VOLUME 160 • 2008

REVIEWS OF Physiology, Biochemistry and Pharmacology

Springer

Reviews of

160 Physiology Biochemistry and Pharmacology

Springer

ISBN 978-3-540-77603-1 e-ISBN 978-3-540-77604-8
DOI 10.1007/978-3-540-77604-8

Reviews of Physiology, Biochemistry and Pharmacology ISSN 0303-4240

Library of Congress-Catalog-Card Number 74-3674

Editor: Simon Rallison, London
Desk Editor: Anne Clauss, Heidelberg
Typesetting and Production: le-tex publishing services oHG, Leipzig
Cover: WMXDesign GmbH, Heidelberg

Printed on acid-free paper

9 8 7 6 5 4 3 2 1 0

springer.com

Rev Physiol Biochem Pharmacol (2006) 160:1 –24
DOI 10.1007/112_0505

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Endocannabinoids: synthesis and degradation

Published online: 29 April 2006

Abstract Endocannabinoids were defined in 1995 as endogenous agonists of cannabinoid receptors, i.e. of the G protein-coupled receptors for cannabis's psychoactive principle, Δ^9-tetrahydrocannabinol. Although there appear to be several endocannabinoids, only two of such endogenous mediators have been thoroughly studied so far: anandamide and 2-arachidonoylglycerol (2-AG). A general strategy seems to apply to the biosynthesis and degradation of anandamide and 2-AG, although the levels of these two compounds appear to be regulated in different, and sometimes even opposing, ways. "Endocannabinoid enzymes", that is to say enzymes that catalyse endocannabinoid biosynthesis or degradation, have been identified and in some cases cloned, and will be described in this review together with their possible pharmacological targeting for therapeutic purposes. The cellular and subcellular localization and the modes for the regulation of the expression and activity of these enzymes play an important role in the functions played by the endocannabinoids under physiological and pathological conditions.

Endocannabinoids

Two subtypes of G protein-coupled receptors for cannabis's psychotropic component, Δ^9-tetrahydrocannabinol (THC), have been cloned to date, the cannabinoid CB_1 and CB_2 receptors (Howlett et al. 2004). Yet, five different types of endogenous agonists for these cannabinoid receptors have been identified so far (Fig. 1). These compounds, named endocannabinoids by analogy with THC (Di Marzo and Fontana 1995), are all derived from long-chain polyunsaturated fatty acids. In particular: (1) the anandamides are amides of ethanolamine with polyunsaturated fatty acids with at least 20 carbon atoms and three 1,4-diene double bonds. The C20:4 homologue in this series, *N*-arachidonoylethanolamine (AEA) (Devane et al. 1992), also known simply as anandamide, has been most studied.

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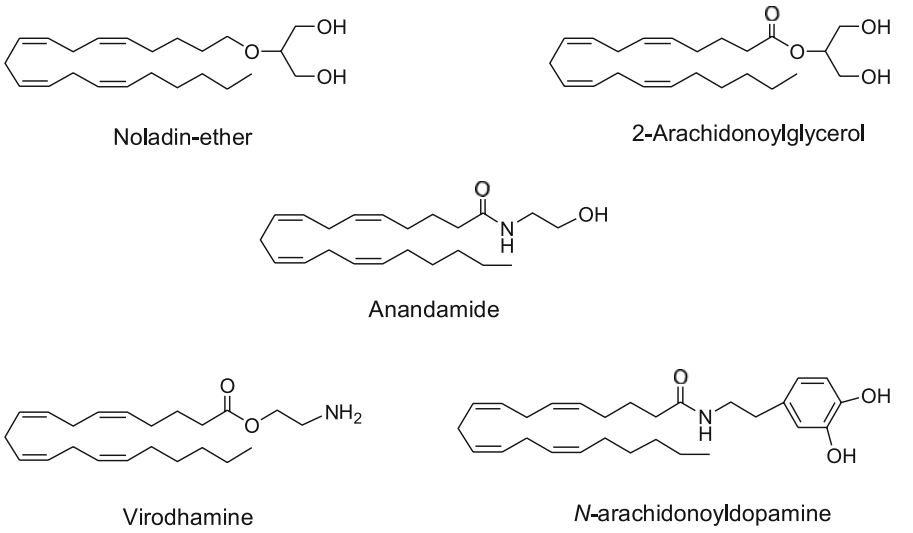

Fig. 1 Chemical structures of the putative endocannabinoids identified to date

We now know that AEA, apart from cannabinoid receptors, can interact with several other plasma membrane proteins (Di Marzo et al. 2002; van der Stelt and Di Marzo 2004); (2) 2-arachidonoyl glycerol (2-AG) (Mechoulam et al. 1995; Sugiura et al. 1995) is considered by some researchers the only selective endogenous cannabinoid receptor agonist (Sugiura and Waku 2002); and (3) 2-arachidonyl glyceryl ether (noladin ether) (Hanus et al. 2001), *O*-arachidonoyl-ethanolamine (virodhamine) (Porter et al. 2002) and *N*-arachidonoylethanolamine (NADA) (Bisogno et al. 2000; Huang et al. 2002) have been described only recently, and their pharmacological activities as endocannabinoids have not yet been fully assessed. Therefore, most endocannabinoids isolated to date are derived from arachidonic acid, which is known to serve as a biosynthetic precursor for many other mediators, the eicosanoids. This structural peculiarity of the endocannabinoids, and the high susceptibility of some of them to be hydrolysed enzymatically to arachidonic acid, raise the possibility that some of the pharmacological actions of these compounds are due to this polyunsaturated fatty acid and its many metabolites. Furthermore, as will be discussed below particularly for AEA and 2-AG, the endocannabinoids can in principle be oxidized, prior to their hydrolysis, by enzymes of the arachidonate cascade, thus generating new series of eicosanoids whose pharmacological properties have not been fully investigated. Finally, the fact that all endocannabinoids are, like THC, lipophilic molecules strongly influences their signalling. In fact, it is now becoming clear that these compounds act as local autocrine or paracrine mediators, and that they are potentially bound to serum albumin in the blood (Bojesen and Hansen 2003).

Commonalities between anandamide and 2-AG anabolic and catabolic reactions

A scenario is now emerging indicating that, although different endocannabinoids share the same molecular targets, their levels are regulated in entirely different ways. This seems to apply particularly to AEA and 2-AG, whose biosynthetic and degradative pathways are

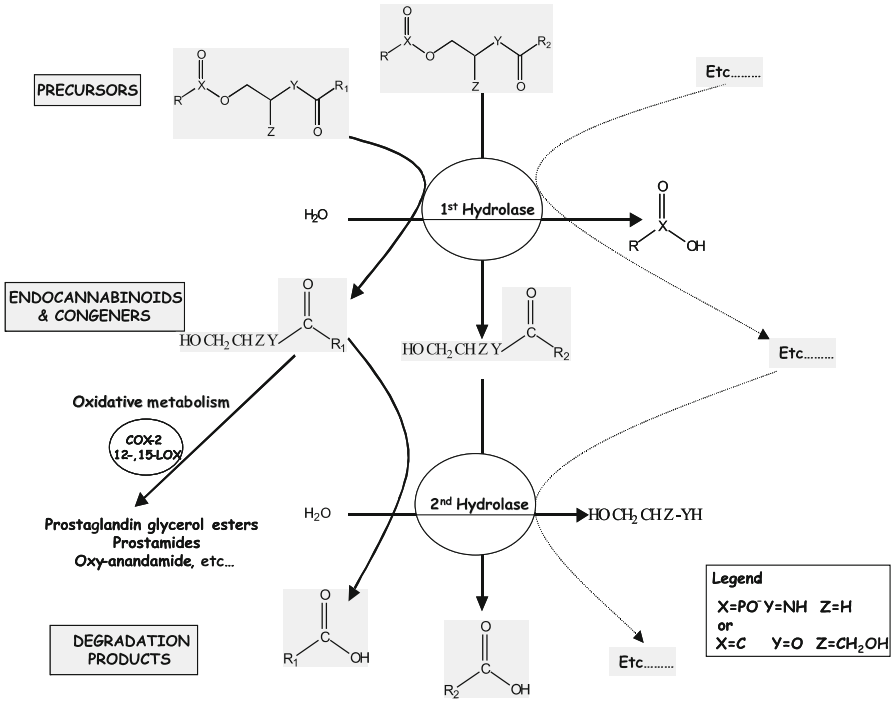

Fig. 2 Commonalities between anandamide and 2-arachidonoylglycerol metabolic pathways. A family of direct biosynthetic precursors, produced from phospholipid remodelling, are all converted into endocannabinoids (R_1=arachidonyl chain) or their cannabinoid receptor-inactive congeners (R_2, etc.) through the catalytic action of a first hydrolytic enzyme (the *N*-acyl-phosphatidylethanolamine-selective phospholipase D—in case of anandamide and other *N*-acylethanolamines—and the *sn*-1-selective diacylglycerol lipases in the case of 2-AG and other 2-acylglycerols). Endocannabinoids and congeners are then degraded to the corresponding fatty acids through the catalytic action of a second hydrolytic enzyme, again common to the same family of compounds [fatty acid amide hydrolase for the *N*-acylethanolamines and possibly the 2-acylglycerols too, and monoacylglycerol lipase(s), which are specific for the 2-acylglycerols]. Members of the same family of congeners (the *N*-acylethanolamines and the 2-acylglycerols) are known to interfere with each other inactivation by substrate competition (Ben-Shabat et al. 1998). Oxidation reaction may serve as "draining reactions" for the potential accumulation of non-polyunsaturated congeners that act also independently from cannabinoid receptors. *COX-2* cyclooxygenase-2, *LOX* lipoxygenases

the ones that have been most studied and utilize entirely different biosynthetic enzymes and precursors. Yet, there are several commonalities between the ways AEA and 2-AG are biosynthesized and degraded (Fig. 2). Since these compounds are eicosanoids, it was reasonable to expect them to be biosynthesized "on demand" and to be immediately released from cells, like with prostaglandins and leukotrienes, and unlike "pre-formed" chemical mediators. Indeed, AEA and 2-AG are produced following the enhancement of intracellular Ca^{2+} concentrations, be it caused by Ca^{2+} influx following, e.g. cell depolarization, or by mobilization of intracellular Ca^{2+} stores subsequent to stimulation of $G_{q/11}$ protein-coupled receptors. Accordingly, as will be discussed herein, the enzymes catalysing the last step of AEA and 2-AG biosynthesis are all Ca^{2+}-sensitive, and extracellular and intracellular Ca^{2+} appear to be differentially involved in the biosynthesis of AEA and 2-AG.

Another common feature of AEA and 2-AG is that they are produced following the hydrolysis of glycero(phospho)lipid precursors, which in turn originate from the remodelling of other membrane phospholipids. Since their most important biosynthetic precursors belong

to two families of lipids, the *N*-acylphosphatidylethanolamines and the diacylglycerols, respectively, and the biosynthesizing enzymes do not appear to be selective for one member of these families over the others, AEA and 2-AG are usually found to occur together with some of their congeners, the *N*-acylethanolamines and the 2-acylglycerols, respectively. The relative amount of a certain congener will reflect the relative amount of its ultimate biosynthetic precursor, and in particular the amount of the corresponding fatty acid esterified on the *sn*-1 and -2 position of phospholipids, respectively. In the case of 2-AG, this compound is the most abundant in its family, and most of its congeners do not seem to possess any biological activity other than that of being capable of potentially enhancing 2-AG life-span or activity at cannabinoid receptors (Ben-Shabat et al. 1998). On the other hand, AEA is one of the least-abundant congeners in its family, and a few of its cognate compounds possess pharmacological actions independent of cannabinoid receptors. The meaning of this seemingly non-selective synthesis of AEA has been debated.

Also the enzymes catalysing AEA and 2-AG hydrolysis are capable of recognizing to some extent as substrates all the unsaturated long-chain congeners of the two endocannabinoids. This means that cannabinoid receptor-inactive congeners can potentially inhibit the degradation of the endocannabinoids by acting as competing substrates, much in the same way the biosynthetic precursors of these congeners can inhibit the conversion of endocannabinoid biosynthetic precursors into endocannabinoids (Fig. 2). A further complication arises from the fact that almost all the enzymes catalysing endocannabinoid biosynthesis and inactivation are hydrolytic enzymes belonging to the large family of serine/cysteine hydrolases. As a consequence, it is difficult to design inhibitors that are selective for each of these enzymes, and this seems particularly true for 2-AG. Although similar to their congeners in their capability of being inactivated by hydrolytic enzymes, AEA and 2-AG are unique in their families because they can be recognized also by some enzymes of the arachidonate cascade, particularly cyclooxygenase-2 (COX-2), and 12- and 15-lipoxygenases (Kozak and Marnett 2002; van der Stelt et al. 2002). These enzymes, if active in the same cell and cell compartment, will catalyse "draining" reactions that may lead to the accumulation of non-polyunsaturated endocannabinoid congeners over polyunsaturated ones, as well as of endocannabinoid oxidation products. The final result would be a shift from cannabinoid receptor stimulation to activation of other molecular targets, i.e. those of saturated or monounsaturated *N*-acylethanolamines and of prostaglandin-ethanolamides and -glycerol esters (see next sections) (Figs. 2, 3). Such a networking of chemical signals provides a typical example of the functional plasticity of lipid mediators, and emphasizes the need of investigating the exact mechanisms regulating endocannabinoid levels in tissues, which will be the subject of the following sections.

Anandamide biosynthesis: one or more pathways?

A study published soon after its discovery suggested that AEA is produced in cell homogenates from the ATP-independent direct condensation of arachidonic acid and ethanolamine (Devane and Axelrod 1994). However, given the high stability of amide bonds, this process must overcome a transition state with very high energy; in fact, it has never been shown to occur in intact cells and can be observed in cell-free systems only when using non-physiological concentrations of arachidonic acid and ethanolamine. Indeed, the enzyme catalysing this most likely non-physiological process is the same that catalyses

AEA hydrolysis (see below), but it works in reverse because of the high concentrations of what would normally be the reaction products (Kurahashi et al. 1997; Arreaza et al. 1997).

Since 1994, it has become widely accepted that AEA is instead produced "on demand" from the hydrolysis of a pre-formed membrane phospholipid precursor, *N*-arachidonoylphosphatidylethanolamine (NArPE) (Di Marzo et al. 1994). This biosynthetic pathway is identical to that through which previously identified long-chain *N*-acyl-ethanolamines are produced from the hydrolysis of the corresponding *N*-acylphosphatidylethanolamines (NAPEs) (Schmid et al. 1990). Measurable levels of NArPE, although relatively low compared to other NAPEs, were found in brain and testis, together with an enzymatic activity capable of converting it into AEA (Di Marzo et al. 1996; Sugiura et al. 1996; Cadas et al. 1997). This enzyme was deemed to be very similar, or identical, to the phospholipase D (PLD)-like enzyme identified in the 1980s in H. Schmid's laboratory (Natarajan et al. 1986; Schmid et al. 1996), which, unlike other PLDs, exhibited no transphosphatidylating activity and low affinity for other phospholipids, and was characterized by little selectivity for any particular NAPE (Sugiura et al. 1996; Petersen and Hansen 1999). The enzyme catalysing the conversion of NAPEs into the corresponding *N*-acylethanolamines was, therefore, termed NAPE-selective PLD (NAPE-PLD). With the recent cloning and expression of the protein (Okamoto et al. 2004), it could also be confirmed that the NAPE-PLD is: (1) chemically and enzymatically distinct from other PLD enzymes; (2) stimulated by Ca^{2+}; and (3) almost equally efficacious with most NAPEs, and hence responsible for the formation of other biologically active *N*-acylethanolamines, such as the C16:0, C18:0 and C18:1 congeners, which all possess pharmacological activity independent of cannabinoid receptors. The amino acid sequence of the enzyme, in fact, revealed no homology with the cloned PLD enzymes and showed it to be a member of the β-lactamase fold of the zinc-metallo-hydrolase family of enzymes (Okamoto et al. 2004). Over-expression of the enzyme in cells led to higher cellular levels of AEA and correspondingly lower levels of NArPE, thus supporting its role in the biosynthesis of this endocannabinoid (Okamoto et al. 2005). Furthermore, it was recently shown that the NAPE-PLD is a fundamental determinant of uterine anandamide levels during mouse pregnancy, and that its expression correlates spatially and temporally with its activity and with anandamide levels, which in turn control embryo implantation (Guo et al. 2005).

Whether or not the direct conversion of NArPE into AEA is the only pathway through which the endocannabinoid is produced in cells still needs final demonstration through the use, for example, of small-interfering RNA (siRNA) technology or of transgenic mice lacking the NAPE-PLD. In fact, although the precursor-product relationship between NArPE and AEA has been substantiated by several studies, including the almost identical distribution of the two molecules in the brain (Bisogno et al. 1999a) and their similar rates of enhancement during brain development (Berrendero et al. 1999), there are additional potential pathways for the conversion of NArPE into the endocannabinoid. Evidence was recently reported in support of another route converting NAPEs into 2-lyso-NAPEs via the action of a group IB soluble phospholipase A_2, and then of 2-lyso-NAPEs into *N*-acylethanolamines via a selective lysoPLD (Sun et al. 2004). In cell-free homogenates this pathways applies to both AEA and its 16:0 congener. Finally, preliminary data suggest that in RAW 264.7 macrophages stimulated with lipopolysaccharide, AEA can be produced from the phosphatase-catalysed hydrolysis of 2′-phospho-AEA, which in turn would derive from phospholipase C-catalysed hydrolysis of NArPE (Liu et al. 2005).

In conclusion, more than one pathway—as is often the case with lipid mediators—may underlie the formation of AEA in cells, depending on the stimulus and on its capability to induce elevations of intracellular Ca^{2+} and/or activate different intracellular hydrolytic en-

zymes. In this context, it is interesting to note that basal AEA levels in mice are under the control of G_q/G_{11} proteins, since transgenic mice lacking these two proteins exhibit significantly reduced brain levels of this endocannabinoid (N. Wettschureck, H.H.H. Tsubokawa, M. van der Stelt, A. Moers, H. Krestel, S. Petrosino, G. Shutz, V. Di Marzo, S. Offermanns, submitted). On the other hand, if these animals are stimulated with kainic acid, which causes neuronal depolarization and Ca^{2+} influx, their brains produce AEA also in the absence of G_q/G_{11} proteins. This suggests that, depending on the conditions, this endocannabinoid, unlike 2-AG (see the following section) can be synthesized following mobilization of either intracellular or extracellular Ca^{2+} alone. Irrespective of the biosynthetic route, AEA always seems to be synthesized inside the cell, to be released into the extracellular milieu only afterwards. In fact, all the biosynthetic enzymes implicated in AEA biosynthesis are intracellular. Since this compound can efficaciously interact with other proteins, in some cases (e.g. as with the vanilloid TRPV1 receptor) using intracellular binding sites, one could foresee that its activation of cannabinoid receptors occurs in some cells after that AEA has influenced the activity of other proteins. Finally, it will be crucial to assess the cellular localization of AEA-synthesizing enzymes, and particularly to verify if, as in the case of 2-AG-synthesizing enzymes, they are post-synaptic in the adult brain and hence in agreement with the possible function as retrograde messengers suggested for endocannabinoids (see below).

2-AG biosynthesis: perhaps less complicated than originally thought

Unlike AEA, 2-AG exhibits selectivity for cannabinoid receptors (Di Marzo et al. 2002) and is also more abundant in almost all tissues analysed so far (Sugiura et al. 2002) (although not, for example, in blood, Maccarrone et al. 2002). Furthermore, this compound, again unlike AEA, behaves as a full agonist in most assays of CB_1 and CB_2 receptor functional activity (McAllister and Glass 2002). Therefore, pharmacological manipulation of the biosynthesis of 2-AG is likely to produce a strong impact on cannabinergic signalling. However, since the first studies on the biosynthesis of this endocannabinoid in intact cells (Bisogno et al. 1997; Stella et al. 1997), it was clear that 2-AG is synthesized through more than one pathway. Furthermore, this lipid is at the crossroads of several metabolic pathways involving glycerophospholipids, triacylglycerols and *sn*-1,2-diacylglycerols (DAGs). However, 2-arachidonate-containing DAGs were immediately identified as the most likely biosynthetic precursors for 2-AG when this compound is used as an extracellular ligand of cannabinoid receptors (Bisogno et al. 1997). In these cases, DAGs appear to be produced in turn from the hydrolysis either of phosphoinositol-bis-phosphate (PIP_2), catalysed by the PIP_2-selective phospholipase C (Stella et al. 1997; Kondo et al. 1998; Berdyshev et al. 2001; Liu et al. 2003) or of phosphatidic acid (PA), catalysed by the PA-selective phosphohydrolase (Bisogno et al. 1999b; Carrier et al. 2004; Oka et al. 2005), and they are converted into 2-AG by the action of *sn*-1-selective DAG lipases (DAGL), which have remained uncharacterized until a couple of years ago. Only recently, two *sn*-1-selective DAGL isoforms, DAGLα and DAGLβ, have been cloned (Bisogno et al. 2003). The molecular characterization and expression of these two enzymes into host cells allowed for the discovery of some of their important features: (1) They contain the typical lipase-3 and Ser-lipase signature sequences; (2) within the Ser-lipase signature sequence, two highly conserved amino acid residues, Ser443 and Asp495—which are normally used to form the catalytic triad of this type of enzyme—were found to be necessary for DAGL activity through site-directed mutagenesis studies; (3) near their N-terminus, the enzymes contain four hydrophobic, and possi-

bly *trans*-membrane, domains, which probably explain why the DAGLs were observed to be localized to the plasma membrane; (4) both DAGLs are stimulated by Ca^{2+} and inhibited by glutathione; (5) although both enzymes exhibit strong selectivity for DAGs over phospholipids, monoacylglycerides, triacylglycerols and fatty acid amides, they do not appear to prefer DAGs with any particular fatty acyl chain in the 2 or *sn*-1 position. Interestingly, DAGLα appeared to be more abundant in the adult brain and DAGLβ in the developing brain. More importantly, the cellular localization of two enzymes shifts during brain development, as they appear to be co-localized with CB_1 in neuronal axons of the peri-natal nervous system, and "move" to a location complementary to CB_1, i.e. to post-synaptic neurons, in the adult brain (Bisogno et al. 2003). This "localization shift" reflects the proposed roles for 2-AG as an autocrine endocannabinoid in axonal guidance (Williams et al. 2003), and as a retrograde messenger in the control of synaptic plasticity in the adult brain (Chevaleyre and Castillo 2003; Melis et al. 2004a).

