases-1/2; **FAAH =** Fatty acid amide hydrolase; **GFP =** Green fluorescent protein; **GIRK =** G protein-coupled inwardly-rectifying potassium (channel); **GP 1a =** *N*-(Piperidin-1-yl)-1-(2,4-dichlorophenyl)-1,4-dihydro-6-methylindeno[1,2-*c*]pyrazole-3-carboxamide; **GP 2a =** *N*-Cyclohexyl-1-(2,4-dichlorophenyl)-1,4-dihydro-6-methylindeno[1,2-*c*]pyrazole-3-carboxamide; **GPCR =** G protein-coupled receptor; **HU-210 =** 3-(1,1'-dimethylheptyl)-6aR,7,10,10aR-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-

methanol; **HU-308 =** 4-[4-(1,1-Dimethylheptyl)-2,6-dimethoxyphenyl]-6,6-dimethylbicyclo[3.1.1]hept-2-ene-2-methanol; **IP$_3$ =** Inositol 1,4,5-trisphosphate; **JTE 907 =** *N*-(1,3-Benzodioxol-5-ylmethyl)-1,2-dihydro-7-methoxy-2-oxo-8-(pentyloxy)-3-quinolinecarboxamide; **JWH 015 =** (2-Methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone; **JWH 133 =** (6a*R*,10a*R*)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b*,*d*]pyran; **L-759,633 =** (6a*R*,10a*R*)-3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1-methoxy-6,6,9-trimethyl-6*H*-dibenzo[*b*,*d*]pyran; **L-759,656 =** (6a*R*,10a*R*)-3-(1,1-Dimethylheptyl)-6a,7,8,9,10,10a-hexahydro-1-methoxy-6,6-dimethyl-9-methylene-6*H*-dibenzo[*b*,*d*]pyran; **LOX =** Lipoxygenase; **MAG =** Monoacylglycerol; **MAPK =** Mitogen-activated protein kinases; **NADA =** *N*-arachidonoyl-dopamine; **PI3K =** Phosphoinositide 3-kinase; **PKC =** Protein kinase C; **PLC =** Phospholipase C; **PTX =** Pertussis toxin; **SER 601 =** *N*-(Adamant-1-yl)-6-isopropyl-4-oxo-1-pentyl-1,4-dihydroquinoline-3-carboxamide; **WIN 55,212-2 =** [(3R)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone, monomethanesulfonate; **SR141716A =** *N*-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride; **SR144528 =** 5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-N-[(1S,2S,4R)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1H-pyrazole-3-carboxamide.

Table 3.1. Binding of endocannabinoids and phytocannabinoids to the human CB_2 receptor.

Endocannabinoid	K_i (nM)	Model	Ref
AEA	371	CHO cells	[8]
	1940	AtT-20 cells	[9]
	795	Sf9 cells	[10]
	3500	CHO cells	[10]
2-AG	949	Sf9 cells	[10]
	650	CHO cells	[10]
Noladin-ether	857[a]	COS-7 cells	[9]
Oleamide	>100 000	HEK-293 cells	[11]
NADA	12000[b]	Rat spleen	[12]
Phytocannabinoid	**K_i (nM)**	**Model**	**Ref**
Δ^9-THC	34.6	CHO cells	[8]
Δ^8-THC	39.3	Mouse spleen	[13]
CBN	96.3	CHO cells	[8]
	301	AtT-20 cells	[9]
CBD	2680	CHO cells	[8]

K_i values were obtained in function of [^{3}H]CP 55,940 displacement unless indicated otherwise: [a] [^{3}H]HU-243 and [b] [^{3}H]WIN55212-2. **NADA =** *N*-arachidonoyl-dopamine; **CBN =** Cannabinol; **CBD =** Cannabidiol.

Table 3.2. CB$_2$ agonists and antagonists.

Agonist	K$_i$ (nM)	Other targets	Ref
AM 1241	3.4	TRPA1	[14, 15]
JWH 133	3.4	TRPV1	[16, 17]
GW 405833	3.6-3.92	-	[18]
JWH 015	13.8	-	[8]
HU 308	22.7	-	[19]
L-759,633	6.4	-	[20]
L-759,656	11.8	-	[20]
SER 601	6.3	-	[21]
GP 1a	0.037	-	[22]
GP 2a	7.6	-	[22]
CB 65	3.3	-	[23]
HU 210	0.061-0.52	CB$_1$, GPR55, 5-HT$_2$	[9, 24, 25]
CP 55,940	0.6 - 5.0	CB$_1$, GPR55	[24, 26]
WIN 55, 212-2	62.3	CB$_1$, TRPA1	[9, 15, 26]
Antagonist	**K$_i$ (nM)**	**Other targets**	**Ref**
SR144528	0.6-4.1	-	[20, 27]
AM 630	5.6-31.2	TRPA1	[20, 28]
JTE907	35.9	-	[29]

(-) This compound is not known to activate other receptors besides CB$_2$. **TRP =** transient receptor potential ion channel.

Table 3.3. CB$_2$ receptor expression in human leukocytes

Cell type	Data	CB$_2$ expression	References
B cells	mRNA	+	[34, 37, 64, 65]
	Protein	+	[46, 64, 66],
Basophils	mRNA	+	[67]
Dendritic cells	mRNA	+	[53]
	Protein	+	[53]
Eosinophils	mRNA	+	[67-70]
	Protein	+	[70]
Mast cells	mRNA	+	[67]
Macrophages	mRNA	+	[71]
	Protein	+	[45, 71, 72]
Microglia	mRNA	+	[57]
	Protein	+	[57]
Monocytes	mRNA	+	[34, 37, 64, 71, 73, 74]
	Protein	+	[64, 71, 74]
NK cells	mRNA	+	[34, 37, 65]
	Protein	+	[46]
Neutrophils	mRNA	+	[34, 37, 67]
		-	[68-70]
	Protein	+	[75]
		-	[70]
Platelets	mRNA	+	[67]
	Protein	+	[76]
		-	[77]
T cells	mRNA	+	[34, 37, 64, 65]
	Protein	+	[46, 64, 78]

Table 3.4. CB$_2$-mediated effects of endocannabinoids on immune cell functions

ANTI-INFLAMMATORY EFFECTS				
Cell type	**Species**	**Endocannabinoid**	**Effects**	**Reference**
Astrocytes	Rat	AEA	↓TNF-α	[99]
Dendritic cells	Human	AEA	↓ IL-6, IL-12 and IFN-α	[100]
Microglia	Mouse (BV-2 cell line)	AEA	↓ Nitric oxide	[101]
	Mouse	AEA	↑ IL-10	[102]
			↑ IL-10 ↓ IL-12p70 and IL-2?	[103]
	Rat	AEA	LPS-induced nitric oxide release	[104]
Neutrophils	Human	2-AG	↓ fMLP-induced migration	[75]
Splenocytes	Human	AEA	↓ Primary and secondary antibody formation	[105]
T cells (not separated)	Human	AEA	↓ Cell proliferation	[106]
		2-AG	↓ SDF-1-induced migration	[107]
CD4+ T cells	Human	AEA	↓ IL-17, IFN-γ and TNF-α	[106]
CD8+ T cells	Human	AEA	↓ IFN-γ and TNF-α	[106]
	Human	AEA	↓ SDF-1-induced migration	[108]
PRO-INFLAMMATORY EFFECTS				
Cell type	**Species**	**Endocannabinoid**	**Effects**	**Reference**
B cells	Human	2-AG	↑ Migration	[109]
	Mouse	2-AG	↑ Migration	[110, 111]
Dendritic cells	Human	2-AG	↑ Migration	[112]
Eosinophils	Human	2-AG	↑ Migration	[70, 113]
	Human	2-AG	↑ Migration ↑ LTC$_4$ and EXC$_4$ synthesis	[97]
Macrophages	Mouse (peritoneal)	2-AG	↑ Zymosan phagocytosis	[114]
	Human (HL-60)	2-AG	↑ Actin polymerization ↑ Adhesion to fibronectin	[115]
			↑ MCP-1 and IL-8	[116]
Microglia	Mouse (BV-2 cell line)	2-AG	↑ Migration	[117]
Monocytes	Human	2-AG	↑ Adhesion to fibronectin	[118]

			↑ Migration	[115]
NK cells	Human	2-AG	↑ Migration	[119]
T cells	Human (Jurkat)	2-AG	↑ L- and P-selectin ↑ Adhesion and transmigration	[120]

TNF = tumor necrosis factor; **IL =** interleukin; **IFN =** interferon; **LPS =** lipopolysaccharide; **fMLP =** formyl-Met-Leu-Phe; **SDF =** stromal cell-derived factor; **LTC₄ =** leukotriene C_4; **EXC₄ =** eoxin C_4; **MCP =** monocyte chemoattractant protein

Table 3.5. Effects of CB$_2$ agonists on immune cell functions

Cell type	Species	Agonist	Effects	Reference
Astrocytes	Human	WIN 55,212-2	↓ Nitric oxide ↓ TNF-α, IL-10, MCP-1 and CCL5	[122]
Dendritic cells	Mouse	Δ^9-THC	↑ NF-κB-dependent apoptosis	[123]
		GP1a	↓ MMP-9 ↓ Migration	[124]
Monocytes	Human	JWH-015	↓ CCL2 and CCL3-induced migration	[74]
		HU-308 JWH-133	↓ TNF-α-induced transendothelial migration	[125]
Macrophages	Human (monocyte-derived)	JWH-133	↓ Expression of 35 genes upregulated by LPS	[126]
		JWH-133 GP1a O-1966	↓ HIV-1 replication	[71]
	Mouse (RAW264.7)	WIN 55,212-2	↓ Reactive oxygen species	[127]
			↓ Nitric oxide	[128]
	Mouse (peritoneal)	Δ^9-THC	↓ RANTES-induced migration	[129]
		JWH-133	↑ IL-10 ↓ IL-12p40	[130]
	Mouse (clone 63)	Δ^9-THC	↓ Activation of CD4+ T cells	[56]
Mast cells	Rat (RBL-2H3)	WIN 55,212-2 CP 55,940	↓ β-hexosaminidase release	[131]
Microglia	Human	WIN 55,212-2	↓ HIV-1 expression	[121]
	Rat	JWH-015	↓ LPS-induced TNF-α production ↓ Migration	[132]
Neutrophils	Mouse	JWH-133	↓ MIP-2α-induced migration	[133]
	Human	JWH-133	↓ TNF-α-induced MMP-9 release	[134]
Splenocytes	Human	Δ^9-THC	↓ Primary and secondary antibody formation	[105]
T cells	Human	Δ^9-THC	↓ Th2 cytokine production	[135]
	Human (Jurkat)	CP 55,940 WIN 55,212-2	↓ SDF-1-induced migration	[136]

		JWH-015		
	Mouse	O-1966	↓ NF-κB activation ↑ SOCS5 expression ↑ IL-10	[137]
CD8+ T cells	Human	JWH-015	↓ SDF-1-induced migration	[136]

CCL = chemokine (C-C motif) ligand ; **NF-κB =** nuclear factor kappa-light-chain-enhancer of activated B cells; **MMP =** matrix metallopeptidase ; **MIP =** macrophage inflammatory protein ; **HIV =** human immunodeficiency virus ; **SOCS =** suppressor of cytokine signaling.

Table 3.6. Anti-inflammatory effects of CB$_2$ receptor deletion in inflammation models

Model	Species	Genotype	Effects	Reference
DNFB-induced hypersensitivity	Mouse	$CB_2^{-/-}$	↑ Neutrophil recruitment ↑ Ear swelling	[139]
Hepatic ischemia-reperfusion injury	Mouse	$CB_2^{-/-}$	↑ Neutrophil recruitment ↑ Inflammatory cytokines ↑ Liver damage	[140]
TNBS-induced colitis	Mouse	$CB_2^{-/-}$	↑ Colitis ↑ TNF-α and IL-1β	[141]
Myocardial ischemia-reperfusion injury	Mouse	$CB_2^{-/-}$	↑ Neutrophil and macrophage infiltration ↓ IL-10	[142]
Traumatic brain injury	Mouse	$CB_2^{-/-}$	↑ TNF-α, iNOS and ICAM mRNA ↑ Blood-brain barrier permeability	[143]
Cecal ligation-induced sepsis	Mouse	$CB_2^{-/-}$	↓ IL-10 ↓ Bacterial invasion ↓ Mortality	[138]

DNFB = 2,4-dinitrofluorobenzene; **TNBS =** Trinitrobenzenesulfonic acid; **iNOS =** inducible nitric oxide synthase; **ICAM =** intercellular adhesion molecule.

Table 3.7. Anti-inflammatory effects of CB$_2$ agonists in animal models of inflammation

Model	Species	Treatment	Effects	Reference
Atherosclerosis	Mouse	Δ^9-THC	↓Atherosclerotic lesions ↓ Macrophage infiltration ↓ Leukocyte adhesion	[145]
		WIN 55,212-2	↓Atherosclerotic lesions ↓ Macrophage infiltration ↓ MCP-1, IL-6 and TNF-α	[144]
Breast cancer cell injection	Mouse	Δ^9-THC	↓ Splenocyte proliferation	[146]
Brain ischemia	Mouse	JWH-133	↓ Microglia and macrophage infiltration ↓ IL-6, MCP-1, MIP-1α, CCL-5 and TNF-α ↓ iNOS	[147]
Experimental autoimmune encephalomyelitis	Mouse	Δ^9-THC JWH-133	↓ Monocyte recruitment ↓ IFN-γ and IL-2 ↓ T cell proliferation	[148]
Hepatic ischemia-reperfusion injury	Mouse	Δ^8-THCV	↓ Hepatic injury ↓ CCL3, CXCL2 and TNF-α ↓ Neutrophil infiltration	[149]
Germinal matrix hemorrhage-induced neuroinflammation	Rat	JWH-133	↓ TNF-α ↓ Microglia accumulation	[150]
L. pneumophila infection	Mouse	Δ^9-THC	↓ IFN-γ and IL-12	[151]
Influenza virus infection	Mouse	Δ^9-THC	↓ Lymphocyte and monocyte recruitment ↓ Viral hemagglutinin	[152]
Myocardial ischemia-reperfusion injury	Mouse	WIN 55,212-2	↓Myeloperoxidase ↓ IL-1β and IL-8	[153]
Ovalbumin-induced asthma	Guinea pig	CP 55,940	↓Myeloperoxidase ↓ Mast cell	[154]

			degranulation ↓ TNF-α and PGD$_2$	
LPS-induced interstitial cystitis	Mouse	JWH-015	↓ Leukocyte infiltration ↓Myeloperoxidase ↓ TNF-α, IL-1α and IL-1β	[155]
Sepsis	Mouse	HU308	↓ Adherent leukocytes in submucosal venules	[156]
Spinal cord injury	Mouse	O-1966	↓ Leukocyte infiltration ↓ CXCL9 and CXCL11 ↓ IL-23p19 and IL-23R ↓ TLR expression	[157]
Stress-induced neuroinflammation	Mouse	JWH-133	↓ TNF-α and MCP-1 ↓ COX-2, iNOS and NF-κB	[158]
Traumatic brain injury	Mouse	O-1966	↓ Microglia and macrophage infiltration ↓ Blood-brain barrier disruption	[159]

PGD$_2$ = prostaglandin D$_2$; **COX-2 =** cyclooxygenase-2.

Table 3.8. Anti-inflammatory effects of CB$_2$ activation by endocannabinoids in mouse models of inflammation

Model	Treatment	Effects	Reference
ConA-induced hepatitis	AEA	↓ Inflammatory cytokines	[140]
Carrageenan-induced acute inflammation	URB602	↓ Edema ↓ Nociception	[163]
Experimental autoimmune encephalomyelitis	WWL70	↓ iNOS, COX-2, TNF-α and IL-1β ↓ T cell infiltration ↓ Microglial activation ↓ NF-κB activation	[164]
LPS-induced acute lung injury	JZL184	↓ Leukocyte infiltration ↓ BALF cytokines and chemokines	[165]
LPS-induced inflammatory pain	FAAH KO	↓ Edema ↓ TNF-α and IL-1β	[166]
	FAAH KO, PF-3845, URB597 or OL-135	↓ Allodynia	[167]
Kaolin and carrageenan-induced osteoarthritis	URB597	↓ Leukocyte rolling ↓ Microvascular perfusion	[168]
TNBS-induced colitis	JZL184	↓ Submucosa edema ↓ Leukocyte infiltration ↓ Mucosal IL-6 and IL-1β ↓ Circulating inflammatory markers	[169]

ConA = Concanavalin A; **BALF =** bronchoalveolar lavage fluid.

Table 3.9. Pro-inflammatory effects of CB$_2$ signaling in mouse models of inflammation

Model	Treatment	Effects	Reference
Primary immunization	2-AG	↑ Delayed-type hypersensitivity ↑ DC migration to draining lymph nodes	[112]
TPA-induced ear inflammation	SR144528	↓ Neutrophil recruitment ↓ Swelling ↓ LTB$_4$ synthesis	[172]
Oxazolone-induced dermatitis	SR144528	↓ Eosinophil recruitment ↓ Swelling ↓ MCP-1, MIP-1 and TNF-α	[171]

TPA = 12-O-tetradecanoylphorbol-13-acetate.

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Chapitre 3. Hypothèses et objectifs

Après une trentaine d'années de recherche sur le système endocannabinoïde, le rôle de celui-ci ne cesse de se définir et son importance dans la santé humaine est devenue indéniable. Pourtant, beaucoup de travail demeure essentiel afin de bien comprendre comment moduler le système endocannabinoïde pour maximiser ses bénéfices dans l'inflammation.

Les deux points suivants résument bien les principaux concepts associés aux propriétés anti-inflammatoires des endocannabinoïdes :

1) Contrairement au récepteur CB_1, le récepteur CB_2 est surtout exprimé en périphérie, plus particulièrement chez les leucocytes. Chez la souris, la délétion génétique ou l'inhibition pharmacologique de ce récepteur engendrent un phénotype d'inflammation exacerbée, lui suggérant un rôle anti-inflammatoire.

2) Les endocannabinoïdes AEA et 2-AG sont considérés comme étant les ligands naturels de ce récepteur, et une augmentation de leurs niveaux mène à une diminution de l'inflammation et de la douleur dans de nombreux modèles animaux d'inflammation.

Puisque que ces deux concepts font l'objet d'un consensus dans la littérature scientifique, plusieurs stratégies sont possibles afin d'augmenter la signalisation aux récepteurs cannabinoïdes et ainsi promouvoir les effets anti-inflammatoires des endocannabinoïdes : administrer des cannabinoïdes (synthétiques ou naturels); administrer des agonistes sélectifs pour le récepteur CB_2 ou bloquer l'hydrolyse des endocannabinoïdes afin d'augmenter leurs niveaux. Ces trois options ont été étudiées dans divers modèles animaux de maladies inflammatoires, qui ont confirmé que ce sont des approches prometteuses. **À mon avis, l'administration d'inhibiteurs d'hydrolyse du 2-AG est l'approche la plus intéressante.** Cette méthode permettrait non seulement d'augmenter les niveaux de 2-AG présents pour activer le récepteur CB_2, mais aussi de diminuer la production d'acide arachidonique et de ses métabolites pro-inflammatoires (leucotriènes, prostaglandines). Par contre, plusieurs questions doivent être adressées avant que cette stratégie ne soit envisageable pour traiter l'inflammation chez l'humain :

1) Existe-t-il des différences fondamentales dans la façon dont les cellules humaines et murines dégradent les endocannabinoïdes ? À cet égard, est-ce que nous disposons d'outils pharmacologiques capables d'inhiber ces voies métaboliques chez les leucocytes humains ?

2) Les leucocytes humains sont-ils des sources significatives d'endocannabinoïdes et si oui, comment les produisent-ils ?

3) Dans un contexte inflammatoire, l'inhibition de l'hydrolyse des endocannabinoïdes risque de favoriser leur métabolisme par d'autres voies, telles que la synthèse de PG-G ou de 15-HETE-G. Quel est l'impact de ces médiateurs sur les fonctions des leucocytes humains ? Les leucocytes sont-ils capables d'hydrolyser ces médiateurs ?

4) Nous avons établi que l'impact du 2-AG sur les fonctions des neutrophiles dépend entièrement de son métabolisme en effecteurs pro-inflammatoires comme le LTB_4. En est-il de même pour les autres leucocytes humains ?

Bien que ces questions soient d'envergure beaucoup trop grande pour un seul projet de doctorat, je me suis fixé des objectifs permettant d'y répondre partiellement en approfondissant considérablement les connaissances à propos du métabolisme et des effets biologiques du 2-AG chez les leucocytes humains. J'ai effectué la majorité des expériences sur des leucocytes humains afin de m'assurer que mes résultats seraient directement transposables à la santé humaine. Les principales cellules que j'ai utilisées comme modèle sont les neutrophiles et les éosinophiles, des cellules critiques dans le développement et le maintien de l'inflammation. J'ai également confirmé certains concepts dans les monocytes et les lymphocytes circulants, ainsi que dans les macrophages alvéolaires humains. La figure 4.1 illustre les mécanismes auxquels je me suis intéressée dans le cadre de mon doctorat.
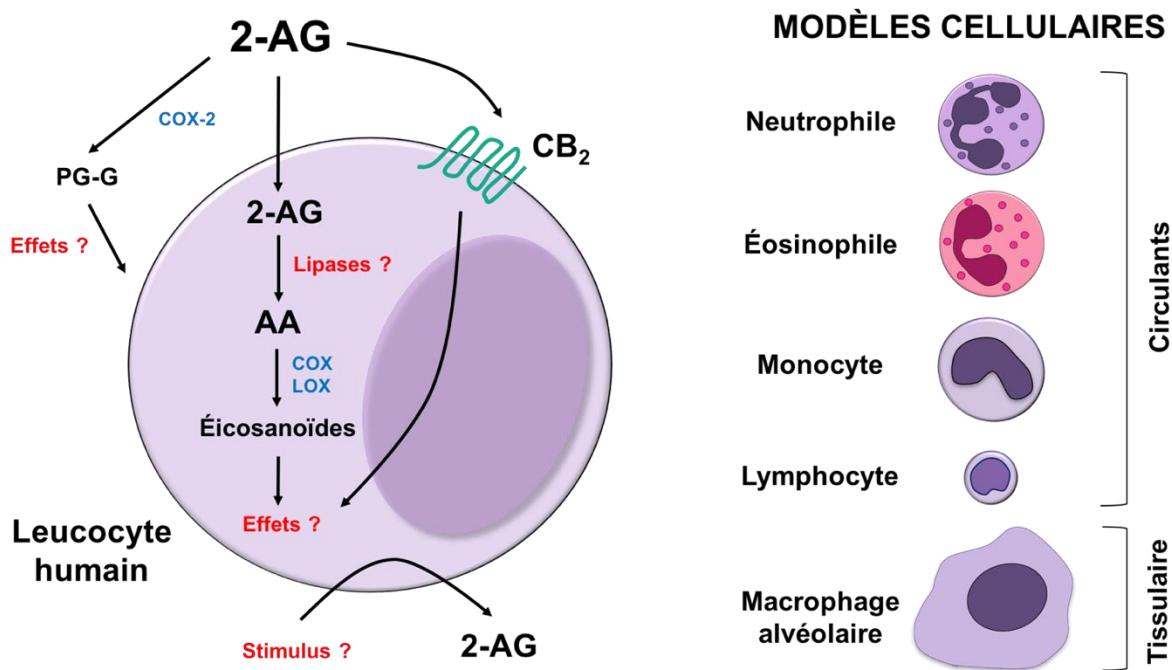

Figure 4.1 : Les mécanismes étudiés dans la présente thèse, ainsi que les modèles cellulaires utilisés.

Les problématiques et les objectifs spécifiques à chacune des études présentées dans cette thèse se détaillent comme suit.

<u>**Objectif du chapitre 4**</u>

Caractériser la dégradation des endocannabinoïdes, de la PGE$_2$-G et du 15-HETE-G par les leucocytes humains.

La première 2-AG hydrolase découverte fut la monoacylglycérol lipase (MAGL). Dans les années suivantes, des inhibiteurs pharmacologiques de cette enzyme ont été développés, dont le JZL184, qui est couramment utilisé en recherche fondamentale. Par conséquent, les recherches visant à étudier l'impact de l'hydrolyse du 2-AG font souvent usage du JZL184, puisque celui-ci bloque efficacement cette hydrolyse chez la souris. Depuis, la liste de lipases possédant une activité 2-AG hydrolase ne cesse de s'allonger, sans que leur patron d'expression dans les tissus humains ou leur sensibilité aux inhibiteurs de MAGL ne soient bien définis. Pourtant, l'importance de ces autres enzymes a été soulignée par des études montrant qu'elles pouvaient être responsables de la majorité de l'hydrolyse du 2-AG dans des types cellulaires n'exprimant pas la MAGL.

Les objectifs du chapitre 4 étaient simples. Nous voulions d'abord définir les patrons d'expression des enzymes impliquées dans le métabolisme du 2-AG chez les leucocytes humains. Puis, nous avons comparé divers inhibiteurs de lipases afin de déterminer quels composés bloquent efficacement l'hydrolyse du 2-AG, de la PGE$_2$-G et du 15-HETE-G dans chaque type leucocytaire. Finalement, nous avons utilisé une méthode de marquage en fluorescence de ces enzymes, afin de visualiser et identifier de potentielles cibles inconnues des inhibiteurs que nous utilisons. Dans l'ensemble, le but de ce chapitre était de bien caractériser la dégradation des endocannabinoïdes par les cellules immunitaires avant d'étudier l'importance biologique de ces voies métaboliques.

<u>**Objectif du chapitre 5**</u>

Déterminer si les leucocytes humains sont des sources significatives d'endocannabinoïdes et si c'est le cas, comprendre comment ils les synthétisent.

La voie classique de biosynthèse du 2-AG implique l'activation d'une phospholipase C et l'hydrolyse du diacylglycérol (DAG) ainsi formé par une DAG lipase. Cette voie a principalement été caractérisée dans le cerveau murin. Par contre, la littérature supporte que chez les leucocytes, les niveaux de 2-AG générés par des agonistes activant cette voie sont généralement sous-optimaux, c'est à dire qu'ils sont trop faibles pour activer le récepteur CB$_2$. L'objectif de cette étude était de déterminer si les leucocytes humains sont des sources d'endocannabinoïdes et si oui, par quel mécanisme ils les synthétisent. Nos expériences nous ont révélé une nouvelle voie engendrant une biosynthèse importante de 2-AG par les leucocytes humains, 1000

fois plus importante que les biosynthèses préalablement documentées. Le reste de l'étude a été consacré à la caractérisation du mécanisme sous-jacent.

Objectif du chapitre 6
Élucider les mécanismes par lesquels le 2-AG induit la migration des éosinophiles, des leucocytes importants dans la physiopathologie de l'asthme.

Notre équipe a démontré précédemment que les neutrophiles humains sont fortement activés en présence de 2-AG, et que cet effet est entièrement attribuable à ses métabolites. D'ailleurs, nos données suggèrent que les neutrophiles humains n'expriment pas le récepteur CB_2, ce qui expliquerait nos résultats. Quant aux éosinophiles, qui jouent un rôle central dans la pathophysiologie de l'asthme, ils expriment abondamment le récepteur CB_2. Ceci suggère que leurs fonctions sont modulées par les endocannabinoïdes, un concept appuyé par des résultats préliminaires que nous avions en main et qui démontraient que le 2-AG induit la migration des éosinophiles humains. Cette étude avait donc pour objectif de comprendre les mécanismes impliqués dans cette migration des éosinophiles induite par le 2-AG. Plus particulièrement, nous avons étudié les rôles respectifs du récepteur CB_2 et du métabolisme du 2-AG en acide arachidonique dans ce processus, ainsi que l'importance de l'IL-5, une cytokine cruciale au développement et à l'activation des éosinophiles.

Objectif du chapitre 7
Déterminer si les fonctions des neutrophiles humains sont régulées par les métabolites issus de l'oxygénation des endocannabinoïdes par la COX-2.

En plus de l'hydrolyse en AA, des voies alternatives de dégradation des endocannabinoïdes existent *in vivo* et peuvent générer des métabolites bioactifs. Dans plusieurs cas, le rôle de ces métabolites ne sont pas bien définis. En effet, le 2-AG et l'AEA peuvent être oxygénés par la COX-2 et ainsi générer des prostaglandines-glycérol (PG-G) et des prostaglandines-ethanolamide (PG-EA). L'importance de la voie des COX dans l'inflammation n'est plus à prouver, puisque ces enzymes sont la cible des anti-inflammatoires non-stéroïdiens. Ces thérapies visent à réduire la production des prostaglandines comme la PGE_2, qui est responsable de certains signes cardinaux de l'inflammation dont la douleur. Ces anti-inflammatoires sont toutefois associés à des effets secondaires importants et ne contribuent pas à résoudre l'inflammation. En plus de causer la douleur et l'œdème observés dans l'inflammation, la PGE_2 peut agir directement sur les fonctions des leucocytes. Les neutrophiles, par exemple, voient leur activation diminuée en présence de PGE_2. En raison de leur ressemblance structurale avec la PGE_2, nous avons posé pour hypothèse que la PGE_2-G et la PGE_2-EA sont également capables de réguler les fonctions des neutrophiles humains. Le but de ce chapitre était de définir l'impact de ces métabolites sur diverses fonctions des neutrophiles comme la biosynthèse des

leucotriènes, l'explosion oxydative, la production de peptides antimicrobiens et la chimiotaxie, et d'étudier les mécanismes cellulaires impliqués.

Chapitre 4.   Human leukocytes differentially express endocannabinoid-glycerol lipases and hydrolyze 2-arachidonoyl-glycerol and its metabolites from the 15-lipoxygenase and cyclooxygenase pathways

Caroline Turcotte, Élizabeth Dumais, Anne-Sophie Archambault, Cyril Martin, Marie-Renée Blanchet, Élyse Bissonnette, Louis-Philippe Boulet, Michel Laviolette, Vincenzo Di Marzo, and Nicolas Flamand.

Résumé

Le 2-arachidonoyl-glycérol (2-AG) est un endocannabinoïde avec des propriétés anti-inflammatoires. L'inhibition de la dégradation du 2-AG, qui vise à augmenter ses effets anti-inflammatoires récepteur-dépendants, s'est montrée efficace dans des modèles murins d'inflammation. Cependant, l'expression des 2-AG hydrolases n'a jamais été étudiée chez les leucocytes humains. Dans cette étude, nous avons déterminé l'expression de sept 2-AG hydrolases chez les divers leucocytes humains circulants ainsi que les macrophages alvéolaires (AMs). Nous avons obtenu les patrons d'expression suivants : MAG lipase (éosinophiles, AMs, monocytes), CES1 (monocytes, AMs), PPT1 (AMs), ABHD6 (principalement les AMs), ABHD12 (tous), ABHD16A (tous), et LYPLA2 (monocytes, lymphocytes, AMs). Puis, nous avons observé que tous les leucocytes hydrolysent le 2-AG et ses métabolites dérivés de la cyclooxygénase-2 (la prostaglandine E_2-glycérol; PGE_2-G et le 15-hydroxy-eicosatetraenoyl-glycérol; 15-HETE-G). Les neutrophiles et les éosinophiles hydrolysent plus efficacement le 2-AG et ses métabolites que les monocytes et les lymphocytes. De plus, le 2-AG était généralement mieux hydrolysé que le 15-HETE-G, et les deux étaient beaucoup mieux hydrolysés que la PGE_2-G. En utilisant les inhibiteurs MAFP, JZL184, palmostatine B, WWL70, WWL113, THL et ML349, nous n'avons pas été en mesure d'identifier une hydrolase spécifique responsable pour l'hydrolyse de chacun de ces lipides par les leucocytes humains. Le JZL184, un inhibiteur sélectif pour la MAG lipase, a bloqué l'hydrolyse des trois lipides par les neutrophiles et l'hydrolyse de la PGE_2-G et du 15-HETE-G par les lymphocytes, deux leucocytes exprimant la MAG lipase de façon très limitée ou ne l'exprimant pas. L'utilisation d'une sonde d'ABPP (*activity-based protein profiling*), qui marque les sérine hydrolases chez les leucocytes, a montré que ces cellules expriment de nombreuses hydrolases sensibles au MAFP, ainsi qu'une hydrolase sensible au JZL184 inconnue, d'une taille d'environ 52 kDa. Dans l'ensemble, nos résultats indiquent que les leucocytes humains sont des experts à hydrolyser le 2-AG et ses métabolites, et qu'ils utilisent plusieurs lipases y compris une lipase non-caractérisée et sensible au JZL184. L'inhibition de l'hydrolyse du 2-AG par ces cellules est susceptible d'augmenter les niveaux de 2-AG in vivo et de maximiser ses effets anti-inflammatoires.

Abstract

2-arachidonoyl-glycerol (2-AG) is an endocannabinoid with anti-inflammatory properties. Blocking 2-AG hydrolysis to enhance CB_2 signaling has proven effective in mouse models of inflammation. However, the expression of 2-AG lipases has never been thoroughly investigated in human leukocytes. Herein, we investigated the expression of seven 2-AG hydrolases by human blood leukocytes and alveolar macrophages (AMs) and found the following protein expression pattern: monoacylglycerol (MAG lipase; eosinophils, AMs, monocytes), carboxylesterase (CES1; monocytes, AMs), palmitoyl-protein thioesterase (PPT1; AMs), α/β hydrolase domain (ABHD6; mainly AMs), ABHD12 (all), ABHD16A (all), and LYPLA2 (lysophospholipase 2; monocytes, lymphocytes, AMs). We next found that all leukocytes could hydrolyze 2-AG and its metabolites derived from cyclooxygenase-2 (prostaglandin E_2-glycerol [PGE_2-G]) and the 15-lipoxygenase (15-hydroxy-eicosatetraenoyl-glycerol [15-HETE-G]). Neutrophils and eosinophils were consistently better at hydrolyzing 2-AG and its metabolites than monocytes and lymphocytes. Moreover, the efficacy of leukocytes to hydrolyze 2-AG and its metabolites was 2-AG ≥ 15-HETE-G >> PGE_2-G for each leukocyte. Using the inhibitors MAFP, JZL184), Palmostatin B, WWL70, WWL113, tetrahydrolipstatin and ML349, we could not pinpoint a specific hydrolase responsible for the hydrolysis of 2-AG, PGE_2-G, and 15-HETE-G by these leukocytes. Furthermore, JZL184, a selective MAG lipase inhibitor, blocked the hydrolysis of 2-AG, PGE_2-G, and 15-HETE-G by neutrophils and the hydrolysis of PGE_2-G and 15-HETE-G by lymphocytes, two cell types with limited/no MAG lipase. Using an activity-based protein profiling (ABPP) probe to label hydrolases in leukocytes, we found that they express many MAFP-sensitive hydrolases and an unknown JZL184-sensitive hydrolase of ~52 kDa. Altogether, our results indicate that human leukocytes are experts at hydrolyzing 2-AG and its metabolites via multiple lipases and probably via a yet-to-be characterized 52 kDa hydrolase. Blocking 2-AG hydrolysis in humans will likely abrogate the ability of human leukocytes to degrade 2-AG and its metabolites and increase their anti-inflammatory effects in vivo.

Introduction

2-Arachidonoyl-glycerol (2-AG) is an endogenous endocannabinoid activating the cannabinoid receptors CB_1 and CB_2 (1). Its involvement in physiological processes such as appetite, adipogenesis and nociception is well established (2-4). Furthermore, the CB_2 receptor is widely expressed by leukocytes (1, 5), implying that 2-AG is also an immunomodulator. As such, numerous studies in CB_2 receptor-deficient mice have reported an enhanced inflammatory phenotype (1, 5), supporting the concept that the CB_2 receptor is mainly anti-inflammatory. However, there is a paucity of evidence supporting the anti-inflammatory potential of the cannabinoid/endocannabinoid system in human (1, 5). Thus, there is an urgent need to decipher the molecular and physiological roles of the cannabinoid system in humans and to confirm whether the data obtained in experimental models are translatable to humans. Two main strategies to increase CB_2 receptor signaling during the inflammatory process could be utilized: the use of selective CB_2 receptor agonists or the use of endocannabinoid hydrolysis inhibitors in order to increase 2-AG levels.

The inhibition of 2-AG hydrolysis is of great interest since it would not only enhance CB_2 receptor signaling, but it would also possibly decrease the production of other pro-inflammatory effectors such as leukotrienes (LT) and prostaglandins (PG). Indeed, 2-AG is very labile and rapidly hydrolyzed into arachidonic acid (AA) by numerous serine hydrolases. This is notably the case in human neutrophils and eosinophils in which the resulting AA is metabolized, in part, by eicosanoid biosynthetic enzymes to form LTB_4 and LTC_4 respectively (6-8). While monoacylglycerol (MAG) lipase is the most recognized lipase involved in 2-AG hydrolysis in the brain of mice (9), other lipases with distinct sensitivities to pharmacological inhibitors can also hydrolyze this endocannabinoid into AA (10-16). The current knowledge about 2-AG lipases is summarized in table I.

Aside from its hydrolysis into AA, 2-AG can be metabolized by eicosanoid biosynthetic enzymes from the cyclooxygenase (COX)-2 and 15-lipoxygenase (15-LO) pathways, leading to eicosanoid-glycerols (G), such as PG-Gs or 15-HETE-G (17-19). In this regard, 15-HETE-G, PGD_2-G and PGE_2-G activate cell surface or nuclear receptors (18, 20-24). Noteworthy, some of these metabolites exert anti-inflammatory effects (1, 25), although additional investigations are required to completely understand their biological effects in health and disease. PG-Gs are hydrolyzed into PGs by human neutrophils (26) and in several murine tissues including the lungs (6-8, 27). In this regard, some 2-AG hydrolases can hydrolyze PG-Gs to some extent in cellulo or in vivo (14, 15, 28, 29). In addition, LYPLA2 can hydrolyze PGE_2-G into PGE_2 while showing no hydrolytic activity towards 2-AG (30), indicating that the complete set of enzymes involved in the hydrolysis of 2-AG and its metabolites from the COX-2 and 15-LO pathway is not totally defined yet.

The importance of each lipase in regulating the levels of 2-AG and its metabolites derived from the COX-2 and 15-LO pathways likely varies among tissues and cell types, due to their different proteomes. Herein, we defined the expression of 2-AG-related lipases in human leukocytes. Using a pharmacological approach, we investigated their possible involvement in the hydrolysis of 2-AG, 15-HETE-G, and PGE$_2$-G. We also used an activity-based protein profiling probe to visualize additional targets for some of the inhibitors we used.

Material and methods

Materials - HBSS, RPMI, Leibovitz's L-15 medium, trypsin and fetal bovine serum were obtained from Wisent Laboratories (St-Bruno, QC, Canada). Ficoll was obtained from Corning (Tewksbury, MA, USA). Dextran was purchased from Fisher Scientific. The CD14 and CD16 magnetic bead-coupled antibody and the MACS magnetic separation system were purchased from Miltenyi Biotec (Auburn, CA, USA). THP-1 and MDA-MB-231 cells were obtained from ATCC (Manassas, VA, USA). Human hypothalamus samples were provided by the Douglas – Bell Canada Brain Bank (Montréal, Québec, Canada). Protease inhibitor cocktail tablets were purchased from Roche (Laval, QC, Canada). Aprotinin, leupeptin and WWL113 were purchased from Sigma-Aldrich (St-Louis, MO, USA). DFP was purchased from BioShop Canada (Burlington, ON, Canada). The HRP-linked anti-mouse IgG and anti-rabbit IgG secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies for α/βhydrolase domain (ABHD) ABHD4, ABHD6, ABHD12 and PPT1 were purchased from Abcam (Toronto, ON, Canada). 1-AG-D$_5$, PGE$_2$-D$_4$, 15-HETE-D$_8$, PGE$_2$-G, 15-HETE-G, 2-AG, MAFP, JZL184, tetrahydrolipstatin (THL), WWL70, as well as the primary antibodies for MAG lipase, were purchased from Cayman Chemical (Ann Arbor, MI, USA). The LYPLA2 primary antibody, the ABHD16A primary antibody, dextran and the FP-TAMRA probe were from Thermo Fisher Scientific (Waltham, MA, USA) and the primary antibody for carboxylesterase (CES)1 was from R&D Systems (Minneapolis, MN, USA). PMSF, Palmostatin B and the ECL detection kit were from EMD Millipore (Billerica, MA, USA). ML349 was a generous gift from Dr Lawrence J. Marnett (Vanderbilt University, USA).

Isolation of human leukocytes - Human eosinophils, neutrophils, lymphocytes and monocytes were isolated from the peripheral blood of healthy volunteers. A sample of 160 ml of blood was collected in tubes containing K$_3$-EDTA as an anticoagulant. Samples were centrifuged at 200 x g for 15 minutes and the supernatant consisting of platelet-rich plasma was discarded. Red blood cells were sedimented using HBSS containing 3% dextran. Leukocytes were centrifuged at 350 x g for 17 minutes on a discontinuous Ficoll-Paque gradient to separate granulocytes from PBMCs. Monocytes and lymphocytes were separated using a CD14 negative selection kit according to the manufacturer's instructions. Neutrophils and eosinophils were separated using a CD16 positive selection kit, according to the manufacturer's instructions. The purities of the resulting cell suspensions were typically >98% for neutrophils and eosinophils, and >95% for lymphocytes and monocytes.

We only utilized cell suspensions in which viability was greater than 99%, as determined by trypan blue exclusion. Cells were either used immediately for 2-AG metabolism assays, or lysed and kept frozen until western blotting experiments were conducted.

Human alveolar macrophages (AMs) were obtained by bronchoalveolar lavage of healthy volunteers as documented before (31). In brief, volunteers underwent local anaesthesia before a total of 300 ml of saline (5 syringes of 60 ml each) was injected in a segmental bronchi of the right middle lobe. The lavages were centrifuged (4ºC, 350 × g, 10 minutes) and cells were washed twice with HBSS. Viability and purity were always greater than 95%, as assessed by trypan blue exclusion and Diff Quick staining, respectively. AMs were either used immediately for 2-AG metabolism assays, or lysed and kept frozen until immunoblotting experiments were conducted.

Ethics approval - All volunteers gave informed consent and protocols were approved by the *Centre de recherche de l'Institut universitaire de cardiologie et de pneumologie de Québec* Ethics Committee.

2-AG, PGE$_2$-G and 15-HETE-G hydrolysis assays - Isolated leukocytes were suspended at a concentration of 2.5 × 10^6 cells/ml in HBSS containing 1.6 mM CaCl$_2$, and preheated at 37°C for 10 minutes. DMSO or serine hydrolase inhibitors (3 μM) were added to cell suspensions 5 minutes prior to the addition of 2-AG, PGE$_2$-G or 15-HETE-G (1 μM) for 5 minutes. Incubations were stopped by adding one volume of ice-cold MeOH containing 2 ng of 1-AG-D$_5$, PGE$_2$-D$_4$ and 15-HETE-D$_8$ as internal standards. For the control condition used to determine the initial amount of 2-AG in cell suspensions (T = 0), 2-AG, PGE$_2$-G or 15-HETE-G were added to a cell suspension already containing MeOH. Samples were kept at -30°C until further processing.

Measurement of endocannabinoids and their metabolites by LC-MS/MS - Samples were thawed and centrifuged to eliminate cellular debris, then supernatants were diluted with water to obtain a final MeOH concentration of 10%. The pH of samples was adjusted at 3 by the addition of acetic acid. Samples were loaded on solid phase extraction cartridges (Strata-X Polymeric Reversed Phase, 60 mg/1ml, Phenomenex), washed with acidified water, and lipids were eluted with 1 ml MeOH. The eluates were dried under a nitrogen stream, and then reconstituted in 25 μl of HPLC solvent A (H2O optima LC/MS + 1 mM ammonium acetate + 0.05% acetic acid) and 25 μl of solvent B (MeCN/H$_2$0, 95/5, v/v containing 1 mM ammonium acetate + 0.05% acetic acid). A 25 μl aliquot was injected onto an RP-HPLC column (Kinetex C8, 150 × 2.1 mm, 2.6 μm, Phenomenex). Samples were eluted at a flow rate of 400 μl/min with a linear gradient of 10% solvent B that increased to 35% in 2 minutes, up to 75% in 10 minutes, from 75% to 95% in 0,1 minute, and held at 98% for 5 minutes before re-equilibration to 10% solvent B for 2 minutes. The HPLC system was directly interfaced into

the electrospray source of a triple quadrupole mass spectrometer (Shimadzu 8050) and mass spectrometric analyses were performed in the positive (+) or the negative (-) ion mode using multiple reaction monitoring for the specific mass transitions of each lipid (2-AG (+), 379.30 → 287.25; 1-AG-D$_5$ (+) 384.50 → 287.20, 15-HETE (-) 319.40 → 301.20; 15-HETE-D$_8$ (-) 327.20 → 226.30; PGE$_2$ (-) 351.20 → 271.15; PGE$_2$-D$_4$ (-) 355.20 → 275.35).

Each compound was diluted to prepare a calibration curve and were spiked with 2 ng of the appropriate deuterated standards. The samples were extracted as described above, and analyzed on the LC-MS/MS system three times. The slope was then calculated using the ratio between the peak areas of the compound and its corresponding deuterated standard. Given that the fatty acid chain of 2-AG rapidly migrates from the *sn*-2 position to *sn*-1 position in aqueous solutions (32), 2-AG levels represent the sum of 1-AG and 2-AG.

Analysis of proteins by immunoblot - Cells were lysed by sonication at 4°C in sucrose buffer containing 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF, 3 mM DFP and 1 tablet protease inhibitor cocktail (for 10 ml of buffer). Laemmli sample buffer (5X; 62.5 mM TRIS-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.01% bromophenol blue) was added to sonicated cell lysates and samples were boiled for 10 minutes. Buffer volumes were adjusted to obtain a final concentration of 2 x 10^6 cells/50 µl of lysate for all cell types with the exception of AMs, which were adjusted to 5 x 10^5 cells/50 µl. Proteins were separated by SDS-PAGE on 12% polyacrylamide gels and transferred onto PVDF membranes. Transfer efficiency and equal protein loading were verified by Ponceau Red staining. Membranes were placed in TBS-Tween buffer (25 mM Tris-HCl [pH 7.6], 0.2 M NaCl, 0.15% Tween 20) containing 5% non-fat dried milk (w/v) for 30 minutes at room temperature, then probed with the primary antibody (4°C, overnight). The membranes were revealed by chemiluminescence using a HRP-coupled secondary antibody and the ECL detection kit.

FP-TAMRA labelling of serine hydrolases – Prewarmed leukocyte suspensions (37°C, 2 × 10^7 cells/ml) in HBSS containing 1.6 mM CaCl$_2$ were incubated with DMSO, MAFP(10 µM) or JZL184 (10 µM) for 10 minutes. FP-TAMRA (3 µM) was then added for 30 minutes, and incubations were stopped using two volumes of ice-cold HBSS before transferring the samples in an ice-water bath. Samples were centrifuged (4°C, 350 × *g*, 5 minutes) and cell pellets were lysed and denatured using the same procedure as for immunoblots. Samples were loaded on a polyacrylamide gel, migrated and FP-TAMRA-stained proteins were visualized using a Typhoon fluorescence imager (GE Healthcare). Enzyme sensitivity for the inhibitors was assessed by comparing the TAMRA fluorescence intensity with that of a labeled control without inhibitors.

Statistical analyses – Statistical analyses (one-way ANOVA with Dunnett's multiple comparisons test) were performed using the GraphPad Prism 7 software. P values < 0.05 were considered significant.

Results

Expression of 2-AG hydrolases in human leukocytes

In the first series of experiments, we assessed the expression of the 2-AG and PGE_2-G hydrolases documented so far (table 1) in human neutrophils, eosinophils, monocytes, AMs, and lymphocytes. Lipase expression was assessed by immunoblot using lysates of freshly isolated leukocytes. While we present the immunoblots from three different donors for each leukocyte population, they were not necessarily obtained from the same individuals. We also included a positive control for each target. Human hypothalamus lysates were utilized for ABHD6 and ABHD12, lysates of the monocytic cell line THP-1 were utilized for ABHD16A, CES1 and PPT1, and lysates from the breast cancer cell line MDA-231 were utilized for LYPLA2. For MAG lipase, we noticed that protein integrity was dramatically affected by freezing (data not shown). For this reason, we used a histidine-tagged recombinant MAG lipase from Cayman Chemicals as a positive control and only probed freshly denatured leukocyte preparations.

Two of the enzymes, ABHD12 and ABHD16A, were found in all cell types tested (figure 5.1). MAG lipase was not ubiquitous among leukocytes: neutrophils and lymphocytes showed very weak expression for this lipase in some donors, and no staining at all in other donors. ABHD6 was also not abundantly expressed in leukocytes, and its expression profile had important variations among donors. AMs are the cell type that express this enzyme most consistently, with two positive samples out of the three samples analyzed. As for LYPLA2, it was detected in all our monocyte, AMs and lymphocyte samples. As for eosinophils and neutrophils, they displayed a weak LYPLA2 signal in some samples and none in others, again underscoring that lipase expression profiles can vary between individuals.

Some of the enzymes displayed more distinct expression pattern. A good example of this is CES1, an enzyme initially described as a 2-AG hydrolase in THP-1 monocytes (14), and that we only detected in human monocytes and AMs. Similarly, PPT1, which was characterized as an endocannabinoid hydrolase in THP-1 cells as well (15), could not be detected in human primary leukocytes with the exception of a weak signal in two out of three AM samples. Granulocytes and lymphocytes were clearly negative for these two enzymes. Overall, each cell type was found to express at least two of the documented hydrolases, suggesting that the hydrolysis of 2-AG by leukocytes might involve more than one lipase and might not be entirely blocked by selective inhibitors.

Pharmacological blockade of 2-AG hydrolysis in human leukocytes.

Knowing that each leukocyte type expresses at least two 2-AG hydrolases, we next performed a series of experiments to confirm that all leukocytes could hydrolyze 2-AG into AA, and to pinpoint which hydrolase(s) is(are) most likely involved in this process. We thus treated leukocyte preparations with the various 2-AG hydrolysis inhibitors (or vehicle) for 5 minutes, added 2-AG (or vehicle) for an additional 5 minutes, and then stopped the reaction to quantitate 2-AG levels by LC-MS/MS. In absence of inhibitors, all leukocyte preparations hydrolyzed 2-AG during the 5-minute incubation time (figure 5.2A). Neutrophils were the most efficient at hydrolyzing 2-AG after 5 minutes (~90%). The hydrolysis of 2-AG was comparable between eosinophils, monocytes and AMs with 25% 2-AG remaining after 5 minutes. Lymphocytes were the least efficient at hydrolyzing 2-AG, with 50% of 2-AG still being detected after 5 minutes.

THL, WWL70, WWL113, and ML349, at 3 µM, did not prevent the hydrolysis of 2-AG in any cell type, indicating that their targets (ABHD6, CES1 and LYPLA2) were likely not involved in the 2-AG hydrolysis we observed. MAFP, the least selective compound, was consistently effective with an increase in 2-AG levels over baseline that was statistically significant in all cell types. JZL184 and Palmostatin B also blocked the hydrolysis of 2-AG, at least partially, in all cell types except for lymphocytes. MAFP was the most effective in neutrophils, eosinophils and lymphocytes while Palmostatin B was more effective in monocytes and AMs. As for JZL184, it was developed as a selective MAG lipase inhibitor (33) and has few documented off target activities at the concentration used in these experiments, although it was reported to potently inhibit CES1 (14, 34). The inability of JZL184 to prevent 2-AG hydrolysis in lymphocytes is thus not surprising given that they do not express MAG lipase nor CES1 (figure 5.1). However, the significant inhibitory effect JZL184 has on human neutrophils was puzzling, given the weak, almost absent, MAG lipase signal we obtained by immunoblot in these cells.

Impact of serine hydrolase inhibitors on PGE$_2$-G and 15-HETE-G hydrolysis by human leukocytes

We previously documented that both PGE$_2$-G and PGD$_2$-G are hydrolyzed into PGE$_2$ and PGD$_2$ by neutrophils, while others showed that JZL184 increased PGE$_2$-G levels in the brain of mice (29). We thus sought to determine if and by which means PGE$_2$-G was also hydrolyzed into PGE$_2$ by leukocytes. PGE$_2$ levels were minimal in all our leukocyte preparations and increased from ~10-200 nM after treating the cells with 1 µM PGE$_2$-G during 5 minutes (figure 5.3A). This represents a 1-20% hydrolysis within 5 minutes. Figure 5.3B-E shows the effect of the inhibitors on the hydrolysis of PGE$_2$-G in each cell type. In neutrophils, eosinophils and monocytes, MAFP, JZL184 and Palmostatin B almost completely inhibited PGE$_2$ formation and thus PGE$_2$-G hydrolysis. This was also the case in lymphocytes, although the effect of JZL184 in these cells was somewhat

less prominent. Unsurprisingly, THL, which also lacks selectivity, significantly reduced PGE_2-G hydrolysis in most leukocytes. WWL113 had a modest but statistically significant effect in neutrophils and monocytes, indicating that CES1, which was only found in monocytes and AMs, may not be its only target in human leukocytes. As for the LYPLA2 inhibitor ML349, it blocked ~35% of PGE_2 production in lymphocytes and also had a partial effect in neutrophils, although this did not reach statistical significance.

The hydrolytic pathway(s) of the PPARα agonist 15-HETE-G is (are) almost unknown. To our knowledge, the only study showing a possible effect of MAFP on15-HETE-G hydrolysis inhibition was published recently by our group when we showed that 2-AG-treated neutrophils and eosinophils synthesized 15-HETE-G from exogenous 2-AG only when MAFP was present (31). However, that study did not explore whether endocannabinoid hydrolysis inhibition with MAFP prevented 2-AG hydrolysis, 15-HETE-G hydrolysis, or both. We investigated that issue in the different leukocyte populations by assessing the impact of each serine hydrolase inhibitor on 15-HETE-G integrity. The hydrolysis of 15-HETE-G by each leukocyte was done by measuring the amounts of 15-HETE-G (1 μM) remaining after a 5 minute incubation with the cells. Although it followed the same trend among leukocytes than for PGE_2-G (eosinophils > monocytes > neutrophils ≈ lymphocytes), 15-HETE-G hydrolysis into 15-HETE was more important than that of PGE_2-G in all leukocyte preparations (figure 5.4A). We next performed similar experiments to evaluate whether the different inhibitors impacted 15-HETE-G hydrolysis as we did for PGE_2-G, i.e. by assessing 15-HETE levels. Once again, MAFP and Palmostatin B almost completely blocked 15-HETE-G hydrolysis in every cell type. JZL184, however, was less effective but had a significant effect in inhibiting the hydrolysis of 15-HETE-G. In all cases, the least selective compounds (MAFP and Palmostatin B) had a greater effect, almost completely blocking the hydrolysis of 15-HETE-G and the subsequent accumulation of 15-HETE. This underscores that 15-HETE-G hydrolysis occurs via numerous enzymes, although the contribution of each lipase appears to vary among cell types. As for THL, WWL70, WWL113 and ML349, they did not have a significant effect on 15-HETE-G hydrolysis in any cell type, in contrast to the hydrolysis of PGE-G (figure 5.3). Of note, PGE_2-G and 15-HETE-G hydrolyses were assessed in parallel, on the same leukocyte preparations, with the same inhibitors solutions.

Visualization of inhibitor targets in leukocytes by activity-based protein profiling

Pharmacological inhibitors are key tools to study metabolic pathways in human cells, but they often hit off-targets, especially when used at higher concentrations. Although the relevant documented secondary targets of each inhibitor are documented in table I, JZL184 inhibited the hydrolysis of 2-AG metabolites despite the lack of the MAG lipase protein in neutrophils and lymphocytes (figures 5.2-5.4). Thus, we could not exclude the possibility that additional enzymes were targeted by the inhibitors utilized in this study. In this respect, we

used the activity-based protein profiling (ABPP) method to visualize the targets of MAFP and JZL184 in human leukocytes. The rationale of this choice of inhibitors was to compare the targets of a nonselective inhibitor (MAFP) with those of a MAG lipase-selective inhibitor (JZL184), and to confirm the possibility that JZL184 has at least one additional target in human leukocytes, especially those that do not abundantly express MAG lipase such as neutrophils and lymphocytes. Figure 5.5A shows the principle of the FP-TAMRA labeling method. FP-TAMRA is, like MAFP, a fluorophosphonate. It covalently binds to serines in the active site of serine hydrolases. When a serine hydrolase inhibitor is added before FP-TAMRA, the latter will no longer label the enzyme, resulting in a decreased FP-TAMRA labeling (and fluorescence). The assay is very sensitive as 2 pg of FP-TAMRA MAG lipase could be detected (data not shown).

To confirm that FP-TAMRA was cell permeable, a series of experiment was undertaken in which we assessed its impact on the 2-AG-and the AA-induced LTB_4 in neutrophils, as we reported previously with MAFP and JZL184 (6). In these experiments, FP-TAMRA inhibited the 2-AG-induced LTB_4 biosynthesis of neutrophils in a concentration-dependent manner, with a complete inhibition at 100 nM. Furthermore, it did not affect the AA-induced LTB_4 biosynthesis (data not shown). Next, serine hydrolases labeling with FP-TAMRA was done in human neutrophils, eosinophils, monocytes, lymphocytes and AMs. Figure 5.5B shows a typical result of a FP-TAMRA labeling in human eosinophils and Figure 5.5C recapitulate the target we found in other leukocytes. MAFP prevented the labeling, by FP-TAMRA, of multiple targets (highlighted in blue). JZL184 also inhibited the labeling of some proteins (highlighted in purple). Of note, none of the labeled lipases was a target of JZL184 without being a target of MAFP. In eosinophils, we only visualized two targets of JZL184. The first band migrated approximately around 30 kDa and was consistent with the height of the band that we usually obtain for MAG lipase (predicted molecular weight of 34 kDa) in immunoblot experiments. Moreover, this band was only present in eosinophils, monocytes and AMs, but not in neutrophils and lymphocytes, which is consistent with the MAG lipase expression profile we established in figure 5.1. The second JZL184 target, which weighs approximately 52 kDa, was observed in all cell types. This does not match the molecular weight of any of the enzymes we studied in this manuscript, strongly pointing to a JZL184-sensitive protein that has yet to be characterized.

Discussion

Over the last decades, many proteins and enzymes have been suggested as promising anti-inflammatory targets. Among them is the MAG lipase which, as a main documented function, degrades the potentially anti-inflammatory 2-AG (1, 5). As such, inhibitors targeting this enzyme were developed and tested in animals for their efficacy at downregulating inflammatory processes and for their safety. One of the most notorious compound fulfilling these requirements is JZL184. It effectively inhibits MAG lipase and also diminishes the

synthesis of eicosanoids such as prostaglandins and leukotrienes both in human cells and in vivo models involving mice, when 2-AG is the source of AA (6-8, 27, 35, 36). Of note, JZL184 does not induce the side effects that non-steroidal anti-inflammatory drugs display on gastrointestinal integrity in mice (37).

It has become clear, over the last decade, that human leukocytes can hydrolyze 2-AG and its metabolites from the COX-2 pathway. Indeed, we and others reported that leukocytes, notably neutrophils and eosinophils, readily and rapidly hydrolyze 2-AG and/or its metabolites (6-8, 26). One way to maximize the effects of 2-AG towards CB receptors is to increase its half-life. This concept has therapeutic potential in animal models of inflammation, as using MAG lipase deficient animals or JZL184 results in decreased inflammation (1). Only one MAG lipase inhibitor, ABX-1431, is currently being investigated in humans (38). Thus, translating this concept to humans requires a clear understanding of the 2-AG metabolic pathways involved in this species. In this study, we addressed the lack of knowledge regarding these pathways by characterizing the hydrolysis of 2-AG and its oxidation metabolites in human leukocytes.

We show that **1)** leukocytes express at least two 2-AG hydrolases; **2)** leukocytes hydrolyze 2-AG, PGE_2-G, and 15-HETE-G; **3)** Serine hydrolase inhibitors impair the hydrolysis of 2-AG, PGE_2-G and 15-HETE-G; **4)** JZL184 inhibits the hydrolysis of 2-AG and its metabolites; **5)** JZL184 displays inhibitory effects in leukocytes that do not express the MAG lipase; and **6)** ABPP profiling indicates that a ~52 kDa JZL184-sensitive protein is expressed in all leukocyte types.

Given that MAG lipase is the best known 2-AG hydrolase and that it is a frequently used genetic or pharmacological target (using JZL184) to increase 2-AG levels in animal models, it was crucial to assess its expression and involvement in human leukocytes. Our data show that neutrophils and lymphocytes do not have detectable amounts of the MAG lipase protein, in contrast to eosinophils, monocytes and AMs. By contrast, other lipases were detected in all leukocyte types (figure 5.1). In this regard, ABHD12 and ABHD16A were detected in all our leukocyte preparations, while CES1 was restricted to monocytes and AMs although its role seems limited, in agreement with Szafran and colleagues (35). Interestingly, LYPLA2 was also found in monocytes, AMs, and in lymphocytes. Finally, PPT1 was undetectable in leukocytes, with the exception of AMs.

This expression profile is consistent with our observation that all leukocyte population hydrolyzed 2-AG, PGE_2-G and 15-HETE-G. In these experiments, we used the same amount of substrates (1 μM) and the same cellular concentration (2.5 million cells/ml) in the various leukocyte preparations. 2-AG hydrolysis was important in all cell types, with 50 – 90% 2-AG being hydrolyzed after 5 minutes. 15-HETE-G hydrolysis was

also important with 50 – 100% hydrolysis after a 5-minute treatment, eosinophils being the most efficient. In contrast, PGE_2-G hydrolytic activity in the same time frame was lower, reaching ~20% in human eosinophils.

Interestingly, the various lipase inhibitors used in this study did not impact the hydrolysis of 2-AG, 15-HETE-G and PGE_2-G in the same manner. For instance, the only compound that maximally and consistently prevented the hydrolysis of 2-AG, 15-HETE-G and PGE_2-G was MAFP, Palmostatin B being second. Both are unselective inhibitors. This supports the notion that 2-AG hydrolysis in leukocytes relies on several hydrolases in each cell types. We also cannot exclude that some enzymes are differentially involved in the hydrolysis of 2-AG, 15-HETE-G, and PGE_2-G. Indeed, while THL, WWL70, WWL113 had a limited, yet significant impact on PGE_2-G hydrolysis in some leukocytes, they did not display significant inhibitory activities for the hydrolysis of 2-AG and 15-HETE-G. Of note, we assessed the hydrolysis of PGE_2-G and 15-HETE-G using the same leukocyte preparation, supporting the concept that the differential inhibitory effects we observed were not the consequence of individual variability or inhibitor solutions. It could reflect the involvement of different lipases, a substrate preference by the hydrolases, or even an inhibitor selectivity toward a given substrate, as previously documented with R-flubiprofen, which selectively inhibits the COX-2-mediated synthesis of PGE_2-G while not affecting that of PGE_2 (39). Finally, the inhibitory effects of THL, WWL70, WWL113 on PGE_2-G were limited compared to MAFP, Palmostatin B and JZL184 and might also be the consequence of the limited PGE_2-G hydrolysis (vs. 2-AG and 15-HETE-G hydrolysis) we were confronted to. We previously showed that PGE_2-G inhibited human neutrophils functions via its hydrolysis into PGE_2, an effect that was prevented by MAFP, palmostatin B, JZL184 and WWL113, but not WWL70, THL or ML349 (26). The data presented herein support our initial findings and rase the possibility that WWL113 targets an additional lipase involved in the hydrolysis of PGE2-G in neutrophils.

Over the last century, research allowed us to appreciate that the selectivity of an inhibitor inversely correlates with the amount of time elapsed since its initial characterization. As such, the inhibitory effects of JZL184 on the hydrolysis of 2-AG, PGE_2-G and 15-HETE-G were, at times, unexpected. For instance, neutrophils and lymphocytes barely or do not express MAG lipase. As a consequence, JZL184 and Palmostatin B did not significantly prevent 2-AG hydrolysis in lymphocytes. In contrast, JZL184 and Palmostatin B did inhibit 2-AG hydrolysis in neutrophils, suggesting that other enzyme(s) or the trace amounts of MAG lipase we found in neutrophils were probably inhibited by JZL184 and Palmostatin B. Even more intriguing is the fact that JZL184 significantly inhibited the hydrolysis of both PGE_2-G and 15-HETE-G hydrolysis by human lymphocytes (figures 5.3 and 5.4), even though MAG lipase was not found in these cells, and where accordingly the inhibitors did not significantly prevent, although diminished the hydrolysis of 2-AG. This indicated to us that

JZL184 had other enzymatic targets in leukocytes, notably in neutrophils and lymphocytes and that the inhibitory effect of JZL184 on 2-AG, PGE$_2$-G and 15-HETE-G reflects its action on multiple targets.

In an attempt to solve that issue, ABPP was performed with MAFP and JZL184 on human leukocytes and JZL184 indeed prevented the labeling of a ~30 kDa protein, very likely the MAG lipase, in the same leukocytes that were positive for MAG lipase expression by immunoblot (figures 5.1 and 5.5), but also prevented the labelling of a ~52 kDa protein by FP-TAMRA in all cell types tested (figure 5.5). While successful at labeling our leukocytes with FP-TAMRA and preventing that labelling with MAFP and JZL194, we were unsuccessful at performing IP experiments with an anti-TAMRA on those labelled-cells to identify the higher molecular weight, JZL184-sensitive protein that we consistently found in our leukocyte preparations.

The inefficacy of WWL113 to inhibit the hydrolysis of 2-AG in monocytes and AMs was surprising because WWL113 is an inhibitor of CES1, which is expressed by these two cell types. This likely reflects the limited role that CES1 plays in the hydrolysis of 2-AG hydrolysis in these cells, at least in our model. However, its significant impact on PGE$_2$-G hydrolysis in neutrophils and monocytes raise the possibility that another lipase, somewhat sensitive to WWL113, is expressed by these two cell types.

The data provided in this paper were obtained with freshly isolated primary leukocytes. Working with freshly isolated primary leukocytes remains a challenge with some limitations, notably the use of chemical inhibitors vs. transfection/silencing. Herein, we utilized a pharmacological approach because: 1) in clinic, enzymes are mostly targeted by chemical compounds; 2) human primary leukocytes are rarely transfectable and, when they are, this requires an overnight treatment; 3) we cannot guarantee that incubating human primary leukocytes will not alter the expression of the several lipases we have been investigating nor their post-translational modifications, which could affect their activity; 4) we cannot exclude that co-factors or steric hindrance competitors are present in different amount during long incubation period, which could even further complicate and or mislead our conclusion.

In conclusion, we herein demonstrate that human leukocytes express at least two documented endocannabinoid hydrolases, are experts at hydrolyzing 2-AG and 15-HETE-G, and have a limited, yet significant impact on PGE$_2$-G hydrolysis (see also (26)). Our data emphasizes the lack of selectivity of the inhibitors that are currently available, and unravel a yet-to-be identified target of JZL184 that appears to be involved in 2-AG hydrolysis by leukocytes. Given that these lipases participate in determining the fate of glycerol-containing endocannabinoids in an inflammatory setting, and that these metabolic pathways regulate the levels of several pro- and anti-inflammatory eicosanoids (6, 26, 27, 29), our findings will help to develop

potent inhibitors that will increase the beneficial effects of endocannabinoids-glycerol and their metabolites in inflammatory diseases.

Acknowledgements

Abbreviations

15-HETE, 15-hydroxy-eicosatetraenate; **15-HETE-G,** 15-hydroxy-eicosatetraenoyl-glycerol; **2-AG,** 2-arachidonoyl-glycerol; AA, arachidonic acid; **AMs,** alveolar macrophages; **ABHD,** α/βhydrolase domain; **CES,** carboxylesterase; **G,** glycerol; **LYPLA,** lysophospholipase; **MAFP,** methylarachidonoyl-fluorophosphonate; **MAG**, monoacylglycerol; **PGE$_2$,** prostaglandin; **PGE$_2$-G,** prostaglandin E$_2$-Glycerol; **PPT,** palmitoyl-protein thioesterase.