Apart from their cellular localization, two more series of experiments confirm the role of these DAGLs in controlling the levels of 2-AG when this metabolic intermediate acts as an endocannabinoid: (1) the expression/activity of the enzymes reflects the tissue concentrations of 2-AG under certain physiopathological concentrations, i.e. when the brain levels of the endocannabinoid change dramatically when passing from the light to the dark phase of the day (Valenti et al. 2004) or following induction of neuronal damage with a β-amyloid peptide (Di Marzo et al. 2005), or when 2-AG uterine concentrations are up-regulated in animals with defective leptin signalling (Maccarrone et al. 2005); (2) pharmacological inhibition of the DAGLs was found to reduce 2-AG levels in several cell types stimulated with ionomycin (Bisogno et al. 2003) and to inhibit at the same time CB_1 receptor-mediated cellular phenomena such as fibroblast growth factor (FGF)-induced axonal sprouting in the developing brain, or depolarization-induced suppression of excitation in adult dopaminergic neurons (Bisogno et al. 2003; Melis et al. 2004a). It must be underlined, however, that although the inhibitor used in these latter studies, tetrahydrolipstatin, is very potent against the DAGLs (IC_{50} ~100 nM), it is also known to interfere with the activity of other lipases, albeit at higher concentrations. Therefore, results obtained with more selective inhibitors, or using other techniques, such as siRNAs, must be seen before conclusively asserting that these enzymes, and hence DAGs, are always and uniquely responsible for the biosynthesis of the "endocannabinoid 2-AG" in intact cells. Indeed, recent data have shown that the PLC-DAG-DAGL pathway is probably not involved in controlling the basal levels of 2-AG in unchallenged mice. In fact, transgenic mice lacking G_q/G_{11} proteins that mediate metabotropic receptor-induced activation of phosphoinositide-selective PLC (PLCβ) do not exhibit reduced brain levels of this endocannabinoid (Wettschureck et al., submitted). It is possible, therefore, that most of the relatively high basal levels of 2-AG are not used to activate cannabinoid receptors (the tissue concentration of this compound in the rat brain approximates 5 μM, and would cause a permanent activation of CB_1 receptors), and are formed via biosynthetic precursors other than DAGs (Nakane et al. 2002) and/or through the action of PLC isoforms different from PLCβ (Di Marzo et al. 1996). On the other hand, unlike what described above for AEA, if mice are stimulated with kainic acid, their brains cannot synthesize "on demand" 2-AG in the absence of G_q/G_{11} proteins, and subsequently cannot protect themselves against kainate-induced excitotoxicity (N. Wettschureck, H.H.H. Tsubokawa, M. van der Stelt, A. Moers, H. Krestel, S. Petrosino, G. Shutz, V. Di Marzo, S. Offermanns, submitted). This finding: (1) is in agreement with the suggestion that PLCβ serves as a coincidence detector directing extracellular and intracellular Ca^{2+} mobilization into the formation of endocannabinoids, and of 2-AG in particular (Hashimotodani et al. 2005), and (2) is probably due to the fact that, unlike AEA, both intracellular Ca^{2+} and

PLCβ-derived biosynthetic precursors (i.e. DAGs) are necessary for the formation of that population of 2-AG which acts as a brain endocannabinoid, at least under certain conditions. It will be interesting to confirm these data by measuring the brain levels of 2-AG in yet-to-be-developed DAGLα/β "double knockout" mice. Furthermore, it will be important to assess the importance of DAGL-dependent, but PLCβ-independent, pathways, such as the one relying on PA and PA-selective phosphohydrolase for the formation of DAG precursors (Bisogno et al. 1999b; Carrier et al. 2004; Oka et al. 2005), in the formation of the "endocannabinoid 2-AG".

Biosynthesis of NADA and other endocannabinoids: more questions than answers

Partly due to the fact that their roles as endocannabinoids have not yet been conclusively confirmed, little information still exists on the biosynthetic and catabolic pathways of NADA, virodhamine and noladin ether. Regarding the latter compound, after the initial work of Hanus et al. (2001), two more studies have reported contrasting results on its actual occurrence in brain tissue (Fezza et al. 2002; Oka et al. 2003), and this may have somehow hampered the performance of further studies on noladin ether's metabolic pathways. However, it was clearly established that this putative endocannabinoid is not produced in intact mouse neuroblastoma cells from arachidonic acid incorporated into phospholipids and after ionomycin stimulation, i.e. using conditions leading to the biosynthesis of large amounts of 2-AG (Fezza et al. 2002). As to NADA, strong evidence has been reported against this compound being produced from the metabolism of *N*-arachidonoyl-tyrosine and using the same enzymes converting tyrosine into dopamine. In fact, although *N*-arachidonoyl-tyrosine is present in small amounts in the brain, it cannot be efficaciously converted into *N*-arachidonoyl-DOPA by tyrosine hydroxylase either in vitro or in vivo, and injection of synthetic *N*-arachidonoyl-DOPA into rat brain does not result in the formation of NADA. Conversely, measurable amounts of NADA can be obtained in brain homogenates from arachidonic acid and dopamine, whereas in vivo the basal levels of NADA in rat striatum are strongly dependent on this brain area receiving dopamine from the substantia nigra (S.S.-J. Hu, J.S.-C. Chen, S.M. Huang, A. Minassi, T. Bisogno, R. Roskoski, V. Di Marzo, J.M. Walker, submitted). It remains to be established if the formation of NADA from the condensation of arachidonic acid with dopamine occurs directly or via the intermediacy of arachidonoyl-coenzyme A (CoA) or other activated forms of this fatty acid. Preliminary data investigating the possibility that arachidonoyl-CoA is converted into NADA yielded contradictory results (V. Di Marzo, T. Bisogno, F. Fezza and M. Maccarrone, unpublished results).

Finally, regarding virodhamine, whether this compound is produced from anandamide via a non-enzymatic reaction at slightly alkaline pH (Markey et al. 2000) or through an independent pathway has not been investigated yet.

The endocannabinoid membrane transporter: an "unknown" between release and re-uptake

Given the intracellular localization of their biosynthetic and catabolic enzymes, and the extracellular/lipid interface localization of cannabinoid receptor binding sites, AEA and 2-AG need to be transported across the membrane in order to activate the receptors and

then to be degraded. The two compounds as well as other putative endocannabinoids are quite lipophilic and therefore there is currently a lively debate regarding the possibility that endocannabinoid uptake by, and release from, cells occurs via facilitated transport across the plasma membrane and not via simple passive diffusion. In the latter case, intracellular metabolism facilitate the uptake by, but would only retard the release from, the cell. The evidence in favour and against the existence of a common carrier, or at least of a specific process, for the bi-directional membrane transport of all endocannabinoids according to the gradient of concentrations across the plasma membrane (Porter et al. 2002; Huang et al. 2002; Beltramo and Piomelli 2000; Bisogno et al. 2001; Hajos et al. 2004; Fezza et al. 2002) has been lately summarized by various authors (Hillard and Jarrahian 2003; McFarland and Barker 2004; De Petrocellis et al. 2004). That fatty acid amide hydrolase (FAAH) cannot account alone for endocannabinoid cellular uptake is supported not only by several indirect data (Bisogno et al. 2005a), but also and most importantly by recent experiments carried out using: (1) cells from FAAH-knockout mice; (2) uptake inhibitors selective vs FAAH; and (3) confocal microscopy to assess the spatial and functional separation between anandamide uptake and hydrolysis (Ligresti et al. 2004; Ortega-Gutierrez et al. 2004; Fegley et al. 2004; Ortar et al. 2003; Oddi et al. 2005). However, the putative endocannabinoid membrane transporter has not been cloned yet, nor has any protein involved in endocannabinoid transport across the membrane been identified to date. Intriguingly, preliminary evidence has been presented recently on the pharmacological characterization of an anandamide membrane transporter (Chesterfield et al. 2005). Using a novel radioligand and a variety of small-molecule inhibitors of AEA uptake, the authors identified a high-affinity plasma membrane binding site whose pharmacology correlated to that of the putative endocannabinoid membrane transporter. It is also possible that endocannabinoid membrane transport is the result of more than one process, perhaps including endocytosis and/or the interaction with more than one protein in more than one subcellular compartment (Hillard and Jarrahian 2003; McFarland and Barker 2004).

The bi-directionality of facilitated endocannabinoid transport mentioned above is suggested, among other things, by the fact that the release of endocannabinoids after their biosynthesis is blocked by the same selective inhibitors that block reuptake (Ligresti et al. 2004; Ronesi et al. 2004). This finding raises an important question: How can substances that block both endocannabinoid uptake and release also elevate the levels of endocannabinoids available for cannabinoid receptor stimulation and subsequently induce beneficial actions in animal models of disorders where endocannabinoids play a protective function? Examples of these disorders include, to date, both central and peripheral disorders (see section “Conclusions: new therapeutic drugs from studies of endocannabinoid synthesis and degradation”). Clearly, if inhibitors of the putative endocannabinoid transporter reach the tissues involved in the disease only after the disease itself has already developed, and hence after the endocannabinoids involved in the disease have been already released, inhibitors of the transporter can prolong endocannabinoid action at cannabinoid receptors. It is also possible that not all cell types are permeable to the endocannabinoid membrane transporter inhibitors developed so far, and that these can only block re-uptake, with no effect on release unless they are previously inserted or allowed to diffuse inside the cell (as shown by Ronesi et al. 2004). In fact, competitive inhibition of release would require that the inhibitors—and not only the endocannabinoid—are inside the cell.

Fatty acid amide hydrolase: AEA degradation and beyond

It was soon clear that AEA is inactivated through the hydrolysis of its amide bond to arachidonic acid and ethanolamine (Di Marzo et al. 1994; Deutsch and Chin 1993), i.e. through the same route previously shown to underlie *N*-acylethanolamine degradation (Schmid et al. 1996). The possibility of using previously obtained information facilitated the identification of the enzyme responsible for this reaction, which was partially purified by Ueda and colleagues (1995), named "anandamide amidohydrolase", and proposed to be identical to *N*-acylethanolamine hydrolase (Schmid et al. 1996). In subsequent studies, it was also observed that this enzyme could also recognize as a substrate oleoylamide (oleamide), a putative sleep factor (Maurelli et al. 1995). This finding allowed for the demonstration that the "oleamide amidase" cloned in 1996 by Cravatt and his collaborators catalysed the hydrolysis also of AEA (Cravatt et al. 1996). Due to its wide selectivity for fatty acid amides, including long-chain *N*-acylethanolamines, primary amides, *N*-acylamino acids and, more recently, *N*-acyltaurines (Fowler et al. 2001; Ueda 2002; Deutsch et al. 2002; Bisogno et al. 2002; Saghatelian et al. 2004), the enzyme was named "fatty acid amide hydrolase" (FAAH). Despite its name, however, FAAH also catalyses quite efficiently the hydrolysis of fatty acyl esters, including 2-AG (Goparaju et al. 1998; Di Marzo et al. 1998) and most probably virodhamine too (Steffens et al. 2005).

Since the several important structural features of FAAH were recently discussed in a comprehensive review (McKinney and Cravatt 2005), they will not be described here. These features were revealed by means of site-directed mutagenesis studies and, more recently, crystallographic X-ray studies carried out on the protein in a complex with a covalent inhibitor (Deutsch et al. 2002; Bracey et al. 2002). On the other hand, several aspects of the function of FAAH under both physiological and pathological conditions were revealed by: (1) the study of the phenotype of transgenic mice lacking the enzyme (the "FAAH-knockout mice") (Cravatt et al. 2001); (2) the design of specific FAAH inhibitors suitable for use in vivo (Bisogno et al. 1998; Kathuria et al. 2003; Lichtman et al. 2004); (3) immunohistochemical studies describing the tissue and cellular distribution of the enzyme and its relationship with CB_1 receptor distribution (Egertova et al. 2003; Gulyas et al. 2004); and (4) the identification of the promoter region of the *Faah* gene and of its regulation by several transcription factors and hormones (Maccarrone et al. 2000b, 2003a, b). As will be discussed herein, we now know that FAAH is an important determinant of endocannabinoid and fatty acid amide levels in several, but not all, physiological and pathological states. In fact, at least in the case of AEA, there is little doubt that FAAH is the major, if not the only, degrading enzyme in the brain since, in this organ, AEA levels are dramatically elevated in FAAH-deficient mice (Cravatt et al. 2001) or following administration of FAAH inhibitors to rats (Kathuria et al. 2003). However, regarding some peripheral organs, such as the duodenum and the liver in rats and mice, there seems to be some discrepancy as to whether or not deletion of the *Faah* gene in mice, or pharmacological inhibition of the enzyme, cause elevation of AEA levels (Fegley et al. 2005; Cravatt et al. 2004). Some studies found that AEA was up-regulated in the liver, kidney and testis of these transgenic mice (Cravatt et al. 2004) as well as in their small intestine (Capasso et al. 2005), where the FAAH inhibitor *N*-arachidonoyl-serotonin administered to wild-type mice also causes an increase of AEA levels (Capasso et al. 2005). By contrast, in another study, the other FAAH inhibitor URB-597, or genetical deletion of *Faah*, did not result in the elevation of small intestine and liver AEA levels (Fegley et al. 2005). Clearly, methodological differences and different pharmacokinetic profiles of the FAAH inhibitors used account for such discrepancies. However, the

possibility that another AEA hydrolysing enzyme may exist in peripheral organs should not be ruled out. This is unlikely to be the recently cloned "*N*-acylethanolamine-hydrolysing acid amidase" (Tsuboi et al. 2005), since this enzyme is specific for long-chain saturated *N*-acylethanolamines. Conversely, it is clearly emerging that FAAH is also involved in the control of fatty acid amides different from AEA. In a recent study (Cravatt et al. 2004), it was elegantly shown how the genetic inactivation of the *Faah* gene, when limited to peripheral tissues, resulted in an anti-inflammatory phenotype that was not due to elevated activation of CB_1 receptors. The authors suggested that other bioactive fatty acid amides that are substrates for FAAH, i.e. oleoylethanolamide, palmitoylethanolamide or *N*-arachidonoylglycine, could be responsible for the elevated threshold to inflammatory pain via peripheral actions.

Despite the wide substrate selectivity of FAAH, this enzyme certainly plays a role in regulating the activity of CB_1 receptors, as shown by the several immunohistochemical data pointing to the complementary distribution of the two proteins in the CNS. In fact, CB_1 and FAAH are mostly localized at the pre- and post-synaptic level, respectively, in many (but not all) brain areas (Egertova et al. 2003; Gulyas et al. 2004). In these regions, FAAH may regulate post-synaptic levels of anandamide (Egertova et al. 2003), whereas in regions such as the globus pallidus and substantia nigra pars reticulata, CB_1 receptors are not associated with FAAH expression. In these brain regions the control over endocannabinoid signalling may be less restricted than in regions enriched with FAAH. Finally, in those brain regions where FAAH-immunoreactive neurons occur in the absence of CB_1, the enzyme may be involved in the regulation of endocannabinoid actions at other endocannabinoid receptors (Di Marzo et al. 2002), or of receptors for other FAAH substrates.

2-AG degradation: redundancy again

Unlike AEA, 2-AG can be metabolized via several different chemical reactions. If one looks just at the enzymatic hydrolysis of this compound, apart from FAAH, another hydrolase, the monoacylglycerol lipase (MAGL), was suggested to be involved in 2-AG inactivation in both nervous and immune cells and tissues; and even with this enzymatic activity, there appears to be some heterogeneity (Goparaju et al. 1999; Di Marzo et al. 1999a; Saario et al. 2004). A MAGL enzyme was cloned first from the human and mouse (Karlsson et al. 1997; Ho et al. 2002) and more recently from the rat (Dinh et al. 2002a). Strong evidence for its role in 2-AG degradation, at least in isolated cells, was provided through the use of the siRNAs, which showed how "silencing" of MAGL results in the impairment of 2-AG degradation and in the enhancement of 2-AG, but not AEA, cellular levels (Dinh et al. 2004). However, other monoacylglycerol lipases are likely to exist. In fact, MAGL was found to account for only 50% of the total 2-AG-hydrolysing activity in soluble fractions of rat brain (Dinh et al. 2004); and the membrane-bound and soluble 2-AG hydrolase(s) previously found in rat circulating macrophages and platelets were shown to be sensitive to down-regulation by lipopolysaccharide in different ways (Di Marzo et al. 1999a). This heterogeneity may also explain why, although the cloned MAGL does not recognize AEA as substrate, MAGL activities in rat macrophage membranes and rat cerebellum cytosolic fractions are sensitive to inhibition by high micromolar concentrations of AEA (Di Marzo et al. 1999a; Ghafouri et al. 2004).

Like FAAH and other "endocannabinoid enzymes", the cloned MAGL, whose other general features have been reviewed recently (Dinh et al. 2002b), is also characterized by

poor substrate selectivity: It recognizes as substrates both *sn*-1- and 2-acylglycerols with almost any long-chain fatty unsaturated acid esterified to the glycerol backbone (see also Di Marzo et al. 1999b). Interestingly, this enzyme appears to be distributed in the CNS in the same brain regions as CB_1 receptors and, unlike FAAH, is mostly a pre-synaptic enzyme (Gulyas et al. 2004; Dinh et al. 2002a). Therefore, from studies on the cellular localization of DAGLα and β, it can be extrapolated that MAGL is localized complementarily to these major 2-AG biosynthesizing enzymes in the adult brain (Bisogno et al. 2003), in agreement with the proposed role of 2-AG in CB_1 receptor-mediated retrograde signalling (Chevaleyre and Castillo 2003; Melis et al. 2004a). In fact, 2-AG can be produced in a Ca^{2+}-sensitive way from the post-synaptic neuron, and diffuse towards the pre-synaptic terminal where it: (1) activates CB_1 receptors, leading to inhibition of neurotransmitter release, and (2) is subsequently inactivated through enzymatic hydrolysis. However, our current knowledge of the role of the cloned MAGL in the control of endocannabinoid "tone" is still limited due to the lack of a "knockout" mouse for this enzyme and of selective inhibitors. A recent article reported the development of a series trifluoromethyl ketone and methyl ketone thioether derivatives as possible specific inhibitors of 2-AG hydrolysis (Nithipatikom et al. 2005). Although the authors showed that some of these compounds effectively inhibit 2-AG hydrolysis by cytosolic fractions of prostate carcinoma (PC)-3 cells, and significantly enhance 2-AG levels in these cells, no direct data on the selectivity of these compounds vs FAAH or the DAGLs were reported. In another study, the *N*-ethyl-maleimide derivative of arachidic acid (*N*-arachidonylmaleimide) was reported to potently inhibit 2-AG hydrolysis (IC_{50}=180 nM) (Saario et al. 2005), but again with no data on the possible selectivity of this compound.

While the role of MAGLs in controlling the rate of 2-AG degradation in vivo is becoming widely recognized, a similar function for FAAH is still controversial. In some studies, FAAH genetic inactivation and/or pharmacological inhibition does not result in the elevation of brain 2-AG concentrations, thus prompting that FAAH may not be important in the control of 2-AG levels (Cravatt et al. 2001; Kathuria et al. 2003; Fegley et al. 2005; Lichtman et al. 2002). However, bearing in mind the high redundancy of pathways and enzymes through which 2-AG is metabolized in vitro, this conclusion is probably not entirely warranted. In fact, one alternative pathway or enzyme might compensate for the loss of FAAH and explain why 2-AG levels are not changed following inactivation of this enzyme. It would require the absence or the genetic or pharmacological inactivation of both FAAH and MAGLs—at least—to observe an elevation of 2-AG levels in an animal. Indeed, previous experiments showing that 2-AG levels are elevated following inactivation of MAGL, as opposed to FAAH, were carried out using experimental conditions where little or no detectable FAAH activity is present, i.e. in: (1) homogenates of rat cerebellum (Saario et al. 2004), (2) HeLa cells (Dinh et al. 2004), or (3) mouse brain cytosolic fractions (Dinh et al. 2004). Instead, a parsimonious interpretation of the available data is that MAGL is not the sole hydrolytic enzyme for 2-AG. In fact, data are now emerging according to which pharmacological blockade of FAAH does produce a significant elevation of 2-AG as well as AEA tissue levels, for example when: (1) a metabolically stable, non-competitive FAAH inhibitor such as *N*-arachidonoylserotonin (Bisogno et al. 1998) is administered systemically and sub-chronically (de Lago et al. 2005); or (2) FAAH inhibitors are administered locally and chronically (Bifulco et al. 2004; Bisogno et al. 2005b); (3) 2-AG levels are measured in peripheral organs (Capasso et al. 2005). Therefore, it is likely that both FAAH and MAGLs might play an important function in the enzymatic hydrolysis of 2-AG. The subsequent formation of arachidonic acid may not only represent the inactivation of an endocannabinoid

signal, but also the formation of arachidonic acid derivatives with altogether different biological functions (Jarai et al. 2000; Kojima et al. 2002; Gauthier et al. 2005).

Finally, to underscore further redundancy as a hallmark of 2-AG inactivation pathways, strong evidence also exists for the direct esterification of this compound into neutral glycerolipids and phospholipids (Di Marzo et al. 1999a). The relevance of these pathways, which were already known when 2-AG was considered a mere intermediate in glycerol(phospho)lipid metabolism, to the regulation of the cannabinergic signal still need to be assessed.

Endocannabinoid oxidation and its possible role

Due to the presence of an arachidonate moiety in their chemical structure, endocannabinoids are in principle easily oxidized through the action of the same enzymes that catalyse the oxidation of arachidonic acid. Indeed, both anandamide and 2-AG are substrates for some enzymes of the arachidonate cascade, in particular the 12- and 15-lipoxygenases, cytochrome p450 oxygenases and COX-2 (Fig. 3). These reactions have been found to occur in intact cell systems only in a few cases, and never in vivo, and their biological meaning is still a matter for speculation (Kozak and Marnett 2002). While lipoxygenase products (Fig. 3) are usually still capable of binding to cannabinoid receptors to some extent and/or to inhibit FAAH (van der Stelt et al. 2002), the prostanoid derivatives of both AEA and 2-AG, obtained from the further metabolism of COX-2-derived, but not COX-1-derived, endoperoxide products of the two compounds, are inactive on all cannabinoid and prostanoid receptors and, in some cases, appear to act instead at novel and yet-to-be-characterized receptors (Ross et al. 2002; Nirodi et al. 2004; Matias et al. 2004). Among these compounds, it is worthwhile mentioning prostaglandin $F_{2\alpha}$-ethanolamide, which can be obtained from prostaglandin $F_{2\alpha}$ through the action of COX-2 and prostaglandin F synthase (Koda et al. 2004), and prostaglandin E_2-glycerol ester, which is formed from COX-2 and prostaglandin E synthase (Kozak et al. 2002a). Interestingly, these prostaglandin-ethanolamides (or prostamides) and prostaglandin-glycerol esters are not subject to FAAH or MAGL-catalysed hydrolysis (Matias et al. 2004; V. Di Marzo and L.J. Marnett, unpublished observations). To date, the evidence in favour of the existence of these compounds in vivo is limited—for prostamides—to data obtained by injecting AEA in FAAH-deficient mice (Weber et al. 2004), or by treating primary amnion tissue explants with endocannabinoids (Glass et al. 2005). We have obtained preliminary mass-spectrometric evidence for the presence of a prostamide $F_{2\alpha}$-like compound in several murine tissues from untreated animals, with the highest concentrations in the eye and lungs (V. Di Marzo, I. Matias, J. Chen and D. Woodward, unpublished data).

While the structural bases of COX-2 interaction with AEA have been thoroughly examined (Kozak et al. 2003), indirect evidence for the participation of this enzyme in the inactivation of the endocannabinoid signal in the hippocampus has been reported. In fact, in this brain area, inhibitors of COX-2, but not FAAH, potentiate both short-term and long-term endocannabinoid-mediated synaptic plasticity (Kim and Alger 2004; Slanina and Schweitzer 2005).