Figures

A

| Enzyme	Molecular weight
ABHD6	38 kDa
ABHD12	45 kDa
ABHD16A	63 kDa
CES1	63 kDa
LYPLA2	25 kDa
MAG Lipase	34 kDa
PPT1	34 kDa
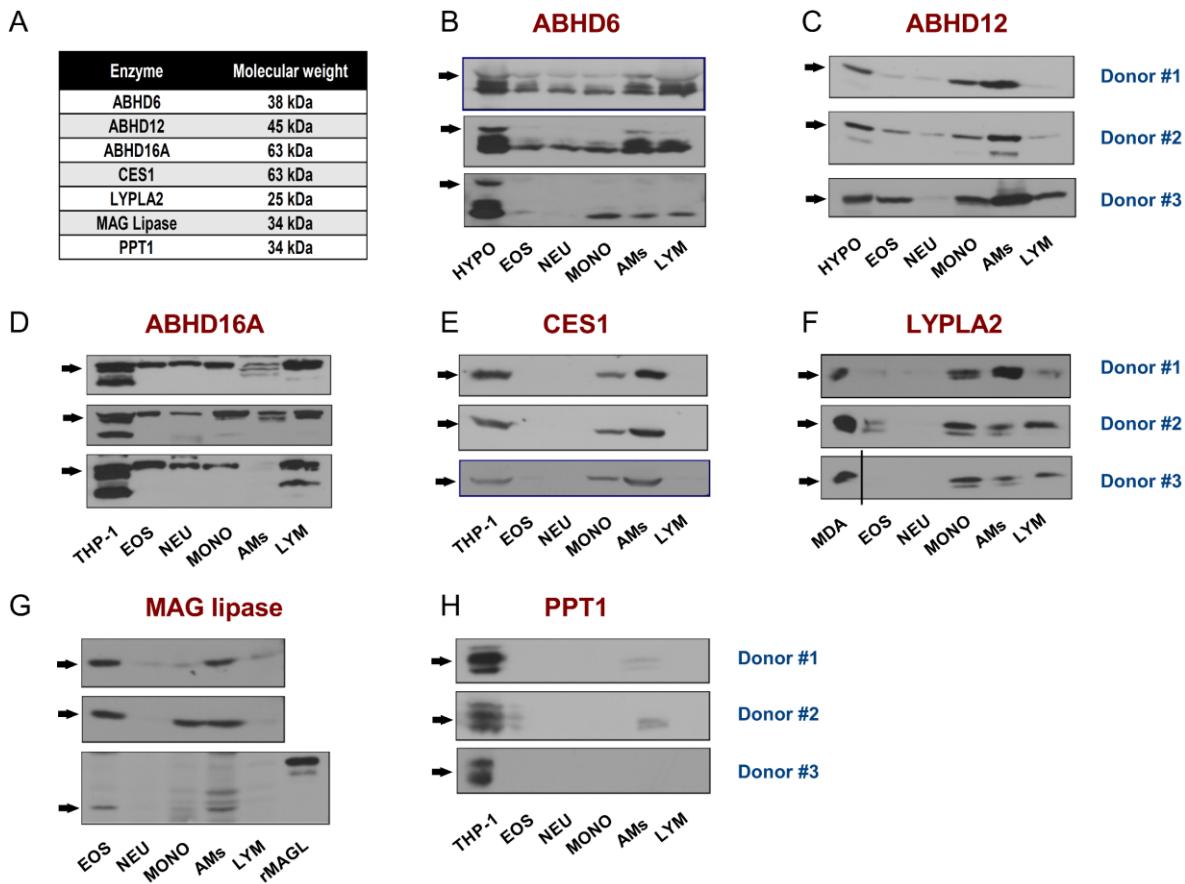
Figure 5.1. Expression of 2-AG hydrolases in human leukocytes. A-G) Protein samples were prepared from freshly isolated human leukocyte preparations, as described in Materials and methods. Immunoblot experiments were conducted as described in Materials and methods by loading the denatured proteins of 2 million cells per well. Each image represent a single experiment and donors are different in the three images. HYPO = hypothalamus; EOS = eosinophils; NEU = neutrophils; MONO = monocytes; LYM = lymphocytes; MDA = MDA-231; rMAGL = His-tagged recombinant MAG lipase.
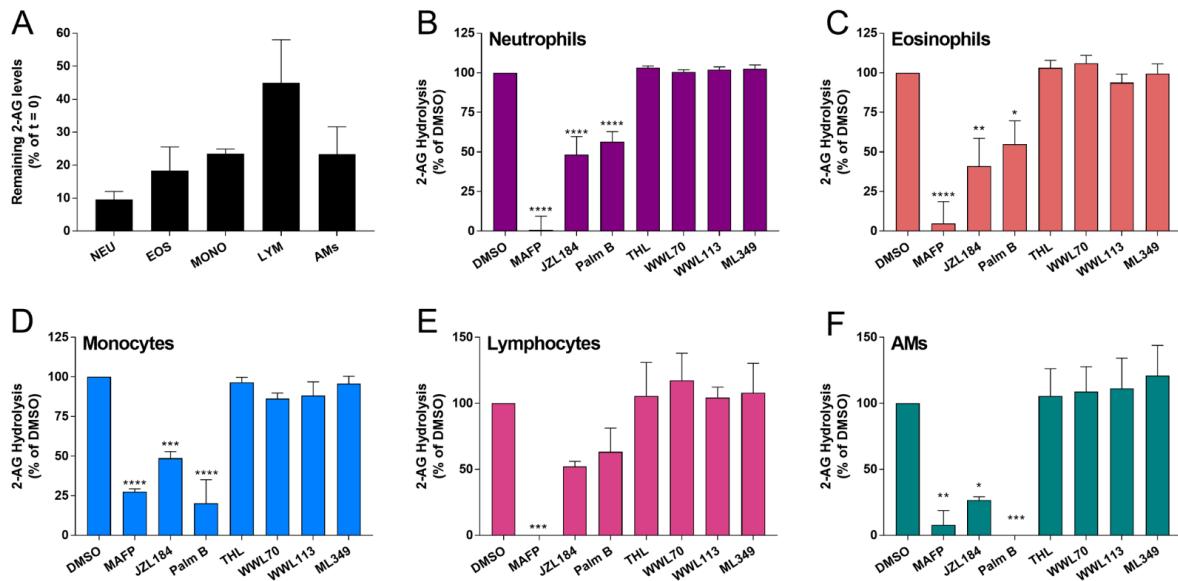
Figure 5.2. Impact of various serine hydrolase inhibitors on 2-AG hydrolysis in human leukocytes. A) Pre-warmed human leukocyte suspensions (37°C, 2.5×10^6 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were treated with 1 µM 2-AG for 5 minutes. **B-F)** Pre-warmed human leukocyte suspensions (37°C, 2.5×10^6 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were treated with DMSO or 3 µM inhibitors for 5 minutes then incubated with 1 µM 2-AG for another 5 minutes. **A-F)** Incubations were stopped by the addition of one volume of cold (-20°C) MeOH containing 5 ng of 1-AG-D_5 as an internal standard. Samples were processed for the analysis of 2-AG by LC-MS/MS as described in *Materials and methods*. Results are the mean (± SD) of 3-5 independent experiments. Statistical analyses (one-way ANOVA with Dunnett's multiple comparisons test) were performed using the GraphPad Prism 7 software. * $p<0.05$; ** $p<0.01$; ***$p<0.001$; ****$p<0.0001$ (vs DMSO, T = 5 min).
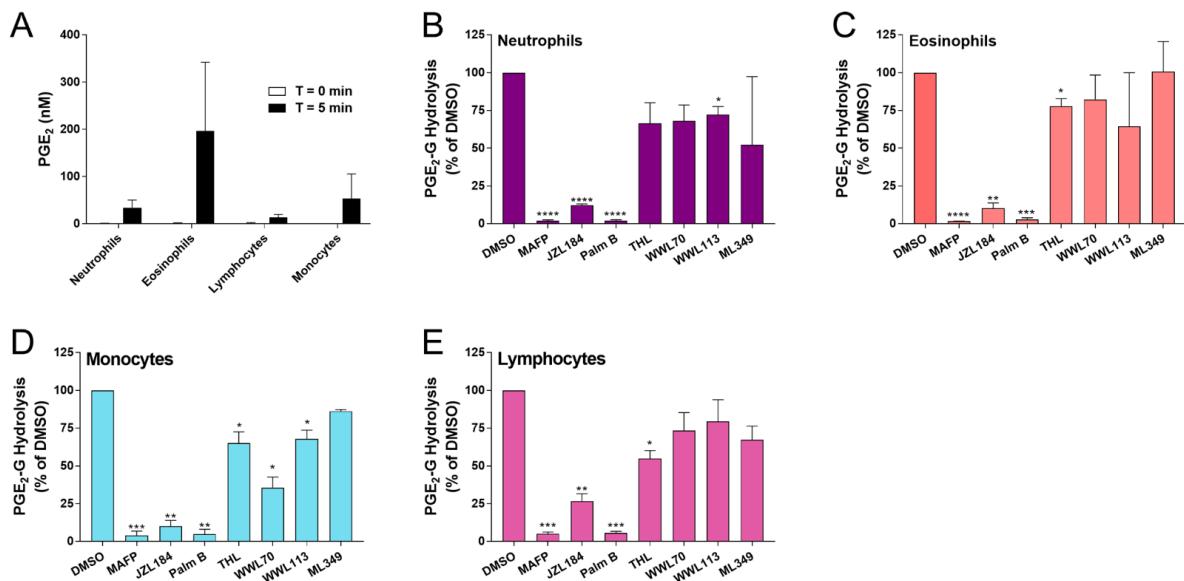
Figure 5.3. Impact of various serine hydrolase inhibitors on PGE$_2$-G hydrolysis in human leukocytes. A) Pre-warmed leukocyte suspensions (37°C, 2.5 × 10^6 cells/ml) in HBSS containing 1.6 mM CaCl$_2$ were treated with DMSO or 1 μM PGE$_2$-G during 5 minutes. **B-E)** Pre-warmed leukocyte suspensions (37°C, 2.5 × 10^6 cells/ml) in HBSS containing 1.6 mM CaCl$_2$ were treated with DMSO or 3 μM inhibitors for 5 minutes then incubated with 1 μM PGE2-G for another 5 minutes. **A-E)** Incubations were stopped by the addition of one volume of cold (-20ºC) MeOH containing 2 ng of PGE$_2$-D$_4$ as an internal standard. Samples were processed and analyzed for PGE$_2$ levels by LC-MS/MS as described in *Materials and methods*. Results are the mean (± SD) of 3-5 independent experiments. Statistical analyses (one-way ANOVA with Dunnett's multiple comparisons test) were performed using the GraphPad Prism 7 software. * $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ (vs DMSO).
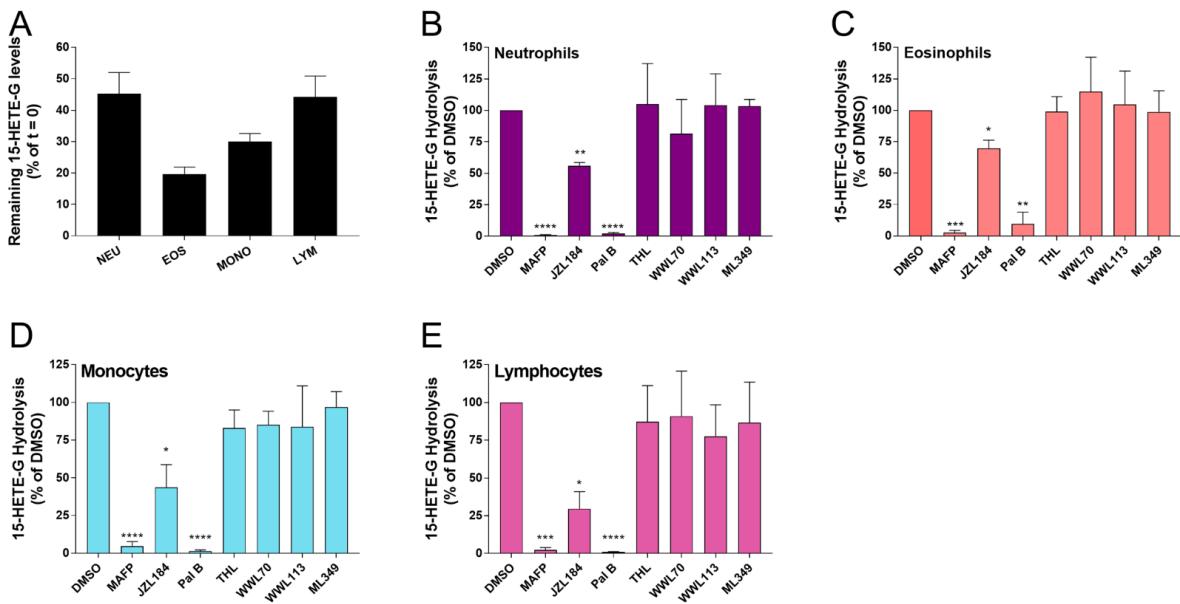
**Figure 5.4. Impact of various serine hydrolase inhibitors on 15-HETE-G hydrolysis in human leukocytes. A)** Pre-warmed leukocyte suspensions (37°C, 2.5×10^6 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were treated with DMSO or 1 µM 15-HETE-G during 5 minutes. **B-E)** Pre-warmed leukocyte suspensions (37°C, 2.5×10^6 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were treated with DMSO or 3 µM inhibitors for 5 minutes then incubated with 1 µM 15-HETE-G for another 5 minutes. **A-E)** Incubations were stopped by the addition of one volume of cold (-20ºC) MeOH containing 2 ng 15-HETE-D_8 as an internal standard. Samples were processed for the analysis of 15-HETE-G (**A**) and 15-HETE (**B-E**) by LC-MS/MS as described in *Materials and methods*. Results are the mean (± SEM) of 3 independent experiments. Statistical analyses (one-way ANOVA with Dunnett's multiple comparisons test) were performed using the GraphPad Prism 7 software. * $p<0.05$; ****$p<0.0001$ (vs DMSO, T = 5 min).
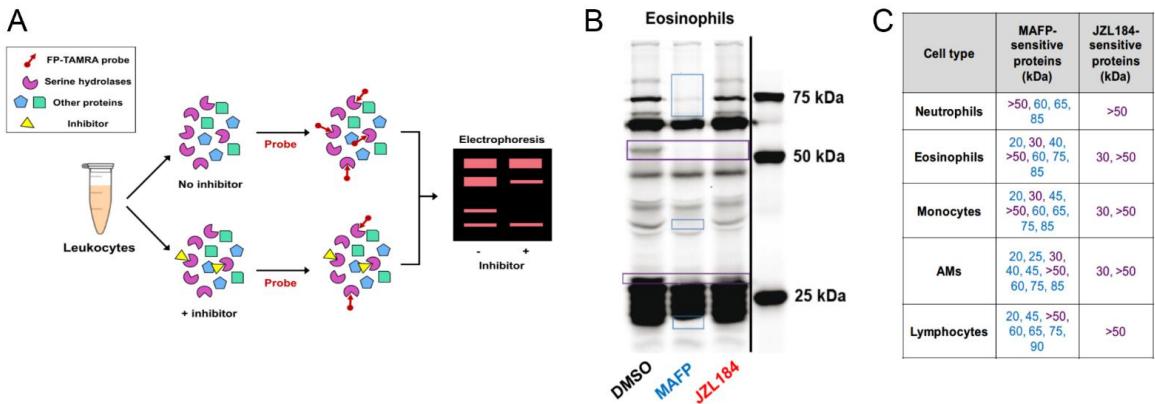
Figure 5.5. Activity-based protein profiling reveals numerous inhibitor targets in human leukocytes. A) Leukocytes were incubated with 10 µM MAFP or JZL184 for 10 minutes. FP-TAMRA was then added to leukocytes for 30 minutes. Incubations were stopped by the addition of one volume of cold incubation buffer. Samples then were processed and analyzed for FP-TAMRA-labeled proteins as described in Material and methods. **B)** Example of the ABPP of human eosinophils, using DMSO, MAFP or JZL184. Data are from one experiment that is representative of three. **C)** Summary of the MAFP- and/or JZL184-sensitive proteins visualized by ABPP in each leukocyte (representative of 3 independent experiments).

Table 5.1. Mains enzymes involved in the hydrolysis of 2-AG, PGE$_2$-G and 15-HETE-G.

Enzyme	Documented to hydrolyze	Documented inhibitors (IC$_{50}$)	REF
ABHD6	2-AG PGE$_2$-G	MAFP (16.9 nM)	(40)
		JZL184 (>10 µM)	(33)
		Palm B (52.5 nM)	(16)
		THL (48 nM)	(40)
		WWL70 (70 nM)	(41)
		WWL113 (ND)	(42)
ABHD12	2-AG PGE$_2$-G	MAFP (87 nM)	(40)
		Palm B (1.8 µM)	(16)
		THL (193 nM)	(40)
ABHD16A	2-AG PGE$_2$-G	Palm B (100 nM)	(16)
		THL (170 nM)	(16)
CES1	2-AG PGE$_2$-G	MAFP (ND)	(14, 15)
		JZL184 (ND)	(14)
		WWL113 (46 nM)	(42)
LYPLA2	PGE$_2$-G	JZL184 (29 µM)	(40)
		Palm B (37.7 nM)	(43)
		ML349 (904 nM)	(40, 44)
MAG lipase	2-AG PGE$_2$-G	MAFP (ND)	(28)
		JZL184 (8 nM)	(33)
		Palm B (93.3 nM)	(16)
PPT1	2-AG PGE$_2$-G	MAFP (ND)	(15)
		Palm B (ND)	(43, 45)

Palm B, Palmostatin B; **ND,** not determined.

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Chapitre 5. Endocannabinoid hydrolysis inhibition unravels that unsaturated fatty acids induce a robust synthesis of glycerol-containing endocannabinoids in human myeloid leukocytes.

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Résumé

Les endocannabinoïdes sont des lipides bioactifs possédant des propriétés analgésiques et anti-inflammatoires. Le 2-arachidonoyl-glycérol (2-AG), un des endocannabinoïdes les mieux caractérisés, peut moduler les fonctions des leucocytes en activant le récepteur cannabinoïde 2 (CB$_2$), ou par le biais de son métabolisme en éicosanoïdes dérivés de l'acide arachidonique (AA). Le 2-AG est typiquement synthétisé à partir des phospholipides membranaires suivant l'activation d'une phospholipase (PL) C et d'une diacylglycérol (DAG) lipase. Cependant, les tentatives d'activer une biosynthèse d'endocannabinoïdes par cette voie chez les leucocytes humains furent sans succès, puisque les concentrations de 2-AG produites étaient largement insuffisantes pour permettre l'activation des récepteurs cannabinoïdes. Dans cette étude, nous décrivons une voie de biosynthèse permettant aux neutrophiles humains de produire des endocannabinoïdes-glycérol suite à une stimulation par des acides gras. Cette voie permet aux neutrophiles de synthétiser des quantités de 2-AG environ 1000 fois supérieures à celles qu'on observe en réponse à des activateurs de PLC. Nous avons observé que ce processus dépend de la réacylation des acides gras dans les phospholipides membranaires, mais le métabolisme subséquent de ces phospholipides en 2-AG est indépendant de la voie des PLC et des DAG lipases. Il serait plutôt dépendant d'un intermédiaire d'acide lysophophatidique (LPA), que nous avons détecté dans nos cellules en réponse à l'AA mais dont la transformation en 2-AG reste à confirmer. La synthèse de 2-AG induite par l'AA chez les neutrophiles est atténuée en présence de médiateurs pro-inflammatoires tels le PAF, le fMLP et le leucotriène B$_4$. De plus, un phénomène similaire a été observé chez les éosinophiles et les monocytes humains, mais est présent de façon très limitée chez les lymphocytes et les macrophages alvéolaires. D'autres acides gras polyinsaturés sont efficacement transformés en leur endocannabinoïde-glycérol par les leucocytes, générant une variété de médiateurs potentiellement anti-inflammatoires. Chacun de ces médiateurs a été détecté dans le plasma de volontaires sains, incluant l'EPA-G, le DHA-G et le DPA-G. À notre connaissance, ces lipides n'ont jamais été détectés dans des tissus humains auparavant. Ces travaux apportent des connaissances cruciales sur la façon dont les leucocytes humains synthétisent des endocannabinoïdes, et soulignent un nouveau rôle des acides gras polyinsaturés dans l'immunité. Nos données guideront sans aucun doute des futures recherches visant à maximiser les effets anti-inflammatoires des endocannabinoïdes pour traiter les maladies inflammatoires.

Abstract

Endocannabinoids (eCB) such as 2-arachidonoyl-gycerol modulate immune responses, either by activating cannabinoid receptors (CB) or through their multiple metabolites, notably eicosanoids. As such, eCB hydrolysis inhibition decreases eicosanoid levels and increases eCB-glycerol levels in humans and mice, and diminishes inflammation in mice. Thus, eCB-glycerol hydrolysis inhibitors might represent a potent anti-inflammatory strategy. eCB synthesis by human leukocytes was ill-defined and the documented syntheses are suboptimal, the measured levels being below the concentrations needed to activate the CB_2 receptor. We thus postulated that eCB hydrolysis inhibition would lead to significant eCB biosynthesis by leukocytes. eCB hydrolysis inhibition dramatically increased 2-AG half-life in neutrophils. Under such settings, neutrophils, eosinophils and monocytes synthesized significant levels of 2-AG and other eCB-glycerols but not eCB-ethanolamides, in response to unsaturated fatty acids. Lymphocytes, alveolar macrophages, platelets and erythrocytes did not generate eCBs in response to unsaturated fatty acids. The obtained eCB-glycerol levels match the documented necessary concentrations to activate the CB2 receptor. The efficacy of unsaturated fatty acids at inducing eCB-glycerol synthesis in leukocytes is ~1000-fold greater than those of GPCR or TLR agonists. Triacsin C, an inhibitor of fatty acyl-CoA synthetases and thimerosal, an inhibitor of acyl-CoA transferases, both inhibited the unsaturated fatty acid-induced eCB-glycerol synthesis in neutrophils, implying that their remodeling is essential in this process. eCB-glycerol biosynthesis was preceded by the synthesis of a LPA intermediate although we could not confirm a causal relationship (LPA dephosphorylation) between the two. While the GPCR agonists PAF, LTB_4 and fMLP did not induce a significant synthesis of 2-AG in neutrophils, they inhibited that induced by AA by 25-50%. This suggests that eCB-glycerol synthesis by leukocytes is decreased when leukocytes are surrounded by a pro-inflammatory entourage, as it is the case in chronic inflammatory diseases. In conclusion, our data support the concept that human leukocytes use unsaturated fatty acids to synthesize biologically significant concentrations of eCB- glycerol and that hijacking the immune system with eCB-glycerol hydrolysis inhibitor might diminish inflammation in humans.

Introduction

2-Arachidonoyl-glycerol (2-AG) is a bioactive lipid and endocannabinoid (eCB) activating the cannabinoid receptors CB_1 and CB_2. It modulates several physiological processes through these receptors, including appetite, pain and adipogenesis (1-3). 2-AG also modulates immune cell functions, usually leading to decreased inflammatory responses (4, 5). Two main strategies are currently investigated to respectively mimic or promote the anti-inflammatory effects of 2-AG: the use of CB_2 receptor agonists or the use of 2-AG hydrolysis inhibitors, which would enhance 2-AG half-life in vivo. While both strategies are attractive, using 2-AG hydrolysis inhibitors in inflammation might have the additional benefit of decreasing eicosanoid levels because leukocytes efficiently hydrolyze 2-AG its metabolites from the cyclooxygenase-2 and 15-lipoxygenase pathways (6-10).

The synthesis of 2-AG by leukocytes is not well documented. Furthermore, efforts to induce 2-AG biosynthesis in leukocytes led to 2-AG levels much lower than those required to activate the CB_2 receptor, which has a Ki of 145 nM (11-14). The classic 2-AG biosynthetic pathway involves two enzymatic steps. First, a phospholipase C (PLC) will cleave a phosphatidylinositol-4,5-bisphospate containing arachidonic acid (AA) in the *sn*-2 position into inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). Next, the obtained DAG will be hydrolyzed by DAG lipase α or β into 2-AG (15-17). While the expression profile of DAG lipase α or β in leukocytes is ill defined, DAG lipase β blockade in murine peritoneal macrophages, led to a significant decrease in 2-AG (18). Two alternative routes for the biosynthesis of 2-AG have been documented. The first one likely involves phospholipase D, as it uses phosphatidic acid as a precursor for DAG synthesis, which is then converted into 2-AG by DAG lipases (19). The second one is the dephosphorylation of lysophosphatidic acid (LPA) into 2-AG (20). The importance of those alternative pathways in 2-AG biosynthesis *in vivo* remains to be elucidated in the periphery.

Herein, we assess whether leukocytes are a significant source of 2-AG, as well as the underlying mechanism involved in its biosynthesis. We report that 2-AG hydrolysis inhibition strikingly prolongs 2-AG half-life, allowing to correctly assess the 2-AG biosynthetic capabilities of leukocytes; that the classic 2-AG biosynthetic pathway (PLC/DAG lipase) does not lead to a significant 2-AG biosynthesis in human leukocytes; that AA stimulates a robust synthesis of 2-AG; and that unsaturated fatty acid stimulate the synthesis of their eCB-glycerol congeners. The unsaturated fatty acid-induced eCB-glycerol pathway was observed in neutrophils, eosinophils and monocytes but not in alveolar macrophages (AMs), lymphocytes, platelets or erythrocytes. This novel biosynthetic pathway is insensitive to DAG lipase inhibitors, sensitive to acyl-CoA synthetase and transferase inhibitors, and coincides with the synthesis of a LPA intermediate.

Materials and methods

Materials – Dextran and mass spectrometry-grade methanol and acetonitrile were purchased from Fisher Scientific. 1-AG-D$_5$, 2-AG, 2-AG-D$_8$, AA, AA-D$_8$, LTB$_4$, PAF, fMLP, A23187, R848, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (n-3) (DPA), linoleic acid (LA), oleic acid (OA), palmitic acid (PA), 2-palmitoyl-glycerol, 2-oleoyl-glycerol, 2-linoleoyl-glycerol, MAFP, triacsin C and JZL184 were obtained from Cayman Chemical (Ann Arbor, MI, USA). Lymphocyte separation medium was obtained from Corning (Corning, NY, USA). Palmostatin B was purchased from EMD Millipore (Billerica, MA, USA). Strata-X columns for solid phase lipid extraction were obtained from Phenomenex (Torrance, CA, USA). The magnetic bead-conjugated anti-CD16 and anti-CD14 mAb and MACS columns were purchased from Miltenyi Biotec (Auburn, CA, USA). Adenosine deaminase (ADA) was purchased from Roche (Laval, QC, Canada). DHA-G, DPA-G and EPA-G were either provided by Dr Samuel Fortin from SCF pharma or purchased from Nu-Chek Prep (Waterville, MN, USA). The primary antibody for DAG lipase α was purchased from Abcam (Toronto, ON, Canada) and the anti-DAG lipase β and anti-mouse HRP-linked secondary antibody were purchased from Cell Signaling Technology (Beverly, MA, USA).). Aprotinin, leupeptin and thimerosal were purchased from Sigma-Aldrich (St-Louis, MO, USA).

Ethics Committee Approval – This work required the use of human cells from healthy volunteers and was approved by our institutional ethics committee. All the experiments were conducted with the understanding and the signed consent of each participant.

Isolation of human leukocytes – For the isolation of neutrophils, eosinophils, lymphocytes and monocytes, human venous blood was obtained from healthy or rhinitic volunteers and collected in tubes containing K$_3$EDTA as anticoagulant. Leukocytes were isolated as described previously (21). In brief, the blood was centrifuged and the plasma was discarded. Erythrocytes were sedimented with 3% dextran, and granulocytes were separated from PBMCs using a discontinuous gradient. The PBMC layer was harvested and monocytes and lymphocytes were separated using a magnetic bead-conjugated anti-CD14, according to the manufacturer's instructions. Residual erythrocytes were eliminated from the granulocyte pellet by hypotonic lysis with sterile water. Eosinophils were separated from neutrophils using anti-CD16-conjugated magnetic beads according to the manufacturer's instructions. The purity and viability of the resulting leukocyte suspensions were always ≥ 98%, as assessed by counting 500 cells by Diff Quik staining and trypan blue exclusion, respectively.

Human AMs were obtained by bronchoalveolar lavage (BAL) of healthy volunteers. In brief, volunteers underwent local anaesthesia before the procedure. A total of 300 ml of saline (5 × 60 ml) was injected in a segmental bronchus of the right middle lobe. The BAL fluid containing leukocytes was recovered and

centrifuged (4°C, 350 x g, 10 minutes). Supernatants were discarded and cells were washed twice with cold HBSS then their viability was assessed by trypan blue exclusion. Viability and purity were always greater than 95%, as assessed by trypan blue exclusion and Diff Quick staining, respectively.

Cell stimulations – Cells were suspended in HBSS containing 1.6 mM $CaCl_2$ and preheated at 37°C for 10 minutes. To better mimic their fate, adenosine deaminase (0.3 U/ml) was added 10 minutes before the addition of the stimuli in all experiments involving neutrophils (8, 22, 23). Inhibitors were added 5 minutes before the stimuli and/or the fatty acids, at the concentrations detailed in the figure legends. In experiments assessing their impact on eCB biosynthesis, PAF, fMLP and LTB_4 were added to the cell suspensions simultaneously with the fatty acids. For the analysis of eCB-glycerol by LC-MS/MS, incubations were stopped by the addition of one volume of cold (-20°C) MeOH containing 0.01% acetic acid and 2 ng of 1-AG-D_5 and as an internal standard. Samples were then kept at -20°C until further processing.

Analysis of DAG lipase expression by immunoblot – For the analysis of DAG lipase protein expression, cells were lysed with NP-40 in a hypotonic lysis buffer containing 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF, 3 mM DFP and 1 tablet protease inhibitor cocktail (for 10 ml of buffer). Laemmli sample buffer (5X; 62.5 mM TRIS-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.01% bromophenol blue) was added to cell lysates and samples were boiled for 10 minutes. Buffer volumes were adjusted to obtain a final concentration of 2×10^6 cells/50 µl of lysate for all cell types except for AMs, which were adjusted to 5×10^5 cells/50 µl. Proteins were separated by SDS-PAGE on 12% polyacrylamide gels and transferred onto PVDF membranes. Transfer efficiency and equal protein loading were confirmed by Ponceau Red staining. Membranes were placed in TBS-Tween buffer (25 mM Tris-HCl [pH 7.6], 0.2 M NaCl, 0.15% Tween 20) containing 5% non-fat dried milk (w/v) for 30 minutes at room temperature, then incubated with the primary antibody (4°C, overnight). The membranes were revealed by chemiluminescence using a HRP-coupled secondary antibody and an ECL detection kit (EMD Millipore; Billerica, MA, USA).

Analysis of DAG lipase expression by qPCR - For gene expression analyses, the mRNA was extracted from freshly isolated leukocytes with TRIzol according to the manufacturer's instructions, followed by a treatment with the RNAse-free DNAse set (Qiagen; Valencia, CA, USA) to eliminate residual genomic DNA. Samples were cleaned with the RNeasy MinElute cleanup kit (Qiagen). cDNA was obtained by reverse transcription using the iScript™ Reverse Transcription Supermix from Bio-Rad, with an RNA input of 1 µg per reaction. Real-time PCR was performed with the SsoAdvanced™ Universal SYBR® Green Supermix on a CFX96 thermal cycler (Bio-Rad; Mississauga, ON, Canada) according to manufacturer's instructions. 18S ribosomal RNA was used as the housekeeping gene and results were reported as ratios over 18S, using the Cq analysis method.

Analysis of eCBs and LPA by liquid chromatography - tandem mass spectrometry (LC-MS/MS) – For the analysis of eCBs, the denatured samples were thawed and centrifuged (1000 × g; 10 minutes) to remove cellular debris, then supernatants were diluted with water to a final MeOH concentration of 10% and maintained at pH 3 by the addition of acetic acid. Samples were loaded on solid phase extraction cartridges (Strata-X Polymeric Reversed Phase, 60 mg/1ml, Phenomenex). Cartridges were washed with 2 ml acidified water and lipids were eluted with 1 ml MeOH. The eluates were evaporated under a stream of nitrogen. For the analysis of LPA, the denatured samples were acidified with acetic acid to obtain a final concentration of 0.1M. Lipids then were extracted from the denatured samples by adding 1 ml chloroform, vortexing for 1 minute and centrifuging at 4000 × g for 5 minutes without brakes. The organic phases were collected, and evaporated under a stream of nitrogen. For the quantification of eCB-glycerols in human plasma, eCB-glycerols were extracted using as documented before (24) with slight modifications. 200 µl plasma samples were mixed with 300 µl TRIS (pH 7.4, 50 mM). Toluene (2 ml) was then added to the samples, which were vortexed for 1 minute and centrifuged at 4000 × g for 5 minutes without brakes. Samples were then placed in an ethanol-dry ice bath (-80°C) to freeze the aqueous phase (bottom). The organic phase (top) was then collected and evaporated under a stream of nitrogen. Samples were reconstituted in 25 µl of HPLC solvent A (H_2O with 0.05% acetic acid and 1 mM NH_4^+) and 25 µl of solvent B (MeCN/H_2O, 95/5, v/v, with 0.05% acetic acid and 1 mM NH_4^+). A 25 µl aliquot was injected onto an RP-HPLC column (Kinetex C8, 150 × 2.1 mm, 2.6 µm, Phenomenex). Quantification was performed on a Shimadzu 8050 triple quadrupole mass spectrometer using the same LC program as described previously (25). In brief, samples were eluted at a flow rate of 400 µl/min with a linear gradient of 35% solvent B that increased to 75% in 12 minutes, from 75% to 95% in 2 minutes, and held at 95% for 5 minutes before re-equilibration to 10% solvent B in 2 minutes.

Quantification was achieved by generating calibration curves using pure standards and analyzed on the LC-MS/MS system three times. The slope was then calculated using the ratio between the peak areas of the compound and its standard (1-AG-D$_5$ for eCB-glycerols, AEA-D$_4$ for AEA and C17:1-LPA for the various LPA species). The mass transition and retention times for each compound we analyzed are provided in table 1.

Statistical analyses – Statistical analyses (one-way ANOVA with Dunnett's multiple comparisons test) were done using the GraphPad Prism 7 software. P values < 0.05 were considered significant.

Results

Expression of DAG lipase α and β

Previous attempts to stimulate 2-AG production by human immune cells were somewhat disappointing, leading to eCB levels below those required to activate the CB$_2$ receptor (11, 13, 15). This raised the possibility that

they might not express the key biosynthetic enzymes. In the first series of experiments, the expression of DAG lipases was assessed by qPCR and immunoblot in human leukocytes. While the mRNA and protein expression patterns were similar, a sharp difference between the expression patterns of the two enzymes was found (figure 6.1). DAG lipase α was detected in the hypothalamus samples, which were included as positive controls. The protein was usually absent from our leukocyte preparations, except for some eosinophil and AM samples in which we detected a weak signal. As for DAG lipase β, it was found in eosinophils, monocytes and AMs. Neutrophils and lymphocytes had little or no signal for either DAG lipase isoforms. These data support the concept that the DAG lipase pathway might not be involved in the biosynthesis of 2-AG by all leukocyte types.

2-AG synthesis by leukocytes stimulated with PLC-activating agonists

Considering the DAG lipase expression patterns we obtained (figure 6.1), we postulated that human leukocytes, especially eosinophils, monocytes and AMs, should be capable of generating 2-AG following PLC activation. Because leukocytes are experts at hydrolyzing 2-AG and its metabolites and that the efficacy of 2-AG hydrolysis inhibitors at enhancing 2-AG half-life overtime is not documented in leukocytes (6, 7, 10), we first compared the effects of three 2-AG hydrolysis inhibitors to determine which one would most effectively increase its half-life in neutrophil suspensions. In absence of inhibitor, ~90% of the added 2-AG (3 µM) had disappeared after one minute. JZL184, Palmostatin B and MAFP all prolonged 2-AG half-life in neutrophils (figure 6.2A). MAFP, at 1 µM, was the most efficient, with ~75% of 2-AG remaining after 15 minutes. We next investigated whether leukocytes could generate 2-AG in response to two agonists previously documented to activate the PLC/DAG lipase pathway: the calcium ionophore A23187 or PAF (11, 26). These stimulations were performed in the presence of MAFP, in order to prolong 2-AG half-life and correctly assess the biosynthetic capabilities of leukocytes. PAF did not stimulate a robust biosynthesis of 2-AG in any cell type. A23187 stimulated the biosynthesis of 2-AG in monocytes and AMs, although the obtained levels were modest. These data indicate that although A23187 has a stimulatory effect on 2-AG biosynthesis, human leukocytes do not generate large amounts of 2-AG via the classic biosynthetic pathway involving the sequential actions of PLC and DAG lipases.

Arachidonic acid stimulates a robust biosynthesis of 2-AG in human neutrophils

We next treated human neutrophils with a larger panel of inflammatory effectors, notably AA (the product of 2-AG hydrolysis), TLR agonists and the recognized neutrophil activator fMLP. In the absence of MAFP, human neutrophils did not synthesize detectable 2-AG levels (detection limit of 25 fmol) in response to fMLP, LPS or the TLR 7/8 agonist R848 while they synthesized modest amounts in response to AA (figure 6.3A). When these experiments were repeated in presence of MAFP, 2-AG was synthesized in very limited amounts upon

stimulation with GPCR or TLR agonists. In sharp contrast, MAFP-treated neutrophils synthesized large amounts of 2-AG in response to AA (figure 6.3B). The effect of AA was concentration-dependent (figure 6.3C) and kinetic experiments unraveled that 2-AG biosynthesis/accumulation was maximal at 15 minutes, after AA was cleared from the incubation media (figure 6.3D). Finally, AA selectively induced the biosynthesis of 2-AG, as no other monoacylglycerols were detected (figure 6.1E). As for the endocannabinoid AEA, it was not detected in large amounts either (from being below detection limit in untreated neutrophils to $0,28 \pm 0.05$ pmol/million cells in AA-stimulated neutrophils), even though MAFP would have blocked its hydrolysis by targeting FAAH (27, 28). Thus, the treatment of human neutrophils with AA led to a robust stimulation of 2-AG while having modest effects on AEA levels. Consequently, we did not explore the biosynthesis of AEA any further.

Inhibition of the AA-induced 2-AG biosynthesis in human neutrophils by reacylation inhibitors

Since 2-AG biosynthesis was maximal after most of the AA was cleared from the incubation media, we postulated that AA was reacylated into cellular membranes then released from the phospholipids to yield a monacylglycerol. To verify this, experiments were performed in which human neutrophils were pre-treated with the fatty acyl-CoA synthetase inhibitor triacsin C (29) or the acyl-CoA transferase inhibitor thimerosal (30-32). In agreement with the working hypothesis, triacsin C and thimerosal both inhibited the AA-induced 2-AG biosynthesis by ~90% (figure 6.4A). To further explore this recycling pathway, another set of experiments was done in which neutrophils were stimulated neutrophils with AA-D_8 instead of AA. This led to an almost inexistent biosynthesis of 2-AG, which was replaced by an equivalent biosynthesis of 2-AG-D_8 (figure 6.4B, C). Altogether, these data indicate that in neutrophils, exogenously-added AA is likely reacylated into phospholipids and then further processed into 2-AG.

Biosynthesis of eCB-glycerols in response to unsaturated fatty acids

While 2-AG is the monoacylglycerol binding to the CB_2 receptor with the highest affinity, other monoacylgycerols were shown to activate HL60 cells, which express the CB_2, with equal or better potency and efficacy than AEA (33). This, combined with the results presented in figure 4, prompted us to determine whether other fatty acids would stimulate the biosynthesis of their eCB-glycerol counterparts. Human neutrophils were thus treated with various fatty acids for 15-minutes then processed for eCB-glycerol quantification. Figure 6.5A shows that neutrophils metabolized unsaturated fatty acids into eCB-glycerols with varying efficacy, with DHA being the most efficiently metabolized. Long chain fatty acids were better transformed than shorter chains fatty acids (C22 > C20 >C18). In addition, for a given fatty acid chain length, an increased in the number of double bonds led to a better efficacy for a given fatty acid chain length (DHA vs.

DPA; EPA vs. AA; LA vs. OA). Of note, the 16-carbon saturated fatty acid palmitic acid did not induce the biosynthesis of palmitoyl-glycerol.

Eosinophils and monocytes, but not AMs nor lymphocytes, also metabolize unsaturated fatty acids into eCB-glycerols

In the next series of experiments, the hypothesis that other leukocytes could also synthesize eCB-glycerols in response to fatty acids was tested. Unsaturated fatty acids also induced the biosynthesis of their eCB-glycerols in eosinophils and monocytes (figure 6.5B, C) but to a lesser extent than neutrophils. In contrast, lymphocytes and AMs only produced trace amounts of eCB-glycerols in response to unsaturated fatty acids (figure 6.5D, E). Finally, AA, LA and OA did not induce the synthesis of their respective monoacylglycerols by platelets or erythrocytes (data not shown). Again, palmitoyl-glycerol was not detected in any cell type stimulated with palmitic acid, suggesting that saturated fatty acids do not undergo this monoacylglycerol biosynthetic pathway. Altogether, these data raise the possibility that the unsaturated fatty acid-mediated synthesis of eCB-glycerols is restricted to leukocytes from the myeloid lineage.

The poor stimulatory effect of GPCR agonists such as PAF and fMLP on the biosynthesis of 2-AG biosynthesis by leukocytes (figure 6.2 and 6.3), and the striking effect of triacsin C and thimerosal on the AA-induced 2-AG biosynthesis in neutrophils suggested that AA stimulated 2-AG biosynthesis independently of the PLC/DAG lipase pathway. As such, the PLC inhibitor U73122 and the DAG lipase inhibitors KT109 and KT172, at 10 µM, did not inhibit the AA-induced 2-AG biosynthesis in neutrophils (data not shown), supporting the concept that AA induces the biosynthesis of 2-AG in a PLC/DAG lipase-independent manner and very likely via its remodeling into phospholipids.

Apart from the PLC/DAG lipase pathway, the biosynthesis of 2-AG (and possibly other eCB-glycerols) was documented to occur via to other pathways: a PLD-mediated cleavage of phosphatidic leading to DAG, which is then converted into 2-AG by DAG lipases (19); and the conversion of LPA into 2-AG by a phosphatase (20). Given the lack of effect of DAG lipase inhibitors, the following experiments were focused on testing whether AA stimulates 2-AG synthesis via an LPA intermediate. Human neutrophils were thus treated with AA for different times and the levels of AA-LPA were assessed. AA stimulated the synthesis of AA-LPA (figure 6.6). Of note, MAFP did not affect the levels of AA-LPA stimulated by AA (data not shown). While 2-AG levels are maximal at 15 minutes (figure 6.3C), AA-LPA levels peaked at 5 minutes then declined overtime (figure 6.6A). Other fatty acids also induced the synthesis of their LPA species in human neutrophils (figure 6.6B). However, the syntheses of the different LPA species did not follow the same trend as their eCB-glycerol counterparts (figure 6.5A). Several phosphatase inhibitors were tested to evaluate whether they could inhibit the putative dephosphorylation of LPA into 2-AG. In that regard, sodium orthovanadate, AlF$_x^-$, propranolol, bromoenol

lactone and XY-14, all of which inhibit LPA dephosphorylation, (34-37) failed to prevent AA-induced 2-AG synthesis (data not shown).

AA-induced 2-AG synthesis is downregulated by pro-inflammatory effectors

Our attempts at stimulating the biosynthesis of 2-AG by agonists stimulating the PLC/DAG lipase pathway did not lead to a robust biosynthesis of 2-AG and other eCB-glycerols, as opposed to the unsaturated fatty acid-stimulated eCB-glycerol synthesis (figures 6.3 and 6.4, respectively). We next examined whether both pathways interacted together by treating human neutrophils with AA in combination with PAF, fMLP, or LTB4. All three mediators significantly decreased the AA-induced 2-AG biosynthesis (figure 6.7). PAF was the most potent with ~45% inhibition, followed by fMLP (~35%) and LTB4 (~20%). This suggests that in an inflammatory setting in which AA is present simultaneously with pro-inflammatory effectors, 2-AG synthesis might be diminished.

Levels of eCB-glycerols in human plasma

The levels of 2-AG in human plasma have been documented in humans, varying from 1 – 20 nM (38). The plasma levels of some of the other eCB-gycerols studied herein have never been documented, although some studies reported the plasma concentrations of OA-glycerol, EPA-glycerol and DHA-glycerol (39, 40). Herein, we quantitated the levels of eCB-glycerols derived from PA, OA, LA, AA, EPA, DPA and DHA in the plasma from fasting healthy volunteers (5 females and 5 males, matched for age and body mass index). All of the eCB-glycerols we measured were detected in the plasma. 2-OG, 2-PG and 2-LG were the most abundant (figure 6.8). There were no statistically significant differences between male (age 30 ± 12) and female (age 29 ± 12) subjects for any of the eCBs we measured.

Discussion

eCBs are natural anti-inflammatory mediators, at least in mice, and the pharmacological blockade of their degradation has great potential to treat inflammatory diseases. However, whether human leukocytes are an important source of eCBs was elusive. In this paper, we show that **1)** DAG lipase expression differs between leukocyte subsets with DAG lipase βbeing the most abundantly expressed; **2)** Leukocytes do not synthesize large amounts of 2-AG via the PLC/DAG lipase pathway; **3)** circulating myeloid leukocytes synthesize eCB-glycerols in response to unsaturated fatty acids; **4)** The unsaturated fatty acid-mediated eCB-glycerol synthesis is not inhibited by DAG lipase inhibitors but is inhibited by acyl-CoA synthetase and an acyl-transferase inhibitors; **5)** the unsaturated fatty acid-mediated eCB-glycerol biosynthesis is preceded by the buildup of its LPA congener; **6)** pro-inflammatory effectors that activate PLCs inhibit AA-induced 2-AG

biosynthesis; and **7)** human plasma contains each of the investigated eCB-glycerols, some of which were never documented before.

The expression of the different DAG lipases by human leukocytes was ill defined. In our experiments, DAG lipase α was only found in trace amounts in leukocytes, notably eosinophils and AMs, in sharp contrast with hypothalamus samples. The latter was expected, as DAG lipase α was described as the main biosynthetic enzyme in mouse brain (16, 17). As for DAG lipase β, it was found in large amounts in eosinophils, monocytes and AMs (figure 6.1) and in low/undetectable amounts neutrophils and lymphocytes. The latter finding is in line with data showing the importance of this enzyme in 2-AG synthesis by murine macrophages (18). The A23187-induced 2-AG synthesis by monocytes is likely explained by the fact that they express DAG lipase β, although this remains to be confirmed using selective DAG lipase inhibitors. However, using this rationale, we would have expected similar findings in eosinophils, which produced less than 0.1 pmol/10^6 cells of 2-AG in response to PLC activation (figure 6.2C). Given that GPCR agonists inhibit 2-AG synthesis by neutrophils (figure 6.7), GPCR activation was bound to fail to induce eCB-glycerol synthesis in leukocytes. We did not detect large amounts of DAG lipase β in neutrophils nor lymphocytes compared to eosinophils, supporting that these leukocytes do not synthesize 2-AG in a DAG lipase-dependent fashion, as underscored in figure 6.2.

Our data underscores the importance of hydrolase inhibition when studying endocannabinoid biosynthesis in leukocytes. This data, combined with our previous work, shows that human leukocytes are experts at hydrolyzing 2-AG (6, 7). Indeed, in the absence of hydrolase inhibitors, we did not observe detectable amounts of 2-AG in stimulated neutrophils with the exception of AA, which led to a minimal 2-AG synthesis (figure 6.2). The use of MAFP unraveled that AA induces a robust biosynthesis of 2-AG by neutrophils. These findings might provide an explanation for the low amounts of 2-AG produced by human immune cells in previous studies, in which 2-AG hydrolysis was not prevented (11, 13, 15). However, we show that most leukocytes don't synthesize substantial amounts of 2-AG in response to agonists known to activate the PLC pathway, even in the presence of MAFP. One study showed that human lung macrophages stimulated with LPS synthesize more 2-AG in the presence of a MAG lipase inhibitor, but this increase of approximately 40% was far from the impact MAFP had in our biosynthetic route involving unsaturated fatty acids (12).

Following the structure elucidation of the main eicosanoids, AA has been typecast as a villain. The stimulatory effect it has on 2-AG biosynthesis by leukocytes might thus be surprising and counter-intuitive for some. However, the data provided herein indicate that the biosynthetic route involved in the AA-induced 2-AG biosynthesis is clearly different from the classical GPCR-PLC-PLA$_2$ pathway involved in eicosanoid biosynthesis. Indeed, the use of the pharmacological inhibitors triacsin C and thimerosal indicates that AA is

transformed into AA-CoA, then incorporated either into a glycerol-moiety or into lysophospholipids, then further metabolized into 2-AG (figure 6.4). This biosynthetic route, while incomplete, is also supported by the data obtained with AA-D$_8$, the latter mainly stimulating the synthesis of 2-AG-D$_8$ (figure 6.4C). Thimerosal is known to effectively block AA reacylation in human neutrophils by inhibiting two acyltransferases, namely MBOAT5 and MBOAT7 (30). Given the sharp inhibitory effect of thimerosal on 2-AG synthesis induced by AA, it is very likely that these enzymes are involved in this process.

The metabolism of AA into 2-AG does not involve the DAG lipase pathway, but might involve LPA as an intermediate (figure 6.6). The dephosphorylation of AA-LPA to produce 2-AG was indeed described in rat brains (20), but its importance in eCB biosynthesis in humans was unknown. In our experimental model, LPA synthesis preceded that of 2-AG, with maximal levels being detected at 5 and 15 minutes respectively (figure 6.3 and 6.6). This suggests than an LPA buildup occurs first, which could be followed by its dephosphorylation into 2-AG over time. However, we were unable to block LPA dephosphorylation, even though we tested a wide array of phosphatase inhibitors. Furthermore, the addition of exogenous AA-LPA did not lead to the synthesis of 2-AG (data not shown). Altogether, the LPA data indicate the following possible outcomes: 1) the buildup of LPA preceding eCB-glycerol synthesis is coincidental; 2) LPA dephosphorylation occurs via a phosphatase that is insensitive to the inhibitor we used; and/or 3) LPA dephosphorylation occurs within the cells and exogenously-added LPA do not enter the cells. Therefore, the involvement of LPA in this process remains elusive and requires further investigation.

We show in figure 6.5 that leukocyte stimulation with other fatty acids produce a robust synthesis of their corresponding eCB-glycerols. This process was not of equal magnitude for every fatty acid, as we observed that fatty acids with more insaturations and a longer fatty acid chain were more effectively converted into monoacylglycerols. The plasmatic levels of 2-AG we report here are in the low nM range, in line with previous studies (24, 38). The eCB-glycerols from the ω-3 fatty acids EPA, DPA and DHA were the least abundant among the compounds we detected. The diet is known to have a direct effect on the fatty acid content found in the circulation and therefore, it could influence the levels of eCB-glycerols as well (41). Given that we hereby show that circulating leukocytes isolated from the peripheral blood convert unsaturated fatty acids into eCBs, the dietary intake of fatty acids is probably a determinant of plasma eCB levels. Another possible determinant of eCB levels in the blood might be their degradation by eCB hydrolases, which is a reaction that occurs rapidly in human plasma (half-life of 16 minutes; (42)). However, we also show that the use of a lipase inhibitor (MAFP), drastically halts this eCB hydrolysis and allows for an impressive build-up in fatty acid-stimulated leukocytes. Therefore, the therapeutic use of hydrolase inhibitors could allow the endocannabinoid tone to be

increased and for endocannabinoids to have higher bioactive potential. In this regard, the use of MAG lipase inhibitors in mouse models were shown to increase plasma levels of 2-AG (43, 44).

Altogether, our data show that human myeloid leukocytes synthesize significant amounts of eCB-glycerols. The eCB-glycerol synthesis is preceded by the synthesis of a LPA intermediate, although its involvement in the biosynthesis the latter remains to be confirmed/infirmed. It also suggests that the development of eCB-glycerol hydrolysis inhibitors might turn human myeloid leukocytes into putative anti-inflammatory effectors by increasing their ability to synthesize anti-inflammatory eCB-glycerols and eventually activate the endocannabinoidome receptors, notably the anti-inflammatory CB_2 receptor.

Acknowledgements

Abbreviations

2-AG, 2-arachidonoyl-glycerol; **2-DHG,** 2-docosahexaenoyl-glycerol; **2-DPG,** 2-docosapentaenoyl-glycerol; **2-EPG,** 2-eicosapentaenoyl-glycerol; **2-LG,** 2-linoleoyl-glycerol; **2-OG,** 2-oleoyl-glycerol; **2-PG**, 2-palmitoyl-glycerol; **AA**, arachidonic acid; **AEA,** anandamide; **AMs**, alveolar macrophages; **BAL,** bronchoalveolar lavage; **DAG,** diacylglycerol; **DHA,** docosahexaenoic acid; **DPA,** docosapentaenoic acid; **eCB;** endocannabinoid; **EPA,** eicosapentaenoic acid; **LA,** linoleic acid; **LPA** lysophosphatidic acid; **LT,** leukotriene; **MAFP,** methoxy arachidonoyl fluorophosphonate; **MAG**, monoacylglycerol; **MBOAT,** membrane bound O-acyl transferase; **OA,** oleic acid; **PA,** palmitic acid; **PAF,** platelet activating factor; **PL**, phospholipase.

Figures
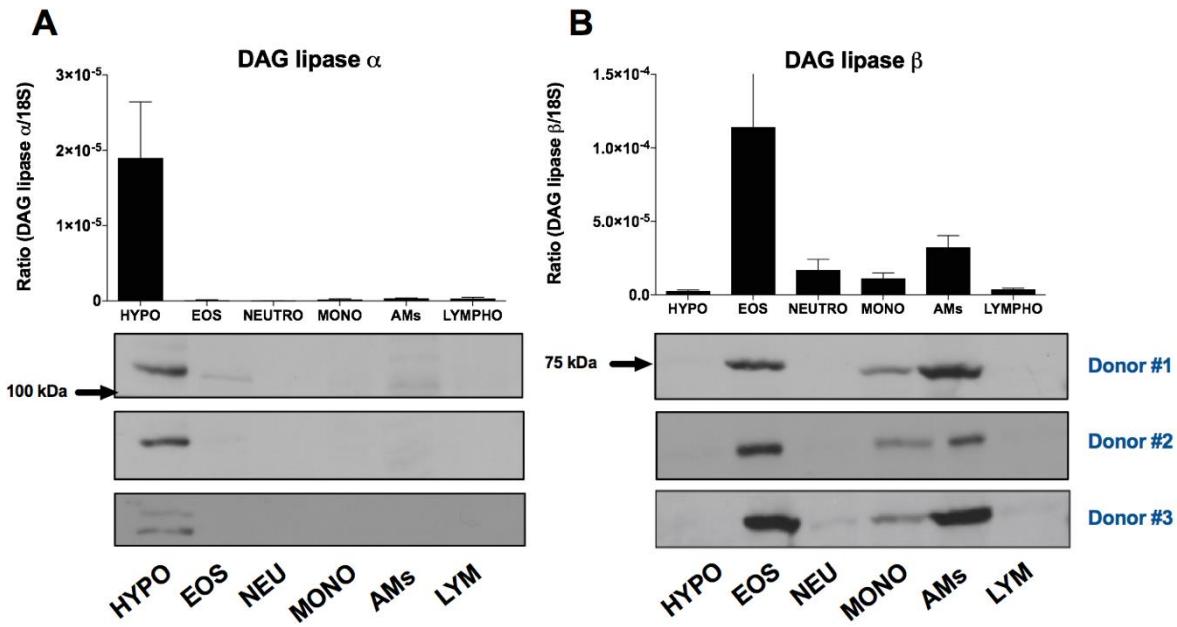
Figure 6.1. Expression of DAG lipases in human leukocytes. Bar graphs show mRNA expression as measured by real-time PCR (qRT-PCR). Cells were isolated, mRNA was extracted and qRT-PCR analyses were conducted as described in *Materials and methods*. The data is presented as the mean $\pm$ SEM for 4 volunteers (3 for hypothalamuses), performed in duplicates. Images below the bar graphs represent the protein expression of DAG lipases in human hypothalamus and leukocytes. Cells were isolated, protein samples were prepared and Western blotting experiments were conducted as described in *Materials and methods*. Experiments were performed on three volunteers and all three gels are shown. **AMs =** alveolar macrophages; **HYPO =** hypothalamus; **EOS =** eosinophils; **NEU =** neutrophils; **MONO =** monocytes; **LYM =** lymphocytes.
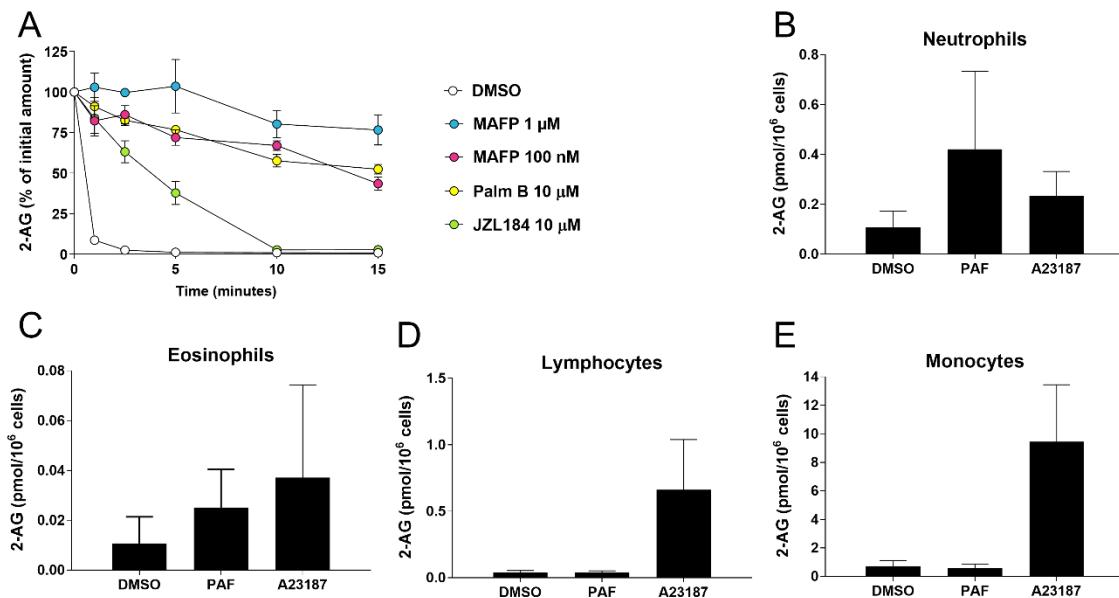
Figure 6.2. Biosynthesis of 2-AG induced by PAF or A23187. A) Pre-warmed neutrophil suspensions (37°C, 5 × 10^6 cells/ml) in HBSS containing 1.6 mM CaCl$_2$ were treated with DMSO, MAFP, Palmostatin B (Palm B) or JZL184 at the indicated concentration for 5 minutes then incubated with 1 µM 2-AG for up to 15 minutes. **B-E)** Pre-warmed leukocyte suspensions (37°C, 5 × 10^6 cells/ml) in HBSS containing 1.6 mM CaCl$_2$ were treated with 1 µM MAFP for 5 minutes then treated with either DMSO, A23187 (100 nM) or PAF (1 µM) for 15 minutes. Incubations were stopped by adding 0.5 volume of cold (4°C) MeOH containin 2 ng 1-AG-D$_5$ as internal standard. Samples were processed and analyzed for 2-AG levels as described in *Materials and methods*. Data are the mean (± sem) of at least three independent experiments.
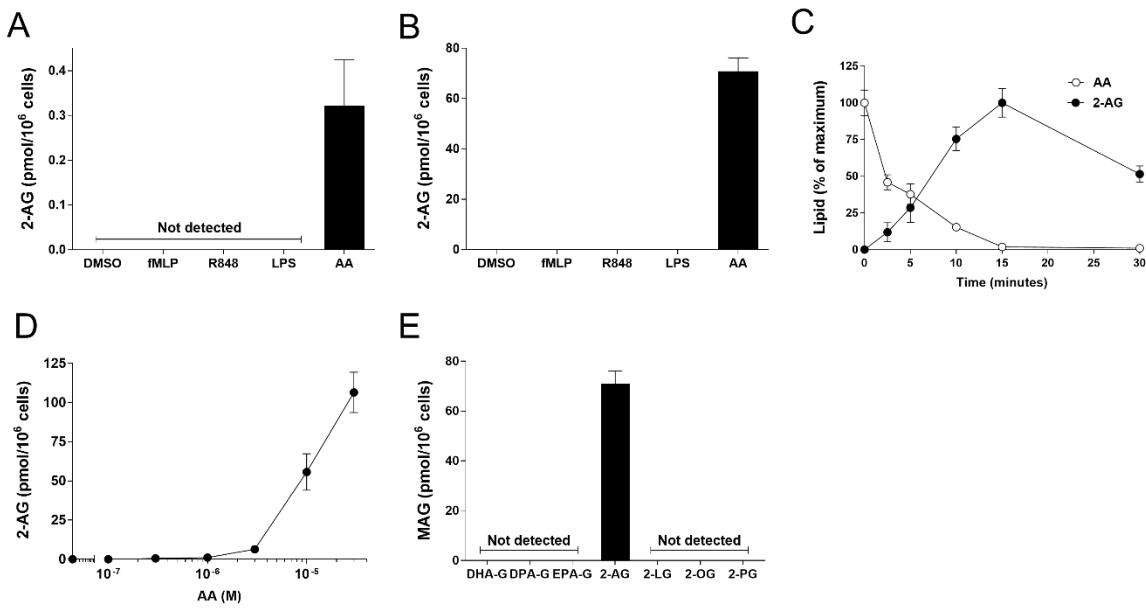
Figure 6.3. Impact of AA on the biosynthesis of 2-AG by human neutrophils. Pre-warmed human neutrophil suspensions (37°C, 5 x 10^6 cells/ml) in HBSS containing 1.6 mM CaCl_2 were used for all experiments. Cells were incubated with **A)** DMSO or **B-E)** 1 μM MAFP for 5 minutes before the addition of the stimulus. **A, B)** Cell were stimulated with fMLP (1 μM), R848 (10 μM), PAF (1 μM), A23187 (100 nM) or AA (10 μM) for 15 minutes. **C)** Cells were stimulated with 10 μM AA for the indicated times. **D)** Cells were stimulated with AA at the indicated concentrations for 15 minutes. **E)** Cells were stimulated with 10 μM for 15 minutes. Incubations were stopped by the addition of 0.5 ml of cold MeOH containing 2 ng 1-AG-D5 as internal standard. Samples then were processed and analyzed for 2-AG and other monoacylglycerols (MAG) by LC-MS/MS as described in *Material and methods*.
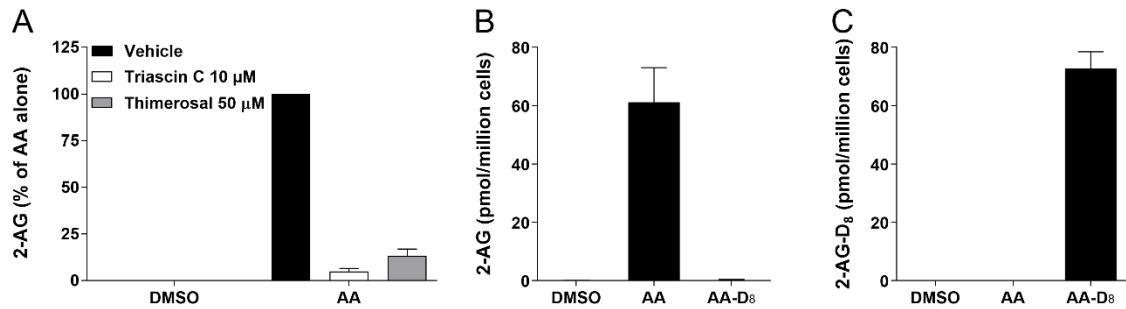
Figure 6.4. Impact of reacylation inhibitors and AA-D$_8$ in the AA-induced 2-AG biosynthesis by neutrophils. Pre-warmed human neutrophil suspensions (37°C, 5 x 10^6 cells/ml) in HBSS containing 1.6 mM CaCl$_2$ were pre-incubated with 1 µM MAFP for 5 minutes then stimulated with 10 µM AA or AA-D$_8$ for 15 minutes. **A)** Triascin C and thimerosal were added 2 minutes before the addition of AA. **A-C)** Incubations were stopped by the addition of 0.5 ml of cold (4°C) MeOH containing 2 ng 1-AG-D$_5$ as internal standard. Samples were processed and analyzed for 2-AG and 2-AG-D$_8$ levels as described in *Material and methods*. The data are the mean (± sem) of at least three independent experiments.
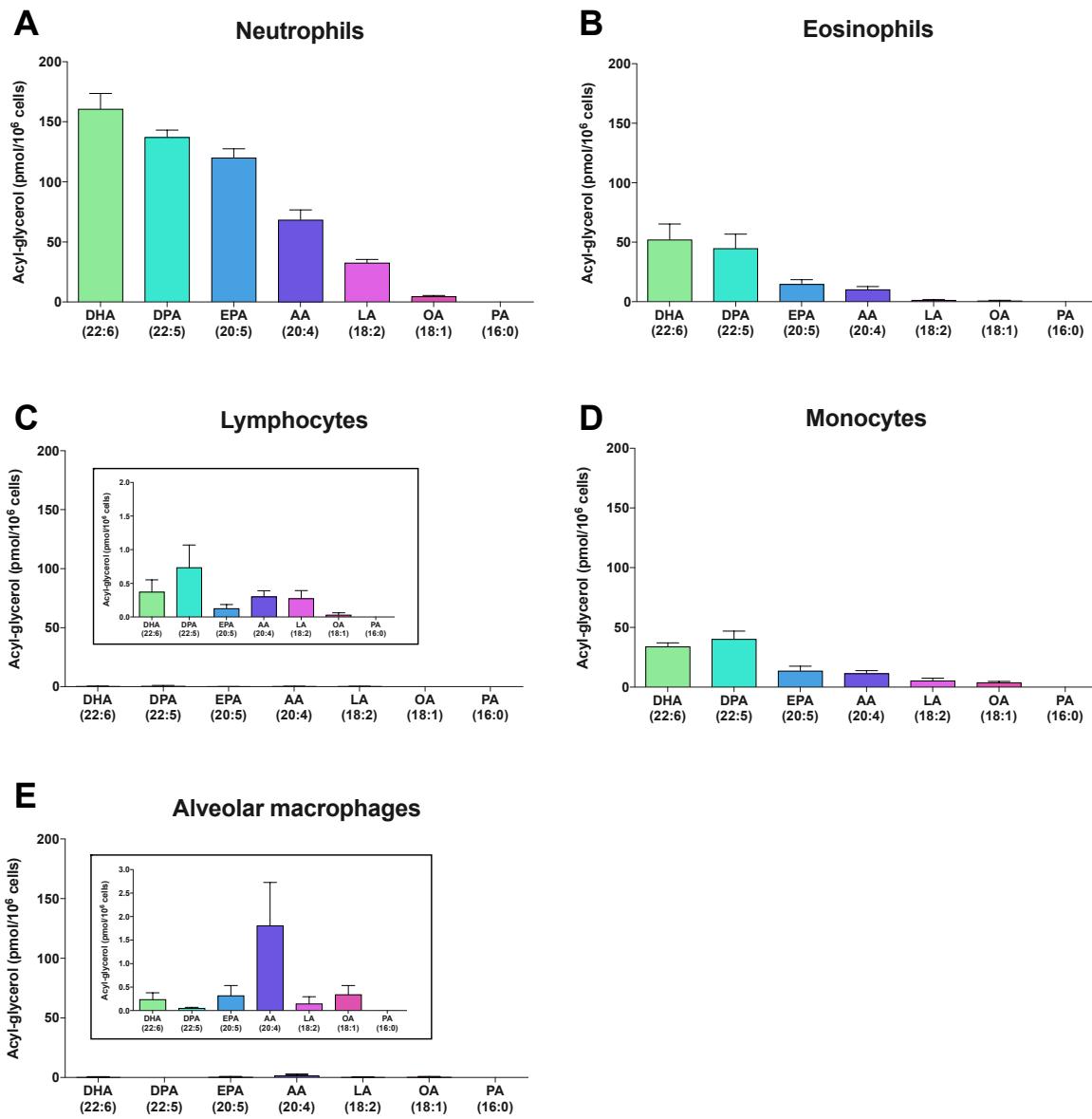
Figure 6.5. Impact of unsaturated fatty acids on the biosynthesis of eCB-glycerols by human leukocytes. Pre-warmed human leukocyte suspensions (37°C, 5 x 10^6 cells/ml) in HBSS containing 1.6 mM CaCl$_2$ were pre-incubated with 1 µM MAFP for 5 minutes then stimulated with 10 µM of the different fatty acids for 15 minutes. Incubations were stopped by the addition of 0.5 volume of MeOH containing 2 ng 1-AG-D$_5$ as internal standard. Samples then were processed and analyzed for monoacylglycerol levels as described in Methods. Data are the mean (± sem) of at least three independent experiments.
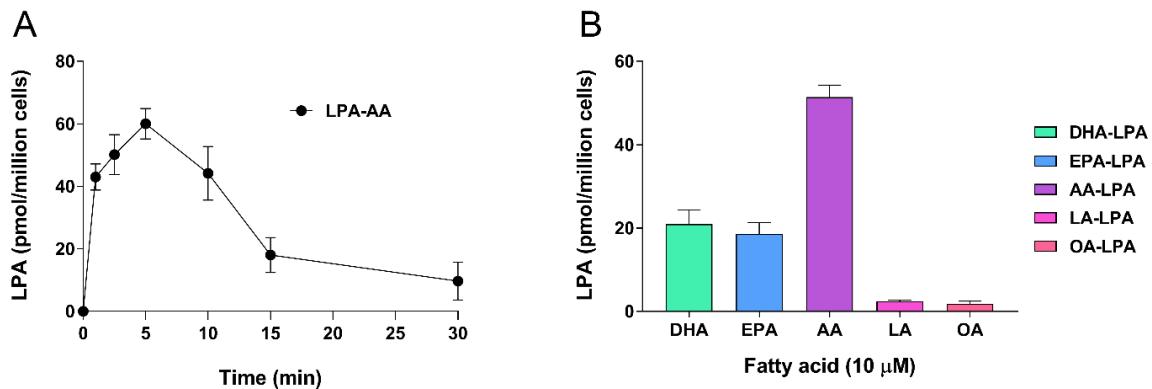
Figure 6.6. Impact of AA and other unsaturated fatty acids on LPA biosynthesis. Pre-warmed human leukocyte suspensions (37°C, 5 x 10^6 cells/ml) in HBSS containing 1.6 mM CaCl$_2$ **A)** were treated with 10 µM AA for the indicated times; or **B)** were treated with 10 µM of fatty acids for 5 minutes. Incubations were stopped by the addition of 0.5 volume of MeOH containing 10 ng C17-LPA. Samples were processed and analyzed for LPA content as documented in *Material and Methods*. Data are the mean (± sem) of at least four independent experiments.
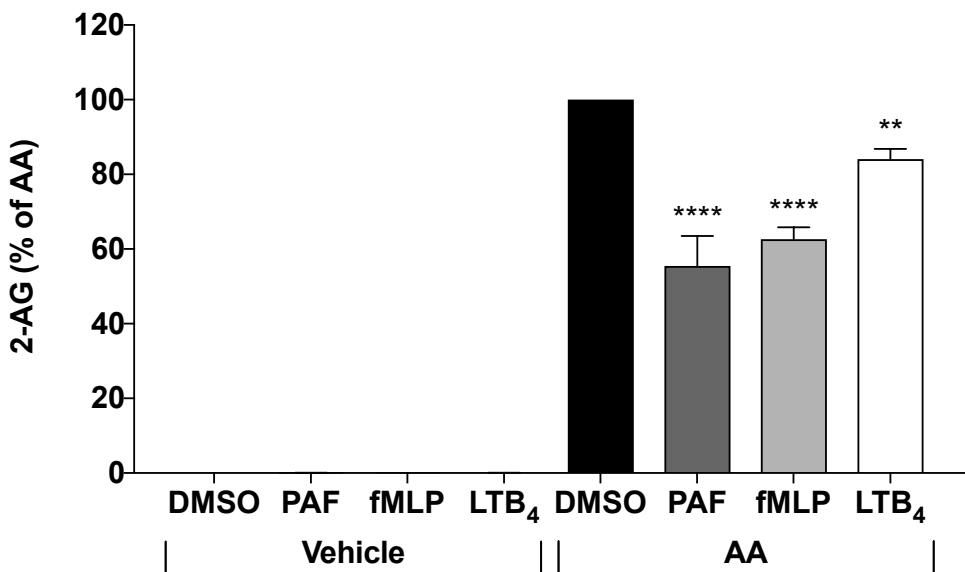
Figure 6.7. Impact of PAF, fMLP and LTB4 on the AA-induced 2-AG biosynthesis by human neutrophils. Pre-warmed human leukocyte suspensions (37°C, 5×10^6 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were treated with MAFP (1 µM) for 5 minutes. AA (10 µM) was then added simultaneously with 1 µM PAF, fMLP or LTB_4 for 15 minutes. Incubations were stopped by the addition of 0.5 ml cold (-20°C) methanol containing 2 ng $1\text{-}AG\text{-}D_5$ as internal standard. Samples were processed for LC-MS/MS analysis of 2-AG as described in *Material and methods*. Statistical analyses (one-way ANOVA with Dunnett's multiple comparisons test) were performed using the GraphPad Prism 7 software. ** $p<0.01$; ***$p<0.001$; ****$p<0.0001$ (vs AA alone).
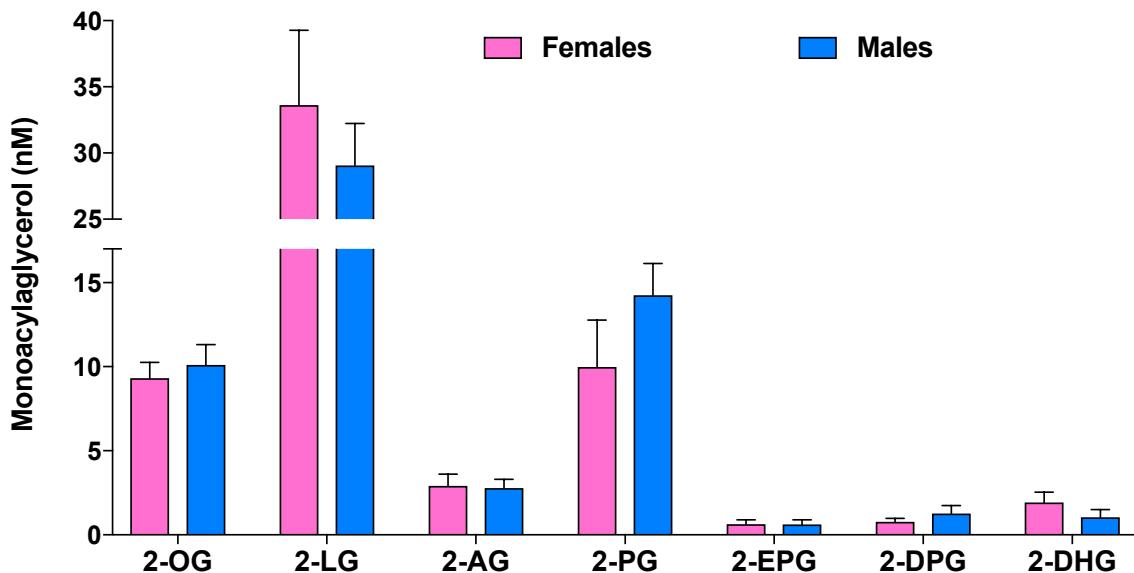
Figure 6.8. Plasma levels of eCB-glycerols in healthy volunteers. Platelet-rich plasma samples were obtained by centrifuging the peripheral blood of healthy volunteers (200 × *g*, 20 minutes, 4°C). The plasmas were harvested and centrifuged (1450 × *g*, 25 minutes, 4°C) to pellet the platelets. eCBs were extracted from the resulting platelet-depleted samples as described in Methods. Results are the mean (± sem) of 5 of five different donors for each group. No statistically significant differences were found between males and females (two-way ANOVA with Sidak's multiple comparison test).