Regarding AEA metabolites obtained from lipoxygenases, it has been suggested, so far only on the basis only of pharmacological evidence, that they might be capable of stimulating vanilloid TRPV1 (Craib et al. 2001). Indeed, a recent study demonstrated the possible action of the leukotriene B_4 derivative of AEA at these receptors (McHugh et al. 2005). Re-

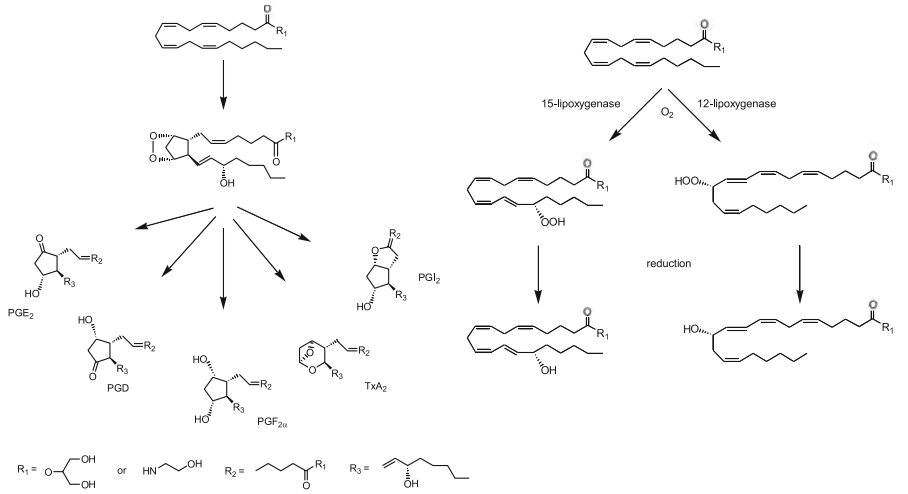

Fig. 3 Enzymatic oxidation of endocannabinoids. The oxidation of anandamide and 2-AG has been suggested to occur through the catalytic action of either cyclooxygenase-2 or 12- and 15-lipoxygenases. The oxidation products of the former, but not latter, reaction are totally inactive on cannabinoid receptors, but may bind to yet-to-be-identified receptors

cent evidence suggested that also the 15-lipoxygenase derivative of 2-AG (Fig. 3), but not 2-AG itself, interacts with peroxisome proliferator-activated receptor (PPAR)-α (Kozak et al. 2002b).

Regulation of endocannabinoid levels: what is the target, biosynthesis or degradation?

The endocannabinoids represent a typical example of mediators whose levels are controlled through the regulation not only of biosynthetic but also of catabolic enzymes (Table 1). Furthermore, these compounds are produced from biosynthetic precursors that in turn derive from phospholipids whose levels are potentially influenced by the diet and by its relative contents in ω3 and ω6 polyunsaturated fatty acids, in particular arachidonic and docosahexaenoic acids (AA and DHA). Indeed, diets defective or rich in DHA will increase or decrease, respectively, the brain levels of 2-AG (Watanabe et al. 2003), whereas milk deprived of both AA and DHA will lead to lower brain levels of AEA in newborns (Berger et al. 2001).

There are several examples of regulation of AEA levels via opposite regulation of FAAH activity and, in particular, expression (Maccarrone et al. 2000b, 2003a, b) under both physiological and pathological conditions. Also the MAGL is subject to regulation, resulting in corresponding opposing changes in 2-AG levels (Di Marzo et al. 1999a; Witting et al. 2004; Maccarrone et al. 2005), although the promoter region of the *Magl* gene has not been identified yet. In some cases, endocannabinoid levels can be regulated by targeting in opposite ways both biosynthetic and degradative enzymes, as in the case of 2-AG in astrocytes following P2X7 receptor stimulation (Witting et al. 2004), and of AEA in mouse uterus during embryo uterine implantation (Maccarrone et al. 2000b; Guo et al. 2005). There are also cases in which the expression and/or activity of both biosynthetic and the degradative enzyme are up-regulated, and these changes lead nevertheless to the up-regulation of endocannabinoid levels, as is the case with rat brain 2-AG when passing from the light to the dark phase of the

Table 1 Possible mechanisms underlying the regulation of endocannabinoid levels. The regulatory events that lead to elevation or decrease of endocannabinoid levels are shown in normal type or in italics, respectively. Examples are mostly limited to physiological conditions, and many more examples of each type of regulation have been found during pathological conditions

	Anandamide	2-AG	Reference(s)
Dietary availability of PUFA precursors	Increased brain levels in piglet and mouse pup brain following increased AA and DHA in milk	Increased and *decreased* brain levels in mice following decreased or increased DHA in diet, respectively	Berger et al. 2001; Watanabe et al. 2003
Regulation of biosynthesis (Mostly exerted by regulating NAPE-PLD and DAGL expression/activity for anandamide and 2-AG, respectively)	In mouse uterus during embryo implantation	In microglial cells after P2X7 receptor stimulation; *In rat brain when passing from light to dark phase of the day*; *In rat hypothalamus following systemic administration of leptin (upstream of DAGL)*; *In the mouse uterus (by leptin)*	Guo et al. 2005; Witting et al. 2004; Valenti etal. 2004; Di Marzo et al. 2001; Maccarrone et al. 2005
Regulation of catabolism (Exerted by regulating FAAH and MAGL expression/activity for anandamide and 2-AG, respectively)	*In the mouse uterus (by leptin)*; *In lymphocytes stimulated with leptin or progesterone*; *In mouse uterus during embryo implantation (by progesterone)*	In macrophages stimulated with LPS; In microglial cells after P2X7 receptor stimulation	Maccarrone et al. 2000b, 2003a, 2005; Di Marzo et al. 1999a; Witting et al. 2004
Regulation of re-uptake (Via the putative membrane transporter)	*In the mouse uterus (by leptin)*	No direct evidence	Maccarrone et al. 2005

AA arachidonic acid, 2-AG 2-arachidonoyl glycerol, DHA docosahexaenoic acid, FAAH fatty acid amide hydrolase, DAGL sn-1-selective diacylglycerol lipase, MAGL monoacylglycerol lipase, NAPE-PLD N-acylphosphatidylethanolamine-selective phospholipase D

day (Valenti et al. 2004) or following induction of neuronal damage with a β-amyloid peptide (Di Marzo et al. 2005). These concurrent changes of the anabolic and catabolic enzymes probably occur when the stimulation of endocannabinoid levels needs to be transient, since they result in an overall increase of endocannabinoid turnover. Finally, there are examples of the same stimulus leading to the same change in the tissue concentrations of AEA and

2-AG, but through different regulatory strategies. This is the case of leptin, which causes down-regulation of AEA levels in the blood and uterus via up-regulation of FAAH expression, whereas it provokes a decrease of 2-AG levels by enhancing its degradation in the uterus and by inhibiting its biosynthesis in both the hypothalamus and the uterus (Di Marzo et al. 2001; Maccarrone et al. 2005). Finally, the possibility that COX-2 over-expression during certain pathological states, such as inflammation and colon carcinoma, may contribute to regulating endocannabinoid levels has not been investigated yet.

Although the endocannabinoid membrane transporter has not been cloned to date, this putative protein too has been shown to be subject to regulation. In particular, nitric oxide stimulates AEA cellular uptake in many cell types, whereas chronic treatment of cells with ethanol inhibits it (Maccarrone et al. 2000a; Basavarajappa et al. 2003). In either case, no effect on FAAH activity was found, whereas leptin inhibits both AEA reuptake by, and FAAH activity in, mouse uterine tissue (Maccarrone et al. 2005).

In conclusion, several possibilities exist for the regulation of endocannabinoid levels, including the one described in the second section of this article and utilizing the concomitant formation of cannabinoid receptor-inactive endocannabinoid congeners capable of acting as competitive substrates for biosynthetic or hydrolytic enzymes. Such a variety of mechanisms indicates that the concentrations of AEA and 2-AG near their molecular targets, and hence the activity of the cannabinoid receptors, are tightly regulated and, therefore, that this signalling system must play a fundamental role under various conditions.

Conclusions: new therapeutic drugs from studies of endocannabinoid synthesis and degradation

The recent development of Rimonabant (Sanofi-Aventis, Paris, France), a selective cannabinoid CB_1 receptor antagonist/inverse agonist, as an efficacious anti-obesity agent with possible use also against the metabolic syndrome (Van Gaal et al. 2005), and the future marketing in Canada of Sativex (GW Pharmaceuticals, Salisbury, UK), a pharmaceutical preparation—based on a *Cannabis* extract—against neuropathic pain in multiple sclerosis, might one day be followed by the use of therapeutic drugs that also manipulate the levels rather than the action of endocannabinoids, thereby influencing the tone of cannabinoid receptors indirectly. For example, given the strong weight of endogenous 2-AG in the activation of cannabinoid receptors, inhibitors of the DAGL might produce a blockade of endocannabinoid signalling similar to that caused by receptor antagonists, and hence, like the latter compounds, find an application in those disorders where, at least in animal models, excessive endocannabinoid signalling appears to contribute to the progress or the symptoms of the disease, i.e Parkinson's disease and levodopa-induced dyskinesia (Di Marzo et al. 2000; van der Stelt et al. 2005; Fernandez-Espejo et al. 2005), Alzheimer's disease (Mazzola et al. 2003; Di Marzo et al. 2005), obesity and metabolic syndrome (Di Marzo and Matias 2005), penile erection (Melis et al. 2004b), nicotine, cocaine and alcohol dependence (Cohen et al. 2002; Soria et al. 2005), and relapse of cocaine and heroin abuse (De Vries et al. 2001; Fattore et al. 2003). On the other hand, inhibitors of endocannabinoid re-uptake and enzymatic hydrolysis might represent an alternative to the use of direct CB_1 and CB_2 receptor agonists, which are more likely to exert undesired psychotropic and immune-suppressive effects, respectively, and to undergo tolerance. Examples of disorders that could be treated with such inhibitors include, to date, both central (experimental allergic encephalomyelitis, kainate-induced excitotoxicity, etc.) and peripheral (cholera-toxin-induced intestinal hypersecretion, cancer, hyperten-

sion, etc.) disorders (Bifulco et al. 2004; Baker et al. 2001; Marsicano et al. 2003; Izzo et al. 2003; Batkai et al. 2004; Mestre et al. 2005). But in principle, in all the diseases—or in all the distinct phases of a certain disease—where endocannabinoids are produced "on demand" to exert a protective function irrespective of their molecular targets, inhibitors of their inactivation should produce therapeutic effects.

Finally, the exact knowledge of the molecular targets of some endocannabinoid metabolic products, such as prostamides and prostaglandin glycerol esters, might lead to new therapeutic agents. Indeed, the 17-phenyl-derivative of prostamide $F_{2\alpha}$, bimatoprost (Lumigan, Allergan, Irvine, CA), is already marketed as an anti-glaucoma agent. This compound exhibits very low affinity for and potency at the FP receptor, although it exerts a powerful contraction of the isolated feline iris sphincter and potently reduces the intraocular pressure in ocular normotensive dogs (Woodward et al. 2001). Indeed, prostamide $F_{2\alpha}$, and hence possibly bimatoprost, may act on an as-yet-uncharacterized novel receptor, since they both exert a strong contraction of feline lung parenchyma at low nanomolar concentrations, and have an entirely different pharmacological profile from prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in all the other available assays for FP receptor-mediated activity, while exerting no activity in a wide range of binding assays for other known prostanoid receptors (Woodward et al. 2001, 2003). The very recent report of a selective antagonist of prostamide, but not $PGF_{2\alpha}$, contractile action on cat iris strips strongly suggests the existence of a specific receptor for these putative COX-2 metabolites of anandamide and their synthetic analogs (Woodward et al. 2005). Based on this experience, it can be foreseen that the full understanding of endocannabinoid synthesis and degradation will lead to more therapeutic drugs for a variety of disorders that still await an efficacious and safe treatment.

Acknowledgements. I am grateful to Dr. Stefania Petrosino for her help during the preparation of this manuscript.

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Rev Physiol Biochem Pharmacol (2006) 160:25–46
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Biological effects of lysophospholipids

Published online: 29 April 2006

Abstract Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are potent biologically active lipid mediators that exert a wide range of cellular effects through specific G protein-coupled receptors. To date, four LPA receptors and five S1P receptors have been identified. These receptors are expressed in a large number of tissues and cell types, allowing for a wide variety of cellular responses to lysophospholipid signaling, including cell adhesion, cell motility, cytoskeletal changes, proliferation, angiogenesis, process retraction, and cell survival. In addition, recent studies in mice show that specific lysophospholipid receptors are required for proper cardiovascular, immune, respiratory, and reproductive system development and function. Lysophospholipid receptors may also have specific roles in cancer and other diseases. This review will cover identification and expression of the lysophospholipid receptors, as well as receptor signaling properties and function. Additionally, phenotypes of mice deficient for specific lysophospholipid receptors will be discussed to demonstrate how these animals have furthered our understanding of the role lysophospholipids play in normal biology and disease.

Introduction

Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are two distinct biologically active lysophospholipid molecules capable of exerting dramatic effects on a wide variety of cell and tissue types. Although both serve as metabolites in membrane phospholipid synthesis, their ability to act as extracellular signaling molecules is their most intriguing function (Moolenaar 1995; Pages et al. 2001b; Spiegel and Milstien 2003). Early studies demonstrated that both LPA and S1P were capable of initiating a variety of cellular responses in diverse cell types. However, it was the identification and subsequent cloning of

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specific G protein-coupled receptors that provided invaluable insight into the biological activities of these molecules in vivo.

Activated platelets are a major source of LPA and S1P; however, these lysophospholipids are produced by many different cell types (Sano et al. 2002; Yatomi et al. 2000). Mature neurons, Schwann cells, adipocytes, and fibroblasts have been implicated in the production of LPA (Fukushima et al. 2000; Pages et al. 2001a, 2001b; Weiner et al. 2001). Likewise, in response to external stimuli, hematopoietic cells such as peripheral mononuclear cells, erythrocytes, and neutrophils can contribute to the level of S1P in the blood (Yang et al. 1999). Together, these multiple sources contribute to LPA and S1P levels in a range of biological fluids, including serum, saliva, and follicular fluid. Interestingly, ovarian cancer cells also produce S1P and LPA, and studies suggest that these molecules may contribute to tumor cell progression and metastasis (Eder et al. 2000; Yatomi et al. 2001).

S1P is produced by sphingosine kinase (SPHK) -mediated phosphorylation of sphingosine, a lipid synthesized through the metabolic conversion of ceramide. Two sphingosine kinase isoforms are known to exist in mice, SPHK1 and SPHK2 (Kohama et al. 1998; Liu et al. 2000a). Deletion of SPHK1 reduces serum and plasma levels of S1P to 50% of those observed in wild type mice, which indicates that SPHK2 or unidentified sphingosine kinases are responsible for the remaining SPHK catalytic activity (Allende et al. 2004). SPHK activity has been described in cytosolic, membrane, and extracellular compartments (Ancellin et al. 2002; Olivera et al. 1999). While SPHKs are involved in S1P production, lipid phosphate phosphatases (LPPs) and S1P lyase (SPL) degrade S1P. To date, two S1P phosphatases have been cloned, SPP1 and SPP2 (Mandala et al. 2000; Ogawa et al. 2003). The presence of these two enzymes in the endoplasmic reticulum (ER) indicates that these molecules dephosphorylate intracellular S1P. SPL is an evolutionarily conserved enzyme, also present in the ER, that catalyses the cleavage of S1P into hexadecanal and phosphoethanolamine (Van Veldhoven and Mannaerts 1994). Platelets lack SPL, which may partially account for their ability to produce large amounts of S1P. In *Dictyostelium discoideum*, *Caenorhabditis elegans*, and *Drosophila melanogaster*, loss of SPL results in abnormal development and differentiation (Herr et al. 2004; Li et al. 2001; Mendel et al. 2003). Mice deficient for SPL have not been generated; however, a targeted deletion of SPL in mouse F9 embryonic carcinoma cells causes an increase in endodermal differentiation in response to retinoic acid (RA) (Kihara et al. 2003). This effect is also observed in F9 cells stably expressing a SPHK (Kihara et al. 2003). Conversely, pretreatment of F9 cells with an S1P synthesis inhibitor blocks differentiation, indicating that an accumulation of S1P is responsible for this effect.

Serum LPA levels are regulated by the secreted lysophospholipases PLA_1 and PLA_2, and lysophospholipase D (lysoPLD). PLA_1 and PLA_2 are also thought to be responsible for the de novo generation of lysophosphatidylcholine (LPC) (Fig. 1), a substrate for lysoPLD, which can then cleave LPC to produce LPA (Aoki et al. 2002; Sano et al. 2002).

The recent identification of lysoPLD as autotaxin (ATX) has been particularly helpful for understanding LPA pathways. ATX was first identified as a protein in melanoma cell culture medium that stimulated cancer cell motility (Murata et al. 1994; Stracke et al. 1992). ATX has a single transmembrane domain, two somatomedin B-like domains, and a catalytic domain (Stracke et al. 1997). ATX is most likely cleaved intracellularly, then released into the extracellular environment. ATX was initially proposed to function as a nucleotide phosphodiesterase, but the role of this activity in ATX function is currently unclear (Bollen et al. 2000; Goding et al. 1998). In addition to its ability to promote cancer cell motility, ATX has also been shown to promote angiogenesis in vivo (Nam et al. 2001). These results support the view that ATX is an important factor in cancer biology.

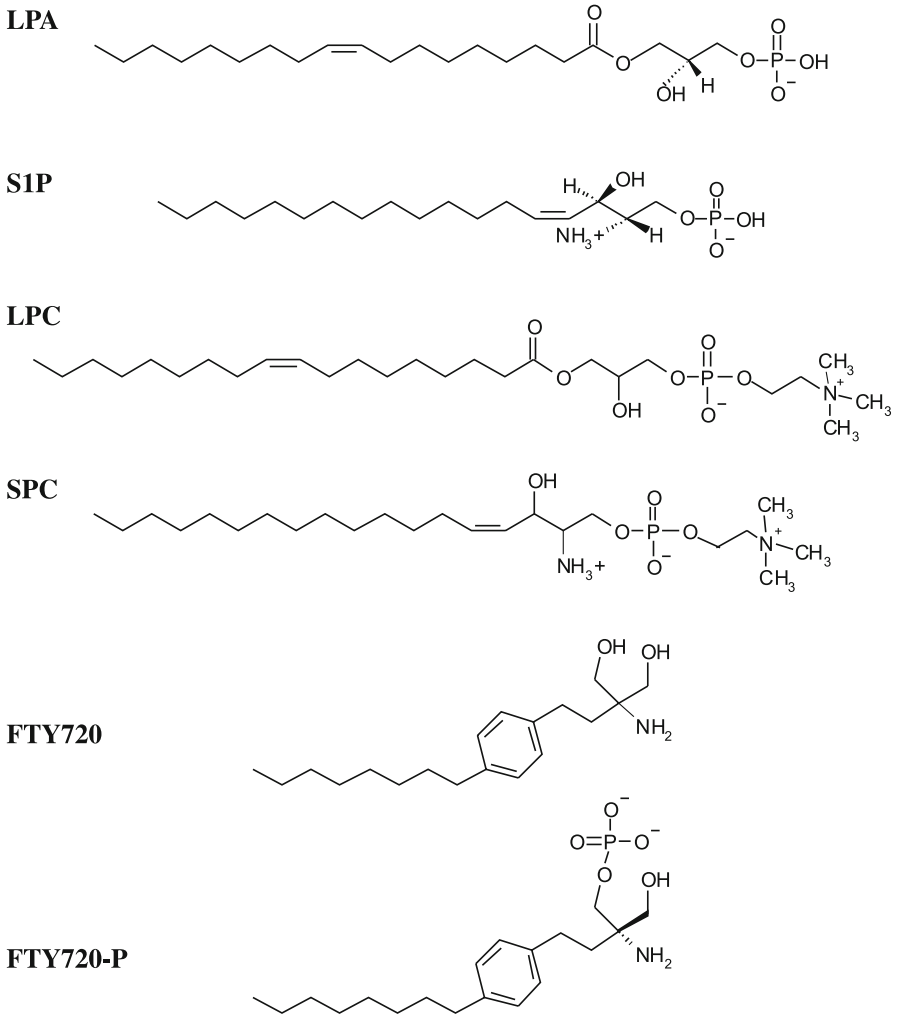

Fig. 1 Chemical structures of some of the bioactive phospholipids and FTY720/FTY720-P. *LPA* lysophosphatidic acid, *S1P* sphingosine 1-phosphate, *LPC* lysophosphatidylcholine, *SPC* sphingosylphosphorylcholine

ATX is important for LPA activity because it can access pools of lysophosphatidyl choline (LPC) that exists in comparatively high concentrations in many bodily fluids, to allow for the synthesis of LPA and subsequent activation of cellular responses through LPA receptors. ATX promotes the proliferation of several cancer cell lines and this increase in proliferation is enhanced in the presence of LPC, suggesting LPA activity is important for the effect (Hama et al. 2004; Umezu-Goto et al. 2002). Another feature shared between LPA and ATX is the ability to stimulate preadipocyte proliferation (Ferry et al. 2003; Pages et al. 2001a). ATX activity and subsequent LPA production also promotes the differentiation of primary preadipocytes and a preadipocyte cell line (Ferry et al. 2003). In vivo, ATX is expressed in mature adipocytes and is upregulated in genetically obese diabetic mice (Ferry et al. 2003). These results, together with the fact that LPA is present in the extracellular fluid of adipose tissue in vivo and released by adipocytes in vitro, implies that an LPA-dependent

paracrine control of adipose tissue is mediated by ATX (Valet et al. 1998). These data show that many LPA-mediated effects are paralleled by ATX, particularly effects mediated by the receptor LPA_1, and that ATX activity creates a microenvironment for LPA activity (Hama et al. 2004). We note that S1P is also generated by the hydrolysis of sphingosylphosphorylcholine (SPC) by ATX (Clair et al. 2003) (Fig. 1). However, this occurs at a lower efficiency than the generation of LPA from LPC, and is currently of uncertain biological significance.

Downregulation of lysophospholipid activity occurs in part, through the action of lipid phosphate phosphatases (LPPs). There are three members of this family of integral membrane glycoproteins, LPP-1, LPP-2, and LPP-3, also known as PAPs (Kai et al. 1996, 1997; Roberts et al. 1998). All three LPP proteins are capable of dephosphorylating and thus attenuating LPA and S1P activity. Byproducts of LPA and S1P degradation are monoacylglycerol and sphingosine, respectively. These LPPs can also dephosphorylate phosphatidic acid (PA) and ceramide 1-phosphate to produce diacylglycerol and ceramide. LPP proteins have six transmembrane domains and a conserved catalytic domain that faces the extracellular side of the plasma membrane or the luminal side of intracellular membranes (Zhang et al. 2000). Because of their ability to dephosphorylate extracellular LPA and S1P, LPPs are also known as "ecto" enzymes (Jasinska et al. 1999; Roberts et al. 1998; Roberts and Morris 2000; Smyth et al. 2003).

Treatment of platelets with an LPP-1 inhibitor results in an increase of LPA-dependent platelet responses, including Rho-dependent actin reorganization, morphological changes, and aggregation (Smyth et al. 2003). Introduction of LPPs into ovarian cancer cell lines increases apoptosis and decreases colony formation (Tanyi et al. 2003). Expression of LPP-3 in an ovarian cancer cell line severely reduces the ability of these cells to form tumors in mice (Tanyi et al. 2003). Interestingly, cells overexpressing LPPs were able to inhibit the growth of adjacent nontransfected cells (Tanyi et al. 2003). Thus, neighboring cells are rendered unresponsive to the proliferative effects of LPA by LPP-expressing cells in the immediate vicinity.

Plasticity related gene 1 (PRG-1) is a neuron-specific LPP that is localized to the membrane of outgrowing axons (Brauer et al. 2003). This protein is thought to inhibit lysophospholipid-induced axon collapse and thus permit axon outgrowth (Brauer et al. 2003). Like other LPPs, PRG-1 activity is dependent on an intact catalytic domain. PRG-1 and a related gene, PRG-2, are similar to other LPPs except they contain a long carboxy terminal tail that may be involved in cell signaling (Brauer et al. 2003). Some debate exists over the catalytic activity of some PRGs (McDermott et al. 2004).

S1P receptors

To date five S1P receptors have been cloned and are designated $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$. All of these receptors are responsive to S1P; however, the temporal and spatial pattern of receptor expression as well as the signaling molecules downstream of each receptor for a diverse variety of responses to S1P. Most receptors were initially cloned by multiple, independent groups as orphan receptors with various names, and the numbering system reflects their initial order in the literature.