Table 6.1: Specific mass transitions and retention times of the metabolites analyzed by LC-MS/MS.

COMPOUND	INTERNAL STD USED	Q1 → Q3	RETENTION TIME (min)
1-AG	1-AG-d$_5$	379.30>287.25	12,201
2-AG	1-AG-d$_5$	379.30>287.25	12,065
1-LG	1-AG-d$_5$	355.20>263.30	12,156
2-LG	1-AG-d$_5$	355.20>263.30	11,976
2-OG	1-AG-d$_5$	357.20>265.40	12,85
1-OG	1-AG-d$_5$	357.20>265.40	12,65
1-PG	1-AG-d$_5$	331.10>313.25	12,635
2-PG	1-AG-d$_5$	331.10>313.25	12,435
AA	AA-d$_8$	303.20>259.30	13,225
DHA-1-G	1-AG-d$_5$	403.20>311.20	12,044
DHA-2-G	1-AG-d$_5$	403.20>311.20	11,94
DPA-1-G	1-AG-d$_5$	405.20>313.30	12,524
DPA-2-G	1-AG-d$_5$	405.20>313.30	12,373
EPA-1-G	1-AG-d$_5$	377.10>285.25	11,194
EPA-2-G	1-AG-d$_5$	377.10>285.25	11,061
1-AA-LPA	17:1-LPA	457.30>152.90	8,087
1-EPA-LPA	17:1-LPA	455.4>153.05	7,370
1-DHA-LPA	17:1-LPA	481.30>152.90	8,085
1-LA-LPA	17:1-LPA	433.3>153.15	8,036
1-OA-LPA	17:1-LPA	435.40>153.05	8,878

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Chapitre 6.   Mechanisms of eosinophil migration induced by the combination of IL-5 and the endocannabinoid 2-arachidonoyl-glycerol.

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Résumé

La migration des éosinophiles vers les tissus, un élément clé dans plusieurs maladies inflammatoires, est un phénomène mal défini. Le rôle de divers médiateurs lipidiques de l'inflammation, incluant le 5-oxo-ETE et la prostaglandine D_2, est bien reconnu. Certains de ces médiateurs lipidiques peuvent recruter les éosinophiles dans les voies respiratoires, soulignant l'importance des lipides dans la pathophysiologie des maladies éosinophiliques. Les endocannabinoïdes, des lipides endogènes dont le rôle dans l'inflammation est établi, ont un profil intriguant d'effets pro- et anti-inflammatoires en raison de la complexité de leur lipidome. Il a toutefois été démontré que le 2-AG, un endocannabinoïde bien caractérisé, induit une migration modeste des éosinophiles. Par conséquent, le but de cette étude était de caractériser l'impact du 2-AG sur le recrutement des éosinophiles humains. Nous démontrons que certaines cytokines, dont l'IL-5, potentialisent l'effet chimiotactique du 2-AG envers les éosinophiles, qui induit alors une migration nette d'environ 40 %. Cette migration est comparable à celle induite par la CCL11, une puissante chimiokine sélective envers les éosinophiles. Des antagonistes du récepteur cannabinoïde CB_2 bloquent complètement la migration des éosinophiles induite par le 2-AG. Cependant, nos tentatives de reproduire cet effet à l'aide d'agonistes du récepteur CB_2 furent sans succès, suggérant que le récepteur CB_2 est seulement partiellement responsable de l'effet chimiotactique du 2-AG. À cet égard, nous avons postulé que le 2-AG est rapidement hydrolysé en acide arachidonique (AA) par les éosinophiles, et qu'un éicosanoïde dérivé de l'AA est formé et participe au recrutement des éosinophiles. Cette hypothèse fut confirmée par des expériences démontrant que l'inhibition pharmacologique de l'hydrolyse du 2-AG ou de l'activité de la 15-lipoxygénase diminuent la migration des éosinophiles induite par le 2-AG. Ces données démontrent que le récepteur CB_2 et le métabolisme du 2-AG via la voie de la 15-lipoxygénase sont tous les deux requis pour que le 2-AG induise une migration des éosinophiles, mais qu'aucun de ces deux éléments n'est suffisant par lui-même pour induire une telle migration. L'effet de l'IL-5 dans ce processus supporte que le 2-AG joue potentiellement un rôle dans le recrutement des éosinophiles dans l'asthme.

Abstract

Eosinophil migration to the tissues, a hallmark of many inflammatory diseases, is not fully understood. The involvement of several lipid mediators in this process, including 5-oxo-ETE and prostaglandin D_2, is well recognized. Some of these mediators are known to recruit eosinophils to the airways in asthma, underscoring the importance of bioactive lipids in the pathophysiology of eosinophilic diseases. Endocannabinoids, although their importance in inflammation is well established, display a puzzling profile of pro- and anti-inflammatory effects due to the complexity of their lipidome. However, 2-AG, one of the best characterized endocannabinoids, was previously reported to modestly induce eosinophil migration. In this respect, this study aimed to characterize the chemotactic effect of 2-AG on human eosinophils. We show that cytokines such as IL-5 potentiate the chemotactic effect of 2-AG, which induced a net migration of eosinophils of ~40%. This migration of eosinophils is comparable to that induced by CCL11, a potent, eosinophil-selective chemokine. CB_2 receptor antagonists completely blocked 2-AG-induced eosinophil migration. However, attempts to replicate this migration using CB_2 receptor agonists were unsuccessful. Thus, CB_2 receptor activation is required, although it appears to be only partially responsible for the chemotactic effect of 2-AG. In this respect, we hypothesized that 2-AG was quickly hydrolyzed into arachidonic acid (AA) by eosinophils, and that an AA-derived eicosanoid was formed and participated in eosinophil recruitment. This was confirmed by experiments showing that the pharmacological blockade of 2-AG hydrolysis or 15-LO activity both decrease 2-AG-induced eosinophil migration. This data shows that although both CB_2 receptor activation and 2-AG metabolism through the 15-LO pathway are required for 2-AG-induced eosinophil migration, neither of these elements alone is sufficient to induce eosinophil migration that is comparable to that induced by 2-AG. The effect of IL-5 in this process supports that 2-AG may play a role in eosinophil recruitment in asthma.

Introduction

How eosinophils migrate to the tissues in eosinophilic diseases is not completely defined but is recognized to involve chemokines and/or bioactive lipids, noteworthy prostaglandin D_2 and 5-oxo-eicosatetraenoate (5-KETE). In mice, eosinophils might play an important role in adipose tissue maintenance (1). The chemoattractants recruiting eosinophils to the latter are unknown, but IL-5 might be involved (1, 2). The endocannabinoid 2-arachidonoyl-glycerol (2-AG), found in the adipose tissue, activates eosinophils (3); is recognized to activate the cannabinoid receptors CB_1 and CB_2; and can regulate leukocytes' functions through its metabolites (4). Herein, we investigated the mechanisms of the 2-AG–induced migration of human eosinophils.

Materials and methods

Materials

15(S)-hydroxyl-eicosatetraenoate (HETE), 1-AG, 2-AG, 2-AG ether, 5-KETE, AA, AEA, CP 55,940, cysteinyl-leukotrienes enzyme immunoassay kit, EXC_4, EXC_4 EIA kit, His-tagged human MAG lipase, JZL-184, L-759,633, lipoxin A_4, methyl-arachidonyl-fluorophosphonate, NDGA, PAF, PD 146176, PD 98059, and pyrrophenone were purchased from Cayman Chemical (Ann Arbor, Mich). AM-630, SR144528, indomethacin, PP2, and PP3 were purchased from Tocris Bioscience (Ellisville, Mo). A23187 and dimethyl sulfoxide were obtained from Sigma-Aldrich (St Louis, Mo). The magnetic bead–conjugated anti-CD16 mAbs for the magnetic cell sorting were purchased from Miltenyi Biotec (Auburn, Calif). HBSS, FBS, and RPMI 1640 were obtained from Wisent Laboratories (St-Bruno, Quebec, Canada). The ECL detection kit was purchased from GE Healthcare (Mississauga, Ontario, Canada). The 5-LO inhibitor L 739,010 and the 5-LO–activating protein antagonist MK-0591 were kindly provided by Dr Denis Riendeau from Merck Frosst (Kirkland, Quebec, Canada). All cytokines and chemokines were purchased from Peprotech (Dollard des Ormeaux, Quebec, Canada). 15(S)-HETE-glycerol (G) was synthesized by Cayman Chemical and represents a mixture of 15(S)-HETE-*sn1*-G (81.6%) and 15(S)-HETE-*sn2*-G (14.6%).

Volunteers and isolation of eosinophils

The study was approved by the Ethic Committee of the Centre de recherche de l'Institut universitaire de cardiologie et de pneumologie de Québec. All volunteers signed an informed consent form for the study. Human eosinophils were purified from the venous blood of rhinitic volunteers as described earlier (4). The purity of the resulting eosinophil suspensions was always 98.5% or more and the viability was 98% or more, as assessed by Diff Quik staining and trypan blue exclusion, respectively.

Migration assay

Migration assays were performed with 3 μm pore inserts (BD Bioscience, Mississauga, Ontario, Canada) as recommended by the manufacturer. In brief, 700 μL of prewarmed (37°C) RPMI containing 10% FBS, the agonist of interest, was put in the lower chambers. Then, 200 μL of prewarmed eosinophil suspensions (37°C, 2.5×10^6 cells/mL) in RPMI containing 10% FBS was added in the upper chamber of the migration apparatus. Eosinophils were allowed to migrate for 2 hours at 37°C unless stated otherwise (see Figures). The upper chambers were then removed, and migrated cells in the lower chambers were enumerated by using the Scepter 2 automated cell counter (EMD Millipore, Billerica, Mass). In experiments in which enzyme inhibitors or receptor antagonists were used, compounds were added to both the eosinophil suspensions and the lower chamber 5 minutes before the migration assay. Experiments were performed in the presence of 10 ng/mL of IL-5, which was added to eosinophils 10 minutes before the beginning of the migration assays (unless stated otherwise). Net migrations were calculated as follows: (stimulus-induced migrated cells – unstimulated cells)/total cells used in the assay. When a cytokine was present, net migration was calculated as follows: (cytokine-treated-stimulus-induced migrated cells) – (cytokine-treated cells). The eosinophil migration observed in the presence of 10 ng/ml of IL-5 alone typically represented approximately 10% of all cells.

Cell stimulations and analyses of cysteinyl-leukotrienes and EXC$_4$ biosynthesis

Prewarmed (37°C), freshly isolated human eosinophil suspensions (10^6 cells/mL) in HBSS containing 1 mM of $CaCl_2$ were stimulated with vehicle (dimethyl sulfoxide) 1-AG, 2-AG, AEA, AA, A23187 alone or in combination with 1 μM of PAF for 15 minutes. Incubations were stopped by the addition of 1 volume of cold (4°C) incubation buffer, then rapidly centrifuged (4°C; 700g). Supernatants were harvested for the analysis of lipid mediator biosynthesis by ELISA according to the manufacturer's instructions.

Statistical analyses

Statistical analyses were done using mixed-effects ANOVA, followed by the Tukey a posteriori test and were considered significant when P values were <.05.

Results and discussion

IL-3, IL-5, and GM-CSF but not IL-13 or IFN-γ robustly increased the 2-AG–induced eosinophil migration from ~3% to ~40% (figure 7.1A). IL-3, IL-5, and GM-CSF receptors share a common $\beta\gamma$ subunit that activates within seconds the Src-like lyn kinase (5). Consistent with this, the effect of IL-5 was dependent on concentration, immediate, and blocked by the Src-like inhibitor PP2 but not its negative control PP3 (figure 7.3A-D). Importantly, IL-5 potentiated the effect of 2-AG but not those of 5-KETE and prostaglandin D$_2$ (figure 7.1B). All

the following migration experiments were performed in the presence of 10 ng/mL IL-5 because 2-AG alone did not induce significant eosinophil chemotaxis. 2-AG induced a migration rate comparable to that induced by CCL11 (eotaxin-1), higher than that induced by arachidonic acid (AA), but lower than that induced by 5-KETE in IL-5–treated eosinophils (figure 7.1C). In contrast, the CB_1/CB_2 receptor agonist CP 55,940 and the selective CB_2 receptor agonist L-759,633 induced minimal eosinophil migrations; AA mimicked 2-AG only at 30 μM (figure 7.1D). When tested at a similar concentration, the 2-AG position isomer 1-AG was as potent as 2-AG whereas the effects of the endocannabinoid (and CB_2 agonist) arachidonyl-ethanolamide (AEA) and of 2-AG ether (nonhydrolysable 2-AG) were minimal (figure 7.1E). Human eosinophils express the CB_2 receptor (3, 4), and both CB_2 receptor antagonists tested in this study inhibited the effects of 2-AG but not those of 5-KETE (figure 7.1F). The contrasting effects of CB_2 agonists and antagonists suggested that additional mechanisms, apart from CB_2 receptor activation, were involved in the effects of 2-AG. Human eosinophils express the monoacyl-glycerol (MAG) lipase, which hydrolyzes 2-AG into AA (figure 7.4A) Consistent with this, 2-AG and AA, alone or in combination with platelet-activating factor (PAF), induced similar cysteinyl-leukotrienes and eoxin C_4 (EXC$_4$) biosynthesis (figure 7.2A-B). Concentration-response experiments in PAF-stimulated eosinophils unraveled an optimal 2-AG concentration of 1 μM (figure 7.4B). Noteworthy, the PAF-/2-AG–induced eicosanoid biosynthesis was blocked by the MAG lipase inhibitors methyl-arachidonoyl-fluorophosphonate and JZL-184 with IC_{50} values of ~3 and ~30 nM, respectively (figure 7.2C). Consequently, we assessed whether 2-AG hydrolysis inhibition affected eosinophil migration. MAG lipase inhibition partially blocked (~40%) the 2-AG–induced eosinophil migration (figure 7.2D) while having no effect on the 5-KETE–induced migration (figure 7.4C). The inability of other CB_2 agonists to mimic 2-AG (figure 7.1D), and the inhibitory effect of CB_2 receptor antagonists (figure 7.1F) and JZL-184 (figure 7.2D), suggested that both CB_2 receptor activation and AA were involved in the regulation of eosinophil migration by 2-AG. In this respect, the combination of the CB_1/CB_2 receptor agonist CP 55,940 and AA led to migration rates similar to those of 2-AG (figure 7.2B). We next assessed whether eicosanoids biosynthetic enzymes were involved in the effects of 2-AG. While 5-lipoxygenase or cyclooxygenase inhibitors did not affect eosinophil migration, the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) and the 15-lipoxygenase inhibitor PD146176 significantly decreased the 2-AG– but not the 5-KETE–induced eosinophil migration (figure 7.2F and figure 7.4D). Altogether these results indicate that the 15-lipoxygenase pathway is very likely involved in the regulation of eosinophil migration induced by 2-AG. However, we could not recapitulate the effect of 2-AG with 15(S)-hydroxyl-eicosatetraenoate, lipoxin A$_4$, EXC$_4$, or 15-(S)-hydroxyl-eicosatetraenoate-*sn1*-glycerol, alone or combined with CP 55,940 (not shown).

We provide herein evidence that 1) 2-AG alone induces minimal eosinophil chemotaxis; 2) the 2-AG–induced migration is blocked by CB_2 receptor antagonists; 3) the 2-AG–induced eosinophil migration is enhanced by

IL-5 in a Lyn-dependent manner; 4) 2-AG, but not AEA, is metabolized into eicosanoids by eosinophils; 5) the migration of eosinophils induced by 2-AG is sensitive to the MAG lipase inhibitor JZL-184; and 6) 15-lipoxygenase inhibitors block the effect of 2-AG on eosinophil migration. Noteworthy, the 2-AG–induced migration of eosinophils occurred at 2-AG concentrations that can be found in vivo (4). 2-AG was shown to induce a modest (~10%) and CB_2-dependent eosinophil migration in the presence of NDGA and in the absence of cytokine (3). Our data demonstrate that 2-AG alone induces minimal chemotaxis (~3%) but underscore that the 2-AG–induced eosinophil migration is complex and involves many mechanisms, notably cytokines, 2-AG hydrolysis, and 15-lipoxygenase metabolites. The inefficacy of natural (AEA) and synthetic CB_2 receptor agonists to mimic the effect of 2-AG indicates that the CB_2 receptor is required but not sufficient for the 2-AG–induced migration of eosinophils. Given the involvement of MAG lipase and 15-lipoxygenase in the 2-AG–induced migration, it is possible that CB_2 engagement on eosinophils activates these enzymes by yet to be documented mechanisms. IL-3, IL-5, and GM-CSF profoundly enhanced the 2-AG–induced eosinophil migration, and this was unique among the tested lipids (figure 7.1A-B). The Lyn inhibitor PP2 inhibited the effect of IL-5, which is in line with its rapid phosphorylation/activation in IL-5–treated eosinophils (6). Feltenmark et al. showed that IL-5 was the best stimulus for EXC4 biosynthesis during short incubation periods, indicating that IL-5 quickly regulates 15-lipoxygenase. Given that the 15-lipoxygenase inhibitors NDGA and PD146176 decreased the 2-AG–induced migration, it is possible that Lyn directly/indirectly regulates 15-lipoxygenase activity by phosphorylation. Forsell et al (7) and Brunnstrom et al (8) recently documented the production of novel 2-AG–derived 15-lipoxygenase metabolites. Thus, the 15-lipoxygenase-derived lipidome of human eosinophils is likely more complex than initially thought. The use of a mixture of 2-AG– and AA-derived 15-lipoxygenase metabolites from eosinophils might have been a better strategy to recapitulate the effect of 2-AG on the migration of eosinophils than testing individual 15-lipoxygenase metabolites. In this regard, we are planning to analyze the 2-AG– and AA-derived lipidomes of eosinophils linked to 15-lipoxygenase and perform migration assays using this lipidome. Finally, we cannot exclude that the inhibitors we used in this study might inhibit off targets.

Conclusion

In conclusion, this study confirms that the endocannabinoid 2-AG, in combination with IL-5, has the ability to activate and modulate eosinophil functional responses and, consequently, to play a significant role in the regulation of inflammatory processes such as asthma and possibly obesity.

Figures
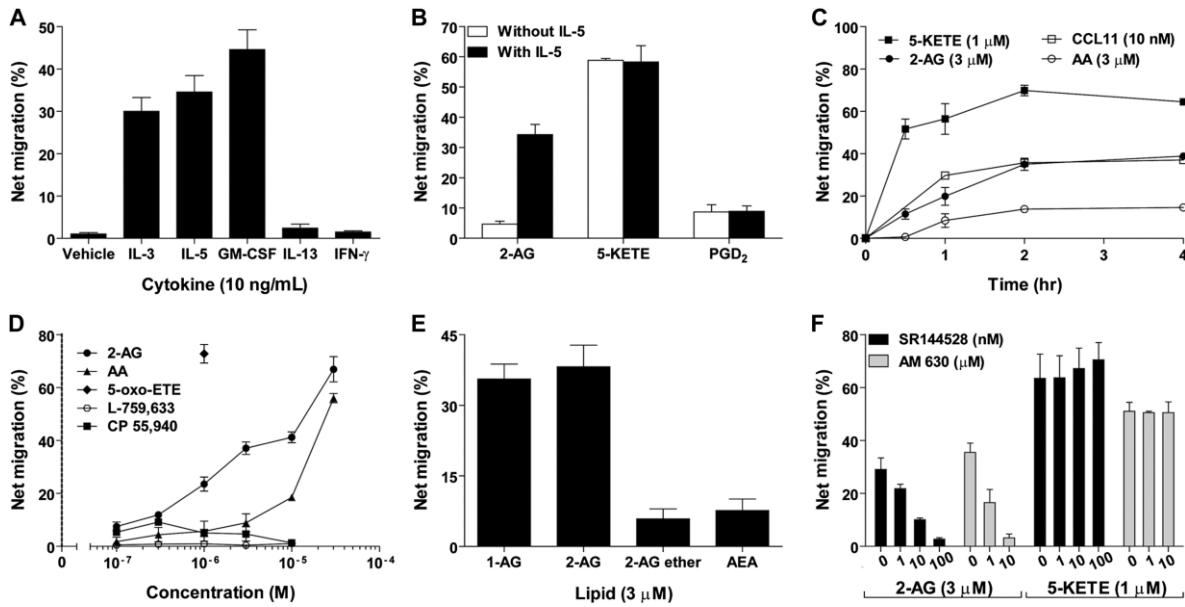
Figure 7.1. Impact of IL-5 and the CB2 receptor on the 2-AG–induced eosinophil migration. A-F, IL-5 (or cytokine)-treated eosinophils migrated for 2 hours unless stated otherwise. Eosinophils were treated or not with IL-5 and then lipid-induced migration was performed (Fig 1, B). Migration of IL-5–treated eosinophils was performed during the indicated times (Fig 1, C). Migration of IL-5–treated eosinophils in response to different agonists (Fig 1, D and E). IL-5–treated eosinophils were incubated for 10 minutes with SR144528 or AM630 and then lipid-induced migration was performed (Fig 1, F).

180
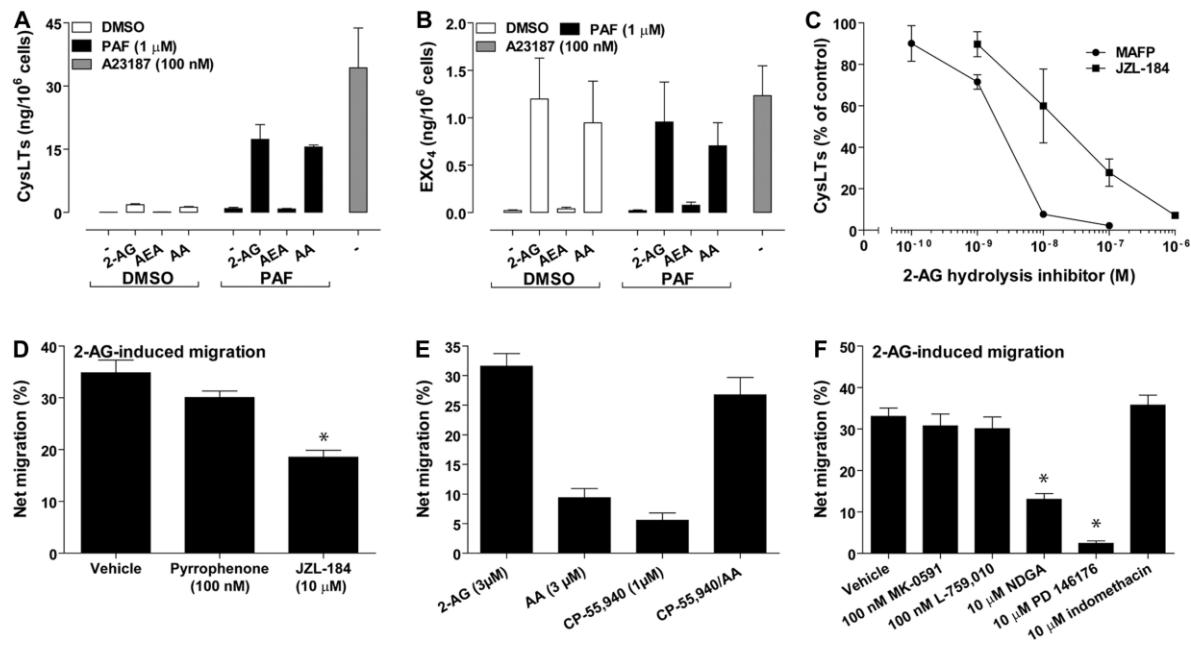
Figure 7.2. Metabolism of 2-AG by eosinophils and its role during migration. A and **B,** Eosinophils were stimulated with 1 μM of 2-AG, AA, or AEA, alone or in combination with PAF. **C,** Eosinophils were stimulated for 15 minutes with 1 μM of PAF and 2-AG in the presence or absence of the indicated inhibitors. **D** and **F,** Migration of IL-5–treated eosinophils in response to 3 μM 2-AG in the presence of different inhibitors. **E,** Migration of IL-5–treated eosinophils in response to the indicated agonists. *$P < .001$. *DMSO*, Dimethyl sulfoxide.
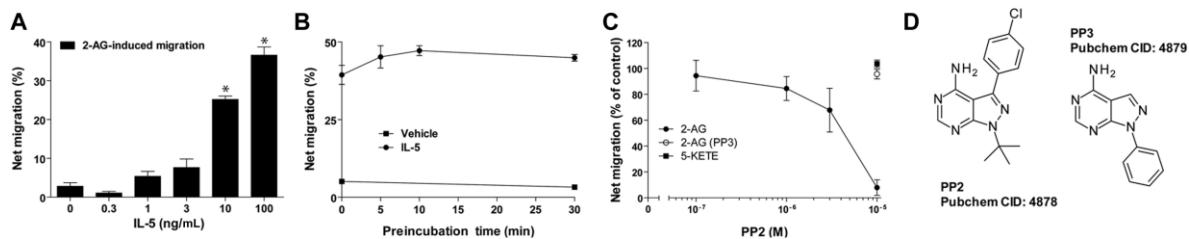
Figure 7.3. Impact of IL-5 and its signaling on the 2-AG–induced eosinophil migration. A, Eosinophils were treated for 10 minutes with IL-5 and then migration was performed for 2 hours. **B,** Eosinophils were treated with IL-5 for the indicated times and then migration was induced by 2-AG for 2 hours. **C,** Eosinophils were treated for 10 minutes with IL-5 (10 ng/mL) and then migration was induced with 2-AG or 5-KETE for 2 hours. PP2 or PP3 was added 10 minutes before the migration. **D,** Chemical structures of PP2 and PP3. *$P <$ .0001.
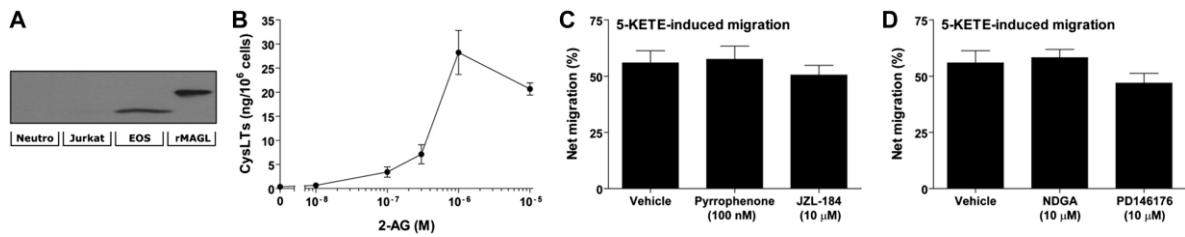
Figure 7.4. Involvement of metabolism for the 2-AG–mediated eosinophil migration. A, Immunoblot of MAG lipase with cell lysates from neutrophils, Jurkat T cells, eosinophils, or human recombinant His-tagged MAG lipase. **B,** PAF-stimulated eosinophils were incubated in the presence of the indicated concentration of 2-AG for 15 minutes. Cysteinyl-leukotriene biosynthesis was then assessed as described in Methods. **C,** Migration of IL-5–treated eosinophils in response to 1 μM of 5-KETE in the presence of the group IVA phospholipase A$_2$ pyrrophenone or the MAG lipase inhibitor JZL-184. **D,** Migration of IL-5–treated eosinophils in response to 1 μM of 5-KETE in the presence of compounds that inhibit the 15-lipoxygenase.

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Chapitre 7. The endocannabinoid metabolite prostaglandin E$_2$ (PGE$_2$)-glycerol inhibits human neutrophil functions: involvement of its hydrolysis into PGE$_2$ and EP receptors

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Résumé

Les endocannabinoïdes 2-arachidonoyl-glycérol (2-AG) et arachidonoyl-éthanolamide (AEA) exercent une variété d'effets pro- et anti-inflammatoires. Ces effets sont causés, en partie, par leur métabolisme par les cyclooxygénases (COX) et les lipoxygénase. Par exemple, le 2-AG et l'AEA peuvent être métabolisés par la COX-2 pour former les prostaglandines-glycérol (PG-G) et les prostaglandines-éthanolamides (PG-EA). Bien que la PGE_2 soit un suppresseur des fonctions des neutrophiles bien connu, les effets des prostaglandines dérivées des endocannabinoïdes sur les neutrophiles sont inconnus. Le but de cette étude était de définir la façon dont ces médiateurs modulent les fonctions des neutrophiles humains et de comprendre les mécanismes sous-jacents. Nos résultats ont démontré que la PGE_2-G, mais pas la PGE_2-EA, inhibe diverses fonctions effectrices des neutrophiles dont la biosynthèse de LTB_4, la production d'anion superoxyde, la migration induite par le LTB_4 et la libération de peptides antimicrobiens. Les effets de la PGE_2-G sont inhibés par un antagoniste des récepteurs EP_1 et EP_2 (AH-6809), mais pas par un antagoniste du récepteur EP_4 (ONO-AE2-227). L'effet inhibiteur de la PGE_2-G requiert son hydrolyse en PGE_2 par les neutrophiles et ne sont pas mimés par la PGE_2 sérinol amide, un analogue non hydrolysable de la PGE_2. Les effets de la PGE_2-G ont été complètement bloqués par deux inhibiteurs de sérine hydrolases, le MAFP et la palmostatine B. Deux autres composés, le JZL184 et le WWL113, ont partiellement prévenu l'effet inhibiteur de la PGE_2-G sur les fonctions des neutrophiles. Parmi les sept PGE_2-G hydrolases documentées, seules ABHD16A et ABHD12 ont été détectées au niveau protéique chez les neutrophiles humains. Nos résultats pharmacologiques, en combinaison avec nos données d'expression des PGE_2-G hydrolases, ne nous ont pas permis d'identifier une seule lipase responsable de l'hydrolyse de la PGE_2-G par les neutrophiles. En conclusion, nos résultats montrent que la PGE_2-G inhibe les fonctions des neutrophiles humains par le biais de son hydrolyse en PGE_2 et de l'activation du récepteur EP_2 par cette dernière. Ces données suggèrent que les neutrophiles pourraient moduler l'inflammation en altérant la balance entre les niveaux de PG-G et de PG in vivo.

Abstract

The endocannabinoids 2-arachidonoyl glycerol (2-AG) and *N*-arachidonoyl-ethanolamine (AEA) mediate an array of pro- and anti-inflammatory effects. These effects are related, in part, to their metabolism by eicosanoid biosynthetic enzymes. For example, AEA and 2-AG can be metabolized by cyclooxygenase (COX)-2 into prostaglandins (PG)-ethanolamide (EA) and PG-glycerol (G), respectively. While PGE_2 is a recognized suppressor of neutrophil functions, the impact of COX-derived endocannabinoids such as PGE_2-EA or PGE_2-G on neutrophils is unknown. This study's aim was to define the effects of these mediators on human neutrophil functions and the underlying cellular mechanisms involved. Our data show that PGE_2-G, but not PGE_2-EA, inhibits LTB_4 biosynthesis, superoxide production, migration, and antimicrobial peptide release. The effects of PGE_2-G were prevented by EP_1/EP_2 receptor antagonist AH-6809 but not the EP_4 antagonist ONO-AE2-227. The effects of PGE_2-G required its hydrolysis into PGE_2, were not observed with the non-hydrolyzable PGE_2-serinol amide, and were completely prevented by methyl-arachidonoyl-fluorophosphonate and palmostatin B, and partially prevented by JZL184 and WWL113. Although we could detect six of the seven documented PG-G hydrolases in neutrophils by qPCR, only ABHD12 and ABHD16A were detected by immunoblot. Our pharmacological data, combined with our protein expression data, did not allow us to pinpoint one PGE_2-G lipase and rather support the involvement of an uncharacterized lipase and/or of multiple hydrolases. In conclusion, we show that PGE_2-G inhibits human neutrophil functions through its hydrolysis into PGE_2, and by activating the EP_2 receptor. This also indicates that neutrophils could regulate inflammation by altering the balance between PG-G and PG levels in vivo.

Introduction

Acute and chronic inflammation are characterized by leukocyte infiltration, pro-inflammatory mediator production, and tissue destruction. Non-steroidal anti-inflammatory drugs have been long utilized to limit pain and inflammatory damages, notably by blocking the production of prostaglandins (PGs) and thromboxane. Moreover, COX-2 inhibitors, which prevent the biosynthesis of PGE_2, have been helpful at decreasing inflammation and inflammatory pain (1).

Historically, COX-2 was perceived as a pro-inflammatory enzyme, because its expression is induced by inflammatory stimuli and leads to PGE_2 synthesis. This was eventually challenged by Gilroy et al, who suggested that COX-2 could have anti-inflammatory properties (2). It was then shown in several murine models that COX-2 blockade worsens inflammation or delays its resolution, at least in mice (3-5). In addition to PGs, COX-2 participates in the synthesis of other lipids that modulate nociception and inflammation, notably PG-glycerol (PG-Gs) and PG-ethanolamides (PG-EAs). These endocannabinoid derivatives are products of the COX-2-dependent oxygenation of 2-arachidonoyl-glycerol and anandamide, respectively. PG-EAs, or prostamides, preferentially activate the prostamide receptor over PG receptors (6). In contrast, PGE_2-G can bind to several PGE_2 receptors, to a lesser extent than PGE_2 (7). Moreover, PGE_2-G induces key signaling events that are not mimicked by PGE_2 (8; 9).

PGE_2 is a well-established inhibitor of human neutrophil functions such as LTB_4 biosynthesis, reactive oxygen species (ROS) production and migration (10-14). These effects are the consequence of elevated cyclic adenosine 3'-5'-monophosphate (cAMP) and involve the EP_2 receptor. Given that PGE_2-G is produced by COX-2 and binds to some of the EP receptors (7), because PGE_2 inhibits neutrophil functions, and because PG-Gs were shown to regulate inflammation (15), we undertook experiments to assess whether PGE_2-G and PGE_2-EA would also modulate human neutrophil functions.

Materials and methods

Materials – 19-OH-PGB_2, 2-AG, AH6809, butaprost (free acid), CAY 10598, H-89, JZL184, L-902 688, MAFP, PGE_2, PGE_2-EA, PGE_2-G, PGE_2-serinol amide (SA), PGD_2-G, PGD_2-SA, $PGF_{2\alpha}$-G, sulprostone, tetrahydrolipstatin, WWL70, and the primary antibody for MAG lipase was purchased from Cayman Chemical (Ann Arbor, MI, USA). Thapsigargin was obtained from Tocris Bioscience (Ellisville, MO). DMSO was purchased from Sigma-Aldrich (St Louis, MO). Protease inhibitor cocktail tablets and adenosine deaminase (ADA) were purchased from Roche (Laval, QC, Canada). Aprotinin, leupeptin, and WWL113 were purchased from Sigma-Aldrich (St-Louis, MO, USA). DFP was purchased from BioShop Canada (Burlington, ON,

Canada). The HRP-linked anti-mouse IgG and anti-rabbit IgG secondary were obtained from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies for ABHD6, ABHD12, and PPT1 were purchased from Abcam (Toronto, ON, Canada). The LYPLA2 primary antibody was purchased from Abnova (Taipei City, Taiwan), the ABHD16A primary antibody was purchased from Thermo Fisher Scientific (Waltham, MA, USA) and the primary antibody for CES1 was purchased from R&D Systems (Minneapolis, MN, USA). PMSF, RO 20-1724, palmostatin B and the ECL detection kit were purchased from EMD Millipore (Billerica, MA, USA). The magnetic bead-conjugated anti-CD16 mAb and MACS were purchased from Miltenyi Biotec (Auburn, CA). HBSS and RPMI were obtained from Wisent Laboratories (St-Bruno, QC, Canada). Dextran and HPLC-grade methanol and acetonitrile were purchased from Fisher Scientific. ML349 was a generous contribution from Dr Lawrence Marnett (Vanderbilt University, Nashville, TN, USA). The HNP1-3 defensin ELISA kit was purchased from Hycult Biotech (Uden, Netherlands). ONO-AE2-227 was a generous gift from Ono Pharmaceutical (Osaka, Japan). Cytochrome C was obtained from Bio Basic (Amherst, NY, USA).

Ethics Committee Approval – This work required the use of human cells from healthy volunteers and was approved by our institutional ethics committee. All the experiments were conducted with the understanding and the signed consent of each participant.

Isolation of human neutrophils – Human venous blood was obtained from volunteers and collected in tubes containing K_3EDTA as anticoagulant. Eosinophil-depleted neutrophils were then isolated as described previously (16). In brief, platelet-rich plasma and erythrocytes were discarded by centrifugation and dextran sedimentation, respectively. Mononuclear cells were then separated from granulocytes by centrifugation on Lymphocyte Separation Medium cushions (Corning), and residual erythrocytes were removed from the granulocyte pellets by hypotonic lysis using sterile water. Neutrophils were separated from eosinophils using anti-CD16–coated magnetic beads, according to the manufacturer's instructions. The purity and viability of the resulting neutrophil suspensions were always ≥ 98%, as assessed by Diff Quik staining and trypan blue exclusion, respectively.

Removal of endogenous adenosine – To better mimic the fate of human neutrophils, adenosine deaminase (0.3 U/ml) was prepared and added 10 minutes before the addition of the stimuli in all experiments (17; 18).

Induction and analysis of LTB$_4$ biosynthesis – Pre-warmed human neutrophils suspensions (37°C, 5 × 10^6 cells/ml) in HBSS containing 1.6 mM CaCl$_2$ were incubated 5 minutes with PGE$_2$, PGE$_2$-G, PGE$_2$-EA, PGE$_2$-SA, PGD$_2$-G, PGD$_2$-SA, PGF$_{2\alpha}$-G, or the different EP receptor agonists (see figure legends for concentrations), then stimulated with either 3 μM 2-AG (5 minutes), 3 μM AA (5 minutes), or 100 nM

thapsigargin (10 minutes). In experiments where inhibitors were used, they were added 10 minutes before the addition of the stimulus. Incubations were stopped by adding 0.5 volume of a cold (-20°C) stop solution (MeOH/MeCN, 1/1 (V/V)) containing 12.5 ng of both 19-OH-PGB$_2$ and PGB$_2$ as internal standards. Samples were denatured overnight (-20°C), centrifuged (700 × g, 10 minutes) to eliminate the denatured proteins, then analyzed by reversed phase high-performance liquid chromatography (RP-HPLC) using an online extraction procedure (19). LTB$_4$, 20-COOH-LTB$_4$, 20-OH-LTB$_4$, and 5(S)-HETE were quantitated using PGB$_2$ as internal standard and are referred to as LTs.

Stimulation and analysis of superoxide anion release – Superoxide anion production by human neutrophils was assessed by cytochrome c reduction exactly as described previously (20). PGE$_2$, PGE$_2$-EA, PGD$_2$-G, PGE$_2$-G, PGF$_{2\alpha}$-G, PGD$_2$-SA, PGE$_2$-SA, or the EP receptor agonists were added 5 minutes before fMLP. EP receptor antagonists or serine hydrolase inhibitors were added 10 minutes before fMLP.

Migration assay – Migration assays were performed using 3 μm pore inserts (Becton Dickinson), as recommended by the manufacturer. In brief, 700 μl of pre-warmed (37°C) HBSS containing 1.6 mM CaCl$_2$ and 100 nM LTB$_4$ were added in the lower chamber and 200 μl of pre-warmed neutrophil suspensions (37°C, 2.5 × 10^6 cells/ml in HBSS containing 1.6 mM CaCl$_2$ and 5% (w/v) FBS) were added in the upper chamber of the transmigration apparatus. Neutrophils were allowed to migrate for two hours at 37°C. The upper chambers were then removed and migrated cells in the lower chamber of the migration apparatus were counted using a Scepter™ 2.0 handheld automated cell counter. In experiments where PGE$_2$, PGE$_2$-G and the type IV phosphodiesterase RO 20-1724 were utilized, they were added to both upper and lower chamber for 5 minutes before the addition of LTB$_4$ in the lower chamber of the migration assay.

Bacterial growth assays – *Escherichia coli* (*E. coli*) (American Type Culture Collection (ATCC) #25922) was grown overnight at 37°C in tryptic soy broth (TSB). The obtained culture was diluted (1:100) in fresh media and incubated at 37°C until an optical density of 0.5 at 600 nm was reached. 500 μl of the *E. coli* culture were then washed, suspended in sodium phosphate buffer at a final concentration of 1000 colony forming unit (UFC)/ml in sodium phosphate buffer. Freshly isolated human neutrophils (37°C, 20 × 10^6 cells/ml) in HBSS containing 1.6 mM CaCl$_2$ incubated for 15 minutes with 10 μM of cytochalasin B then activated with 1 μM 2-AG for 5 minutes. PGE$_2$ (1 μM) or PGE$_2$-G (1 μM) were added 5 minutes before the addition of 2-AG. Incubations were stopped by transferring the tubes in an ice-water bath. Samples were centrifuged (500 × *g*; 4°C; 5 minutes) and the resulting supernatants were mixed with the bacterial suspensions (1:1) and incubated for 4 hours on a rotating plate at 37°C. The mixtures then were diluted (1:300) and plated on Luria-Bertani (LB)-agar plates. Colonies were counted after the incubation of the agar plates overnight at 37°C.

Analysis of antimicrobial peptide release – Following cell stimulation, samples were rapidly centrifuged; the supernatants were collected and stored at -80ºC until further analysis. Quantitation of α-defensins in the supernatants was performed using a commercially available ELISA kit (Hycult, Uden, Netherlands), according to the manufacturer's instructions.

Analysis of receptor and lipase expression by qPCR - Total RNA extracts were prepared using TRIzol according to the manufacturer's instructions. cDNA was obtained by reverse transcription using the iScript™ Reverse Transcription Supermix from Bio-Rad, with an RNA input of 1 µg per reaction. For the analysis of EP receptor expression, qPCR assays were performed on a 7900 Fast-Real-Time PCR system (Applied Biosystems) using customs RT$_2$ Profiler qPCR Multiplex Array Kit (Qiagen). For the analysis of the dfferent endocannabinoid hydrolases, qPCR was performed with the SsoAdvanced™ Universal SYBR® Green Supermix on a CFX96 thermal cycler (Bio-Rad; Mississauga, ON, Canada) according to manufacturer's instructions. Primers for the 18S housekeeping gene were obtained from Qiagen (QT00199367). Primers for all other target genes were designed using the Primer-BLAST tool and synthetized by IDT (Coralville, IA, USA). Primer sequences are as followed (5'$\rightarrow$ 3'): ABHD6 forward CATCTGGGGGAAACAAGACCA, ABHD6 reverse TTTCCATCACTACTGAGTGCCC, ABHD12 forward CGGATACTGAGGGAATTCCTGG, ABHD12 reverse AGGTCTTCATGCTTCCTTCCC, MAG lipase forward TGCCTACCATGTTCTCCACA, MAG lipase reverse CCTCCAGTTATTGCAGTCTGG, CES1 forward TGCCTTTATCCTGGCCACTC, CES1 reverse CTTGGGTGCACATAGGAGGG, PPT1 forward TGGCATGGGATGGGTGTTTT, PPT1 reverse GGCGTTCCTGAACAACTTTGG, LYPLA2 forward AAGAAGGCAGCAGAGAACATC, LYPLA2 reverse CTCCCAGCACGATTCGATTG, ABHD16A forward CCCCCGGCTCTACAAAATCTAC, ABHD16A reverse GATAGTACGTATCCCAGGAGCTG. Results are expressed in relative quantification normalized to the 18S rRNA as reference gene with the $2^{-\Delta\Delta CT}$ method.

Liquid chromatography - tandem mass spectrometry (LC-MS/MS) analyses – For the analysis of PGE$_2$-G, PGE$_2$ and D$_4$-PGE$_2$, incubations were stopped by the addition of one volume of cold (-20°C) MeOH and samples were kept frozen until further processing. Samples were centrifuged (700 × g; 10 minutes) after addition of 2 ng of the internal standards (D$_4$-PGE$_2$ and D$_4$-PGE$_2$-G). The supernatants were diluted with water to a final MeOH concentration of 10% and loaded on solid phase extraction cartridges (Strata-X Polymeric Reversed Phase, 60 mg/1ml, Phenomenex). Cartridges were washed with 2 ml water then lipids were extracted with 1 ml MeOH. The eluates were dried down and reconstituted in 40 µl of HPLC solvent A (8.3 mM acetic acid buffered to pH 5.7 with ammonium hydroxide) and 20 µl of solvent B (MeCN/MeOH, 65/35, v/v). A 25 µl aliquot was injected onto an RP-HPLC column (Ascentis C18, 150 × 2.1 mm, 5 µm, Supelco) eluted at a

flow rate of 200 µl/min with a linear gradient from 45% solvent B, increased to 75% in 12 min, from 75% to 98% in 2 min, and held for 10 min at 98% B before re-equilibration to 45% B in 10 min. The HPLC system was directly interfaced into the electrospray source of a triple quadrupole mass spectrometer (API 3000, AB Sciex) and mass spectrometric analysis was performed in the negative ion mode using multiple reaction monitoring (MRM) for the specific mass transitions of PGE_2 (m/z 351.3→271.2) and D_4-PGE_2 (m/z 355.3→275.2) and in the positive ion mode for the ammonium adducts $[M+NH_4]^+$ of PGE_2-G (m/z 444.4→391.3) and D_4-PGE_2-G (m/z 448.4→395.3). Quantitation was performed using standard isotope dilution curves, as previously described (21).

In experiments in which concentrations of JZL-184 higher than 1 µM were utilized, LTB_4 biosynthesis was analyzed by LC-MS/MS and multiple reaction monitoring. In brief, incubation were stopped by the addition of one volume of cold (-20°C) MeOH and samples were kept frozen until further processing. Samples were centrifuged (700 × g; 10 minutes) after addition of 2 ng of the internal standard (D_4-PGB_2). The supernatants were diluted with water to a final MeOH concentration of 10% and loaded on solid phase extraction cartridges (Strata-X Polymeric Reversed Phase, 60 mg/1ml, Phenomenex). Cartridges were washed with 2 ml water then lipids were extracted with 1 ml MeOH containing 2% formic acid. The eluates were dried down and reconstituted in 25 µl of HPLC solvent A (0.05% Formic acid in water) and 25 µl of solvent B (0.05% Formic acid in acetonitrile). A 25 µl aliquot was injected onto an HPLC column (Kinetec C8, 150 × 2.1 mm, 2.6 µm, Phenomenex) eluted at a flow rate of 400 µl/min with a discoutinuous gradient (from 10% solvent B, increased to 25% in 15 min, from 25% to 35% in 5 min, from 35% to 75% in 10 min, from 75% to 95% in 0.1 min and held for 5 min at 95% B before re-equilibration to 10% B in 5 min. The HPLC system was directly interfaced into the electrospray source of a triple quadrupole mass spectrometer (Shimadzu 8050) and mass spectrometric analysis was performed in the negative ion mode using multiple reaction monitoring (MRM) for the specific mass transitions of D_4-PGB_2 (m/z 337.20→179.05), LTB_4 (m/z 351.2→195.1), 20-OH-LTB_4 (m/z 351.2→195.1), 20-COOH-LTB_4 (m/z 365.3→169.1), and 5-HETE (m/z 319.2→115.1). Quantitation was performed using standard isotope dilution curves, as previously described (21).

Analysis of proteins by immunoblot - Cells were lysed by sonication at 4°C in sucrose buffer containing 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF, 3 mM DFP and 1 tablet protease inhibitor cocktail (for 10 ml of buffer). Laemmli sample buffer (62.5 mM TRIS-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.01% bromophenol blue) was added to sonicated cell lysates and samples were boiled for 10 minutes. Buffer volumes were adjusted to obtain a final concentration of 2 x 10^6 cells/50 µl of lysate for all samples. Proteins were separated by SDS-PAGE on 12% polyacrylamide gels and transferred onto PVDF membranes. Transfer efficiency and equal protein loading were verified by Ponceau Red staining. Membranes were placed in TBS-Tween buffer

(25 mM Tris-HCl [pH 7.6], 0.2 M NaCl, 0.15% Tween 20) containing 5% non-fat dried milk (w/v) for 30 minutes at room temperature, then probed with the primary antibody (4°C, overnight). The membranes were revealed by chemiluminescence using a HRP-coupled secondary antibody and an ECL detection kit.

Statistical analyses – The effect of the lipase inhibitors on PGE_2-G hydrolysis was analyzed with GraphPad Prism 6. The software was used to perform one-way ANOVA with Dunnett's test. P values of < 0.05 were considered significant.

Results

PGE_2-G inhibits human neutrophil functions

We first performed a series of experiments in which we assessed the impact of PGE_2, PGE_2-G and PGE_2-EA on LT biosynthesis. A 5-minute pre-treatment of neutrophils with PGE_2-G, but not PGE_2-EA, prevented LTB_4 biosynthesis by thapsigargin-stimulated neutrophils in a concentration-dependent manner (figure 8.1A). Under that experimental setting, the inhibitory effect of PGE_2-G on LTB_4 biosynthesis was less potent than that observed with PGE_2 by one order of magnitude (IC_{50} of 3 nM and 30 nM for PGE_2 and PGE_2-G, respectively). A similar pattern was obtained when we assessed ROS production induced by fMLP (figure 8.1B). PGE_2-G also inhibited the migration of human neutrophils *ex vivo*, although this required a higher concentration (10 µM) and the use of the type IV phosphodiesterase inhibitor RO 20-1724 (Figure 8.1C). Finally, PGE_2-G and PGE_2 also inhibited the ability of 2-AG-activated neutrophil supernatants to kill *E. coli* (figure 8.1D). This inhibitory effect of PGE_2 and PGE_2-G correlated with an inhibition of antimicrobial peptide release by 2-AG- or fMLP-stimulated neutrophils (Figure 8.1E). Altogether, the results presented in figure 8.1 indicate that in contrast to PGE_2-EA, PGE_2-G exerts potent inhibitory effects on human neutrophil functions. Of note, neither PGE_2-G or PGE_2-EA stimulated any of the functional responses investigated above (data not shown).

Involvement of EP receptors in the inhibitory effects of PGE_2 and PGE_2-G

PGE_2 mediates its effects by activating the EP receptors 1 to 4. We thus analyzed EP receptor expression in human neutrophils by qPCR array and confirmed that they mainly express the EP_2 and EP_4 receptors (figure 8.2A). To establish the contribution of the EP_2 and EP_4 receptors in the inhibitory effect of PGE_2-G and PGE_2 on human neutrophils, we performed experiments with the EP_1/EP_2 receptor antagonist AH-6809 and the EP_4 antagonist ONO-A2E-227. The inhibitory effect of PGE_2 and PGE_2-G on the fMLP-induced ROS production was completely prevented by the EP_1/EP_2 receptor antagonist AH-6809 (figure 8.2B). In contrast, the EP_4 antagonist ONO-A2E-227 did not prevent the inhibitory effects of PGE_2 or PGE_2-G. Of note, the EP_2 receptor agonist butaprost mimicked the effects of PGE_2 and PGE_2-G on neutrophil activation as previously described (22), whereas the $EP_{1/3}$ agonist sulprostone had no effect (data not shown), supporting the involvement of the

EP$_2$ receptor in the effect we observed. Finally, we confirmed that like those of PGE$_2$, the inhibitory effects of PGE$_2$-G were prevented by the PKA inhibitor H-89, underscoring similar downstream signaling events for both lipids (figure 8.2C).

Hydrolysis of PGE$_2$-G into PGE$_2$ by human neutrophils

In contrast to PGE$_2$, which binds to all EP receptors, PGE$_2$-G only binds to the EP$_1$, EP$_3$, and the EP$_4$ receptors (7). Given that PGE$_2$-G inhibits neutrophil functions in an EP$_2$-dependent manner, we investigated whether PGE$_2$-G was hydrolyzed into PGE$_2$ by human neutrophils. As shown in figure 8.3A, the incubation of human neutrophils with 300 nM PGE$_2$-G resulted in a time-dependent decrease in its levels (half-life of ~60 minutes) and a concomitant buildup of PGE$_2$. To confirm that this PGE$_2$ buildup originated from PGE$_2$-G hydrolysis rather than *de novo* biosynthesis, we incubated neutrophils with D$_4$-PGE$_2$-G. We observed a time-dependent increase of D$_4$-PGE$_2$ levels, while those of PGE$_2$ did not change (figure 8.3B), demonstrating that PGE$_2$-G is hydrolyzed into PGE$_2$ by neutrophils. In agreement with the hydrolysis of PGE$_2$-G into PGE$_2$ in our neutrophil suspensions, the inhibitory effect of 300 nM PGE$_2$-G occurred over time, and reached its maximal effect after 5 minutes of pre-incubation (figure 8.3C), which corresponds to the buildup of mid-nM concentrations of PGE$_2$ (figure 8.3A). In contrast, PGE$_2$ and the EP$_2$ receptor agonist butaprost did not require any pre-incubation time to inhibit the AA-induced LT biosynthesis (figure 8.3C). Altogether, this suggests that PGE$_2$-G is hydrolyzed into PGE$_2$ and that PGE$_2$ mediates the inhibitory effects of PGE$_2$-G on human neutrophil functions. Noteworthy, PGE$_2$, PGE$_2$-G and PGE$_2$-EA did not modulate the expression of COX-2 or mPGES-1, the main proteins involved in PGE$_2$ biosynthesis in neutrophils (data not shown; (23)).

PG-Gs can by hydrolyzed to some extent by several lipases that are more or less sensitive to lipase inhibitors (table 8.1). In this respect, we assessed, by qPCR and immunoblot, which of these hydrolases are expressed by freshly isolated neutrophils. We were able to detect, by qPCR, the mRNA for all of these lipases, with the exception of CES1. In contrast, our immunoblot data, which includes a positive control for each target, indicates that only ABHD12 and ABHD16A are expressed at a level that allows their detection with this technique (figure 8.4). As for LYPLA2, we found mRNA levels comparable to those of the documented positive control, the MDA-231 cell line (24). However, we were unable to detect the protein in our neutrophil lysates, despite getting a strong band for MDA-231 cells. Altogether, these experiments underscore that the expression of PG-G-hydrolyzing lipases is limited to ABHD12 and ABHD16A in neutrophils.

In an attempt to pinpoint whether ABHD12 and ABHD16A are involved in the hydrolysis of PGE$_2$-G into PGE$_2$, we next undertook experiments using numerous hydrolase inhibitors and assessed if they could prevent the inhibitory effect of PGE$_2$-G on human neutrophils. Knowing that neutrophils display a strong 2-AG-hydrolyzing

activity that is involved in the 2-AG-induced LTB$_4$ biosynthesis (25), we also assessed the impact of these inhibitors on that biosynthetic pathway, in order to compare the pharmacological profiles of both hydrolytic reactions. Palmostatin B, JZL184, and MAFP were the only compounds we tested that completely inhibited the 2-AG-induced LTB$_4$ biosynthesis in a concentration-dependent fashion (figure 8.5A-B). In contrast, although the effect PGE$_2$-G was sensitive to MAFP and palmostatin B, it was only partially sensitive to WWL113 and JZL184 at higher concentrations (figure 8.5C). We obtained essentially similar results when we attempted to prevent the inhibitory effect of PGE$_2$-G on thapsigargin-induced LTB$_4$ biosynthesis (data not shown). In addition to showing these effects on neutrophil functions, we sought to confirm that the inhibitors indeed prevented PGE$_2$-G hydrolysis into PGE$_2$. We thus incubated neutrophils with PGE$_2$-G for 10 minutes, in presence of 10 µM MAFP, palmostatin B, MAFP or WWL113 and measured the accumulation of PGE$_2$ in supernatants by LC-MS/MS (figure 8.5E). We found that MAFP, JZL184 and palmostatin B almost completely blocked the buildup of PGE$_2$, whereas WWL113 had a partial, statistically significant inhibitory effect ($p < 0.001$). Of note, none of the inhibitors prevented the inhibitory effects of PGE$_2$ on the fMLP-induced ROS production or the AA-induced LTB$_4$ biosynthesis (data not shown).

Impact of other PG-Gs and their non-hydrolysable analogs on neutrophil functions

Finally, we investigated if the inhibitory effect of PGE$_2$-G is mimicked by other PG-Gs. We found that like PGE$_2$-G, PGD$_2$-G also inhibits the AA-induced LT biosynthesis as well as the fMLP-induced ROS production, while PGF$_{2\alpha}$-G was ineffective (figure 8.6). Importantly, the structurally similar but non hydrolysable PGE$_2$-SA and PGD$_2$-SA had no effect, again underscoring the importance of PG-G hydrolysis for their inhibitory effect to occur.

Discussion

Endocannabinoids have been classified as anti-inflammatory lipids, mainly because of the pro-inflammatory state that CB$_1$/CB$_2$ deficient mice usually display in experimental models of inflammatory disease (26; 27). However, AEA and 2-AG do not only modulate leukocyte functions by activating the CB receptors, but also through their numerous metabolites, notably eicosanoids, prostamides, and PG-Gs (26; 28). The cellular and molecular mechanisms involved in the immunomodulatory effects of prostamides and PG-Gs remain ill defined. In this study, we provide evidence that **1)** the 2-AG metabolites PGE$_2$-G and PGD$_2$-G inhibit human neutrophil functions; **2)** The effects of PGE$_2$-G and PGD$_2$-G require their hydrolysis into PGE$_2$ and PGD$_2$, respectively; **3)** the PGE$_2$-G hydrolases ABHD12 and ABHD16A were detected by qPCR and immunoblot in human neutrophils; **4)** the hydrolysis of PGE$_2$-G into PGE$_2$ likely involves more than one hydrolase; and **5)** the effects of PGE$_2$-G are blocked by the EP$_2$ receptor antagonist AH-6809 and the PKA inhibitor H-89.

A limited number of studies have previously evaluated the bioactivity of COX-2 metabolites of endocannabinoids. Hu *et al.* observed that in rats, PGE_2-G and PGE_2 had similar effects on NF-κB activity, mechanical allodynia and thermal hyperalgesia (29). Interestingly, the cocktail of EP receptor antagonists they used completely blocked the effects of PGE_2, but only partially blocked the effects of PGE_2-G, suggesting different mechanisms of action. In this regard, Nirodi *et al.* observed that PGE_2-G (but not PGE_2) induces a quick, dose-dependent Ca^{2+} mobilization in RAW264.7 cells (7). These results indicate that PGE_2 and PGE_2-G can exert either similar or opposite effects, a phenomenon that is likely attributable to differential patterns of EP_{1-4} and PG-G receptor expression and activation.

Our data shows that PGE_2-G, but not PGE_2-EA, inhibits every neutrophil functions we tested in a similar fashion than PGE_2 (figure 8.1). The EP_2 antagonist AH-6809 (10 µM) and the PKA inhibitor H-89 prevented the inhibitory effects of both PGE_2 and PGE_2-G, suggesting the involvement of EP_2 and PKA (figure 8.2). Given that PGE_2-G has some binding affinity for EP_4 but practically none for EP_2 (7), this indicates that the EP_2-dependent effects we observed is not caused by PGE_2-G but is rather the results of PGE_2-G hydrolysis into PGE_2. This is supported the fact that: **1)** PGE_2-G is hydrolyzed into PGE_2 by neutrophils (figure 8.2); **2)** the non-hydrolysable version of PGE_2-G, PGE_2-SA, does not inhibit human neutrophils (figure 8.5); and **3)** the effect of PGE_2-G is mediated by the only EP receptor not activated by PGE_2-G (figure 8.2).

PGE_2-EA and PGE_2-G are relatively stable in biological systems. Indeed, in human plasma, PGE_2-EA undergoes slow dehydration/isomerisation into PGB_2-EA, while PGE_2-G is hydrolyzed with a half-life of over 10 minutes (30). However, the enzymatic pathways involved in the hydrolysis of PGE_2-G into PGE_2 remain unclear. Of the seven candidate lipases that were reported to hydrolyze PGE_2-G (table 8.1), six were detected by qPCR, but only two were detected by qPCR and immunoblot, namely ABHD12 and ABHD16A (figure 8.4). Of note, we were unable to detect ABHD6, MAG lipase, PPT1 and LYPLA2 in neutrophils by immunoblot, despite the presence of mRNA. Given that our experiments included documented positive controls that did yield a band at the expected size, we can conclude that the antibodies we used are reliable in this setting. It is possible, however, that our failing to detect these lipases at the protein level was caused by artifacts during cell lysis and protein denaturation, even though we tried three different lysis methods (sonication, immediate solubilization in boiling Laemmli buffer, and hypotonic lysis) which yielded similar results. While we also found LYPLA2 mRNA in cells that contaminate our neutrophil suspensions (eosinophils and monocytes), we could not detect any protein from those two cell types either (data not shown). This raises the interesting possibility that perhaps a miRNA or another post-transcriptional modification prevents the translation of the LYPLA2 mRNA we detected.

We also utilized numerous inhibitors (summarized in table 8.1) in order to establish the pharmacological profile linked to the hydrolase(s) involved. MAFP, palmostatin B and JZL184 were potent inhibitors of 2-AG hydrolysis with IC_{50} values in the mid-nM range, as assessed by 2-AG-induced LTB_4 biosynthesis (25). Furthermore, WWL70, WWL113, tetrahydrolipstatin, and ML349 did not prevent the hydrolysis of 2-AG. In contrast, the inhibitory effect of PGE_2-G was completely prevented by MAFP and palmostatin B, and partially prevented by micromolar concentrations of JZL184 and WWL113. WWL70, tetrahydrolipstatin, and ML349 did not have any effect. This suggests that the lipases involved in 2-AG and PGE_2-G hydrolysis are likely different. In addition, none of the documented PGE_2-G hydrolases match the pharmacological profile we found. Thus, the data we gathered did not allow us to confirm that one enzyme among our candidates (table 8.1) is responsible for the hydrolysis of PGE_2-G by human neutrophils. Therefore, it is likely that the PGE_2-G hydrolytic activity that we observed is catalyzed by more than one lipase, and/or by a lipase that has yet to be characterized.

A recent study showed that the inhibition of 2-AG hydrolysis led to decreased inflammation in mice (15). This was due, at least in part, to the endocannabinoid metabolite PGD_2-G, which prevented the production of IL-1β Of note, PGE_2-G and $PGF_{2\alpha}$-G did the opposite. Thus, it is possible that PG-Gs regulate inflammation through multiple mechanisms of actions and thus participate in the inflammatory process in a coordinated and timely manner. Our data indicate that human neutrophils have the ability to hydrolyze PG-Gs, notably PGE_2-G and PGD_2-G. While our data support that PG-G hydrolysis leads to PG production and dampens neutrophil functions, it remains possible that neutrophils prevent the anti-inflammatory effects of PG-Gs by eliminating those putative pro-resolving mediators in vivo.

In conclusion, we provide clear evidences that PGE_2-G, but not PGE_2-EA, inhibits numerous functions of human neutrophils in a concentration-dependent manner similar to PGE_2. This inhibitory effect requires the hydrolysis of PGE_2-G into PGE_2, the subsequent activation of the EP_2 receptor and PKA. These results also support the view that the anti-inflammatory effects related to the endocannabinoid 2-AG might be due, at least in part, to its metabolism by the COX pathway.

Abbreviations

2-AG, 2-arachidonoyl-glycerol; **AA**, arachidonic acid; **ADA**, adenosine deaminase; **AEA**, arachidonoyl-ethanolamide; **cAMP**, cyclic adenosine 3'-5'-monophosphate; **EA**, ethanolamide; **G**, glycerol; **LT**, leukotriene; **MAFP**, methyl arachidonoyl fluorophosphonate; **PG**, prostaglandin; **PKA**, cAMP-dependant protein kinase; **ROS**, reactive oxygen species; **SA**, serinol-amide; **THC**, (-)-Δ-tetrahydrocannabinol.