$S1P_1$

$S1P_1$ was identified as a gene, edg-1, that was upregulated during phorbol 12-myristate 13-acetate (PMA) -induced differentiation of human endothelial cells in vitro (Hla and Maciag 1990). The $S1P_1$ receptor is expressed in a large number of cell types and tissues. By Northern blot analysis, high levels of $S1P_1$ expression are detected in mouse adult brain, heart, lung, liver, and spleen (Ishii et al. 2001, 2002; Liu and Hla 1997; McGiffert et al. 2002; Zhang et al. 1999). In the embryonic brain, high levels of $S1P_1$ expression are found in cerebellum, neocortical areas, intersomitic arteries, capillaries and developing blood vessels, aorta, and skeletal system (Liu and Hla 1997; McGiffert et al. 2002). $S1P_1$ expression has also been reported in a variety of immune system cells including $CD4^+$ and $CD8^+$ T cells, macrophages, dendritic cells, and NK cells (Goetzl et al. 2004; Graeler and Goetzl 2002; Jin et al. 2003; Matloubian et al. 2004; Wang et al. 2004).

Signaling via S1P and LPA receptors is mediated through at least three families of G proteins: G_i, G_q, and $G_{12/13}$. Co-immunopreciptation experiments using intracellular domains of $S1P_1$ and cell extracts showed that $S1P_1$ could couple to G_i proteins (Lee et al. 1996). It was shown in both Sf9 insect cells and in a *Xenopus* oocyte system that $S1P_1$ couples exclusively to the pertussis toxin (PTX) -sensitive G_i pathway (Ancellin and Hla 1999; Windh et al. 1999) (Fig. 2). Stimulation of the G_i-coupled $S1P_1$ receptor results in ERK activation, phospholipase C (PLC) activation, and inhibition of adenylyl cyclase (AC) (Lee et al. 1996; Okamoto et al. 1998; Zondag et al. 1998). S1P induces calcium mobilization in Chinese hamster ovary (CHO) cells but not in Sf9 insect cells, HEK293 cells, Cos-7 cells, or *Xenopus* oocytes overexpressing $S1P_1$ (Ancellin and Hla 1999; Okamoto et al. 1998; Zondag et al. 1998). S1P activates Rho in CHO cells expressing $S1P_1$ and Rho activation is required for S1P-induced migration and clustering of integrins into focal contact sites (Paik et al. 2001) (Fig. 2). S1P signaling and Rho activation through $S1P_1$ also mediate cell–cell contacts by upregulating cadherin expression and adherens junction assembly (Lee et al. 1998).

In endothelial cells, S1P-induced cortical actin assembly and cell migration requires the phosphorylation of $S1P_1$ (Lee et al. 2001). Interestingly, S1P induces PI-3-kinase-dependent activation of AKT in endothelial cells, and this activation may lead to phosphorylation of $S1P_1$ and subsequent activation of Rac (Lee et al. 2001).

The generation of $S1P_1$ receptor-deficient mice was essential in helping elucidate the in vivo function of $S1P_1$ (Liu et al. 2000b) (Table 1). Targeted deletion of the $S1P_1$ gene results in embryonic lethality between embryonic day 12.5 (E12.5) and E14.5 of development due to hemorrhaging (Liu et al. 2000b). $S1P_1$-deficient mice do not display defects in vasculogenesis or angiogenesis; however, vascular smooth muscle cells in the $S1P_1$-deficient mice fail to envelop completely the developing blood vessels (Liu et al. 2000b). Vascular maturation requires interactions between endothelial cells and vascular smooth muscle cells. Specific deletion of $S1P_1$ in endothelial cells showed that expression of the $S1P_1$ receptor in these cells is essential for proper vascular maturation (Allende et al. 2003). $S1P_1$ also mediates S1P-dependent migration of vascular smooth muscle cells. It is likely that without this receptor, recruitment of these cells into developing blood vessel walls is impaired. S1P-induced activation of Rac was ablated in fibroblasts derived from $S1P_1$-deficient mice, resulting in an inability of these $S1P_1$ null fibroblasts to migrate in response to S1P (Liu et al. 2000b).

In the immune system, $S1P_1$ is expressed in a variety of cell types, including B and T cells (Goetzl et al. 2004; Graeler and Goetzl 2002; Jin et al. 2003; Matloubian et al. 2004; Wang et al. 2004). $S1P_1$ expression levels are highest on the most mature T cells in the thymus, and are downregulated on activated T cells that are normally retained in peripheral

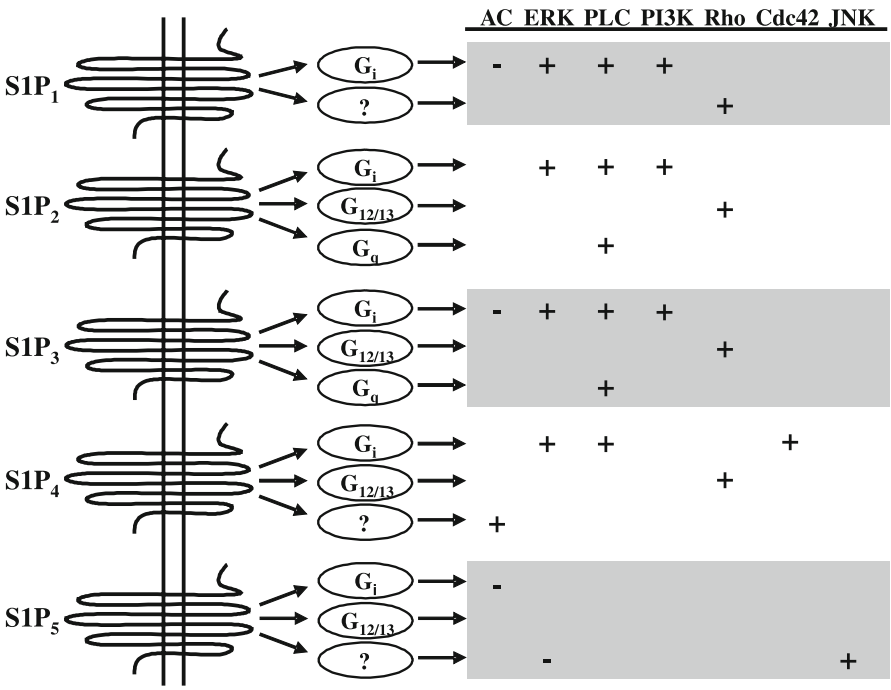

Fig. 2 Signaling pathways regulated by the S1P family of G protein-coupled receptors. The five known S1P receptors and the G proteins that couple to them are indicated. Cell signaling pathways downstream of receptor signaling are also indicated. *AC* adenylyl cyclase, *ERK* mitogen-activated protein kinase, *PLC* phospholipase C, *PI3K* phosphoinositol 3-kinase, *JNK* c-Jun N-terminal kinase

lymphoid organs (Matloubian et al. 2004). To ascertain the role of $S1P_1$ in hematopoietic cells, fetal liver cells derived from $S1P_1$-deficient mice were transferred into lethally irradiated hosts (Matloubian et al. 2004). Surprisingly, developing T cells from donor mice could not exit the thymus, and donor-derived B cells were present in peripheral lymphoid organs but dramatically reduced in blood and lymph. When donor-derived T cells were reintroduced into wild-type recipients, the T cells homed to secondary lymphoid organs; however, they could not recirculate to blood and lymph (Matloubian et al. 2004) (Table 1). These results were similar to the effects observed with FTY720, an agonist for $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ receptors (Fig. 1) (Brinkmann et al. 2000, 2002). FTY720 downregulates $S1P_1$ receptor expression, thus mimicking an $S1P_1$-deficient state and inhibits developing T cells from exiting the thymus while retaining T cells in peripheral lymphoid organs (Graler and Goetzl 2004; Matloubian et al. 2004). It should be noted that other interpretations of these data exist and further studies are required to delineate the receptor mechanisms involved in these phenomena (Rosen and Goetzl 2005).

$S1P_2$

Two independent groups identified $S1P_2$ in screens of rat cDNA libraries (MacLennan et al. 1994; Okazaki et al. 1993). One group isolated $S1P_2$ from a hippocampal cDNA library while the second group identified $S1P_2$ by PCR of an aortic smooth muscle library with G protein-coupled receptor degenerate oligonucleotide primers. In the developing embryo,

Table 1 LPA and S1P receptor null mutantmice phenotypes

Receptor	In vivo phenotype	Cell signaling defects
$S1P_1$	Embryonic lethality between E12.5 and 14.5 Hemorrhage due to defective blood vessel formation Failure of null thymocyte egress in radiation chimeras	Reduced migratory response of MEFs in response to S1P
$S1P_2$	Slightly reduced viability Seizures in certain genetic backgrounds Neuronal hyperexcitability	Decreased S1P-induced Rho activation in MEFs
$S1P_3$	Slightly reduced viability Severe lethality in $S1P_2$-null background	Decreased S1P-induced PLC activation and AC inhibition in MEFs In $S1P_2/S1P_3$ double-null MEFs, S1P-induced Rho activation abolished, reduction of PLC activation
$S1P_5$	No obvious phenotype	Diminished S1P-induced pre-oligodendrocyte process retraction
LPA_1	Partial neonatal lethality due to impaired suckling Craniofacial deformities Frontal hematoma Increased apoptosis of Schwann cells of sciatic nerve	Reduced PLC activation and complete block of AC inhibition in MEFs treated with LPA; reduced morphological changes and proliferation of neuronal cells in response to LPA
LPA_2	No obvious phenotype	Reduction of MEF PLC activation in response to LPA Loss of PLC, JNK, and Akt activation, as well as proliferative responses in LPA_1/LPA_2 double-null MEFs in response to LPA Reduced stress fiber formation in LPA_1/LPA_2 double-null MEFs in response to LPA
LPA_3	No obvious phenotype Reduced fertility in female null mice due to implantation abnormalities	

Abbreviations: *MEF*, mouse embryo fibroplast; *JNK*, c-Jun kinase; *PLC*, phospholipase C; *AC*, adenylyl cyclase

$S1P_2$ RNA is present at high levels in the embryonic brain (MacLennan et al. 1994). In situ hybridization localized $S1P_2$ expression specifically to the midbrain and choroids plexus (McGiffert et al. 2002). Immunohistochemistry analysis suggested that $S1P_2$ protein was present in young differentiating neurons and in axons during a period of outgrowth, although the specificity of the antisera employed is not clear (MacLennan et al. 1997). $S1P_2$ RNA expression is high in a variety of human and rodent adult tissues, including lung, heart, kidney, thymus, and spleen (Ishii et al. 2001, 2002; MacLennan et al. 1994; McGiffert et al. 2002; Okazaki et al. 1993; Yamaguchi et al. 1996; Zhang et al. 1999). High $S1P_2$ RNA levels in the thymus are likely due to a relatively high level of $S1P_2$ expression in double- and single-positive thymocytes (Matloubian et al. 2004). The expression of $S1P_2$ in monocytes and dendritic cells may account for high $S1P_2$ levels in the spleen (Goetzl et al. 2004).

When co-expressed in Sf9 cells, $S1P_2$ was found to couple to G_i, G_q, and $G_{12/13}$ protein families (Windh et al. 1999) (Fig. 2). When overexpressed in *Xenopus* oocytes, $S1P_2$ mobilizes calcium in response to S1P stimulation (An et al. 1997a; Ancellin and Hla 1999). An $S1P_2$-dependent calcium response to S1P was also observed in human and rat cell lines (An et al. 1999). The calcium response was partially PTX-sensitive, indicating that $S1P_2$

coupling to G_i in part mediates this effect. In Jurkat cells overexpressing $S1P_2$, S1P induced activation of a serum response element (SRE) promoter (An et al. 1997a). Activation of the SRE requires the G_i-Ras-Raf-ERK pathway and the GTPase Rho. S1P-induced rounding of HEK293 and PC12 cell lines transfected with $S1P_2$ provided further evidence that $S1P_2$ is coupled to $G_{q/o}$ and $G_{12/13}$ proteins (Van Brocklyn et al. 1999) (Fig. 2).

Three independent groups generated $S1P_2$-deficient mice (Ishii et al. 2002; Kono et al. 2004; MacLennan et al. 2001) (Table 1). $S1P_2$-deficient mice from two groups were found to be viable, fertile, and displayed no obvious defects (Ishii et al. 2002; Kono et al. 2004). However, when mice deficient in both $S1P_2$ and $S1P_3$ were crossed together, the number of pups obtained was dramatically reduced compared to the number of viable pups obtained from wild-type crosses (Ishii et al. 2002; Kono et al. 2004). The high incidence of embryonic death observed in $S1P_2$/$S1P_3$-double-null embryos may be due to endothelial cell abnormalities that lead to hemorrhage (Kono et al. 2004). Interestingly, the loss of $S1P_2$ also leads to an exacerbation of the $S1P_1$-null phenotype. $S1P_1$/$S1P_2$-double-null mice have a vasculature that appears less mature than that observed in $S1P_1$ null mutant mice, and this defect may account for the slightly earlier incidence of embryonic lethality of $S1P_1$/$S1P_2$-null mutant mice compared to mice deficient for $S1P_1$ only (Kono et al. 2004). A few weeks after birth, $S1P_2$-null mice from the third group developed severe and sometimes lethal seizures (MacLennan et al. 2001). Neurons derived from these $S1P_2$-null mutant mice were hyperexcitable in vitro (MacLennan et al. 2001). It is likely that genetic background strain differences or differences in embryonic stem cells used to create the mice account for the differences between the two $S1P_2$-deficient mice.

Mouse embryonic fibroblasts (MEFs) derived from $S1P_2$ null mutant mice proved quite useful in further understanding the role $S1P_2$ plays in S1P signaling. In response to S1P, MEFs derived from $S1P_2$ null mutant mice showed a significant decrease in Rho activation but had relatively normal PLC, calcium mobilization, and AC responses (Ishii et al. 2002).

$S1P_3$

$S1P_3$ was isolated by PCR of human genomic DNA with degenerate primers to cannabinoid receptors (Yamaguchi et al. 1996). In human tissues, high levels of $S1P_3$ RNA are found in the heart, lung, kidney, and pancreas (Yamaguchi et al. 1996). The pattern of tissue expression overlaps with adult mouse tissues, where high $S1P_3$ levels are also seen in the spleen and embryonic brain (McGiffert et al. 2002; Zhang et al. 1999). $S1P_3$ expression during embryonic development was determined using in situ hybridization (McGiffert et al. 2002). In the embryonic brain, $S1P_3$ localizes to the olfactory epithelium, choroids plexus, and developing blood vessels (Ishii et al. 2001; McGiffert et al. 2002). $S1P_3$ RNA was also detected in the embryonic lung, kidney, intestine, submandibular gland, and cartilaginous regions (Ishii et al. 2001; McGiffert et al. 2002; Zhang et al. 1999).

Similar to $S1P_2$, $S1P_3$ was also found to couple to the G_i, G_q, and $G_{12/13}$ G protein families in Sf9 co-expression experiments (Windh et al. 1999) (Fig. 2). When expressed in Jurkat T cells or *Xenopus* oocytes, activation of $S1P_3$ by S1P induces calcium mobilization (An et al. 1997a, 1999; Ancellin and Hla 1999). In rat hepatoma cells overexpressing $S1P_3$, S1P stimulation initiates a PLC-mediated sustained calcium mobilization response and IP_3 production (An et al. 1999). The calcium response and IP_3 production were partially inhibited by PTX, indicating that these responses are mediated in part by coupling to G_i proteins (An et al. 1999). Stable $S1P_3$ expression in these cells also inhibited forskolin-induced cAMP accumulation (An et al. 1999). This AC inhibition was abolished when the

cells were treated with PTX, indicating that this is a G_i-mediated response (An et al. 1999). G_i-mediated coupling of $S1P_3$ to the Ras-Raf-ERK pathway was also demonstrated in Jurkat T cells in response to S1P (An et al. 1997a).

S1P also induces activation of Rho in $S1P_3$-expressing CHO cells, and this activation is thought to mediate CHO cell migration (Paik et al. 2001). Downregulation of $S1P_3$ with antisense oligonucleotides inhibits activation of Rho, and Rho-dependent endothelial cell migration in response to S1P (An et al. 1997a). S1P also induces activation of Rho in $S1P_3$-expressing CHO cells, and this activation is thought to mediate CHO cell migration (Paik et al. 2001).

Similar to mice deficient for $S1P_2$, $S1P_3$-deficient mice displayed no obvious phenotype (Ishii et al. 2001; Kono et al. 2004) (Table 1). Mice deficient for $S1P_3$ are viable, fertile, and develop normally. When $S1P_3$-null mice were bred together, resultant litter sizes were slightly smaller than those obtained from wild-type crosses (Ishii et al. 2001). However, when mice deficient for both $S1P_2$ and $S1P_3$ were bred together, litter sizes were severely reduced (Ishii et al. 2002; Kono et al. 2004) (Table 1). MEFs generated from both $S1P_3$-deficient mice and $S1P_2$/$S1P_3$-double-null mice were used to analyze the roles of these receptors in transducing S1P signals (Ishii et al. 2001, 2002). PLC activation in response to S1P treatment was severely reduced in $S1P_3$-deficient MEFs (Ishii et al. 2001). Residual PLC activity was blocked by PTX treatment. In contrast, when $S1P_3$-deficient MEFs were infected with $S1P_3$-expressing retrovirus, PTX treatment did not block S1P-induced PLC activation (Ishii et al. 2001). This result shows that the S1P-induced PLC activity in MEFs occurs largely through $S1P_3$ and couples to PTX-insensitive G proteins. $S1P_3$-deficient MEFs also have a slight reduction in G_i-mediated inhibition of cAMP accumulation (Ishii et al. 2001). The loss of $S1P_3$ in MEFs did not have an effect on S1P-induced Rho activation (Ishii et al. 2001). However, in MEFs deficient for $S1P_2$, S1P-induced activation of Rho was severely reduced, and in $S1P_2$/$S1P_3$-null MEFs, Rho activation was abolished (Ishii et al. 2002). This indicates that $S1P_3$ has a minor role in Rho activation in MEFs. An increasing number of studies have revealed roles for this receptor in cardiovascular and respiratory systems (Gon et al. 2005; Levkau et al. 2004; Nofer et al. 2004; Sanna et al. 2004; Tolle et al. 2005).

$S1P_4$

$S1P_4$ was originally cloned from in vitro differentiated human dendritic cells using chemokine G-protein-coupled receptor (GPCR) degenerate primers (Graler et al. 1998). The $S1P_4$ receptor expression is unique compared to other S1P receptors, as its expression is confined to lymphoid cells and tissues. High levels of $S1P_4$ RNA are present in the thymus, spleen, lymph nodes, as well as peripheral blood lymphocytes (Contos et al. 2002b; Graler et al. 1998; Ishii et al. 2001). In thymus, $S1P_4$ transcripts are detected in immature CD4 and CD8 double-positive as well as mature single-positive thymoctyes (Matloubian et al. 2004). $S1P_4$ is expressed in an incredibly broad range of lymphoid cells, including $CD4^+$ and $CD8^+$ T cells, B cells, NK cells, dendritic cells, and macrophages (Goetzl et al. 2004; Graeler and Goetzl 2002; Jin et al. 2003; Matloubian et al. 2004; Wang et al. 2004). Although high $S1P_4$ RNA levels are also detected in the lung, it is not clear if this is due to expression in alveolar macrophages (Ishii et al. 2001, 2002; McGiffert et al. 2002; Zhang et al. 1999).

S1P-mediated cell signaling occurs, in part, through G protein subunits G_i and $G_{12/13}$ that have been shown to couple directly to the $S1P_4$ receptor (Graler et al. 2003) (Fig. 2). In

cell lines overexpressing $S1P_4$, PTX-sensitive responses to S1P include activation of PLC, the mitogen-activated protein kinase signal transduction pathway, and the activation of the small Rho family GTPase Cdc42 (Graler et al. 2003; Kohno et al. 2003; Van Brocklyn et al. 2000; Yamazaki et al. 2000). This indicates that G_i coupling to $S1P_4$ mediates all of these responses.

In CHO cells overexpressing $S1P_4$, S1P induces cell rounding, stress fiber formation and cell motility (Graler et al. 2003; Kohno et al. 2003). The S1P-induced migratory response of CHO cells overexpressing $S1P_4$ is abolished by a dominant negative Cdc42 or PTX treatment (Kohno et al. 2003). Interestingly, S1P also induces Rho activation in CHO cells overexpressing $S1P_4$ (Graler et al. 2003). However, it is not clear if Rho activation is required for cell migration, stress fiber formation, and morphological changes in these cells. In response to S1P, $S1P_4$-overexpressing Jurkat T cells also undergo a cell motility response (Graler et al. 2003).

In response to S1P, MEFs infected with $S1P_4$-expressing retrovirus show a robust AC response (Ishii et al. 2001). This is in contrast to $S1P_1$, $S1P_3$, and $S1P_5$, which all inhibit AC activity in response to S1P (Im et al. 2000a, 2001; Ishii et al. 2001; Malek et al. 2001; Niedernberg et al. 2002). It has not been determined which G protein subunit couples with $S1P_4$ to activate this response.

Mice deficient for $S1P_4$ have not yet been reported. However, given the lymphoid-specific expression pattern of this receptor, defects in lymphocyte development and/or function can be expected. Based on expression patterns and experiments done in vitro, it is likely that the loss of $S1P_4$ will have an effect on T cell proliferation, chemotaxis, and immune response localization. In support of this hypothesis, $S1P_1$ and $S1P_4$ are expressed on $CD4^+$ and $CD8^+$ splenic T cells, which undergo chemotaxis in response to S1P (Graeler and Goetzl 2002). RNA and protein levels of these receptors are both downregulated upon treatment with the anti-T cell receptor (TCR) antibodies CD3 and CD28 (Graeler and Goetzl 2002). Downregulation of $S1P_1$ and $S1P_4$ receptors renders the cells unable to undergo a chemotactic response to S1P (Graeler and Goetzl 2002). FTY720, an S1P receptor agonist, also blocks T cell chemotactic responses, downregulates S1P receptor expression, and prevents lymphocytes from exiting peripheral lymphoid organs in vivo (Brinkmann et al. 2000, 2001; Graeler and Goetzl 2002; Mandala et al. 2002). Thus, $S1P_1$ and $S1P_4$ may act to recruit naïve T cells to areas necessary for an immune response, whereas activated T cells may be retained in the same areas due to a decreased chemotactic response to S1P.

$S1P_1$ and $S1P_4$ are also expressed on $CD4^+CD25^+$ regulatory T cells (Wang et al. 2004). In response to S1P treatment, these cells decrease the ability of naïve $CD4^+$ T cells to produce IL-2 and undergo proliferation (Wang et al. 2004). The inhibitory effects of S1P on regulatory T cells are also blocked by FTY720 treatment, indicating that S1P receptors are responsible for this effect (Wang et al. 2004). In addition S1P can inhibit T cell proliferation in response to PMA and ionomycin treatment, anti-CD3 plus anti-CD8 antibodies, and dendritic cell stimulation (Jin et al. 2003).

One major caveat to these experiments is that FTY720 does not appear to downregulate $S1P_4$ expression, indicating that chemotactic and immunoregulatory responses are mediated by $S1P_1$ and not $S1P_4$ (Fig. 1) (Wang et al. 2004). Furthermore, chemotaxis in response to S1P occurs in a rat hepatoma cell line when $S1P_1$ is overexpressed but not $S1P_4$ (Graeler and Goetzl 2002). The generation of $S1P_4$ null mice will help to determine whether $S1P_4$ has a role of mediating these responses in vivo.