Figures
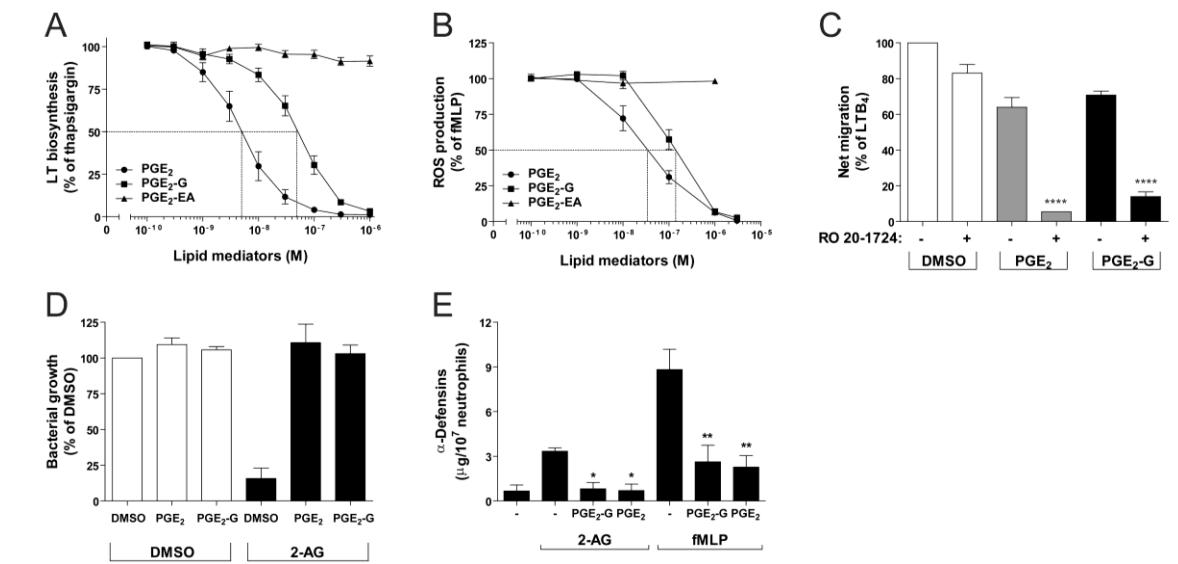
Figure 8.1. Impact of PGE$_2$-G on human neutrophil functions. A) Pre-warmed neutrophils suspensions were treated with PGE$_2$, PGE$_2$-G, or PGE$_2$-EA at the indicated concentrations for 5 minutes, and then stimulated with 100 nM thapsigargin for 10 minutes. Samples were then processed and analyzed for LTB$_4$ biosynthesis by HPLC, as described in *Methods*. **B)** Neutrophil suspensions were treated with PGE$_2$, PGE$_2$-G, or PGE$_2$-EA at the indicated concentrations for 5 minutes, and then stimulated with 100 nM fMLP for 10 minutes. ROS production was determined as described in *Methods*. **C)** Human neutrophil suspensions were added to the upper chambers of the transmigration apparatus, and neutrophils were allowed to migrate toward 30 nM LTB$_4$ for 2 hours into the lower chambers, as described in *Methods*. RO 20-1724 (10 µM), PGE$_2$ (10 µM), PGE$_2$-G (10 µM), or vehicle were added to the neutrophil suspensions and the lower chamber medium 5 minutes before the addition of LTB$_4$ in the lower chamber. **D, E)** Pre-warmed neutrophils suspensions were treated with 3 µM of PGE$_2$ or PGE$_2$-G for 5 minutes before stimulation with 3 µM 2-AG or 100 nM fMLP for another 5 minutes. Incubations were stopped and samples were processed **D)** to assess *E. coli* killing or **E)** to measure antimicrobial peptide release by ELISA, as described in *Methods*. **A-E)** Results are the mean (± SEM) of at least 3 individual experiments, each performed in duplicate. **C) ****** p < 0.0001 vs DMSO + RO 20-1724. **E) *** p < 0.05 vs 2-AG alone; ****** p < 0.01 vs fMLP alone.
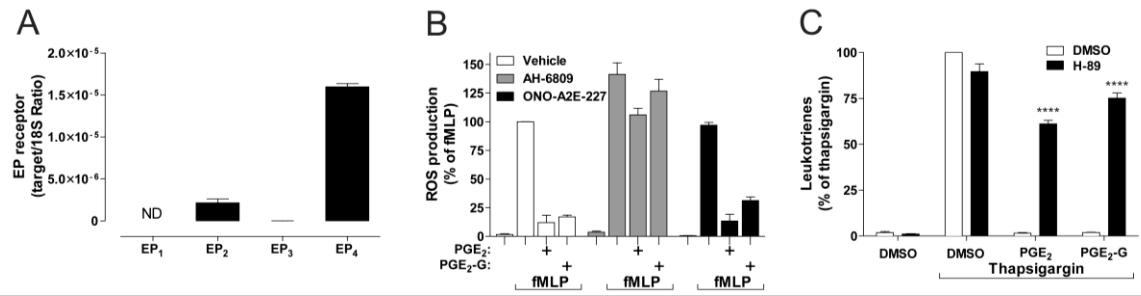
Figure 8.2: Involvement of EP receptors on the effect of PGE$_2$-G on neutrophils. A) mRNA was extracted from freshly isolated neutrophils and qPCR reactions were done as described in methods. Results are expressed in relative quantification, with 18S rRNA as a housekeeping gene using the 2$^{-\Delta CT}$ method. **B)** Neutrophil suspensions were treated with 1 µM PGE$_2$ or PGE$_2$-G for 5 minutes then were stimulated with 100 nM fMLP for 10 minutes. ROS production was determined as described in *Methods*. AH6809 (10 µM) or ONO-AE2-227 (10 µM) were added 5 minutes before PGE$_2$ or PGE$_2$-G. **C)** Freshly isolated neutrophils suspensions were treated with the PKA inhibitor H-89 (10 µM), then with DMSO, PGE$_2$, or PGE$_2$-G, and finally stimulated with 100 nM thapsigargin for 10 minutes. H-89 and PGE$_2$/PGE$_2$-G were respectively added 10 and 5 minutes before the addition of thapsigargin. LT biosynthesis was analyzed as described in *Methods*. **A-C)** Results are the mean (± SEM) of at least 3 individual experiments, each performed in duplicates. **C)** **** p < 0.0001 vs PGE$_2$ or PGE$_2$-G without H-89.
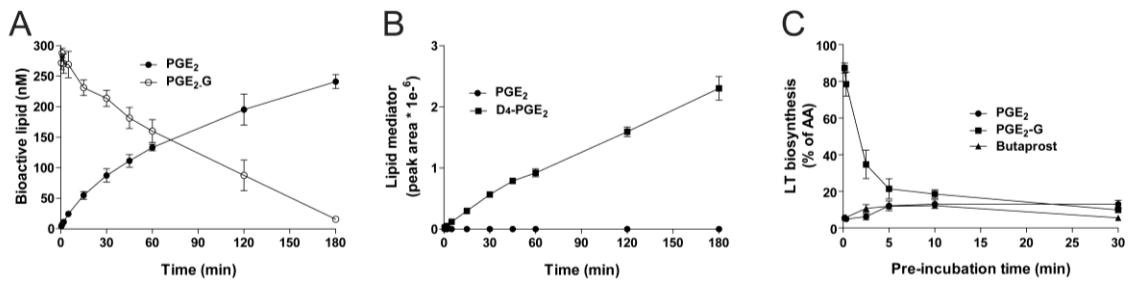
Figure 8.3: Hydrolysis of PGE₂-G into PGE₂ by neutrophils. Pre-warmed neutrophil suspensions were treated with 300 nM of **A)** PGE₂-G or **B)** D₄-PGE₂-G during the indicated time. Incubations were stopped with 0.5 ml ice-cold MeOH containing 2 ng of D₄-PGE₂ and/or D₄-PGE₂-G as an internal standard. Lipid extractions and analyses by LC-MS/MS were performed as detailed in *Methods*. **C)** Pre-warmed neutrophil suspensions were treated with PGE₂ (300 nM), PGE₂-G (300 nM), or butaprost (3 µM) for the indicated times before being stimulated with 100 nM thapsigargin for 10 minutes. Samples were analyzed for LT biosynthesis as described in *Methods*. Data are the mean (± SEM) of at least 3 individual experiments, each performed in duplicate.
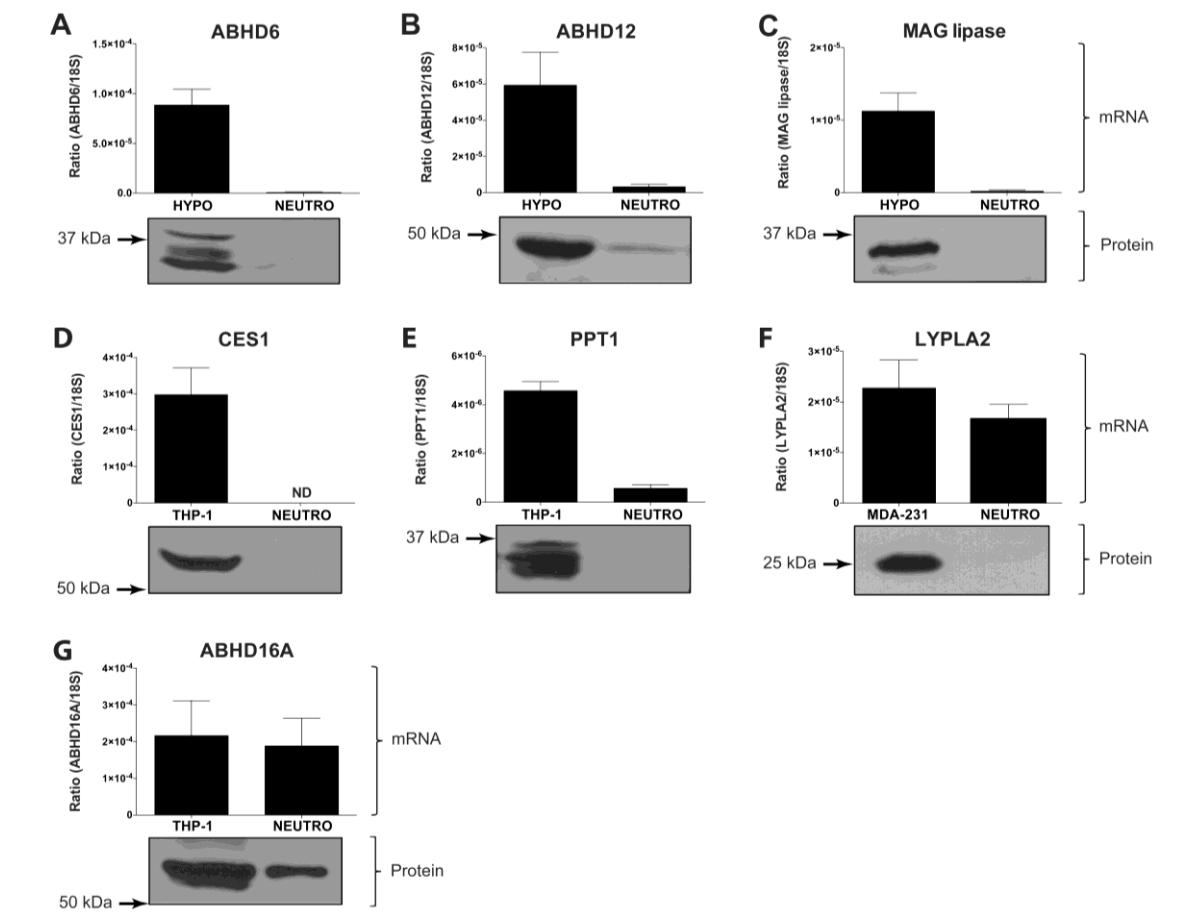
Figure 8.4: Expression of documented PGE₂-G hydrolases in human neutrophils. Each graph and immunoblot shows data for a documented positive control (left) and human neutrophils (right). mRNA was obtained from tissues and cells with TRIzol and qPCR were performed as described in *Methods*. Results are expressed in relative quantification normalized to the 18S rRNA as reference gene with the $2^{-\Delta CT}$ method. For immunoblots, cells or hypothalamus samples were disrupted and analyzed as described in *Methods*. The input per well is the equivalent of 2 million cells for neutrophils and cell lines, and 30 µg of protein per well for hypothalamus lysates (HYPO). The qPCR data is the mean (± SEM) of at least 4 experiments, and the western blotting images are representative of 3 separate experiments.
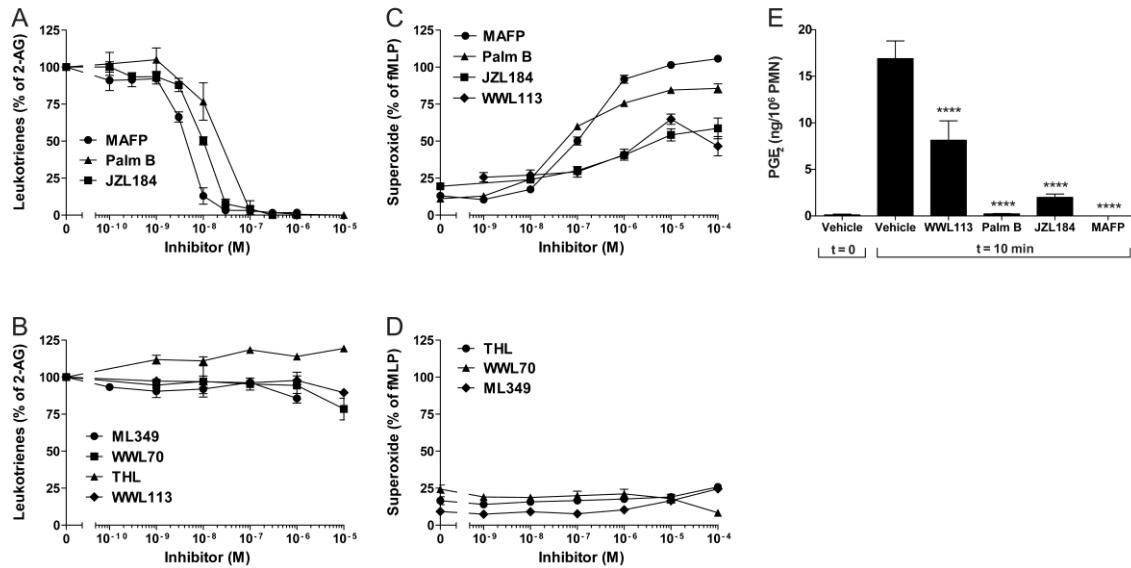
Figure 8.5. Pharmacological profiles of 2-AG and PGE$_2$-G hydrolysis in human neutrophils. A, B) Pre-warmed neutrophil suspensions were treated with the different inhibitors 5 minutes prior to the addition of 3 µM 2-AG for 5 minutes. Incubations were then stopped and LTB$_4$ biosynthesis was analyzed as indicated in Methods. **C, D**) Pre-warmed neutrophil suspensions were treated with the different inhibitors 5 minutes prior to the addition of 1 µM PGE$_2$-G for 5 minutes. Cells then were stimulated with 100 nM fMLP for 10 minutes. **E**) Pre-warmed neutrophil suspensions were treated with 10 µM of the various inhibitors during 5 minutes before adding 1 µM PGE$_2$-G for 10 minutes. Incubations were stopped and PGE$_2$ levels were measured by LC-MS/MS, as described in Methods. **A-E**) Data are the mean (± SEM) of at least 3 independent experiments performed in duplicate. **E**) **** $p < 0.0001$ vs vehicle (t = 10 min).
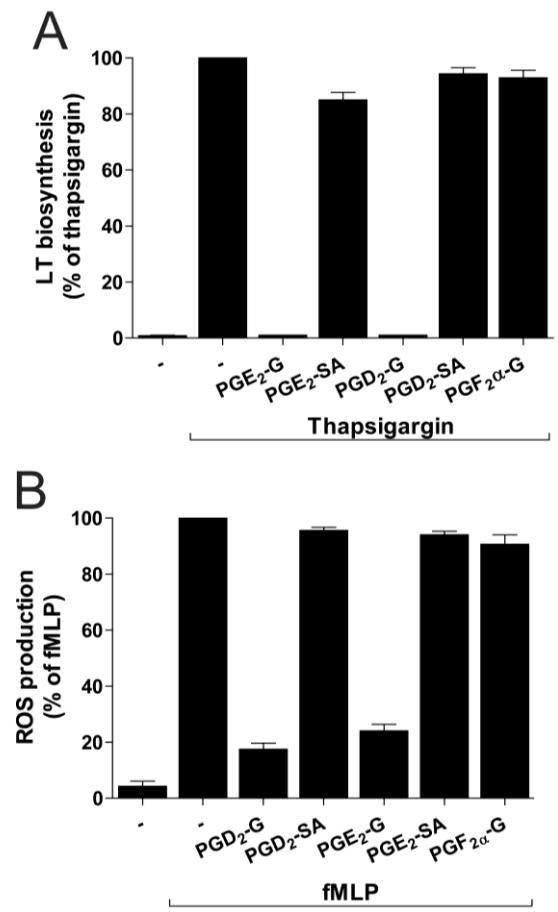
Figure 8.6. Impact of other PG-Gs and their non-hydrolysable analogs on neutrophil functions. A) Pre-warmed neutrophils suspensions were treated with 1 μM of PGE$_2$-G, PGE$_2$-SA, PGD$_2$-G, PGD$_2$-SA, or PGF$_{2\alpha}$-G for 5 minutes, then stimulated with 100 nM thapsigargin (100 nM) for 10 minutes. Incubations were then stopped and LTB$_4$ biosynthesis was analyzed as indicated in *Methods*. **B)** Pre-warmed neutrophils suspensions were treated with 1 μM of PGE$_2$-G, PGE$_2$-SA, PGD$_2$-G, PGD$_2$-SA, or PGF$_{2\alpha}$-G for 5 minutes, then stimulated with 100 nM fMLP for 10 minutes. ROS production was analyzed as described in *Methods*. **A, B**) Results are the mean (± SEM) of at least 3 individual experiments, each performed in duplicate.

Table 8.1: Summary of PGE₂-G hydrolyzing enzymes

Enzyme (Reference)	Inhibitor	Sensitive?	IC$_{50}$ value*	Reference
ABHD6 (31)	MAFP	Yes	16.9 nM	(32)
	JZL184	Yes	>100 µM	(33)
	Palmostatin B	Yes	52.5 nM	(34)
	WWL70	Yes	70 nM	(35)
	Tetrahydrolipstatin	Yes	48 nM	(32)
	ML349	No	n/a	(36)
	WWL113	Yes	-	(37)
ABHD12 (31)	MAFP	Yes	87 nM	(32)
	JZL184	No	n/a	(33)
	Palmostatin B	Yes	1.8 µM	(34)
	WWL70	No	n/a	(35)
	Tetrahydrolipstatin	Yes	193 nM	(32)
	ML349	No	n/a	(36)
	WWL113	Unknown	n/a	n/a
ABHD16A (34)	MAFP	Unknown	n/a	n/a
	JZL184	No	n/a	(33)
	Palmostatin B	Yes	100 nM	(34)
	WWL70	No	n/a	
	Tetrahydrolipstatin	Yes	170 nM	
	ML349	Unknown	n/a	n/a
	WWL113	Unknown	n/a	n/a
CES1 (38)	MAFP	Yes	-	(38; 39)
	JZL184	Yes	-	(38)
	Palmostatin B	Unknown	n/a	n/a
	WWL70	Unknown	n/a	n/a
	Tetrahydrolipstatin	Unknown	n/a	n/a
	ML349	Unknown	n/a	n/a
	WWL113	Yes	46 nM	(37)
LYPLA2 (24)	MAFP	Unknown	n/a	n/a
	JZL184	Yes	29 µM	(24)
	Palmostatin B	Yes	37.7 nM	(40)
	WWL70	No	n/a	(35)
	Tetrahydrolipstatin	No	n/a	(41)
	ML349	Yes	904 nM	(24; 36)
	WWL113	No	n/a	(37)
MAG lipase (31; 38)	MAFP	Yes	-	(31)
	JZL184	Yes	8 nM	(33)
	Palmostatin B	Yes	93.3 nM	(34)
	WWL70	No	n/a	(33; 35)
	Tetrahydrolipstatin	No	n/a	(34; 42)
	ML349	Unknown	n/a	n/a
	WWL113	No	n/a	(37)
PPT1 (39)	MAFP	Yes	-	(39)
	JZL184	No	n/a	
	Palmostatin B	Yes	-	(40; 43)

WWL70	Unknown	n/a	n/a
Tetrahydrolipstatin	Unknown	n/a	n/a
ML349	Unknown	n/a	n/a
WWL113	Unknown	n/a	n/a

(-) Not determined, (n/a) Not applicable; *Determined either by activity-based protein profiling.

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Chapitre 8.   Discussion

Nos travaux ont apporté de nouvelles connaissances sur les voies de biosynthèse et de dégradation des endocannabinoïdes par les leucocytes humains, en plus de définir leurs effets ou ceux de leurs métabolites sur les fonctions des neutrophiles et des éosinophiles. Ce chapitre comprendra d'abord une discussion plus détaillée de chacune des quatre études de la thèse, puis se terminera par une discussion et des perspectives faisant le lien entre l'ensemble des résultats présentés et les questions soulevées dans le chapitre *Hypothèses et objectifs*.

Le métabolisme du 2-AG, de la PGE$_2$-G et du 15-HETE-G par les leucocytes humains

Les objectifs principaux du chapitre 4 étaient de définir l'expression des enzymes impliquées dans le métabolisme du 2-AG chez les leucocytes humains et de caractériser des outils pharmacologiques nous permettant de bloquer ces voies de métabolisme. En effet, aucune étude n'était présente dans la littérature afin de connaître l'expression de ces enzymes chez ces cellules. En élaborant les objectifs de mon doctorat, nous avons constaté que ce manque de connaissances affecterait notre capacité à bien comprendre les mécanismes de régulation des fonctions des leucocytes par les endocannabinoïdes, puisque leurs métabolites semblaient souvent être impliqués dans le phénomène. Ceci était basé sur deux publications du laboratoire, dont une à laquelle j'avais activement participé (154, 155), dans lesquelles notre équipe démontrait que l'effet activateur du 2-AG sur les neutrophiles humains dépend entièrement de son hydrolyse en acide arachidonique. De plus, des résultats suggérant que les neutrophiles n'expriment pas de MAG lipase, la principale 2-AG-hydrolase dans le CNS des souris, ont suggéré que nous devions nous intéresser aux autres enzymes impliquées dans cette voie de métabolisme.

Une revue de la littérature nous a permis de constater que plusieurs enzymes sont capables d'effectuer l'hydrolyse du 2-AG en acide arachidonique (voir tableau 5.1). Certaines de ces enzymes, ABHD6 et ABHD12, sont responsables de la majorité de l'activité d'hydrolyse du 2-AG chez des cellules n'exprimant pas la MAG lipase, comme il a été démontré dans la lignée cellulaire microgliale BV-2 (156, 157). Nous avons d'abord entrepris de déterminer l'expression de ces lipases chez les neutrophiles, éosinophiles, monocytes, lymphocytes et macrophages alvéolaires humains. Ces résultats nous ont montré que chacun que ces types cellulaires exprime un minimum de deux lipases capables d'hydrolyser le 2-AG et/ou la PGE$_2$-G et le 15-HETE-G. Les données obtenues à l'aide d'inhibiteurs pharmacologiques de ces lipases ont également souligné que chacune de ces réactions d'hydrolyse est effectuée par plusieurs lipases dans chaque type cellulaire. En effet, seuls les inhibiteurs les moins sélectifs ont permis d'inhiber la quasi-totalité de l'activité d'hydrolyse de ces endocannabinoïdes.

Nous avons remarqué que certaines cibles présentent des différences indéniables entre les volontaires, et nous avons d'ailleurs choisi de montrer chacune des trois expériences effectuées pour chaque cible afin de bien souligner ces différences, qui illustrent bien la complexité du système. Il existe peu de données publiées au sujet d'une possible altération des niveaux d'expression de ces enzymes dans des maladies inflammatoires chez l'humain, mais quelques études ont rapporté une augmentation de la MAG lipase dans des conditions inflammatoires chez la souris (158, 159). Puisque le but de notre étude était de déterminer l'expression basale de ces enzymes par les leucocytes, nous avons uniquement recruté des volontaires sains, c'est-à-dire qu'ils n'avaient pas de maladie inflammatoire connue et qu'ils ne prenaient pas d'anti-inflammatoires. Les lymphocytes, monocytes et neutrophiles, qui sont des cellules abondantes dans le sang, pouvaient facilement être isolés en grandes quantités à partir de ces échantillons. Les nombres d'éosinophiles obtenus étaient plus restreints puisque leur concentration dans la circulation se retrouvait dans les valeurs normales chez ces volontaires, mais nous avons néanmoins réussi à effectuer ces expériences sur des prélèvements sanguins provenant de volontaires sains. Il demeure impossible de déterminer ce qui explique les différences que nous observons, qui pourraient aussi bien être génétiques que causées par une condition inflammatoire dont nous n'étions pas au courant lors du recrutement.

Nous avons par la suite établi des profils d'inhibition de l'hydrolyse du 2-AG, de la PGE_2-G et du 15-HETE-G par divers inhibiteurs documentés. L'utilisation d'inhibiteurs pharmacologiques, bien qu'efficace, apporte souvent des problèmes liés au manque de spécificité des molécules. À cet égard, les inhibiteurs de 2-AG hydrolases connus et couramment utilisés en recherche fondamentale sont de sélectivité variable. Le MAFP, qui est un inhibiteur de sérine hydrolases non-sélectif, est souvent efficace en raison de sa capacité à bloquer la réaction peu importe la (ou les) lipase(s) impliquée(s). Quant au JZL184, il est considéré comme l'inhibiteur de MAG lipase commercialement disponible le plus sélectif. Par contre, plusieurs études ont rapporté une sensibilité d'autres lipases au JZL184 (160-162). Bien que certaines de ces enzymes soient uniquement inhibées par le JZL184 à très hautes concentrations, nos résultats montrent qu'à une concentration de 3 µM, le JZL184 inhibe indéniablement une cible additionnelle, que nous soupçonnons d'être une 2-AG hydrolase non-identifiée.

Une expérience additionnelle aurait pu bonifier grandement cette étude : l'identification des protéines marquées à la sonde TAMRA-FP par spectrométrie de masse. L'ABPP est d'ailleurs fréquemment utilisée à des fins d'identification d'enzymes et d'optimisation d'inhibiteurs (163-165). L'identification par spectrométrie de masse nécessite que les protéines marquées soient immunoprécipitées à l'aide d'un anticorps reconnaissant la molécule de TAMRA. Cette immunoprécipitation sert à enrichir significativement les protéines marquées, ce qui réduit le bruit de fond lorsque les bandes d'intérêt sont analysées en spectrométrie de masse. Cependant, un seul anticorps anti-TAMRA est présentement disponible sur le marché, et nos efforts

de purification des protéines marquées dans nos lysats à l'aide de cet anticorps furent sans succès. Nous avons rencontré deux principaux problèmes lors de ces expériences : un manque de spécificité de l'anticorps, qui capturait beaucoup de protéines non-marquées, et une capture incomplète des protéines marquées. Ces problèmes n'ont pas pu être éliminés malgré de nombreuses tentatives de mise au point du protocole. Néanmoins, plusieurs équipes ont publié des résultats obtenus grâce à cet anticorps, mais dans des protéomes différents du nôtre, notamment le cerveau murin. Nous savons que les granulocytes humains ont la particularité d'être extrêmement riches en sérine protéases, et que ces enzymes sont marquées par la sonde TAMRA-FP. Nous pouvons d'ailleurs visualiser sur nos gels de neutrophiles des bandes correspondant probablement à l'élastase, qui produit des bandes de haute intensité à une hauteur approximative de 25 kDa (figure 5.5). Il est donc probable que la méthode ne soit pas adaptée à ce type de protéome, et que ce soit le contenu riche en protéases de nos cellules qui nous empêche de compléter cette étape avec succès.

Lorsque nous regardons les profils d'inhibition des marquages présentés dans l'article, nous pouvons déduire, par le poids moléculaire des protéines et leur sensibilité connue à certains inhibiteurs, l'identité de certaines hydrolases. Par contre, quelques cibles, notamment une protéine d'une taille approximative de 52 kDa retrouvée chez tous les types cellulaires étudiés et montrant une sensibilité au JZL184, ne correspondent à aucune 2-AG hydrolase connue. Nous ne pouvons exclure qu'il s'agisse de nouvelles 2-AG hydrolases, et même si ce n'était pas le cas, leur sensibilité à des composés couramment utilisés pour bloquer la dégradation des endocannabinoïdes en fait des cibles importantes à identifier. Ces expériences d'identification en spectrométrie de masse font partie des perspectives du projet et seront effectuées dans le futur, dans la mesure où un anticorps permettant une meilleure purification des enzymes marquées deviendrait disponible.

Peu de temps après que nous ayons complété l'étude, un article évaluant l'expression de la MAG lipase et de la CES1 dans les PBMC humains est paru (166). Les auteurs rapportent que la MAG lipase et la CES1 sont toutes les deux exprimées au niveau protéique chez les PBMC humains, mais que leur expression est beaucoup plus forte chez les monocytes que chez les lymphocytes. De plus, des différences d'expression entre volontaires ont été observées, comme c'est le cas dans nos expériences. Finalement, l'étude montre que le JZL184 bloque efficacement l'hydrolyse du 2-AG par les PBMC humains, mais que le WWL113 n'a pas d'effet significatif. Somme toute, cette étude appuie bien nos résultats du chapitre 4.

La synthèse d'endocannabinoïdes-glycérol par les leucocytes humains

Après avoir étudié la dégradation du 2-AG et de ses métabolites par les leucocytes humains, nous nous sommes intéressés à la façon dont les endocannabinoïdes sont synthétisés par ces cellules. À cet égard, une voie de biosynthèse du 2-AG, qui implique l'activation d'une PLC et l'action d'une DAG lipase, est bien caractérisée (167). Cette voie est considérée comme étant la voie classique de biosynthèse du 2-AG en raison

de son importance démontrée dans la biosynthèse 2-AG dans le cerveau murin (168, 169). Il existe cependant au moins deux autres voies de biosynthèse du 2-AG (170, 171), dont le rôle biologique demeure inconnu. Le but du chapitre 5 était de comprendre comment les leucocytes humains synthétisent du 2-AG.

Nos données d'expression des DAG lipases, ainsi que les niveaux de 2-AG produits par les leucocytes humains en réponse à un stimulus activant les PLC, nous ont permis de comprendre que les voies de biosynthèse du 2-AG varient d'un type cellulaire à l'autre. Les figures 6.1 et 6.2 montrent que certains types cellulaires n'expriment aucune DAG lipase, et que la plupart des leucocytes humains ne synthétisent pas de 2-AG en réponse à l'activation des PLC. En effet, les monocytes et les macrophages alvéolaires sont les seuls leucocytes à avoir synthétisé des quantités biologiquement significatives de 2-AG en réponse à une stimulation au calcium ionophore (A23187), un activateur connu des PLC. Ils ne sont toutefois pas les seuls leucocytes à exprimer la DAG lipase β, qui est également présente au niveau protéique chez les éosinophiles. Il est possible que ces derniers soient en mesure de synthétiser du 2-AG de façon DAG lipase-dépendante, mais que nous n'ayons tout simplement pas élucidé la façon optimale d'activer cette voie. Nous avons été intrigués par l'absence d'une voie PLC-DAG lipase certains leucocytes dont les neutrophiles, et nous avons décidé d'approfondir la question en testant d'autres stimuli et en évaluant la production de 2-AG par ces cellules.

Nos résultats soulignent l'importance de l'utilisation d'inhibiteurs d'hydrolases pour étudier la synthèse du 2-AG, vu sa demi-vie extrêmement courte dans nos suspensions de leucocytes. En utilisant une variété de stimuli afin de tenter d'activer la production de 2-AG par les neutrophiles, nous n'avons obtenu aucune synthèse en absence d'inhibiteur. Nous avons par contre obtenu un résultat complètement différent en présence de 1 µM de MAFP, puisque l'acide arachidonique a induit une synthèse massive de 2-AG par les neutrophiles. Étant donné que le MAFP n'est pas un inhibiteur sélectif, nous pouvons présumer que toutes les enzymes capables d'hydrolyser le 2-AG chez les neutrophiles sont bloquées par ce composé, et que la dégradation de 2-AG par les cellules pendant l'essai est minimale. Les niveaux mesurés, estimés à une moyenne de 80 pmol par million de neutrophiles, sont suffisants pour activer les récepteurs cannabinoïdes *in vivo*. En effet, cette quantité correspond à une concentration de ~300 nM dans les conditions de l'essai, et l'affinité de liaison (K_i) du récepteur CB_2 par le 2-AG est de 145 nM (172). Il est important de souligner que l'essai a été effectué à une concentration cellulaire de 5 x 10^6 neutrophiles/ml, qui se situe dans la zone normale de la concentration de neutrophiles circulants chez un humain en santé (6). Les quantités produites par les neutrophiles dans notre essai sont donc susceptibles d'avoir des effets importants sur les fonctions des leucocytes dans le sang et autres tissus humains.

Nous avons élucidé une portion du mécanisme de biosynthèse d'endocannabinoïdes-glycérol en réponse aux acides gras, c'est-à-dire l'étape indispensable de réacylation de l'acide gras avant son métabolisme en

endocannabinoïdes. Une expériences clé nous a mené à cette conclusion : l'utilisation d'inhibiteurs pharmacologiques bloquant deux différentes étapes de l'incorporation d'un acide gras dans un phospholipide. La triacsine C est un inhibiteur d'acyl-coenzyme A synthétase, ce qui signifie qu'elle bloque l'association de l'acide gras libre à un coenzyme A (CoA), une étape vitale à son incorporation dans un lysophospholipide. Quant au thimérosal, il inhibe les acyl-CoA-transférases, bloquant ainsi la deuxième étape de la réacylation des acides gras. Puisqu'il est connu que ces inhibiteurs inhibent la réacylation des acides gras (notamment l'AA) chez les neutrophiles (173-176), nous avons choisi de tester leurs effets dans ces cellules. L'inhibition marquée observée pour les deux inhibiteurs nous a confirmé que sans la réacylation de l'AA, celui-ci ne peut être métabolisé en 2-AG par les neutrophiles.

La façon dont l'AA est métabolisé en 2-AG après avoir été réacylé dans les membranes est encore inconnue. Nous avions postulé que cette production de 2-AG pourrait être DAG lipase dépendante, une hypothèse que nous avons testée en utilisant divers inhibiteurs de DAG lipase. Ces derniers n'ont eu aucun effet sur la synthèse de 2-AG induite par l'AA, supportant que cette voie biosynthétique est entièrement indépendante des DAG lipases. Parmi les voies de biosynthèse du 2-AG documentées par la littérature, une seule voie est indépendante des DAG lipases, et cette voie dépend de la synthèse d'acide lysophosphatidique (LPA) en tant qu'intermédiaire (171). Le LPA est ensuite déphosphorylé par une phosphatase pour générer le 2-AG. Sachant qu'une biosynthèse d'endocannabinoïdes par les neutrophiles via cette voie nécessiterait une production importante de l'intermédiaire LPA, nous avons mesuré la synthèse des diverses espèces de LPA par les neutrophiles stimulés avec des acides gras, et constaté que le LPA est présent dans les suspensions cellulaires (figure 6.6B). Une cinétique nous a permis d'établir que les neutrophiles stimulés à l'AA synthétisent une quantité maximale d'AA-LPA après 5 minutes, alors que la synthèse maximale de 2-AG survient après 15 minutes (figure 6.6A). Ces données suggèrent qu'une accumulation de LPA survient avant que celui-ci ne soit transformé en endocannabinoïdes par les cellules. Par contre, nous n'avons pas été en mesure de bloquer la déphosphorylation du LPA par un inhibiteur, bien que nous ayons testé plusieurs inhibiteurs de phosphatases de sélectivité variable (XY-14, AlFx⁻, propranolol, bromoenol lactone, orthovanadate de sodium). Par conséquent, les preuves dont nous disposons sont circonstancielles et le rôle du LPA dans notre voie biosynthétique demeure incertain. Advenant que notre hypothèse quant au rôle d'un intermédiaire LPA soit infirmée, le phénomène que nous observons serait une voie complètement nouvelle, jamais documentée auparavant.

Bien que nous ayons utilisé le neutrophile comme modèle pour étudier le nouveau mécanisme que nous avons mis en évidence, nous avons par la suite établi qu'il était également présent chez les éosinophiles et les monocytes humains. L'absence du mécanisme de biosynthèse du 2-AG chez les lymphocytes suggère fortement qu'il s'agit d'une voie restreinte aux leucocytes de la lignée myéloïde. Puisque nous n'avons pas

encore élucidé la totalité du mécanisme impliqué, il est difficile de comprendre avec certitude ce qui détermine la capacité d'un type cellulaire à synthétiser des endocannabinoïdes par cette voie. Par contre, nous savons que le mécanisme est dépendant de la réacylation de l'acide gras ajouté, et qu'une inhibition des enzymes responsable de cette réacylation empêche la synthèse d'endocannabinoïdes-glycérol. Par conséquent, il est possible que les lymphocytes ne possèdent pas la machinerie nécessaire pour réacyler une aussi grande quantité d'acides gras et que ceci cause une incapacité de ces cellules à transformer les acides gras en endocannabinoïdes. À cet égard, il est connu que les lymphocytes humains au repos ont une faible activité de remodelage des acides gras, comparativement aux lymphocytes en prolifération (177). Une étude a démontré que les lymphocytes T en prolifération ont une expression augmentée de plusieurs enzymes impliquées dans le remodelage des acides gras polyinsaturés et que par conséquent, ils les réacylent plus efficacement que les lymphocytes au repos (178). Considérant que nos expériences ont uniquement été effectuées sur des lymphocytes fraîchement isolés et au repos, il serait pertinent d'effectuer des expériences supplémentaires afin de vérifier si les lymphocytes en prolifération acquièrent une capacité à produire des endocannabinoïdes-glycérol en réponse aux acides gras.

Le 2-AG et la migration des éosinophiles

L'infiltration d'éosinophiles au tissu est un facteur crucial au maintien de l'inflammation dans de nombreuses conditions inflammatoires chroniques comme l'asthme. Dans l'asthme, le nombre d'éosinophiles présents dans les bronches corrèle avec la sévérité de la maladie (179). Cette composante inflammatoire de la maladie est habituellement traitée par des corticostéroïdes (180). Dans plusieurs cas, cette thérapie suffit à contrôler les symptômes et à réduire le recrutement d'éosinophiles et du même coup, à diminuer l'inflammation. Dans certains cas, même de fortes doses de corticostéroïdes ne permettent pas de contrôler la maladie, et l'éosinophilie persiste. Considérant les dommages bronchiques que peut engendrer une inflammation chronique, plusieurs thérapies émergentes visent directement le développement et le recrutement des éosinophiles en bloquant l'action de certaines cytokines (181, 182). Il est donc important de mieux comprendre les processus par lesquels les éosinophiles sont recrutés et activés, afin de trouver de nouvelles cibles permettant de contrôler l'inflammation éosinophilique dans l'asthme. Puisque nous savons que les endocannabinoïdes peuvent participer à l'inflammation en modulant les fonctions de nombreuses cellules, nous nous sommes intéressés à la façon dont ils pourraient potentiellement influencer l'inflammation éosinophilique dans un contexte d'asthme.

Il avait déjà été rapporté que le 2-AG induit une très modeste migration des éosinophiles (183, 184). Cependant, ces expériences ont été effectuées en l'absence de cytokines pouvant conditionner les éosinophiles, comme l'IL-5. Nous avons donc entrepris de déterminer si le 2-AG aurait un effet chimioattractant plus puissant en présence d'IL-5. Nous démontrons au chapitre 6 que c'est effectivement le

cas chez les éosinophiles humains, puisque le prétraitement avec l'IL-5 fait passer la migration nette induite par le 2-AG de ~5% à ~35%. Il est à noter que cet effet n'était pas limité à l'IL-5, et d'autres cytokines dont le récepteur partage la même sous-unité $\beta\gamma$ (IL-3, GM-CSF) ont eu un effet similaire. Ce concept a été confirmé par l'utilisation de PP2, un inhibiteur des Lyn kinases activées par cette sous-unité $\beta\gamma$, qui a complètement bloqué l'effet de ces cytokines. Il est important de souligner que cet effet de l'IL-5 n'est pas présent lorsque des éosinophiles migrent en réponse à la PGD$_2$ ou au 5-oxo-ETE, suggérant que l'effet chimiotactique du 2-AG se produit via un mécanisme distinct.

Sachant maintenant que le 2-AG est un puissant agent chimiotactique de l'éosinophile en présence d'IL-5, nous avons déterminé les mécanismes par lesquels il exerce cet effet. Nos principaux constats sont les suivants :

1) Les agonistes du récepteur CB$_2$ n'induisent pas à eux seuls la migration des éosinophiles en présence d'IL-5. Pourtant, la migration a été presque complètement inhibée par deux antagonistes du récepteur CB$_2$. Ces données suggèrent que l'activation du récepteur CB$_2$ est nécessaire aux effets du 2-AG, mais pas suffisante.

2) L'utilisation d'un analogue non-hydrolysable du 2-AG, ainsi que d'inhibiteurs d'hydrolyse du 2-AG, nous ont confirmé qu'un métabolite de l'AA était impliqué dans l'effet chimiotactique du 2-AG.

3) Ce métabolite est forcément issu de la voie de la 15-LO, puisque deux inhibiteurs de cette voie (NDGA et PD146176) ont bloqué la migration des éosinophiles induite par le 2-AG.