$S1P_5$

The $S1P_5$ receptor was identified in expressed sequence tag data bases and cloned from rat cDNA (Glickman et al. 1999; Im et al. 2000a). This gene has a relatively restricted expression pattern with high RNA levels present in the spleen, skin, lung, and brain (Glickman et al. 1999; Im et al. 2000a, 2001; Ishii et al. 2001, 2002; Niedernberg et al. 2002). Northern blot analysis showed that $S1P_5$ transcripts are highly expressed in a number of brain regions (Glickman et al. 1999; Im et al. 2000a, 2001). In situ hybridization further localized $S1P_5$ expression to white matter tracts throughout the brain at every stage of oligodendrocyte differentiation (Im et al. 2000a; Jaillard et al. 2005; Terai et al. 2003; Yu et al. 2004). This expression pattern suggests that $S1P_5$ plays a role in myelination.

The $S1P_5$ receptor has been found to couple to G_i and $G_{12/13}$ proteins (Fig. 2). In several experiments, expression of $S1P_5$ in cell lines was shown to inhibit AC activity in response to S1P through a G_i regulated pathway (Im et al. 2000a, 2001; Malek et al. 2001; Niedernberg et al. 2002). In addition, S1P stimulation of the $S1P_5$ receptor repressed ERK activation while inducing c-Jun NH_2-terminal kinase (JNK) activation (Malek et al. 2001). It is not known at the present time how the activation states of ERK and JNK are regulated by $S1P_5$. Serum-stimulated proliferation of $S1P_5$ overexpressing CHO cells was inhibited with S1P treatment (Malek et al. 2001; Niedernberg et al. 2002).

Although $S1P_5$ is expressed at all stages of oligodendrocyte development, mice deficient for $S1P_5$ do not display obvious myelination abnormalities, and they have no other apparent phenotypic defects (Table 1) (Jaillard et al. 2005). However, Rho kinase-dependent process retraction in response to S1P was abolished in cultured preoligodendrocytes derived from S1P-null mutant mice (Jaillard et al. 2005). At more mature stages of oligodendrocyte development, repression of $S1P_5$ expression by siRNAs negated the ability of S1P to act as a survival factor in response to differentiation signals in vitro (Jaillard et al. 2005). Interestingly, S1P treatment of mature oligodendrocytes induces phosphorylation of AKT (Jaillard et al. 2005). The ability of S1P to protect mature oligodendrocytes from cell death and to promote AKT phosphorylation is diminished upon PTX treatment (Jaillard et al. 2005). It should be noted that oligodendrocyte survival experiments were not done with oligodendroctyes cultured from $S1P_5$-deficient mice. It is possible that functional redundancies with other lysophospholipid receptors account for the absence of myelination defects in $S1P_5$-deficient mice. The generation of $S1P_5$-deficient mice crossed with mice null for other lysophospholipid receptors will help further our understanding of the role lysophospholipids play in the myelination process.

LPA receptors

There are four known LPA receptors that mediate LPA signaling in a wide variety of tissues and cell types. These receptors are designated LPA_1, LPA_2, LPA_3, and LPA_4. The numbering system reflects their initial order in the literature.

LPA_1

The LPA_1 receptor was the first lysophospholipid receptor identified. It was cloned from neural progenitor cell lines using degenerate primers designed against transmembrane do-

mains in G protein-coupled receptors (Hecht et al. 1996). In adult mouse tissues, high levels of RNA are also detected in the lung and testis (Contos et al. 2002a; Hecht et al. 1996). The LPA_1 receptor also has a biphasic expression pattern in the developing brain. LPA_1 levels are high in the developing cerebral cortex throughout development of the embryonic brain followed by a decrease in expression at or near birth, then reemergence of gene expression soon after birth (Hecht et al. 1996; Weiner et al. 1998). In situ hybridization experiments show that LPA_1 expression is localized to proliferative zones of the embryonic cerebral cortex (Hecht et al. 1996). LPA_1 expression in the postnatal brain correlates with white matter tracts (Weiner et al. 1998). In situ hybridization experiments with oligodendrocyte-specific and LPA_1 probes shows that LPA_1 is expressed in oligodendrocytes during a period that coincides with the onset of myelination (Weiner et al. 1998). LPA_1 transcripts are also present in Schwann cells (Weiner and Chun 1999).

LPA exerts diverse effects on a variety of cell types through several signal transduction pathways (Fig. 3). For example, LPA inhibits AC activation in neuronal cell lines overexpressing LPA_1 (Ishii et al. 2000). This response is inhibited by PTX treatment, indicating that LPA_1 is coupled to G_i proteins (Ishii et al. 2000). LPA_1-overexpressing cells also activate a SRE element reporter and MAPK in response to LPA (An et al. 1997b; Fukushima et al. 1998; Ishii et al. 2000). However, this response is sensitive to PTX and C3 exoenzyme, indicating that G_i proteins and the GTPase Rho mediate this effect (Fukushima et al. 1998; Ishii et al. 2000). Overexpression of LPA_1 in cell lines also leads to Rho-dependent morphological changes such as stress fiber formation, neurite retraction, and cell rounding in response to LPA (Fukushima et al. 1998; Ishii et al. 2000). In addition to G_i and Rho-mediated responses, LPA can activate PLC in LPA_1-overexpressing cells (Ishii et al. 2000). The role of LPA_1 in these signaling pathways was confirmed in MEFs derived from LPA_1-null mutant mice (Contos et al. 2002a). In addition to decreases in other responses that will be discussed below, MEFs from LPA_1-deficient mice displayed a marked reduction in PLC activation, and a minimal inhibition of forskolin-induced cAMP accumulation in response to LPA (Contos et al. 2002a).

The importance of the LPA_1 gene in mammalian development is evident by the reduced viability of LPA_1-null mutant mice (Contos et al. 2002a) (Table. 1). The loss of LPA_1 in mice results in an approximately 50% reduction of the expected number of pups homozygous for the LPA_1 mutant allele. The majority of LPA_1 null mutant mice die between birth and three weeks of age. One reason for this reduced viability is an abnormal suckling behavior that appears to be due to an olfaction defect. LPA_1-null mutant mice that survive to adulthood have craniofacial defects that include flattened faces, shortened snouts, an increased distance between the eyes and reduced body mass. Some LPA_1-deficient embryonic and neonatal mice also have frontal hematomas or exencephaly.

In agreement with the finding that LPA is a Schwann cell survival factor, an increased number of apoptotic Schwann cells are present in the sciatic nerve of LPA_1-deficient mice (Contos et al. 2002a; Weiner and Chun 1999). In contrast to Schwann cells derived from wild-type mice, Schwann cells from LPA_1-deficient mice fail to undergo normal actin rearrangements in response to LPA treatment (Weiner et al. 2001). LPA_1 was originally identified as a gene present in the ventricular zone of the cerebral cortex (Hecht et al. 1996). LPA treatment induces morphological changes and enhances the proliferation of neuroblasts derived from this region (Contos et al. 2000; Fukushima et al. 2000). In response to LPA, cortical neuroblast cultures derived from LPA_1-deficient mice fail to undergo LPA-dependent cell rounding and compaction and show slightly reduced proliferation compared to cultures derived from wild-type mice (Contos et al. 2000). Elegant experiments conducted using ex vivo cultured mouse embryonic cortices showed that LPA induces dramatic folding

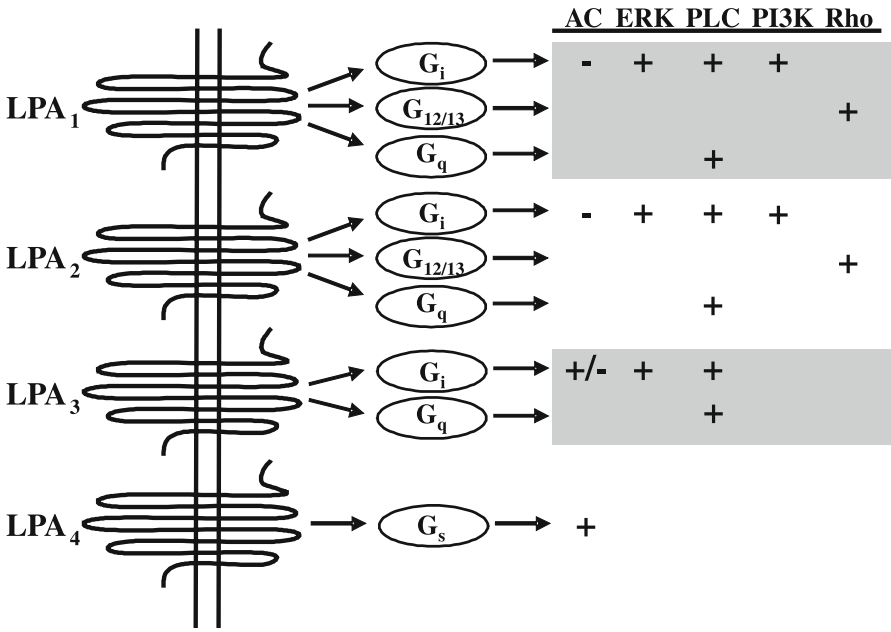

Fig. 3 Signaling pathways regulated by the LPA family of G-protein coupled receptors. Shown are the four known LPA receptors and the G proteins coupled to them. Cell signaling pathways downstream of receptor signaling are indicated. *AC* adenylyl cyclase, *ERK* mitogen-activated protein kinase, *PLC* phospholipase C, *PI3K* phosphoinositol 3-kinase

and thickening of the developing cerebral cortex (Kingsbury et al. 2003). This remarkable morphological change is due to an LPA-dependent decrease in cell death and increased terminal mitosis of neural progenitor cells (Kingsbury et al. 2003). These changes are not apparent in LPA treated cortices of mice deficient for both LPA_1 and LPA_2 (Kingsbury et al. 2003), demonstrating the necessity for these two receptors in this response.

MEFs isolated from LPA_1-null mutant mice also display abnormal responses to LPA signaling (Contos et al. 2002a). When LPA_1 is overexpressed in cell lines, LPA can activate PLC, inhibit AC activity, and induce morphological changes such as cell rounding (Fukushima et al. 1998; Hecht et al. 1996; Ishii et al. 2000). LPA_1-deficient MEFs show a substantial but incomplete loss of PLC activation, and a near complete loss of AC inhibition when treated with LPA (Contos et al. 2002a). These data argue that other LPA receptors contribute to LPA-dependent activation of PLC and that LPA_1 is the only LPA receptor involved in AC inhibition in MEFs. Stress fiber formation in response to LPA treatment is also reduced in MEFs derived from the meninges of LPA_1 knockout mice as compared to wild-type controls (Contos et al. 2002a). LPA_1 mutant mice have been extremely useful in demonstrating the importance of this receptor in mammalian development and cell function (Table 1). In addition, these mice have indicated that other lysophospholipid receptors are likely important for Schwann cell development, cortical neuroblast development, and cellular responses to LPA signaling.

LPA_2

The LPA_2 receptor was discovered in a search for genes similar to LPA_1, although the first published sequence contained a non-wild-type mutation in the carboxy terminus (An et al. 1998). In contrast to LPA_1, LPA_2 has a more restricted pattern of expression. LPA_2 RNA levels are high in adult human peripheral blood lymphocytes, thymus, spleen, and testis (An et al. 1998). In mice, LPA_2 RNA transcripts are abundant in testis, kidney, $CD4^+$ and $CD8^+$ T cells, and B cells (Contos et al. 2002a; Goetzl et al. 2004).

Similar to LPA_1, overexpression of LPA_2 in cell lines leads to an inhibition of AC activity in response to LPA treatment (Ishii et al. 2000). Like LPA_1, AC activation is sensitive to PTX treatment, indicating that this response occurs through a G_i-mediated pathway (Ishii et al. 2000) (Fig. 3). Also, similar to LPA_1 activation, LPA appears to induce activation of the SRE in cell lines overexpressing LPA_2, and this response is partially inhibited by both PTX and C3 exoenzyme, suggesting mediation of the response by both G_i- and Rho-dependent pathways (An et al. 1998) (Fig. 3). In neuroblastoma cell lines infected with LPA_2 expressing virus, LPA causes Rho-dependent cell rounding and processes retraction (Ishii et al. 2000). LPA signaling through LPA_2 also activates PLC (Ishii et al. 2000) (Fig. 3).

Since MEFs express LPA_1 and LPA_2 but not LPA_3, MEFs deficient for LPA_2 were useful in helping to determine the extent that LPA signaling through LPA_2 receptor contributes to these responses in vivo (Contos et al. 2002a). For example, LPA_2-deficient MEFs have a greater reduction in PLC activation than MEFs deficient for LPA_1 (Contos et al. 2002a). However, PLC activation in response to LPA signaling is nearly abolished in LPA_1/LPA_2 double knockout MEFs (Contos et al. 2002a). This result shows that both LPA_1 and LPA_2 receptors play a role in PLC activation but that PLC is activated by LPA_2 to a greater extent in MEFs. While overexpression of LPA_2 in cell lines can result in an inhibition of AC, the loss of LPA_2 in MEFs does not have a major effect on AC activity (Contos et al. 2002a; Ishii et al. 2000). AC activity was not further inhibited in double knockout MEFs, nor was it activated in double knockout MEFs infected with LPA_2-expressing retrovirus (Contos et al. 2002a). These data show that in MEFs, the LPA receptor responsible for LPA-mediated inhibition of AC is LPA_1. LPA_1 and LPA_2 were found to have redundant roles in LPA-induced stress fiber formation, as well as activation of JNK, AKT, and MAPK (Contos et al. 2002a).

Mice deficient for LPA_2 have no obvious phenotype (Contos et al. 2002a) (Table 1), and mice deficient for both LPA_1 and LPA_2 display the same abnormalities as LPA_1 deficient mice except for an increase in the incidence of frontal hematomas and a slight decrease in survival rate (Contos et al. 2002a).

LPA_3

The gene for LPA_3 was cloned using two different PCR approaches. Reverse transcription was used to amplify a cDNA sequence with similarities to LPA_1 and LPA_2 in one approach, while PCR with degenerate primers to LPA_1 and LPA_2 transmembrane domains was used in the other (Bandoh et al. 1999; Im et al. 2000b).

High LPA_3 RNA levels are found in the heart, pancreas, prostate, testis, and brain in humans (Bandoh et al. 1999; Im et al. 2000b). An overlapping expression pattern is observed in rodent tissues (Contos and Chun 2001; Im et al. 2000b). In the mouse brain, postnatal expression of LPA_3 is higher than that observed in embryonic development (Contos and Chun 2001).

LPA-mediated stimulation of LPA_3 activates signal transduction pathways that both overlap and are distinct from those activated by LPA_1 and LPA_2 (Table 1). While LPA_1 and LPA_2 mediate LPA-induced cell rounding and processes retraction, LPA_3 inhibits cell rounding and causes neurites to elongate in the B103 neuroblastoma cell line (Ishii et al. 2000). Like LPA_1 and LPA_2, LPA_3 activation causes an upregulation of IP_3 production, an inhibition of AC activity, and an activation of MAP kinase when overexpressed in B103 cells (Ishii et al. 2000). AC inhibition, and MAP kinase activation are both sensitive to PTX treatment (Ishii et al. 2000). Previous reports demonstrated that overexpression of LPA_3 in cell lines or *Xenopus* oocytes activates a calcium response in response to LPA treatment (Bandoh et al. 1999; Im et al. 2000b). This is consistent with LPA_3 coupling to G_q and activating PLC and IP_3 accumulation (Fig. 3). The PTX sensitivity of AC inhibition and MAP kinase activation indicates that LPA_3 couples to G_i proteins to mediate these effects (Ishii et al. 2000). It should be noted that forskolin-induced cAMP accumulation is not affected in Sf9 insect cells or RH7777 cells overexpressing LPA_3 (Bandoh et al. 1999). This difference may reflect different downstream signaling components or signaling properties in these cells.

Mice deficient for LPA_3 have recently been generated and show a surprising phenotype (Ye et al. 2005) (Table1). LPA_3-null mutant females have reduced litter sizes compared to wild-type and LPA_3 heterozygous females. When the uteri of pregnant LPA_3 deficient mice were analyzed, it was discovered that the number of implantation sites was reduced and there were multiple embryos per placenta. The implantation sites that were present were predominantly located proximal to the cervix. On average, the embryos recovered from these implantation sites were smaller in size relative to embryos isolated from wild-type female mice. Pregnancy was slightly prolonged in LPA_3-deficient mice, and although the number of newborn pups was reduced, they were slightly larger than wild-type pups of the same age.

LPA_3 expression is upregulated in the luminal endometrial epithelium during a period critical for implantation and the data suggest that maternal LPA_3 signaling is crucial for proper embryo implantation and spacing (Ye et al. 2005). These abnormalities are similar to those observed in mice deficient for phospholipase $A_{2\alpha}$, cyclooxygenase 2 (COX2), and mice treated with indomethacin, an inhibitor of cyclooxygenases (COXs) (Frenkian et al. 2001; Kennedy 1977; Kinoshita et al. 1985; Lim et al. 1997; Reese et al. 1999; Song et al. 2002). Cytoplasmic phospholipase $A_{2\alpha}$ ($cPLA_{2\alpha}$) is involved in arachidonic acid synthesis, while COX proteins are involved in the conversion of arachidonic acid to prostaglandins. Thus, LPA signaling through LPA_3 appears to regulate prostaglandin synthesis (Fig. 4). In support of this model, COX2 expression, as well as prostaglandin synthesis, is reduced in the uteri of LPA_3-deficient mice (Ye et al. 2005). Administration of exogenous prostaglandins partially rescues implantation abnormalities in mice lacking LPA_3 (Ye et al. 2005). In support of a role for LPA signaling in the regulation of COX2, it was recently reported that LPA induces COX2 expression in ovarian cancer cell lines (Symowicz et al. 2005).

LPA_4

A novel LPA receptor has recently been identified in a project to de-orphan known G protein-coupled receptors (Noguchi et al. 2003). This receptor previously known as p2y/GPR23, is the most divergent member of the LPA receptor family and shares only 20–24% amino acid identity with all known LPA receptors (Noguchi et al. 2003). LPA_4 is expressed predominantly in the ovary and moderately in the thymus (Noguchi et al. 2003). CHO cells stably expressing LPA_4 accumulate cAMP in response to LPA treatment either in the presence or absence of forskolin (Noguchi et al. 2003). PTX treatment does not attenuate the cAMP re-

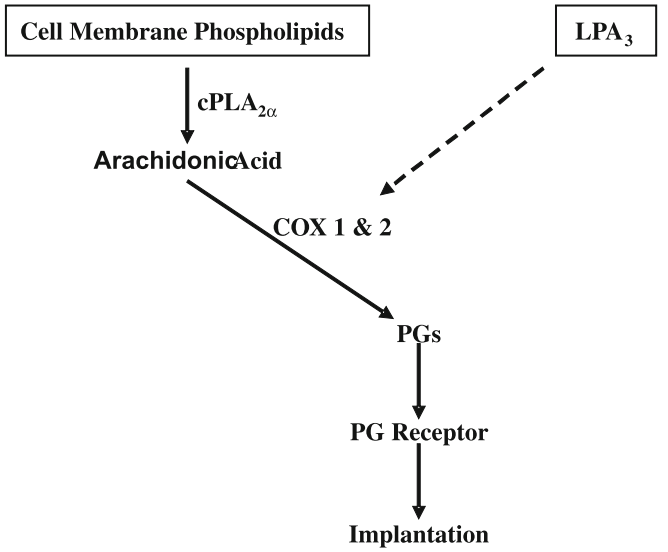

Fig. 4 Model for LPA_3 in the regulation of prostaglandin synthesis. LPA_3 signaling upregulates COX expression, leading to the formation of prostaglandins (PGs) from arachidonic acid. PGs bind to receptors in the epithelium and provide signals for implantation

sponse, indicating that this pathway is mediated by LPA_4 coupling to G_s proteins, although it is likely that other G proteins are activated as well (Noguchi et al. 2003) (Fig. 3). LPA also induces a dose-dependent increase in intracellular calcium levels in CHO cells stably expressing LPA_4.

A more extensive analysis of this receptor and signal transduction pathways downstream of this receptor is needed to provide further insight into the function of this receptor in vivo.

Conclusions and future prospects

The identification and characterization of lysophospholipid receptors has provided new insights into our understanding of how lysophospholipids exert their biological effects. In addition, mice deficient for specific lysophospholipid receptors have provided important clues about the function and significance of lysophospholipid signaling in vivo. However, there are still many questions left to answer. For example, functional redundancies between receptors likely exist and the generation of mice deficient for multiple lysophospholipid receptors will help determine if this is indeed the case. Also, given the overlapping expression pattern of many lysophospholipid receptors, combinatorial deletions could elucidate how these receptors might cooperate in physiological and pathophysiological conditions. Furthermore, receptor-specific agonists and antagonists that are currently being developed will be useful in determining the functional properties of lysophospholipid receptors in vitro and in vivo, particularly when combined with defined receptor null animals. The coming years should see an increasing depth of basic and medicinal biology related to the signaling of this novel group of simple lipids.

Acknowledgements. We thank Brigitte Anliker and Christine Paczkowski for critical reading of the manuscript. This work was supported by NIH grants MH51699, MH01723, and NS048478 to J.C. from the National Institute of Mental Health and the National Institute of Neurological Disorders and Stroke.

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Rev Physiol Biochem Pharmacol (2007) 160:47 –69
DOI 10.1007/112_2006_0606

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Lipoxins: update and impact of endogenous pro-resolution lipid mediators

Published online: 12 January 2007

Abstract Lipoxins (LXs) are endogenously produced eicosanoids that are typically generated by transcellular biosynthesis. These trihydroxytetraene-containing lipid mediators and their stable synthetic analogues possess a wide spectrum of anti-inflammatory and pro-resolution bioactions both in vitro and in vivo. More recently, LXs have emerged as potential anti-fibrotic mediators that may influence pro-fibrotic cytokines and matrix-associated gene expression in response to platelet-derived growth factor (PDGF). Here we review the biosynthesis, metabolism and bioactions of LXs and LX analogues and their therapeutic potential.

Introduction

Bioactive eicosanoids play critical roles regulating pleiotropic processes. These well-established mediators include prostaglandins, thromboxanes, leukotrienes and hydroxyeicosatetraenoic acids (HETEs) (Goetzl et al.1995). More recently, the lipoxin (LX) family of eicosanoids has been described (Serhan et al.1984). The term lipoxins refers to the provenance of these mediators, i.e. lipoxygenase interaction products. LX generation plays a pivotal role in both physiologic and pathologic responses (Serhan et al.1984). More specifically, these eicosanoids have emerged as prominent lipid/chemical mediators whose synthesis is "switched on" in the resolution phase of an inflammatory response and can function as "braking signals" in inflammation (McMahon et al. 2001; Serhan 2002). Recently, a second class of novel mediators generated from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been identified and, like LXs, display potent anti-inflammatory and immunoregulatory actions (Hong et al. 2003; Serhan et al. 2000, 2002). These data suggest potential roles for such mediators in diseases such as intestinal

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inflammation, respiratory disease, glomerulonephritis and rheumatoid arthritis, where chronic inflammation underlies the pathology (McMahon and Godson 2004; McMahon et al. 2001; Serhan 2002).

Lipoxin biosynthesis and metabolism

LXs are bioactive eicosanoids that possess a tetraene structure and are formed by dual lipoxygenation of arachidonic acid. First identified in 1984 from mixed fractions of purified human leukocytes (Serhan et al.1984), LXs are now known to be generated by one of three pathways depending on their cellular context (Samuelsson et al.1987). The first of these pathways involves the sequential lipoxygenation of arachidonic acid (AA) (C20:4) by 15-lipoxygenase and 5-lipoxygenase (5-LO) in activated granulocytes (Serhan et al.1984). More specifically, 15-LO inserts molecular oxygen at the carbon-15 position of AA predominately in the *S* configuration to generate 15S-hydroperoxyeiscosatetraenoic acid (15S-H(p)ETE), or the reduced alcohol form 15S-hydroxyeicosatetraenoic acid (15S-HETE). Neutrophil-derived 5-LO in turn converts these reaction products to unstable epoxide intermediates, 5–6-epoxytetraenes, which are subsequently hydrolysed to lipoxin A_4 (LXA_4; which carries hydroxyl groups at carbons 5*S*, 6*R*, and 15*S*) and lipoxin B_4 (LXB_4; which carries hydroxyl groups at carbons 5*S*, 14*R*, and 15*S*) by epoxide hydrolases (Samuelsson et al.1987; Serhan et al. 1984).

The second pathway for LX synthesis predominately occurs within the vasculature and involves interactions between 5-LO present in cells of myeloid lineage and 12-LO present in platelets (Edenius et al. 1988; Fiore and Serhan 1990; Serhan and Sheppard 1990). During cell—cell interactions, activated platelets convert neutrophil-derived LTA_4 to 5–6-epoxytetraene via a 12-LO-dependent mechanism. Like that of the previous pathway, the resulting unstable intermediate is further metabolized to LXA_4 and LXB_4 by epoxide hydrolases (Edenius et al. 1988; Fiore and Serhan 1990; Serhan and Sheppard 1990). The cell—cell interaction and cell adhesion occurring in this pathway are crucial in this transcellular biosynthetic pathway (Kantarci and Van Dyke 2003). A study on P-selectin knockout mice, for example, suggested that adhesion is selectin-dependent and when blocked by specific antibodies, transcellular LX biosynthesis was attenuated (Mayadas et al. 1996). This in vitro observation was also demonstrated in a model of ConA-F glomerular nephritis (Papayianni et al. 1995). In this model, neutrophil infiltration was enhanced during acute nephrotoxic serum nephritis in P-selectin-deficient mice. Moreover, P-selectin deficient mice had a reduced efficiency of transcellular LXA_4 generation; however, LXA_4 levels were restored and polymorphonuclear neutrophils (PMN) infiltration was normalized when the mice were injected with wild-type platelets (Papayianni et al. 1995). These observations point to the importance of platelet-neutrophil adherence in the transcellular biosynthesis of LX and the subsequent fate of these lipid mediators in determining cellular events in inflammation.

The third pathway for LX generation involves aspirin and the actions of cyclooxygenase (COX) 2 and 5-LO (Claria and Serhan 1995). In a cytokine-enriched environ, aspirin-acetylated COX-2 shunts its activity from a prostaglandin synthesizing endoperoxide to an LO, thereby transforming arachidonate to 15-HETE. This intermediary carries its C15 alcohol in the *R* configuration. Upon release from endothelial and epithelial cells, 15 (R)-HETE is converted by leukocyte 5-LO to either 15-epimer lipoxinA_4 (15-epi-LXA_4) or 15-epimer lipoxinB_4 (15-epi-LXB_4), otherwise known as aspirin-triggered LXs (ATL) (Claria and Serhan 1995). These ATLs retain many of the bioactions of native LXs albeit at greater potency

and may contribute to the well-documented prostaglandin-independent anti-inflammatory actions of aspirin (Clish et al. 1999; Serhan 2002).

In addition to the transcellular routes outlined above, primed neutrophils are another source of LX biosynthesis (Brezinski and Serhan 1990). This mechanism is mediated by the esterification and accumulation of 15-HETE in PMN cell membranes. Once stimulated by various agonists, PMN release 15-HETE, which is subsequently transformed into LXs in the absence of transcellular LO activity. This pathway suggests that inflammatory cells can be primed for action and subsequent resolution by their ability to be stored as precursors within cell membranes (Brezinski and Serhan 1990).

The route of LX biosynthesis is dependent on cells and enzymes present therein and can be subject to modulation by various factors (Serhan et al. 1996). For example, interleukin 4 (IL-4) and IL-13, putative negative regulators of inflammatory and immune responses, promote transcellular LX generation through enhanced expression of 15-LO in blood monocytes and epithelial cells (Munger et al. 1999; Nassar et al. 1994). Cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-3 up-regulate 5-LO transcripts (Murakami et al. 1995; Ring et al. 1996; Stankova et al. 1995), while hypoxia and pro-inflammatory cytokines such as IL-1β, IL-6 and tumour necrosis factor (TNF-α) have been shown to induce COX-2, thus potentially contributing to the formation of ATLs in vivo (Parente and Perretti 2003). More recently, *Toxoplasma gondii*, a protozoan parasite, has been shown to activate LXA_4 biosynthesis (Aliberti 2005; Aliberti et al. 2002). In this setting, supraphysiological levels of LXA_4 are generated during infection, which in turn reduce IL-12 production by dendritic cells, thus dampening Th1-type cell-mediated immune responses and thus increasing host immunopathology (Aliberti 2005; Aliberti et al. 2002). Further analysis pointed to a *T. gondii* tachyzoite (the rapidly multiplying and invasive stage of the parasite) that possessed a 15-lipoxygenase that initiated LX biosynthesis and in turn mediated host immune system deflection (Bannenberg et al. 2004).

Resolvins

Recently, novel lipoxygenase- and cyclooxygenase-2-derived mediators generated from EPA and DHA (omega-3 fatty acid constituents of fish oils) were identified (Serhan et al. 2000). These bioactive di- and trihydroxy-containing lipid autocoids named resolvins (resolution phase interaction products) and docosatrienes displayed potent anti-inflammatory and immunoregulatory actions (Hong et al. 2003; Serhan et al. 2000). In addition, aspirin therapy can lead to the creation of 17 (*R*) resolvins generated from DHA by COX-2. First identified in murine brain and human microglial cells treated with aspirin, COX-2 converted DHA to 17R-hydroxydocosahexaenoic acid (17R-HDHA) (Serhan et al. 2002), which was transformed by human neutrophils (PMN) to di- and trihydroxy products; one initiated via oxygenation at carbon 7 and the other at carbon 4. These compounds inhibited microglial cell cytokine expression and ameliorated experimental models of dermal inflammation and leukocyte accumulation in peritonitis at nanogram doses (Serhan et al. 2002).

Metabolism

LXs are produced rapidly in response to various physiological and pathological stimuli, function in an autocrine or paracrine fashion, and are rapidly metabolized to their oxo and

dihydro derivatives (Clish et al. 2000; Maddox and Serhan 1996; Serhan et al.1993, 1995). The major route of LXA_4 involves dehydrogenation of LXA_4 by a monocyte/macrophage lineage to yield 15-oxo-LXA_4 (Maddox and Serhan 1996; Serhan et al.1993). More specifically, 15-hydroxyprostaglandin dehydrogenase (15-PGDH) catalyses the dehydrogenation of the C15 hydroxyl group to an oxo group (Maddox and Serhan 1996; Serhan et al. 1995). This biologically inactive compound is further metabolized to 13,14-dihydro-15-oxo-LXA_4 by LXA_4/PGE 13,14-reductase/LTB_4 12-hydroxydehydrogenase (PGR/LTB_4DH) (Clish et al. 2000). In addition, 15-PGDH can also reduce the 15-oxo group to 13,14-dihydro-LXA_4 (Clish et al. 2000). Similarly, LXB_4 is dehydrogenated by 15-PGDH at carbon 5 to oxo- and dihydro-LXB_4 (Maddox et al. 1998). In contrast to native LX, ATLs are less susceptible to dehydrogenation at C15 in vitro and in vivo, suggesting an enhanced biological half-life (Clish et al. 1999; Maddox et al. 1997; Serhan et al. 1995). Indeed, the relative short half-life and rapid metabolism of these autocoids have promulgated the development of novel LX analogues designed to resist metabolism and maintain their structural integrity and bioavailability as well as their potential beneficial bioactions in vitro and in vivo (Clish et al. 1999; Maddox et al. 1997; Serhan et al. 1995). Such stable analogues of both native LXs and ATLs utilize modifications such as addition of methyl groups to C5 and C15 of LXA_4, and phenoxyl or *para*-fluoro-phenoxyl groups at C16 of both LXA_4 and ATL. In essence, these modifications confer protection against dehydrogenation by sterical hindrance (Clish et al. 1999; Maddox et al. 1997; Serhan et al. 1995). Similarly, in human PMN RvE1 is metabolized to 20-hydroxy-RvE1 by ω-oxidation at carbon 20. Like the LX stable analogues, addition of a *para*-fluoro-phenoxyl group generated an analogue that was resistant to inactivation but proved to retain much of the biological activity of native RvE1 (Arita et al. 2006). Thus, the generation of these stable analogues affords us new avenues of approach in considering therapies for inflammation, cardiovascular diseases and cancer, as will be outlined later in this review.

Bioactions of lipoxins

LXs are generated in vivo during the resolution phase of an inflammatory response. This is a rapid process that is activated by pro-inflammatory components such as IL-4, IL-13 or granulocyte–macrophage colony-stimulating factor (GM-CSF) through increases in LO activity (Levy et al. 2001). The role for LXs as anti-inflammatory molecules is well defined, with bioactions involving the inhibition of chemotactic responses of PMN and stimulating the activation of monocytes and macrophages (Serhan 2002). In conjunction with their anti-inflammatory bioactions, there is a growing appreciation that LX biosynthesis coincides with the resolution phase of inflammation, and so mediates numerous pro-resolution effects such as stimulation of macrophage clearance of apoptotic PMN from an inflammatory focus, modulation of cytokine-stimulated metalloproteinase activity and inhibition of cellular proliferation (Fig. 1) (McMahon et al. 2001). Most recently, we have developed an in vitro model of renal fibrosis typified by epithelial mesenchymal *trans*-differentiation (EMT). Supernatants derived from mesangial cells treated with PDGF caused a morphological change in murine renal tubular cells, typified by loss of the epithelial tight junction E-cadherin and a gain of α-smooth muscle actin. LXA_4 pre-treatment abrogated these effects, thus implying that in addition to the anti-inflammatory and pro-resolution bioactions associated with LXA_4, LXA_4 possess anti-fibrotic activity, preventing growth factor-induced mesangial matrix production and the progression of renal disease characterized by EMT (Rodgers et al.

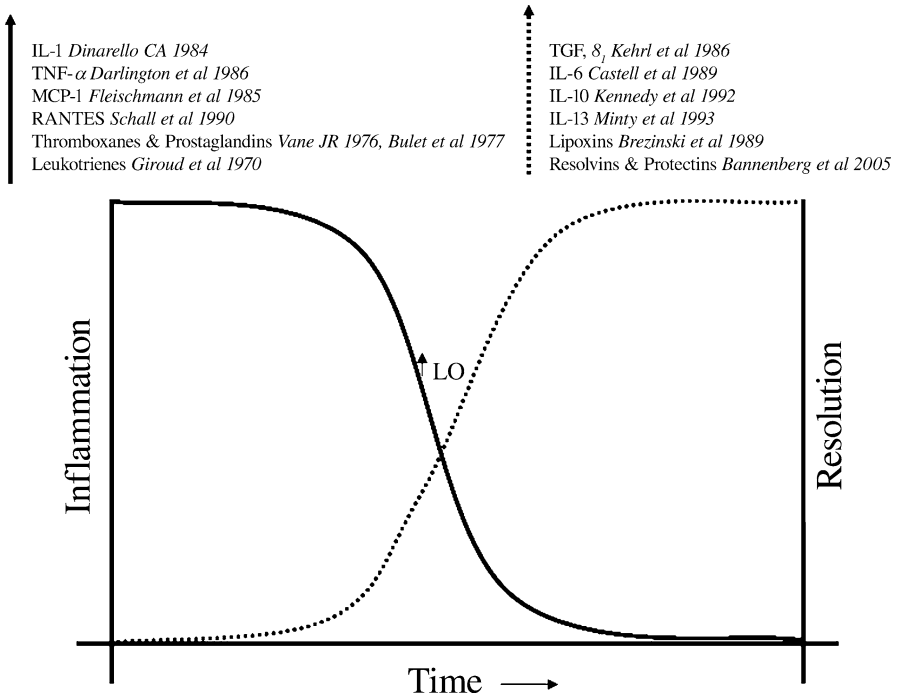

Fig. 1 Proposed homeostatic regulation of inflammation in effective host defence. The initial phase of inflammation is initiated by a host of pro-inflammatory cytokines and eicosanoids which subsequently become diminished with time, and are coupled with the likely activation of anti-inflammatory and pro-resolution mediators, including the lipoxins, resolvins and protectins. Typically, IL13 up-regulates 15-LO gene expression of human blood monocytes for example (Nassar et al. 1994). The associated increase in enzyme activity results in an increase in LX generation which promotes the resolution phase of inflammation. *IL*, interleukin; *TNF-α*, tumour necrosis factor-α; *MCP-1*, monocyte chemotactic protein-1; *RANTES*, regulated upon activation, normal T-cell expressed and secreted; $TGF\beta_1$, transforming growth factor β_1

2005). Table 1 summarizes the current literature on the biological effects of LX and ATL analogues in vitro and in vivo.

Mechanisms of lipoxin action: receptors and signalling pathways

Three potential mechanisms have been proposed through which LXs exert their bioactions. These include activation of high-affinity LX-specific G-protein-coupled receptors (GPCRs), activation of subclasses of peptide-leukotriene (LT) GPCRs and/or cellular uptake of LX which in turn facilitates interactions with intracellular targets such as nuclear receptors (Chiang et al. 2000; Fiore et al. 1992; McMahon et al. 2001; Planaguma et al. 2002; Schaldach et al. 1999; Simchowitz et al. 1994). In 1992 Fiore identified a distinct LX recognition site on PMN that was likely to mediate many of the selective actions of LX in these cells (Fiore et al. 1992). Binding of [11, 12-^{3}H]-LXA_4 radiolabel to intact PMN was localized to membrane, intracellular and nuclear fractions and showed a high affinity dissociation constant (K_d) of 0.5±0.3 nM, representing approximately 1,830 site per PMN cell. This binding was shown to be stereoselective in that other hydroxyl-containing eicosanoids such as LTB_4, LXB_4, or LXA_4 stereoisomers did not specifically compete for [^{3}H]-LXA_4 (Fiore et al.

Table 1 The bioactions of lipoxin in vivo

	Lipoxin bioactions	Reference
Ageing	A reduction in urinary levels of LXA was detected in elderly individuals	Gangemi et al. 2005
	Positive correlation between 15-epi-L.XA synthesis and age in females only	Chiang et al. 2006
Angiogenesis	Prophylactic treatment with ATL-1 protected against angiogenesis and CD-31 expression in a murine air pouch model of angiogenesis	Fierro et al 2002
	ATL-1 potently inhibits endothelial cell proliferation	Fierro et al 2003
	ATL-1 inhibits VEGF-induced endothelial cell chemotaxis	
Glomerulonephritis	ATL is protective in a renal model of ischemic-reperfusion injury	Kieran et al. 2003
	ATL attenuated neutrophil accumulation and expression of interferons in a model of anti-glomerular basement membrane nephritis	Ohse et al. 2004
	LXA4 protects against PDGF or LTD4-induced human mesangial cell proliferation	McMahon et al. 2002
	LXA4 prevents the expression of fibrosis-associated genes; fibronectin, thrombospondin, collagens and the TGFβ1 cytokine in human mesangial cells	Rodgers et al. 2005
	LXA4 attenuates TNF-α-induced proliferation and cytokine expression in rat mesangial cells	Wu et al. 2005
Intestinal inflammation	In an animal model of Crohn's disease (hapten-induced colitis), ZK-192 administered after disease onset displayed anti-inflammatory bioactions reducing TNF-, interferon-γ and interleukin-8	Fiorucci et al. 2004
	LX modulates neutrophil transmigration across the intestinal epithelial monolayer, inhibits leukocyte rolling and adherence, modulates interleukin-8 release chemokine secretion	
	LX inhibits TNF-α-induced neutrophil-enterocyte interaction, chemokine release and colonocyte apoptosis	

LX, lipoxin; ATL, aspirin-triggered lipoxin; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; LTD4, leukotriene D4; TGFb1, transforming growth factor b1; TNF-α, tumor necrosis factor-a; ALXR, lipoxin receptor; GvHD, graft versus host disease; COPD, chronic obstructive pulmonary disease; IL, interleukin; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of MMP

Table 1 (continued)

	Lipoxin bioactions	Reference
Ocular damage	Interruption of lipoxygenase activity delayed corneal epithelial wound healing	Gupta et al. 1993
	Corneal epithelial cells express LXA4, 12/15-lipoxygenases and the ALXR	Gronert et al. 2005
	LXA4 promotes re-epithelialization and PMN infiltration in damaged comeal epithelium	
	Systemic administration of ATL resolved eye inflammation observed in a murine model of bone marrow transplant-induced GvHD	Devchand et al. 2005
Respiratory disease	Alveolar macrophages synthesize lipoxins	Kim et al. 1988
	Reduced levels of endogenous lipoxin observed in asthma and COPD	Vaichier et al. 2005
	LX administration to a murine model of asthma blocked airway hyper-responsiveness and pulmonary inflammation	Levy et al. 2002
Rheumatoid arthritis	LXA4 prevents the expression of IL-1β-induced interleukins 6&8 and MMP-3 synthesis	Sodin-Semrl et al. 2000
	LX acts on it's receptor in fibroblast-like synovial cells to promote TIMP2 expression and inhibits IL-1β-induced NF-κB and AP-1 DNA binding activity	Sodin-Semrl et al. 2004b
	15-lipoxygenase over-expressing transgenic models have reduced bone damage in microbe-associated inflammation and leukocyte-mediated bone destruction	Serhan et al. 2003
Mycobacterium tuberculosis	LX are negative regulators of the TH1 response to mycobacterial infection	Bafica et al. 2005
	Transgenic animals deficient in producing lipoxins have elevated levels of interleukin-12, interferon-γ and nitric oxide synthase 2 mRNA, as well as decreased bacterial burden in lung tissue	

LX, lipoxin; ATL, aspirin-triggered lipoxin; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; LTD4, leukotriene D4; TGFb1, transforming growth factor b1; TNF-α, tumor necrosis factor-a; ALXR, lipoxin receptor; GvHD, graft versus host disease; COPD, chronic obstructive pulmonary disease; IL, interleukin; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of MMP

1992). In addition, [^{3}H]-LXA_4 binding was shown to be time-dependent, saturable, specific, and reversible and was modulated by guanosine analogues, suggesting the involvement of G proteins (Fiore et al. 1994, 1992). In subsequent studies, cDNA encoding a high-affinity G-protein-coupled receptor for LXA_4 was identified and cloned from human PMN (Fiore et al. 1994). Interestingly, while this receptor (now designated the ALXR) was originally identified as a formyl peptide receptor given its homology with members of the chemokine and chemotactic peptide receptor superfamily, it binds [^{3}H]-fMLP poorly and proves to be selective for LXA_4 by 3 orders of magnitude (Fiore and Serhan 1995). The murine and rat ALX receptors have also been cloned from a spleen cDNA library (Takano et al. 1997) and peripheral blood leukocytes (Chiang et al. 2003), respectively. Indeed, the overall homology between the these receptors is relatively high, with murine and human ALXR, for example, sharing 76% nucleotide sequence and 73% amino acid identity, respectively (Takano et al. 1997). In terms of tissue/cell distribution, the human ALXR is highly expressed in lung, placenta, peripheral blood leukocytes, spleen and kidney (Fiore et al. 1994; Takano et al. 1997). This pattern of tissue distribution is mimicked by both the murine and rat ALXRs (Takano et al. 1997).

Recently, a second murine LXA_4 GPCR has been identified from a macrophage cDNA library and shares 89% sequence identity with the previously described murine homologue (Vaughn et al. 2002). Like that of the previously described mouse ALXR, activation of this receptor mediates biological activity typified by LXA_4-induced production of inositol-1,4,5-triphosphate (Vaughn et al. 2002). The existence of a distinct human ALXR subtype has been supported by the variable binding properties of the ALXR in endothelial cells and endothelial-like cells, human umbilical vein endothelial cells (HUVECs) and ECV304 cells, respectively (Maderna et al. 2000). Maderna et al. (2000) evaluated the influence of LXs on the expression of tissue factor (TF), a key regulator of coagulation, in both HUVECs and ECV304 (Maderna et al. 2000). These studies demonstrated that LXA_4 stimulated TF activity as assessed by a one-stage clotting assay in both cell types. Interestingly, induction of TF activity was specific for native LXA_4 and was not observed with LXB_4. Furthermore, ECV304 cell TF expression was not activated by either 15(*R/S*)-methyl-LXA_4 or 16-phenoxy-LXA_4 nor modulated by pobilukast, which blocks LXA_4 bioactivities transduced through the putative shared LXA_4/LTD_4 receptor (Maderna et al. 2000). These observations suggested that in certain cell types distinct ALX receptor subtypes exist.

Transcription of ALXR had been shown to be controlled by various cytokines of which IL-13, IL-1β and interferon gamma (IFN-γ) were the most notable (Gronert et al. 1998; Sodin-Semrl et al. 2000). Sodin-Smerl et al. established that IL-1β and, to a lesser extent, LXA_4 up-regulate ALXR expression in both synovial fibroblasts (SF) and in human fibroblast like synoviocytes (FLS), respectively (Sodin-Semrl et al. 2004a, , 2000). Furthermore, they demonstrated that LXA_4 inhibited the synthesis of inflammatory cytokines and matrix metalloproteinases (MMP) and stimulated tissue inhibitor of metalloproteinase (TIMP) production in activated SF. These findings suggest that LXA_4 and up-regulation of its receptor may be involved in a negative feedback loop opposing inflammatory cytokine-induced activation of SF (Sodin-Semrl et al. 2004a, 2000). To date, the promoter of the ALXR remains elusive. Its description may confer an important insight as to the receptor's expression and activity, which in contrast to most other GPCRs is thought to be regulated at transcription level as opposed to post-translation level (Marinissen and Gutkind 2001). The role of transcription and transcription factors involved in LXA_4 signalling will be examined more thoroughly later in this review.

Presently, little is known regarding the putative receptor through which LXB_4 signals. In contrast to LXA_4, LXB_4 does not bind ALXR and although functional studies have sug-

gested the presence of a receptor activated by LXB_4 (Romano et al. 1996), this receptor remains to be cloned.

The partial antagonism of a subclass of peptide-leukotriene receptors (cysLT1) is a potential mechanism through which LXs may contribute to the anti-inflammatory bioactions of LXs (Badr et al. 1989; Brady et al. 1990; Clish et al. 1999; Lee et al. 1989; McMahon et al. 2000; Papayianni et al. 1996). LX antagonism at the LT receptors has been demonstrated in PMN, endothelial, bronchial smooth muscle, intestinal epithelial and mesangial cells (McMahon et al. 2001). McMahon et al. demonstrated that LXA_4 inhibits LTD_4-induced proliferation by modulating LTD_4-induced transactivation of the PDGF receptor and subsequent phosphotidylinositol 3 (PI3)-kinase activation and mitogenic responses (McMahon et al. 2002, 2000). The counter-regulatory responses identified for LX were mediated in part by competition of the cysLT1 receptor (McMahon et al. 2002). These data confirm and extend earlier work by Badr et al. which suggested that LTD_4 and LXA_4 interacted at a common site on rat mesangial cells and at which LXA_4 provoked partial agonist responses and competitively antagonized both the cellular and physiological actions of LTD_4 (Badr et al. 1989). Moreover, Gronert et al. observed that both aspirin-triggered 15-epi-LXA_4 and LTD_4 bind to and compete with equal affinity at cysLT1 receptors in human vascular endothelial cells (Gronert et al. 2001). In contrast, LTD_4 was an ineffective competitive ligand for recombinant ALXR with a [^{3}H]-ATL analogue (Gronert et al. 2001). The basis for this common interaction at the LTD_4 receptor may be related, in part, to the shared spatial orientation [(*S*), (*R*)] of the hydroxyl group at C5 and C6 in both eicosanoids, given that the reversal of this configuration leads to loss of inhibitory interaction as well as biological activities for both eicosanoids (Lee et al. 1991; Serhan et al. 1996).