Puisque le lipidome de la 15-LO chez les éosinophiles est complexe et que nous ne disposions pas de l'équipement nécessaire pour l'étudier à ce moment, cette étude n'a pas poussé plus loin l'élucidation des métabolites impliqués. Nous avons toutefois déterminé dans cette publication que les éosinophiles sont capables de transformer l'AA et le 2-AG en éoxine C$_4$, un métabolite de la 15-LO. Nous avons également démontré, dans une autre publication ne faisant pas partie de cette thèse, que les éosinophiles humains transforment efficacement l'AA en 15-HETE, et que cette voie métabolique est significativement inhibée par le NDGA (185). Par contre, dans cette publication, le PD146176 n'a pas inhibé la conversion de l'AA en 15-HETE par les éosinophiles. Considérant l'effet inhibiteur du PD146176 sur la migration des éosinophiles induite par le 2-AG, il est probable que le mécanisme sous-jacent implique l'éoxine C$_4$. Ces questions seront adressées dans des études futures du laboratoire, dans lesquelles le lipidome de la 15-LO et son rôle dans l'asthme seront étudiés par spectrométrie de masse.

Cette étude souligne un point très important : les endocannabinoïdes, plus particulièrement le 2-AG, peuvent exercer des effets biologiques qui ne peuvent être reproduits par des cannabinoïdes synthétiques ou des agonistes des récepteurs CB. Il s'agit de la combinaison de leur activation des récepteurs cannabinoïdes et de

leur métabolisme en éicosanoïdes qui résulte en un profil unique d'effets pro- ou anti-inflammatoires. Ce concept a d'ailleurs été renforcé par une expérience de l'étude dans laquelle nous avons combiné l'AA et un agoniste des récepteurs cannabinoïdes, le CP55,940. La combinaison de ces deux éléments a engendré une migration des éosinophiles comparable à celle induite par le 2-AG. Donc, la nature de l'effet du 2-AG diffère selon sa capacité à être dégradé en AA. Par conséquent, si du 2-AG est présent dans un tissu, par exemple dans un contexte d'asthme, des éosinophiles pourront y migrer, ce qui sera néfaste en bout de ligne. Par contre, si on bloque pharmacologiquement l'hydrolyse du 2-AG, ce dernier n'induira pas la migration des éosinophiles par faute d'être métabolisé par la voie de la 15-LO. Les niveaux de 2-AG demeureront élevés et ce dernier pourra exercer localement ses effets anti-inflammatoires CB_2-dépendants. Cette étude souligne donc un potentiel intéressant de la voie du 2-AG dans le traitement de l'inflammation éosinophilique, que ce soit dans l'asthme ou dans une autre maladie. L'étude des effets biologiques du blocage pharmacologique de l'hydrolyse du 2-AG dans un contexte d'inflammation éosinophilique n'a pas été approfondie dans le cadre de cette thèse, mais est une perspective prometteuse.

L'effet inhibiteur de la PGE_2-G sur les fonctions des neutrophiles humains

Nos données présentées au chapitre 4, ainsi que nos études antérieures, montrent que les neutrophiles humains fraîchement isolées métabolisent principalement le 2-AG en acide arachidonique, qui est ensuite converti en LTB_4 (154, 155). Cependant, dans un contexte inflammatoire, le profil de métabolites des endocannabinoïdes qui sont produits est différent, notamment en raison de l'expression d'enzymes inductibles comme la COX-2. Ce phénomène est susceptible de favoriser la biosynthèse des métabolites de l'oxygénation du 2-AG, comme les prostaglandines-glycérol (PG-G) et les prostaglandines-éthanolamide (PG-EA). Les effets biologiques de ces métabolites sont mal définis, et les seuls effets documentés ont été présentés dans l'article de revue du chapitre 1. L'objectif de cette étude était donc d'étudier l'impact de deux de ces métabolites, soit la PGE_2-G et la PGE_2-EA, sur les fonctions des neutrophiles. Nous avons choisi ces métabolites pour trois raisons principales :

1) La littérature suggère qu'ils sont bioactifs, mais le récepteur en cause était toujours inconnu au moment d'entamer l'étude (186, 187).

2) Les deux métabolites sont structurellement semblables à la PGE_2, un médiateur de l'inflammation pouvant moduler les fonctions des neutrophiles.

3) Les prostaglandines-glycérol ont été détectées dans plusieurs tissus chez le rat et la souris, confirmant qu'elles peuvent être formées *in vivo* et justifiant l'étude de leurs fonctions biologiques (188-190).

Dans cette étude, nous avons démontré que la PGE$_2$-G exerce un effet inhibiteur sur diverses fonctions des neutrophiles humains. Plus précisément, la PGE$_2$-G, lorsque pré-incubée dans une suspension de neutrophiles, prévient l'activation subséquente de diverses fonctions effectrices par d'autres agonistes. Parmi les effets inhibés par la PGE$_2$-G, on compte la biosynthèse de leucotriènes, la production de peptides antimicrobiens, la migration induite par le LTB$_4$ ainsi que l'explosion oxydative.

Le mécanisme que nous avons mis en évidence est le suivant : la PGE$_2$-G est hydrolysée en PGE$_2$ par les neutrophiles, puis cette PGE$_2$ inhibe l'activation des neutrophiles en liant le récepteur EP$_2$, ce qui mène à une augmentation des taux intracellulaires d'AMP cyclique. Trois constats principaux nous permettent d'être certains de ce mécanisme :

1) Nos expériences avec la PGE$_2$-G-D$_4$ montrent clairement qu'en présence de neutrophiles, la quantité de celle-ci diminue dans le temps, pendant que la quantité de PGE$_2$-D$_4$ augmente.

2) L'effet inhibiteur de la PGE$_2$-G est bloqué par un inhibiteur d'hydrolyse, et n'est pas reproduit par un analogue non-hydrolysable de la PGE$_2$-G.

3) Les effets de la PGE$_2$-G ont été reproduits par la PGE$_2$ et par un agoniste du récepteur EP$_2$, et bloqués par un antagoniste du même récepteur.

Quant à la PGE$_2$-EA, elle n'a eu aucun effet. La PGE$_2$-EA est beaucoup plus stable que la PGE$_2$-G, tel que démontré dans le plasma de rats (191). Elle n'est pas hydrolysée en PGE$_2$, mais plutôt isomérisée en PGB$_2$-EA, une réaction qui se fait plus lentement. Puisque notre mécanisme requiert l'hydrolyse en PGE$_2$, ceci pourrait expliquer pourquoi la PGE$_2$-EA n'a pas inhibé les fonctions des neutrophiles humains dans notre modèle. La PGE$_2$-EA peut se lier aux quatre récepteurs des prostaglandines, ce qui suggère qu'elle est capable moduler les fonctions des neutrophiles de façon EP-dépendante (192). Cependant, l'affinité de la PGE$_2$-EA pour les récepteurs EP est en moyenne 500 fois plus faible que celle de la PGE$_2$, expliquant potentiellement l'absence d'effet sur les fonctions des neutrophiles. Puisque nous avons reproduit l'effet inhibiteur de la PGE$_2$-G en utilisant un agoniste du récepteur EP$_2$, la PGE$_2$-EA devrait forcément reproduire cet effet si elle activait efficacement le récepteur EP$_2$ à la surface des neutrophiles, suggérant que ce n'est pas le cas.

La principale faiblesse de notre étude est notre impossibilité d'identifier la lipase responsable de l'hydrolyse de la PGE$_2$-G par les neutrophiles. Nous avons d'abord établi que les neutrophiles expriment un nombre limité de PGE$_2$-G hydrolases parmi celles qui ont été documentées. En effet, seules ABHD12 et ABHD16A sont présentes chez les neutrophiles au niveau protéique. Lorsque nous avons vérifié la capacité de sept inhibiteurs pharmacologiques à bloquer l'hydrolyse de la PGE$_2$-G par les neutrophiles, plusieurs d'entre eux se

sont avérés efficaces. Par contre, le MAFP et la palmostatine B inhibent la majorité des PGE$_2$-G hydrolases connues (tableau 8.1), nous donnant donc peu d'information sur l'identité de l'enzyme impliquée. Selon les informations trouvées dans la littérature, l'absence d'effet du THL, du WWL70 et du ML349 nous permet d'éliminer la LYPLA2, la CES1, ABHD6, ABHD12 et ABHD16A. Les deux enzymes présentes chez les neutrophiles au niveau protéique, ABHD12 et ABHD16A, font partie de cette liste. Nous nous sommes donc retrouvés dans une impasse quant à l'identification de la PGE$_2$-G hydrolase chez les neutrophiles, les outils dont nous disposions ne nous permettant pas d'apporter une réponse claire. **Nous soupçonnons qu'une lipase inconnue jusqu'à ce jour soit impliquée dans l'hydrolyse de la PGE$_2$-G par les neutrophiles humains.** Nous avons déjà établi, dans le chapitre 4, que les leucocytes possèdent une 2-AG hydrolase non-caractérisée, sensible au JZL184. Puisque l'hydrolyse de la PGE$_2$-G par les neutrophiles est partiellement sensible au JZL184, cette lipase inconnue pourrait être impliquée dans la réaction, du moins partiellement. Toutefois, la confirmation de cette hypothèse est expérimentalement laborieuse. Il faudrait d'abord procéder à l'immunoprécipitation de l'enzyme par ABPP et à son identification par spectrométrie de masse, un processus présentant des obstacles (décrits plus haut, dans la section dédiée au chapitre 4). Il faudrait ensuite caractériser l'enzyme identifiée, et bloquer sélectivement son activité ou son expression afin de démontrer son rôle dans l'hydrolyse du 2-AG et/ou de la PGE$_2$-G. Bien que je n'aie pas eu l'occasion de poursuivre dans cette voie pendant mon doctorat, ces expériences pourraient mener à la découverte d'une nouvelle hydrolase qui deviendrait une cible pharmacologique intéressante pour moduler les niveaux d'endocannabinoïdes et de PGE$_2$-G dans un contexte d'inflammation.

L'identification du récepteur de la PGE$_2$-G et impacts potentiels

Au moment où nous avons réalisé cette étude, le récepteur de la PGE$_2$-G était inconnu. La bioactivité de la PGE$_2$-G envers un récepteur inconnu avait été documentée dans des lignées cellulaires macrophagiques. Il était également connu que la PGE$_2$-G active certains des récepteurs EP. Après la publication de notre article, le récepteur de la PGE$_2$-G a été identifié comme étant le récepteur P2Y$_6$, un récepteur purinergique (193). Avant cette découverte, le meilleur ligand de ce récepteur était l'uridine diphosphate (UDP), un nucléotide, d'où la classification initiale du récepteur. Cependant, la PGE$_2$-G peut activer ce récepteur à des concentrations ~50 fois plus faibles que l'UDP (~1 pmol vs ~50 pmol), ce qui en fait un deuxième agoniste endogène du récepteur. Sachant maintenant que c'est ce récepteur qui cause l'élévation du calcium intracellulaire par la PGE$_2$-G dans les lignées macrophagiques, nous pouvons postuler que la PGE$_2$-G modulerait certaines fonctions des leucocytes via ce récepteur.

À cet égard, notre équipe s'est intéressée par le passé à la régulation des fonctions des leucocytes par les nucléotides extracellulaires. J'ai obtenu des résultats d'expression des récepteurs P2Y en PCR classique, qui n'ont jamais été publiés et qui sont présentés à la figure 9.1. Comme le montre la figure, nous avons détecté

l'ARNm du récepteur P2Y$_6$ chez les éosinophiles et en quantité plus limitée, chez les PBMC. L'expression des récepteurs P2Y par les éosinophiles humains n'a jamais été documentée auparavant, mais un article a montré que les éosinophiles de souris expriment le récepteur P2Y$_6$ au niveau protéique (194). Dans deux autres études visant à définir l'expression des récepteurs P2Y chez divers leucocytes, les auteurs ont détecté l'ARNm du récepteur P2Y$_6$ dans les neutrophiles humains (195, 196). Par contre, il s'agissait de neutrophiles non-déplétés en éosinophiles, qui peuvent définitivement contenir un pourcentage d'éosinophiles suffisant pour causer un faux-positif. Dans nos expériences, les neutrophiles humains déplétés en éosinophiles ne montrent aucun signal d'ARNm pour le récepteur P2Y$_6$. Il serait intéressant de mieux définir l'expression protéique du récepteur chez les leucocytes humains afin de comprendre comment la PGE$_2$-G pourrait moduler leurs fonctions. Pour ce qui est de notre mécanisme chez les neutrophiles, nos résultats montrent de façon assez robuste que les effets de la PGE$_2$-G sont récepteur-indépendants.
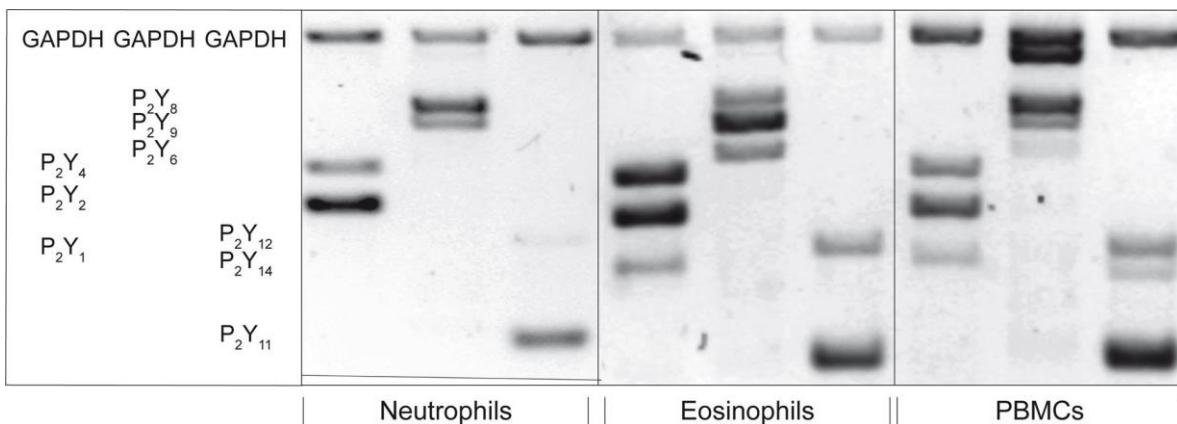

Figure 9.1. Expression génique des récepteurs P$_2$Y par les leucocytes humains. L'expression a été déterminée par PCR sur l'ADN complémentaire total de chacun des types cellulaires et séparation des amplicons sur gel d'agarose. La figure est représentative de trois expériences indépendantes.

Bien que le récepteur P2Y$_6$ ne semble pas être impliqué dans la régulation des fonctions des neutrophiles par la PGE$_2$-G, il serait intéressant d'étudier les effets de cette dernière sur les fonctions des éosinophiles, qui eux, semblent exprimer son récepteur. Il a d'ailleurs été démontré que l'activation du récepteur P2Y$_6$ par l'UDP désensibilise le récepteur CysLT$_1$ dans une lignée cellulaire co-exprimant les deux récepteurs (197). Ceci signifie que la PGE$_2$-G pourrait potentiellement atténuer n'importe quel effet CysLT$_1$-dépendant des cystéinyl-leucotriènes en désensibilisant leur récepteur. Il est bien connu que les cystéinyl-leucotriènes exercent une grande variété d'effets sur les éosinophiles, dont la chimiotaxie et la sécrétion de protéines granulaires (198). Nos résultats du chapitre 4 montrent que la PGE$_2$-G est relativement stable dans des suspensions de leucocytes humains, puisque chaque type cellulaire convertit moins de 5% de la PGE$_2$-G en PGE$_2$ après 5

minutes. Par conséquent, la PGE$_2$-G pourrait assurément agir *in vivo* pour réguler le recrutement et les fonctions des éosinophiles via l'activation du récepteur P2Y$_6$. Une étude très récente a d'ailleurs démontré qu'un agoniste du récepteur P2Y$_6$ diminue l'inflammation pulmonaire éosinophilique causée par un allergène chez la souris, appuyant cette hypothèse (199).

La PGE$_2$-G et la réaction inflammatoire

Les résultats présentés dans cette thèse montrent que la PGE$_2$-G atténue les fonctions des neutrophiles associées à la phase aiguë de l'inflammation (migration, production de ROS et de leucotriènes, production de peptides antimicrobiens). Considérant que la PGE$_2$-G peut uniquement être produite lorsque la COX-2 est exprimée, il est logique d'assumer que sa présence coïnciderait non seulement avec la phase aiguë de l'inflammation, mais aussi avec l'initiation de la phase de résolution. Dans ce contexte, la PGE$_2$-G est susceptible d'agir non seulement sur les neutrophiles, mais également sur les monocytes et les macrophages. Il serait donc important d'évaluer son impact sur les fonctions de ces cellules afin de comprendre comment elle pourrait moduler la réaction inflammatoire. J'ai eu l'occasion d'effectuer quelques expériences préliminaires sur des monocytes humains et des macrophages alvéolaires, dont les résultats sont présentés en figure 9.2. Le panneau A de cette figure montre que la PGE$_2$-G semble exercer un effet chimiotactique sur les monocytes humains. Le recrutement des monocytes et leur différenciation en macrophages est un évènement clé dans l'initiation de la résolution de l'inflammation et ces résultats suggèrent que la PGE$_2$-G pourrait contribuer à ce processus. En figure 9.2, nous avons stimulé avec divers agonistes des macrophages alvéolaires humains, fraîchement isolés. Les résultats montrent que la PGE$_2$-G induit une biosynthèse de LTB$_4$ par ces macrophages qui est comparable par celle induite par le PAF. Puisque la biosynthèse du LTB$_4$ est grandement facilitée par une élévation des taux de calcium intracellulaire (200), ces résultats concordent avec les études montrant une mobilisation de calcium chez les cellules macrophagiques RAW 264.7 en présence de PGE$_2$-G (186, 187). Cette hypothèse pourrait facilement être vérifiée en confirmant la présence du récepteur P2Y$_6$ à la surface de ces macrophages, et en testant l'impact d'un antagoniste du récepteur sur la biosynthèse de LTB$_4$. Il serait également important de tenir compte de l'état d'activation des macrophages, et de garder en tête que *in vivo*, les macrophages alvéolaires acquièrent une polarisation en fonction des cytokines et autres médiateurs présents dans leur milieu (201, 202). À cet égard, il est possible que les macrophages répondent différemment à la PGE$_2$-G selon leur état d'activation.

Dans un contexte de résolution de l'inflammation, le recrutement et les fonctions des neutrophiles sont freinés, alors que le recrutement des monocytes et leur différenciation en macrophages possédant un phénotype pro-résolution augmentent. Le terme « immunorésolvant » désigne les médiateurs participant à la résolution active de l'inflammation en contribuant tant l'atténuation de la phase aiguë qu'à l'initiation de la phase de résolution. À la lumière des effets de la PGE$_2$-G que nous observons dans nos modèles, nous croyons qu'il est possible

qu'il s'agisse d'un médiateur immunorésolvant. Un tel concept doit être testé *in vivo* dans un modèle animal d'inflammation afin d'être validé, ce qui constitue une perspective intéressante au projet. Il faut également garder en tête que d'autres prostaglandines-glycérol existent et qu'elles pourraient également avoir un effet dans l'inflammation. À cet égard, des articles publiés au cours des dernières années ont démontré que la PGD$_2$-G avait des effets bénéfiques dans des modèles murins de colite (190, 203). Un tel impact des prostaglandines-glycérol s'aligne bien avec les études montrant que l'inhibition de la COX-2 prévient la résolution de l'inflammation (145-150) et vaut la peine d'être investigué davantage.
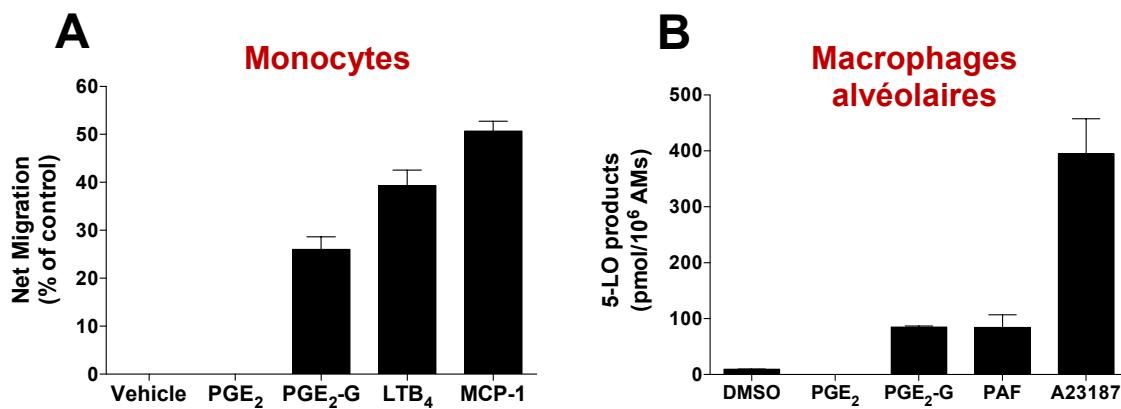

Figure 9.2. Effets de la PGE$_2$-G sur les monocytes et macrophages alvéolaires humains. A) Migration des monocytes humains en réponse à la PGE$_2$, la PGE$_2$-G, le LTB$_4$ ou la MCP-1. **B)** Synthèse de produits de la 5-LO par des macrophages alvéolaires fraîchement isolés et stimulés à la PGE$_2$, la PGE$_2$-G, le PAF ou le A23187.

Le rôle (ou l'absence de rôle) de l'AEA

Bien que cette thèse porte principalement sur le 2-AG et non sur tous les endocannabinoïdes connus, il est important de souligner que nous avons également étudié l'AEA et ses métabolites. À la lumière des résultats présentés dans cette thèse et d'autres résultats obtenus au cours de mon doctorat, nous pouvons suggérer que l'AEA ne régule pas les fonctions des leucocytes humains, du moins pas par les mécanismes que nous avons mis en évidence pour le 2-AG.

Tout d'abord, des résultats publiés dans le passé par notre équipe ont démontré que contrairement au 2-AG, l'AEA n'est pas hydrolysé en AA puis transformé en LTB$_4$ par les neutrophiles humains (154, 155). Il est évident que la capacité d'une cellule à hydrolyser l'AEA dépend premièrement de son expression des FAAH-1 et -2, des hydrolases dégradant spécifiquement l'AEA en AA. Il serait donc important d'analyser l'expression protéique des FAAH chez ces cellules afin de confirmer qu'elles n'y sont pas présentes. Tout comme c'est le cas pour le 2-AG, il est possible que d'autres hydrolases puissent effectuer cette réaction et ainsi compenser

pour une absence de FAAH dans un type cellulaire donné, mais la liste d'enzymes capables d'effectuer cette réaction est beaucoup plus courte.

Pour ce qui est des éosinophiles, notre étude sur leur migration induite par le 2-AG comportait également des expériences visant à comparer l'effet du 2-AG avec celui de l'AEA. Nous avons rapidement constaté que l'AEA induisait très faiblement la migration des éosinophiles et ce, même en présence d'IL-5 (migration nette <10%). Cette migration est équivalente à celle induite par le 2-AG-ether, un analogue du 2-AG capable d'activer le récepteur CB$_2$ mais incapable d'être hydrolysé en AA. Ceci suggère que l'effet modeste de l'AEA sur la migration des éosinophiles est dû à l'activation du récepteur CB$_2$. De plus, nous démontrons que l'hydrolyse du 2-AG en AA et la synthèse de dérivés de la 15-LO sont responsables de la majeure partie de l'effet chimiotactique du 2-AG envers l'éosinophile. Il est donc possible que l'éosinophile présente la même incapacité que le neutrophile à hydrolyser l'AEA, ce qui expliquerait son manque d'un effet significatif dans ce modèle de migration. Finalement, le manque d'effet chimiotactique de l'AEA envers les éosinophiles ne signifie pas nécessairement qu'il n'exerce aucun rôle dans leur migration au tissu. À cet égard, une étude a démontré que l'AEA augmente la perméabilité des cellules épithéliales bronchiques, une étape clé dans la transmigration des leucocytes vers les bronches (204). Bien que cette étude ne confirme en rien que l'AEA joue un rôle dans le recrutement et l'activation des leucocytes *in vivo*, il peut s'agir d'une piste suggérant que l'AEA module l'inflammation sans nécessairement réguler directement les fonctions des leucocytes.

Conclusion

La figure 9.3 montre le schéma présenté en figure 4.1, mis à jour pour y intégrer les réponses apportées par nos travaux.
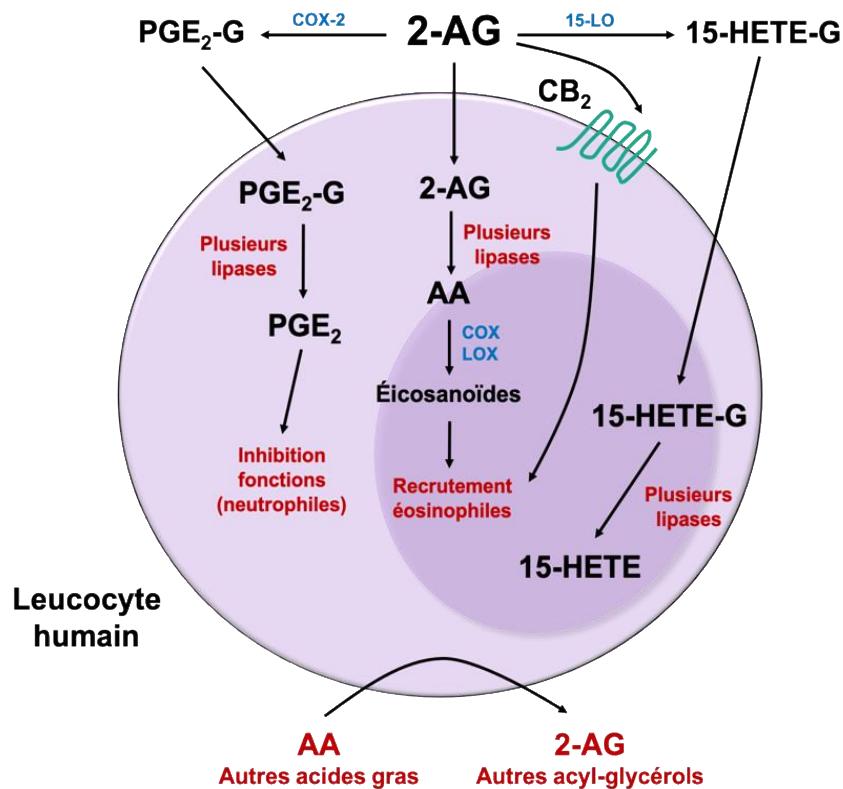

Figure 9.3. Voies de métabolismes des endocannabinoïdes chez les leucocytes humains et leurs effets biologiques. Le contenu en rouge désigne les nouvelles connaissances apportées par les travaux de cette thèse.

Pour récapituler, voici comment cette thèse a apporté des éléments de réponse à chacune des grandes questions posées au chapitre 3.

Y existe-t-il des différences fondamentales dans la façon dont les cellules humaines et murines dégradent les endocannabinoïdes ?

Oui. À la lumière de nos résultats, nous pouvons stipuler que les leucocytes humains sont des experts à dégrader le 2-AG, et que les sentiers métaboliques qu'ils utilisent ne sont pas toujours les mêmes que ce qui a été documenté dans le cerveau murin. La MAG lipase, par exemple, joue un rôle central dans la dégradation du 2-AG dans le cerveau des souris alors qu'elle n'est même pas présente dans les neutrophiles humains, des

leucocytes cruciaux dans l'inflammation aiguë. De plus, nous rapportons des différences majeures entre les divers types leucocytaires en termes de profils d'expression des 2-AG hydrolases.

À cet égard, est-ce que nous disposons d'outils pharmacologiques capables d'inhiber ces voies métaboliques chez les leucocytes humains ?

Oui. Nous avons consacré le chapitre 4 à bien définir l'effet de nombreux inhibiteurs de 2-AG hydrolases sur l'hydrolyse du 2-AG, de la PGE$_2$-G et du 15-HETE-G par chaque type leucocytaire. Par contre, un des constats que nous en avons dégagé est que les inhibiteurs bloquant le plus efficacement ces voies chez les leucocytes humains sont les moins sélectifs parmi ceux que nous avions à notre disposition. Nos résultats soulignent donc un besoin flagrant de développer des inhibiteurs plus sélectifs, tant pour la MAG lipase que pour d'autres cibles.

Les leucocytes humains sont-ils des sources significatives d'endocannabinoïdes et si oui, comment les produisent-ils ?

Oui, les leucocytes humains sont définitivement une source significative d'endocannabinoïdes. Nous sommes, à notre connaissance, les premiers à démontrer une biosynthèse de 2-AG aussi importante par les leucocytes humains, en plus de montrer que des endocannabinoïdes dérivés d'autres acides gras insaturés sont également produits. Nous mettons en évidence une voie de biosynthèse du 2-AG complètement distincte de la voie des DAG lipases, bien caractérisée chez la souris. Cette voie de biosynthèse semble être restreinte aux leucocytes de la lignée myéloïde, et absente des cellules structurales humaines. Par conséquent, les voies de biosynthèse du 2-AG semblent non seulement être différentes entre l'humain et la souris, mais également différentes entre les différents tissus humains.

Dans un contexte inflammatoire, l'inhibition de l'hydrolyse des endocannabinoïdes risque de favoriser leur métabolisme par d'autres voies, telles que la synthèse de PG-G ou de 15-HETE-G. Quel est l'impact de ces médiateurs sur les fonctions des leucocytes humains ?

Nos résultats du chapitre 7 montrent que dans le cas des neutrophiles, les PG-G exercent un effet inhibiteur. Nous avons également des résultats préliminaires effectués sur les monocytes et les macrophages alvéolaires humains, qui suggèrent que l'effet de la PGE$_2$-G sur ces cellules serait plutôt activateur. L'identification récente du récepteur de la PGE$_2$-G permettra d'approfondir nos connaissances par rapport à cette question, notamment en investiguant les effets P2Y$_6$-dépendants des PG-G sur les leucocytes humains.

Les leucocytes sont-ils capables d'hydrolyser ces médiateurs afin de les utiliser comme sources de prostaglandines et de 15-HETE ?

Oui. Nos résultats du chapitre 4 montre que chaque type leucocytaire humain est capable d'effectuer l'hydrolyse de la PGE$_2$-G et du 15-HETE-G pour produire la PGE$_2$ et le 15-HETE, respectivement. Par contre, nous savons aussi que cette hydrolyse peut être effectuée par les mêmes lipases que celles qui dégradent le 2-AG, et que certains inhibiteurs bloquent les trois réactions. Cela signifie que dans un contexte thérapeutique, un inhibiteur de l'hydrolyse du 2-AG diminuerait potentiellement l'hydrolyse de la PGE$_2$-G et du 15-HETE-G également. Donc, nous pouvons avancer que l'inhibition de l'hydrolyse du 2-AG pourrait du même coup augmenter la demi-vie et le potentiel bioactif de la PGE$_2$-G et du 15-HETE-G, en plus d'empêcher la cellule de les utiliser comme source d'éicosanoïdes pro-inflammatoires.

Nous avons établi que l'impact du 2-AG sur les neutrophiles dépend entièrement de son métabolisme en effecteurs pro-inflammatoires comme le LTB$_4$. En est-il de même pour les autres leucocytes humains ?

Non. Pour le moment, nous avons uniquement étudié l'éosinophile, qui présente un profil différent du neutrophile. En effet, l'effet chimiotactique du 2-AG envers l'éosinophile est causé par une combinaison de l'activation du récepteur CB$_2$ ainsi que le métabolisme du 2-AG par la voie de la 15-LO par l'éosinophile. Dans le futur, il sera primordial de bien définir l'expression du récepteur CB$_2$ au niveau protéique chez les leucocytes humains, et de bien caractériser les effets du 2-AG sur les fonctions de ces cellules.

Perspectives

Maintenant que nos connaissances des voies métaboliques du 2-AG chez les leucocytes humains sont mieux étoffées, il est envisageable de bloquer l'hydrolyse du 2-AG pour traiter l'inflammation. Nos résultats nous permettent d'avoir une meilleure compréhension de ce qui se passe chez les leucocytes humains, en présence ou en absence d'inhibiteurs d'hydrolyse du 2-AG. Ces scénarios sont schématisés à la figure 9.4, qui illustre le changement entre un phénotype pro- et anti-inflammatoire qui aurait lieu en présence d'un inhibiteur d'hydrolyse du 2-AG.
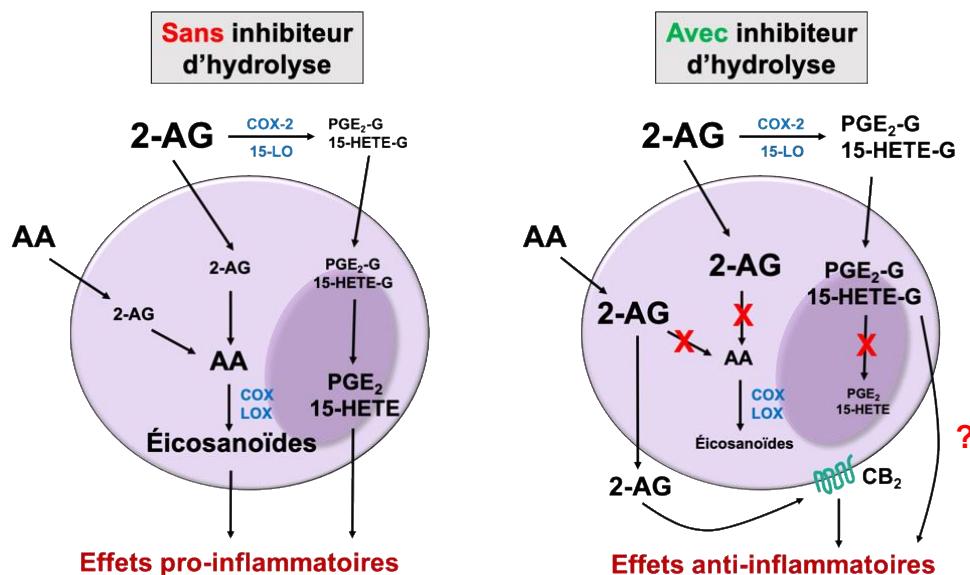

Figure 9.4. Métabolisme du 2-AG et ses effets chez les leucocytes humains, en présence ou en absence d'inhibiteur d'hydrolyse. La taille du texte est proportionnelle à l'abondance du médiateur.

Ce concept a été validé dans de nombreux modèles murins d'inflammation, malgré la présence de différences importantes entre les deux espèces en matière de voies de métabolisme des endocannabinoïdes. Nos travaux informent sur la façon de transposer ce concept à l'humain, et trois principales avenues d'exploration future permettront d'avancer dans cette direction :

1) De nouveaux inhibiteurs d'hydrolases doivent être développés, en fonction des profils d'expression des lipases et des profils d'hydrolyse du 2-AG, de la PGE$_2$-G et du 15-HETE-G décrits au chapitre 4. Les effets de ces composés sur leucocytes humains devront être étudiés afin de choisir un composé qui aura les effets recherchés.

2) Les effets récepteurs-dépendants des PG-G devront être mieux documentés, ce qui sera possible grâce à l'identification récente du récepteur de la PGE_2-G.

3) Les effets des endocannabinoïdes-glycérol autres que le 2-AG sur les leucocytes humains devront être étudiés. Nos résultats rapportent pour la première fois que les leucocytes humains synthétisent ces médiateurs et qu'ils sont retrouvés dans la circulation chez l'humain, mais leurs effets biologiques demeurent mal définis.

Ces recherches permettront de mieux comprendre la façon dont il faudrait bloquer l'hydrolyse des endocannabinoïdes, ainsi que tous les effets biologiques qui en découleront. Le but ultime est de développer de nouveaux traitements contre les maladies inflammatoires chroniques, qui maximiseront les effets analgésiques et anti-inflammatoires des endocannabinoïdes tout en limitant leurs effets néfastes.

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