In addition to signalling via GPCRs, further studies have established that LXs can act as ligand for the aryl hydrocarbon receptor (AhR), which is primarily involved in xenobiotic metabolism (Schaldach et al. 1999). Binding of LXA_4 to the AhR at micromolar concentrations mediates the transformation of this receptor to a form that binds to the cognate DNA response element (DRE) and activates transcription of the associated gene, *CYP1A1*. In addition, LXA_4 acts as a substrate for CYP1A1 and given that the expression of cytochrome P450 (CYP) 1A1 isoenzyme is regulated by the activated Ah receptor, the metabolism of LXA_4 potentially represents a means of LX auto-regulation (Schaldach et al. 1999).

Aside from LXA_4, several pleiotropic ligands have been shown to activate the ALXR. These include synthetic peptides, N-formylated hexapeptides, serum amyloid A (SAA) protein, amyloid β (Aβ42), HIV-1 envelope protein domains (gp120), major histocompatibility complex (MHC) binding peptides, urokinase-type plasminogen activator receptor (uPAR) fragment, a mitochondrial peptide fragment (MYFINILTL) derived from NADH dehydrogenase subunit, a neurotoxic prion peptide fragment (PrP106–126) and the 37-kDa protein annexin 1 (ANXA1) (McMahon et al. 2001). Intriguingly, the ALXR may mediate ligand-specific responses. Engagement of SAA protein with ALXR in human neutrophils, for example, generates a pro-inflammatory phenotype, triggering PMN chemotaxis, IL-8, and TNF-α production as a consequence of NF-κB activation (Sodin-Semrl et al. 2004b; Su et al. 1999). However, the production of this pro-inflammatory response in PMNs by peptide agonists can be blunted by LXA_4 (Chiang et al. 2000).

Another ligand of particular interest is that of annexin 1 given that similar to LXA_4, it is generated within an inflammatory milieu. Annexin 1A is a glucocorticoid-inducible protein that mediates many of the inflammatory actions of glucocorticoids in models of acute and chronic inflammation including modulation of leukocyte trafficking, ischaemic damage, pain and fever (Maderna et al. 2005). Perretti et al. first identified that ANXA1-derived peptides directly interact with human ALXR/FPRL1 to inhibit PMN diapedesis (Perretti et

al. 2002). In addition, the combination of both ATL and ANXA1-derived peptides limited PMN infiltration and reduced production of inflammatory mediators (i.e. prostaglandins and chemokines) in vivo. These studies therefore suggested that these two structurally distinct endogenous systems, namely lipid-derived (ATL) and protein-derived (ANXA1) mediators, act in concert at ALXR to down-regulate PMN recruitment to inflammatory loci (Perretti et al. 2002).

In renal mesangial cells, LXA_4 blocked LTD_4-stimulated phosphatidylinositol 3-kinase (PI 3-kinase) activity in parallel to inhibition of LTD_4-induced cell proliferation (McMahon et al. 2002, 2000). LXA_4 may mediate these responses through the activation of MAP kinase superfamily via two distinct receptor: one shared with LTD_4 and coupled to a PTX-sensitive G protein (G_i) which leads to p38 activation and the other coupled via an alternative PTX-insensitive G protein, such as G_q or G_{12}, and leads to erk activation (McMahon et al. 2002, 2000; Mitchell et al. 2004). Further studies demonstrated that LX can regulate mesangial cell proliferation in response to pleiotropic growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (McMahon et al. 2002, 2000). Activation of mesangial PDGF receptor-β by PDGF-BB induces significant proliferative responses in human mesangial cells and has been linked to the aetiology of glomerulonephritis (Abboud 1995; Cybulsky 2000). Indeed, targeting of this growth factor signalling pathway through receptor antagonism and/or inhibition of PDGF-β tyrosine kinase activity ameliorated mesangial cell proliferation and renal scarring in an experimental model of Thy-1.1 glomerulonephritis (Gilbert et al. 2001). Later studies demonstrated that LTD_4 *trans*-activates the PDGFRβ, a process associated with c-src recruitment and ras activation (McMahon et al. 2002). Furthermore, LX was shown to inhibit LTD_4-stimulated activation of the PDGFRβ via modulation of the PI-3-kinase pathway, which prevented mitogen-elicited G_1-S phase progression, and through alteration of PDGF-BB-stimulated PDGFRβ activation (McMahon et al. 2002; Mitchell et al. 2004). Intriguingly, the activity of LXA_4 in modulating RTK (PDGFR, EGFR) is not only restricted to this so-called receptor *trans*-inactivation but can also directly modulate the RTK itself. This inhibitory action appears to be coupled to modulation of recruitment of SH2 domain-containing proteins to the activated PDGF receptor, which in turn decreases the phosphorylation of the PDGFRβ (Mitchell et al. 2006). The exact mechanism through which this inactivation occurs is thought to be mediated through the coupling of the LXA_4 receptor (ALXR) to the activation of the protein tyrosine phosphatase (PTP), SHP-2. More specifically, it is proposed that the association of the PDGFRβ with lipid raft microdomains renders it susceptible to LXA_4-mediated dephosphorylation by possible reactivation of oxidatively inactivated SHP-2 (Mitchell et al. 2006).

In addition to the regulation of mesangial cell proliferation, LX or more specifically the synthetic LX analogue 15-epi-16-(*para*-fluoro)-phenoxy-lipoxin A_4 (ATL-1), has proved a potent inhibitor of vascular endothelial growth factor (VEGF) and LTD_4-stimulated angiogenesis in vitro and in vivo (Fierro et al. 2002). The means by which this is achieved are thought to reside in the ability of LX to shift the cytokine/chemokine axis involved in angiogenesis from a pro-angiogenic to an anti-angiogenic process (Fierro 2005). In addition, LX has been shown to regulate the proteolytic activity necessary to digest basement membrane, a key event in angiogenesis (Fierro 2005). More recently, Fierro and colleagues showed that VEGF-induced EC migration, another essential component for the formation of the vessel sprout, was modulated by ALT-1 by the combined inhibition of actin polymerization and proper assembly of focal adhesions via impairment of stress-activated protein kinase (SAPK2/p38) and focal adhesion kinase (FAK) phosphorylation, respectively, in an ALXR-dependent manner (Cezar-de-Mello et al. 2006). Interestingly, while ATL-1 alone had no apparent effect on EC proliferation or migration per se, LXB_4 stable analogues sig-

nificantly increased proliferation (Cezar-de-Mello et al. 2006; Fierro et al. 2002). Because these are related structures, the separate actions of ATL and LXB_4 analogues within these cells indicate that the ATL-1 response is highly stereoselective (Fierro et al. 2002), thus making it a potentially attractive anti-angiogenic therapeutic.

As previously outlined, LXs are endogenous "stop signals" but also actively promote the resolution of inflammation (Levy et al. 2001). To facilitate resolution, removal of apoptotic cells is vital for the protection of tissue from exposure to noxious and immunogenic contents of necrotic cells. Clearance of apoptotic cells is largely mediated by professional and non-professional phagocytes such as macrophages and immature dendritic cells (Maderna and Godson 2005; Serhan and Savill 2005). In recent years, the role of endogenous mediators that actively promote phagocytosis of apoptotic cells has become apparent (Maderna and Godson 2005). In the context of this review, LX and LX analogues have been shown to stimulate phagocytosis of apoptotic PMN in vitro (Godson et al. 2000) and in vivo (Mitchell et al. 2002), priming the phagocytic macrophages through rearrangement of the active cytoskeleton (Fig. 2) (Maderna et al. 2002). More recently, research efforts have focused on mechanisms whereby ANXA1 and its derivative peptide Ac2–26 can stimulate phagocytosis and clearance of apoptotic cells (Maderna et al. 2005). These investigations have indicated that both ANXA1 and Ac2–26 promote actin cytoskeleton rearrangement (Fig. 2) and sig-

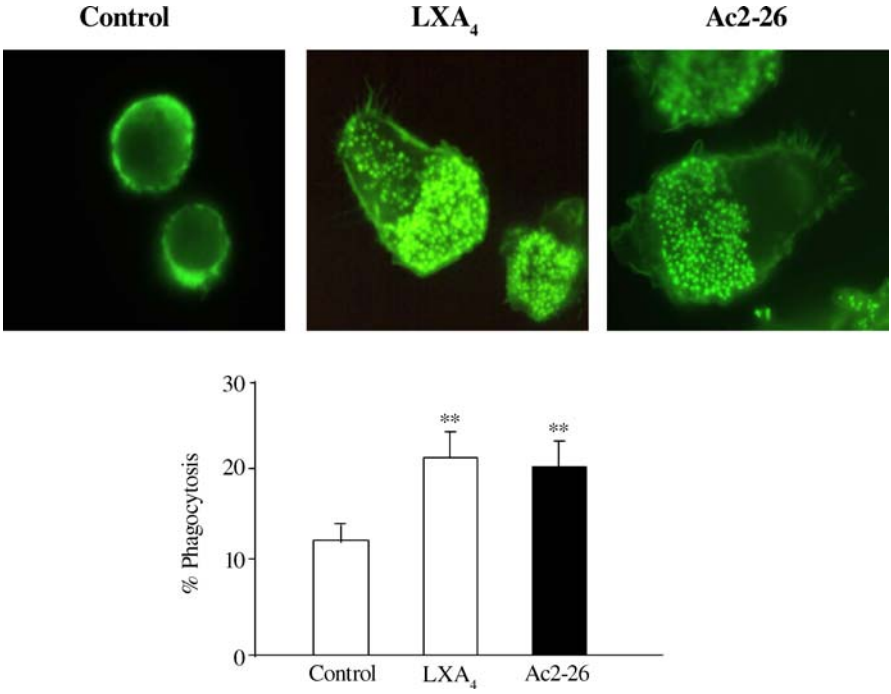

Fig. 2 LXA_4, its stable analogue 15-(*R/S*)-methyl-LXA_4 and Ac2–26 stimulate macrophage (*Mφ*) actin rearrangement and phagocytosis of apoptotic leukocytes. Mφ were exposed to vehicle, LXA_4 (1 nM), 15-(*R/S*)-methyl-LXA_4 (10 pM) and Ac2–26 (32 μM) for 15 min at 37°C. F-actin rearrangement was assessed by staining with Oregon Green Phalloidin and visualized by fluorescence microscopy (Maderna et al.2002, 2005). Phagocytosis of apoptotic PMN was determined by myeloperoxidase staining of co-cultures of Mφ and PMN. For each experiment, the number of Mφ containing one or more PMN in at least five fields (minimum of 400 cells) was expressed as a percentage of the total number of Mφ and an average between duplicate wells was calculated

nificantly stimulate non-phlogistic phagocytosis of apoptotic PMNs by human monocyte-derived macrophages (φ) (Maderna et al. 2005). Moreover, a significant decreased phagocytosis of apoptotic PMNs was observed in bone marrow-derived Mφ ANXA1-null mice compared to cells taken from littermate controls (Maderna et al. 2005). In addition to stimulating macrophage phagocytosis of apoptotic PMN, LX has also been shown to stimulate the phagocytosis of apoptotic lymphocytes by THP-1 cells differentiated into a macrophage-like phenotype. This mechanism is associated with decreased phosphorylation of non-muscle myosin heavy-chain IIA, myosin rearrangement and cellular polarization coupled to Cdc42, PKB/Akt, PKCζ and GSK-3β activation (Reville et al. 2006). Mediators such as LX and LX mimetics may prove useful therapeutics in chronic inflammatory conditions where impaired phagocytosis contributes to disease (Serhan and Savill 2005).

The spectrum of biological activities of LX and its receptor is ever rapidly expanding. However, the true intracellular signalling pathway through which it elicits its diverse actions raises many intriguing possibilities. For example, does LXA_4 binding to the ALXR trigger distinct anti-inflammatory signals or does it merely reflect the ability of the activated ALXR to quench pro-inflammatory signals? How do multiple ligands (lipids and peptides), acting through identical ALXRs, evoke such varied and contrasting biological responses? Despite considerable efforts and the major inroads made in tackling these questions, our overall current understanding of LX receptor signalling remains incomplete.

Transcription factors involved in lipoxin A_4 signalling

As previously discussed, LX signalling via a G-protein-coupled receptor is thought to be partly regulated at the transcription level, but it may also govern the regulation of other genes associated with the resolution of inflammation. Indeed, many complementary oligonucleotide microarray and bioinformatics analyses have indicated numerous anti-inflammatory, pro-resolution and, more recently, pro-fibrotic genes modified by LXA_4 in various disease settings (Gewirtz et al. 2002; Kieran et al. 2003; Leonard et al. 2002; Rodgers et al. 2005). Given that transcription is dependent on a plethora of transcription factors, co-activators and co-repressors, it is quite difficult to assign a direct "LX-dependent" effect that might be involved in the modulation of the above genes. However, several publications have suggested the involvement of NF-κB, AP-1, Sp-1 and the co-repressor NAB1.

In an attempt to elucidate a mechanism by which LXs, ATL and their stable synthetic analogues exert their anti-inflammatory effects, Gewirtz et al. (2002) examined the global gene expression of intestinal epithelium using cDNA microarrays. 15-(*R/S*)-methyl-LXA_4 pre-treatment attenuated almost 50% of the 125 genes up-regulated in response to the gastroenteritis-causing pathogen *Salmonella typhimurium* (Gewirtz et al. 2002). Interestingly, a major subset of those genes affected by LXA_4 analogue was NF-κB regulated and included TNF-α-induced protein 3, IL-1α and apoptosis inhibitors 1 and 2. This anti-inflammatory effect has been suggested to be mediated by the ALXR, whereby nanomolar concentrations of LXA_4 analogue reduced NF-κB-mediated transcriptional activation in an ALXR-dependent manner and inhibited degradation of IκBα. Moreover, Ca^{2+} mobilization and IκBα phosphorylation induced by *S. typhimurium* was not affected by any of the tested concentrations of LXA_4 analogues, suggesting that they act subsequent to initial Ca^{2+} signal and resulting IκB phosphorylation in the reduction of the IκBα degradation that attenuates pro-inflammatory gene activation (Gewirtz et al. 2002). Interestingly, while Sodin-Semrl

et al. observed that LXA_4 inhibited IL-1β-induced activation of NF-κB and consequently attenuated IL-8 production in human fibroblast-like synoviocytes, SAA interaction with the ALXR elicited opposite IL-8 and NF-κB responses (Sodin-Semrl et al. 2004b). More recently, Wu et al. demonstrated that LXA_4 dose-dependently reduced TNF-α-stimulated IL-1β and IL-6 synthesis in rat glomerular mesangial cells and that these reductions were related to SHP-2 and NF-κB pathway-dependent signal transduction (Wu et al. 2005). SHP-2 is an integral component of the IKK complex and plays a positive role in mediating the induction of NF-κB activity and IL-6 synthesis by TNF-α (You et al. 2001). Thus the ability of LXA_4 to inhibit SHP-2 and NF-κB activities induced by TNF-α suggested that LXA_4 down-regulated NF-κB by inhibiting activation of SHP-2-IKK complex (Wu et al. 2005).

Jozsef et al. (2002) examined the impact of ATL (15(*R/S*)-methyl LXA_4 and 15-epi-16-*p*-fluorophenoxy-LXA_4) and LXA_4 (16-phenoxy-LXA_4) on LPS-stimulated $ONOO^-$ formation, NF-κB and AP-1 activation, and subsequent IL-8 gene expression in human leukocytes. LXA_4/ATL analogues attenuated nuclear accumulation of AP-1 and NF-κB in both polymorphonuclear and mononuclear leukocytes in a dose-dependent manner. This in turn inhibited IL-8 mRNA expression and IL-8 release by 50%–65% in response to LPS (Jozsef et al. 2002). They later postulated that LXA_4/ATL analogues may protect IκB from nitration by $ONOO^-$, thereby preventing activation of NF-κB, and may attenuate nuclear accumulation of AP-1 by suppressing p38 MAPK activation (Filep et al. 2005).

In a recent study undertaken to define specific transcription factors activated in human mesangial cells when exposed to PDGF and/or LXA_4, promoter analysis identified several ubiquitously expressed transcription sites including cAMP response element binding site (CREB), early growth response factor (EGRF) and stimulating protein 1 (Sp1). Of all the PDGF-stimulated genes identified, however, Sp1 was present on almost half of all PDGF-stimulated genes (Rodgers et al. 2005). Nuclear Sp1 binding measured using a TransAM assay showed that stimulation of mesangial cells with PDGF caused an increase in Sp1 expression, while LXA_4 pre-treatment attenuated this effect (Rodgers et al. 2005).

Qui et al. (2001) conducted a study to identify a subset of LXA_4/ATLa-selective early responsive genes in human PMN. ATLa treatment resulted in the rapid induction of Nab protein 1 (NAB1), a co-repressor that interacts with and represses transcription mediated by three members of the NGFI-A family of immediate early gene transcription factors (Swirnoff et al. 1998), via a pertussis toxin-sensitive, LXA_4 receptor-mediated pathway (Qiu et al. 2001). In addition to this, Devchand et al. (2005) observed elevated NAB1 expression levels in murine bone marrow cells treated with 100 nM ATLa (Devchand et al. 2005). These studies highlight the additional protective transcriptional involvement of LX anti-inflammatory signalling that is initiated at the surface membrane receptor. Current data suggest a strong case for LX in regulating transcription factors and provide a rationale for further exploration of the efficacy of LXA_4 and its analogues in a range of inflammatory disorders.

Lipoxins in inflammation and disease

Under healthy conditions the inflammatory process is “self-regulating”, where stop signals such as LX are generated to maintain homeostasis (Serhan and Savill 2005). These signals may, however, become absent or dysregulated and thus contribute to the pathogenesis of many disease states that reflect “chronic inflammation”, including intestinal inflammation,

respiratory disease, glomerulonephritis, rheumatoid arthritis, angiogenesis and wound healing (McMahon and Godson 2004).

Intestinal inflammation

Chronic inflammatory diseases of the intestine, including Crohn's disease and chronic ulcerative colitis, are associated with a local over-production of pro-inflammatory cytokines while central immune cell activation causes the production of non-specific inflammatory mediators, which amplify the local immune response and promote tissue destruction (Fiorucci et al. 2004). In this context LXs exert their anti-inflammatory effects through several diverse mechanisms including the modulation of neutrophil transmigration across the intestinal epithelial monolayer, inhibition of leukocyte rolling and adherence, modulation of IL-8 release chemokine secretion, inhibition of TNF-α-induced neutrophil–enterocyte interaction, chemokine release and colonocyte apoptosis—all of which are central to the physiological process of intestinal inflammation (Colgan et al. 1993; Goh et al. 2001; Gronert et al. 1998; Scalia et al. 1997)

Several animal models of IBD have been developed (Elson et al. 1995). Fiorucci et al. examined the effect of several LX analogues in a hapten-induced colitis animal model, in which trinitrobenzene sulfonic acid (TNBS) was delivered intrarectally to rodents (Fiorucci et al. 2004). This model shares many characteristics with Crohn's disease including the activation of $CD4^+$ T cells and transmural mononuclear cell-driven inflammation. ZK-192, an LX β-oxidation-resistant 3-oxa-analogue, was orally administered after the onset of disease and displayed counter-regulatory or "resolving" actions as well as anti-inflammatory bioactions. Indeed, ZK-192 attenuated weight loss, macroscopic and histologic colon injury, mucosal neutrophil infiltration and colon wall thickening. In addition, it decreased mucosal mRNA levels for several inflammatory mediators such as inducible nitric oxide synthase, COX-2, and macrophage inflammatory protein 2. Moreover, mucosal mRNA, protein levels and overall systemic levels of T helper 1 effector cytokines including TNF-α, IL-2 and IFN-γ were also diminished. Additionally, ZK-192 also prevented colitis in lymphocyte-deficient severe combined immunodeficient mice in which innate immune cells such as neutrophils, macrophages/monocytes, dendritic cells and natural killer cells can operate as triggers for hapten-induced colitis. Given that neutrophils, macrophages/monocytes, dendritic cells and natural killer cells are known to respond to LX/ATL, they hypothesized that LX/ATL exert their potent anti-inflammatory effects and immunomodulatory effects on these innate and adaptive immune cell that drive mucosal pathology and hence hapten-induced colitis (Fiorucci et al. 2004).

A growing body of evidence supports the notion that locally generated ATL limit leukocyte recruitment in the gastric microcirculation, an event that is thought to play a critical role in the pathogenesis of aspirin-induced gastric injury (Fiorucci et al. 2002, 2003). Consistent with this view, Souza et al. demonstrated that COX-2 inhibition suppresses ATL formation and exacerbates aspirin-induced gastric damage in rats (Souza et al. 2003). More recent clinical evidence demonstrated that these events translate to healthy human volunteers taking aspirin, where Celecoxib induced inhibition of COX-2 activity and hence ATL formation exacerbated gastric mucosal injury within these subjects (Fiorucci et al. 2003). These studies indicate a role for LX in resolving the inflammatory components of IBD, thereby preventing the amplification of the local immune responses and the development of such chronic diseases.

Respiratory disease

Disorders of the respiratory tract include asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis. Common to all of these disorders is an ongoing inflammatory component caused by the interaction of resident epithelium with infiltrating inflammatory cells (Bonnans et al. 2004). Kim first detected the ability of alveolar macrophages to generate LXs (Kim 1988). Since then, several investigators have detected decreased LX levels in various respiratory tract disorders including asthma and COPD (Levy et al. 2005; Vachier et al. 2005). Asthmatic patients possess the capacity to generate both LX and 15-epi-LXs. In contrast, aspirin-intolerant asthmatics display a lower biosynthetic capacity than aspirin-tolerant asthmatics, and so it has been proposed that the severity of the disease correlates with defects in LX biosynthesis capacity. Vachier et al. indicated that pro-inflammatory IL-8, LTB_4 and 15-epi-lipoxin A_4 were increased significantly in the severe asthma and COPD patients, while native LXA_4 was elevated in mild asthma patients only (Vachier et al. 2005). Moreover, imbalances in mean LXA_4 levels and pro-phlogistic cysteinyl leukotrienes levels have been shown to correlate with the degree of airflow obstruction within similar asthmatic patients (Levy et al. 2005). These observations further demonstrate that the generation of endogenous LX and the balance between pro- and anti-inflammatory mediators are essential in maintaining airway homeostasis (Vachier et al. 2005).

Current therapies for respiratory disorders involve the use of corticosteroids and/or β_2 adrenergic agonists; however, rather than inhibiting a single class of airway mediators, LXs and their stable synthetic analogues are suggested to promote the resolution of inflammation via multiple mechanisms (Levy et al. 2002). Administration of a stable analogue of LXA_4 blocked both airway hyper-responsiveness and pulmonary inflammation in a murine model of asthma (Levy et al. 2002). Moreover, transgenic expression of human ALXR in murine leukocytes led to significant inhibition of pulmonary inflammation and eicosanoid-initiated eosinophil tissue infiltration (Levy et al. 2002). Furthermore, a recent publication highlighted the significance of LX in an in vivo model of gastric acid injury. LXA_4 generated in vivo during acute lung injury modulated injury of normal bronchial epithelial cells by increasing basal epithelial cell proliferation and blocking acid-triggered IL-6 release and preventing neutrophil transmigration across well-differentiated normal human bronchial epithelial cells. In addition, acid triggered increases in COX-2 expression and PGE_2 production, and acid-induced PGE_2 significantly increased epithelial LXA_4 receptor (ALX) expression. Together, these events limited pro-inflammatory responses induced by acid and helped to promote resolution of the inflammatory disorder (Bonnans et al. 2006). These findings suggest that LX and their stable synthetic analogues offer a novel therapeutic approach for human asthma and related respiratory disorders.

Glomerulonephritis and renal diseases

The anti-inflammatory spectrum of activity of LXs is well documented in in vivo models of glomerulonephritis (GN) and acute renal failure (Badr et al. 1989; Ohse et al. 2004; Papayianni et al. 1995), as well as in in vitro models (McMahon et al. 2002, 2000; Mitchell et al. 2004; Rodgers et al. 2005). LXs are (a) potent intrarenal vasodilators; (b) inhibitors of PMN chemotaxis, adhesion, and migration across glomerular endothelial cells (Papayianni et al. 1996); (c) promoters of apoptotic PMN clearance from inflamed glomeruli (Godson et al. 2000); (d) inhibitors of mesangial cell proliferation in response to mitogens LTD_4 and

PDGF (McMahon et al. 2002, 2000); and (e) modulators of cytokine production (Kieran et al. 2003). The therapeutic potential of LXs has been demonstrated in animal models of renal disease. Exposure of PMNs to LXA_4 ex vivo attenuates their recruitment to inflamed renal glomeruli (Papayianni et al. 1995), while the over-expression of 15-LO in rat kidney has been shown to be protective in immune-mediated glomerulonephritis and is paralleled with enhanced LX formation (Munger et al. 1999). In a murine model of ischaemic renal injury (IRI) disease, administration of the LX analogue 15-epi-16-(FphO)-LXA_4-Me (ATL) before onset of ischaemia markedly reduced PMN infiltration to the IRI kidney while maintaining glomerular function and morphology and attenuating chemokine and cytokine responses (Leonard et al. 2002). Subsequent to this study, oligonucleotide microarray-based comparisons of mRNA expression profiles in IRI kidneys identified specific cohorts of genes whose expression was altered in renal IRI and modulated by 15-epi-16-(FphO)-LXA_4-Me (ATL) (Kieran et al. 2003). Some of these genes included chemoattractants, cytokines (e.g. IL-6), chemokines, and chemokine receptors (e.g. IL-1 receptor); growth factors and their receptors (e.g. thrombospondin-1, TGF-1 receptor); adhesion molecules (e.g. ICAM-1, VCAM-1); and other genes (claudin-1, claudin-7) (Kieran et al. 2003). Furthermore, a model of anti-glomerular basement membrane nephritis demonstrated that ATL ameliorated the expression of several interferon-related genes as well as neutrophil accumulation in mice (Ohse et al. 2004).

There is increasing evidence to suggest that prosclerotic growth factors such as PDGF contribute to the development of diabetic nephropathy (DN) (Lassila et al. 2005). In the diabetic kidney, for example, up-regulation of the PDGF pathway has been shown in experimental DN (Kelly et al. 2001; Nakagawa et al. 2000; Nakamura et al. 1993) and in the kidneys from patients with diabetes (Langham et al. 2003). Further data demonstrated a protective role of LXA_4 in PDGF-mediated $TGF\beta_1$ release in mesangial cells and additionally in preventing the expansion of the mesangial matrix, a histological hallmark of DN (Rodgers et al. 2005). In this study, matrix-associated proteins, fibronectin, thrombospondin and collagens enhanced by growth factor stimulation were diminished by LXA_4 pre-treatment. Moreover, PDGF-treated renal mesangial cells were shown to secrete factors that may cause the onset of tubulointerstitial damage, as observed by EMT in proximal tubular epithelial cells. Pre-treatment with LXA_4 attenuated an equivalent pro-fibrotic response and thus change in cell morphology encountered by EMT (Rodgers et al. 2005). Further to these data, Wu et al. demonstrated that TNF-α-induced proliferation and cytokine release and CTGF-mediated release of fractalkine, monocyte chemoattractant protein-1 (MCP-1), and RANTES were also modulated by LXA_4 in rat mesangial cells (Wu et al. 2005, 2006). Given, the powerful anti-inflammatory, pro-resolution and more recently appreciated potential anti-fibrotic properties (McMahon et al. 2002; Mitchell et al. 2004; Rodgers et al. 2005) reported for LXs in vitro and in vivo, LXs represent a novel potential therapeutic for the various pathobiological processes associated with GN and other various renal diseases.

Angiogenesis

Angiogenesis, the formation of new blood vessels from pre-existing ones, occurs through induction of endothelial cell (EC) proliferation, migration and maturation by various growth factors and cytokines. Angiogenesis is paramount to various embryonic processes such as vascular development, organ regeneration and wound healing (Folkman and Shing 1992). However, it may also contribute to the progression of destructive pathologic processes that

depend on neovascularization, including diabetic retinopathies, rheumatoid arthritis and tumour growth (Folkman 1995). As reviewed by Fierro (2005), native LXA_4 inhibits the release of pro-angiogenic factors IL-6 an IL-8, stimulates the production of anti-angiogenic IL-4 and prevents IL-1β-induced matrix metalloproteinases (MMP), required for basement membrane digestion. In an in vivo model of inflammatory angiogenesis, murine air pouches were raised by the subcutaneous injection of sterile air, ATL-1 or vehicle that were added locally prior to the injection of VEGF (Fierro et al. 2002). Vascular casts were examined indicating that ATL-1 has a protective role in angiogenesis. Due to the vasodilatory effect of LXs, staining for a cell adhesion molecule (CD-31), a vascular endothelial cell marker that is held to play a role in angiogenesis, was subsequently carried out in order to demonstrate that the mode of action of LXs was not dependent simply on dilation of blood vessels. Strong specific staining of endothelial cells was observed in VEGF-treated animals, ATL-1 markedly decreased this expression (Fierro et al. 2002). These data strongly suggest a role for LXs in attenuating the pathological responses associated with angiogenesis, but also suggest a function for LXs in tumour growth and metastasis, all of which have an angiogenic component.

Tuberculosis

The anti-inflammatory profile of LXs is very well defined, its beneficial effects in a number of pathological diseases have already been discussed. Recently, however, LXs have been found to be negative regulators of Th1 responses against mycobacterial infection. Furthermore, inhibition of LX synthesis has been suggested as a strategy for increasing host resistance to infection (Bafica et al. 2005). Mycobacterium tuberculosis infection leads to a prolonged type 1 immune response in the lung. A balance between infection and inflammation may be maintained for the life of the host; however, breakdown of this balance results in tissue damage, bacterial growth and recrudescent disease (Karp and Cooper 2005). Animals deficient in synthesizing LXs were shown to have elevated levels of IL-12, IFN-γ and nitric oxide synthase 2 mRNA and to have decreased bacterial burden in lung tissue; this inferred better control of mycobacteria and increased survival. Application of LX to these animals reversed the otherwise protective effects observed with the knockout of 5-lipoxygenase, pulmonary bacterial loads and IFN-γ levels were increased to match those of wild-type infected animals (Bafica et al. 2005). It is suggested that modulation of LX biosynthesis may be a potential immunopharmacologic intervention for the control of mycobacteria replication in tuberculosis patients.

Ocular wound healing

The cornea of the eye is an important mucosal barrier, prone to infection and injury. It is therefore essential that this tissue can conduct a self-resolving inflammatory-reparative response when challenged (Bazan 2005). Regeneration of epithelial cells in the cornea is of importance in wound healing. Gupta et al. (1993) found that inhibition of lipoxygenases delays corneal epithelial wound healing rates, inferring that lipoxygenase activity is important in corneal epithelial healing perhaps by influencing epithelial cell migration (Gupta et al. 1993). Moreover, these cells were subsequently shown to express LXA_4. Gronert et al. (2005) demonstrated the presence of endogenous LXA_4 in murine corneal epithelial cells.

12/15 lipoxygenase and ALXR mRNA as well as LXA_4 formation were abrogated by epithelial cell removal and subsequently restored during wound healing. Topical treatment with LXA_4 increased re-epithelialization, induced PMN infiltration and reduced the inflammatory insult. These data highlight the importance of this eicosanoid in maintaining the integrity of the cornea.

A murine model of bone marrow transplant (BMT)-induced graft-vs-host disease (GvHD) examined the effects of ATLa in inflammation-associated multifactorial disease, and in particular eye inflammation was monitored (Devchand et al. 2005). A single prophylactic dose of ATLa caused the resolution of eye inflammation post-BMT. Ocular inflammation and damage to the cornea may perhaps benefit from therapeutic uses of LXs.

Conclusions

The potential therapeutic applications of LXs and their stable synthetic analogues are striking. Whereas they are implicated as potential modulators of inflammation in the initial phases of inflammatory disease, more recent data indicate their potential as endogenous drivers of resolution and as potential anti-fibrotic mediators (Devchand et al. 2005; Rodgers et al.2005). As the cellular and molecular basis for the array of LXs actions unfold, new avenues of approach in considering therapeutics for inflammation, cardiovascular diseases and cancer will become more apparent.

Acknowledgements. Work in the authors' laboratory is supported by the Health Research Board, Ireland, Enterprise Ireland, The Welcome Trust, European Commission FP6 funding (LSHM-CT-2004-005033), and The Government of Ireland Programme for Research in Third Level Institutions.

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Rev Physiol Biochem Pharmacol (2008) 160:71 –92
DOI 10.1007/112_2008_801

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Multi-photon excitation imaging of dynamic processes in living cells and tissues

Published online: 17 April 2008

Abstract Over the past decade, two-photon microscopy has successfully made the transition from the laser laboratory into a true biological research setting. This has been due in part to the recent development of turnkey ultrafast laser systems required for two-photon microscopy, allowing ease of use in nonspecialist laboratories. The advantages of two-photon microscopy over conventional optical sectioning techniques are for greater imaging depths and reduced overall phototoxicity, as such enabling noninvasive intra-vital imaging of cellular and subcellular processes. Greater understanding of these advantages has allowed this technique to be more effectively utilized in a biological research setting. This review will cover the recent widespread uses of two-photon microscopy and highlight the wide range of physiological studies enabled in fields such as neurosciences, developmental biology, immunology, cancer biology, and endocrinology.

Keywords Two-photon microscopy · Optical sectioning · Fluorescence · Intra-vital imaging · Photoactivation · Ablation · Intrinsic fluorophores · Second harmonic generation

Abbreviations

2PM	Two-photon microscopy
fl	Femtoliter
fs	Femtosecond
NA	Numerical aperture
NDD	Non-descanned detection
GFP	Green fluorescent protein
$[Ca^{2+}]_i$	Intra-cellular calcium concentration
DCs	Dendritic cells
QD	Quantum dot
NAD(P)H	β-Nicotinamide adenine dinucleotide (phosphate)

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SHG Second harmonic generation
THG Third harmonic generation

Introduction

Over the past few decades, developments in fluorescence microscopy have enabled biological imaging studies to move from the single cell level to the tissue level and even to whole animals. This has been in part due to the use of genetically engineered green fluorescent proteins (GFPs) and their use in transgenic animal models. Another key technological development has been that of the optical sectioning microscope. This allows the investigator to acquire a 3D imaging dataset from the intact, live sample without resort to fixation and mechanical slicing.

The most widespread optical sectioning microscopy used is confocal microscopy. In this method, excitation light is focused at the sample and a pinhole just before the detector spatially rejects all the fluorescence except that originating from the focus. When imaging live samples, however, there are some deleterious effects associated with confocal microscopy; in particular due to phototoxicity of the excitation light. Fluorescence is detected from only a single optical plane, but fluorescence is being excited throughout the sample, and most of that fluorescence is rejected by the pinhole. When utilizing blue and UV excitation, this can be particularly damaging in causing rapid photodestruction of the fluorophore in question as well as exciting autofluorescent compounds that can contribute to the killing of the live sample.

A more recently developed optically sectioning microscopy technique is two-photon excitation microscopy (Denk et al. 1990) (also known interchangeably as multi-photon microscopy or nonlinear microscopy). As described in the following sections, this technique provides several advantages over conventional optical sectioning microscopy techniques when imaging in live samples. The major advantage is that imaging depth is increased many times over that possible with conventional confocal microscopy: essential for intravital microscopy. Additionally, two-photon microscopy is less phototoxic in 3D imaging compared to conventional fluorescence microscopy, especially when imaging UV-excitable fluorophores.

It is important to note that while this technique was first demonstrated approx. 15 years ago, its use has been limited to more specialist laboratories. This is because sophisticated ultra-fast laser technology is required for a two-photon microscope, which has in the past required one or two laser physicists residing in the lab to maintain the system. In the past few years, however, reliable “turnkey” laser systems have been developed, allowing a hands off approach to using the two-photon microscope and rendering it as easy to drive as commercial confocal microscopes. This has meant the two-photon microscope can now move into a true biological research setting and investigators can concentrate on studying the biological question at hand without recourse to worrying about driving and maintaining the system.

This review will cover the recent widespread deployment of two-photon microscopy and highlight the wide range of studies and attainable information now possible. We will begin with describing the anatomy of the current two-photon microscopes suitable for deployment in a biological setting. We will then move onto describing the studies that have been enabled following the recent technological advances, covering a wide range of areas including neu-

rosciences, cancer biology, immunology, and developmental biology. We will conclude by looking toward current developments that can further the use of two-photon microscopy.

Two-photon absorption and the two-photon microscope

In order to explain the principals of two-photon absorption and two-photon microscopy, it is useful to consider single-photon absorption and fluorescence emission that occurs in conventional fluorescence microscopy. Fluorescence can occur when a single excitation photon (typically of UV, blue or green wavelengths) is absorbed by a fluorescent molecule (a fluorophore) causing a transition of the molecule into a more energetic, excited state. The molecule then relaxes back to the ground state by emitting a single photon of a less energetic, longer wavelength than the excitation wavelength (see Fig. 1a). Under low to moderate excitation intensities, at any point in time each fluorophore absorbs a single excitation photon and reemits a single fluorescent photon.

Under very high excitation intensities, it is possible that a fluorophore can simultaneously absorb two excitation photons, each at half the excitation energy required for a single-photon absorption event (typically red to infrared wavelengths, see Fig. 1a). Due to the high intensities at which this occurs it is not practical to simply turn up the excitation power in order to obtain significant amounts of two-photon absorption. In order to obtain powers at the sample sufficient for two-photon absorption the excitation photons need to be tightly localized in both space and time. To obtain the temporal localization of photons, ultra-short pulsed lasers are used. These lasers concentrate the infrared excitation light into discrete pulses,

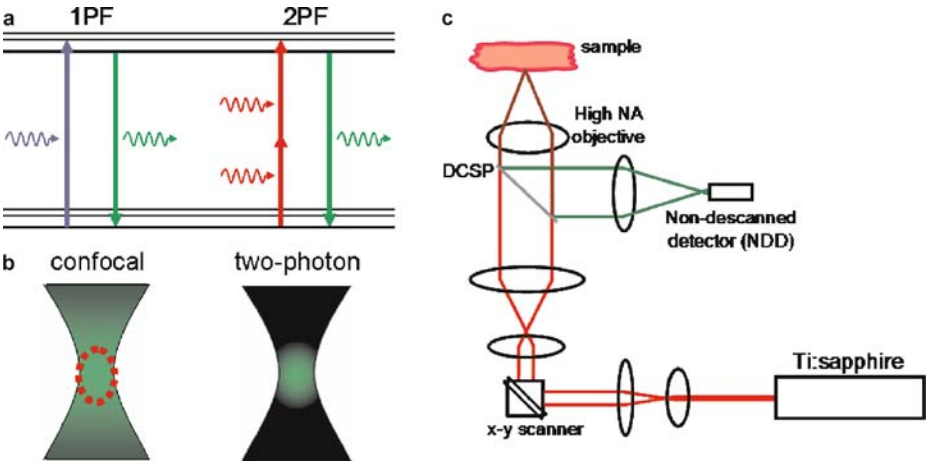

Fig. 1 a Energy level (Jabłoński) diagram illustrating one- and two-photon excited fluorescence. Two-photon excited fluorescence results from the simultaneous absorption of two photons, each of half the energy of that from single-photon absorption. **b** Schematic representation of the fluorescence distribution in the focus resulting from single- and two-photon excitation. Under single-photon excitation fluorescence is generated throughout the sample; in a confocal microscope only fluorescence generated at the focal plane is detected (*red dotted line*). Two-photon excited fluorescence results in fluorescence being generated solely at the focal plane. The lack of out-of-focus excited fluorescence reduces overall phototoxicity in the sample as well as enhancing contrast in deep tissue imaging. **c** Schematic of a typical two-photon microscope setup. Most of the apparatus is the same as that of a confocal microscope with the exception of those components labeled: the *Ti:sapphire* laser source, the *high NA objective*, the dichroic short-pass mirror (*DCSP*), the lack of detection pinhole, and *non-descanned detection (NDD)*

typically of approx. 100-femtosecond (fs) duration. This enables about 1 million times the number of photons to be present at once than would be possible with the type of lasers used in confocal microscopes. It was in fact the development of these ultra-fast pulsed lasers (Valdmanis and Fork 1986; Wise et al. 1988) that initially enabled two-photon microscopy (Denk et al. 1990). Additionally, high numerical aperture (NA) lenses are used to focus the excitation beam into a very small volume, on the order of femtoliters (fl). This region, called the focal volume, or focus, is the only location at which significant two-photon absorption occurs due to the tight focusing of the high NA lens. This means that fluorescence originates only from this small focal region and not from other depths through which the excitation light passes. It is this localization of excitation that enables inherent optical sectioning to be achieved (see Fig. 1b). To make the analogy; if conventional fluorescence microscopy is like examining the inside of a house by shining a powerful spotlight in from the outside, two-photon microscopy is like an investigator shining a flashlight around inside the house to examine its contents. All the light used for the investigation is generated inside the sample of interest.

This analogy brings us to one of the principle advantages of two-photon microscopy: since the excitation of fluorescence is localized to the focal volume, this is the only region where any photobleaching will occur. Conversely, in the confocal microscope excitation and thus photobleaching occurs throughout the sample, but only fluorescence originating from the focal volume is detected. This brings us to another principle advantage of two-photon microscopy. In confocal microscopy, the scattering of excitation light can also excite fluorescence outside of the focal volume. In a weakly scattering sample this will generally be spatially filtered and only negligible amounts are detected. In a more strongly scattering sample, however, the increased amount of excitation scattering will both reduce the power exciting fluorescence at the focus and result in finite amounts of "background" fluorescence, being detected (washing out the contrast in the sectioned image). With two-photon excitation, scattered excitation photons will not contribute to the fluorescence signal since the probability of two photons scattering to the same location *at the same time*, to be subsequently absorbed, is practically zero. Finally, a further advantage is associated with the fact that fluorescence is excited solely at the focus. In the confocal microscope fluorescence emission needs to be passed back through the scanning mirror optics in order for the confocal pinhole to track the position of excitation. Passing back through these optics inevitably leads to signal losses. In the two-photon microscope since the fluorescence originates solely from the focus, all the fluorescence emission can be collected without the use of a pinhole. An external detector can be used immediately after the objective increasing collection efficiency. Hence we can see how phototoxicity to the sample is reduced and collection efficiency and imaging depth are increased when using two-photon microscopy over confocal microscopy.

A two-photon microscope is generally configured in a similar way to a confocal microscope. In fact, many microscope manufacturers provide the option of using the confocal microscope as a two-photon microscope. Three additional key components are required, however: the ultra-fast femtosecond infrared laser, the tight focusing high NA objective, and an external detector, as previously discussed. A schematic of a typical two-photon microscope setup can be seen in Fig. 1c. The laser source most commonly used is the Titanium:sapphire laser oscillator. This typically provides 100-fs pulsed infrared laser illumination with a wavelength range between 680 nm and 1050 nm (approximately equivalent to 340 nm and 525 nm single-photon absorption wavelength range). This wide tunable wavelength range brings with it a further advantage not yet discussed: that it is possible to select a continuum of excitation wavelengths with two-photon excitation, whereas the currently available confocal microscopes are restricted to several discrete wavelength lines.

As discussed in the introduction, in the early days of two-photon microscopy these Ti:sapphire lasers, while being relatively easy to use for a laser expert, were far from being turnkey systems. A certain level of knowledge of laser physics was required—as well as continual optical alignment of the laser cavity for the laser to be operated—which is not generally available in a cell biology or physiology laboratory. Recently, however, laser manufacturers have developed Ti:sapphire laser systems in which the alignment and wavelength tuning is automatic, and the laser system is essentially a turnkey system like the visible lasers on the confocal microscope. As well as allowing ease of use of the two-photon microscope in a nonspecialist laboratory, excitation wavelength scanning can also be achieved with relative ease.

The major microscope manufacturers have developed microscope objectives that are optimized for two-photon microscopy. These account for the need to efficiently transmit both infrared excitation and visible emission wavelengths and to image into a variety of media without introducing significant image aberrations. Finally, to take advantage of the fact no pinhole is required, a method of detection known as non-descanned detection (NDD) can be utilized with the two-photon microscope. This requires the placement of an external detector immediately after the microscope objective, as indicated in Fig. 1c, maximizing the signal collected, which is especially useful in deep tissue imaging where large amounts of fluorescence emission scattering can occur.

There are some challenges and limitations to two-photon microscopy that should be appreciated. There have been reports that the infrared excitation light can cause localized heating effects in the sample when excessive powers are used (Chirico et al. 2003; Schonle and Hell 1998), although this is rarely a problem with the intensities required for imaging experiments. Additionally, there can be accelerated photobleaching of the fluorescence *at the focal plane* for some fluorophores under two-photon excitation (Hopt and Neher 2001; Patterson and Piston 2000). For these reasons, proper control of the excitation power is essential in two-photon microscopy so as not to damage the sample. It is also important, for this reason, to realize that two-photon excitation will not always lead to significant improvements over confocal microscopy when imaging into thin samples. Another challenge when using two-photon microscopy is that the two-photon excitation spectrum of a fluorophore can often be very different from that of the one photon excitation spectrum due to the nature of the two-photon absorption process. Fortunately, in this respect, many commonly used fluorophores and fluorescent proteins have now had their excitation spectra characterized (e.g., Albota et al. 1998; Bestvater et al. 2002; Spiess et al. 2005; Wokosin et al. 2004; Xu and Webb 1996; Xu et al. 1996). Despite these challenges, however, many important studies have been enabled by two-photon microscopy, ones that would not have been possible utilizing conventional microscopy or other imaging techniques.

Applications of two-photon microscopy

Localized photoactivation of “caged” compounds

One of the first applications for two-photon microscopy was for highly localized photoactivation of “caged” compounds such as calcium or the neurotransmitter glutamate. These compounds are natively inactive, but can be rendered active through intense irradiation with UV-visible light. One now widespread use is to uncage neurotransmitter to activate a specific neuron or neuronal region and observe the resultant electrical activity in neuronal regions

for the mapping of electrical networking. When utilizing UV-visible light for uncaging, the neurotransmitter does not photoactivate in a well-defined location due to out-of-focus light photoactivating neurotransmitter throughout the sample. Hence, conventional microscopy precludes the ability of the photoactivation to stimulate a well-defined neuronal region and hinders the ability to quantitatively map the neuronal networking. Since two-photon excitation is localized to the femtoliter focal volume, uncaging can be highly localized. The application of two-photon microscopy for well-localized photoactivation was first shown by Denk, who used two-photon uncaging of carbamoylcholine to map ligand-gated ion channel distribution in combination with electrophysiology (Denk 1994).

Some more recent examples of two-photon uncaging include utilizing uncageable glutamate to activate excitatory potentials in multiple well-defined locations in the cortical neuronal network (Araya et al. 2006a,b). The tight femtoliter two-photon excitation volume allowed uncaging to be localized within 1 μm, which is sufficient to excite individual dendritic spines, as described in Fig. 2a. Through subsequent observation of the resulting electrical activity, it was possible to determine how the network processes excitatory inputs via dendritic spines and shafts, in terms of membrane potential summation and filtering. Similarly, two-photon uncaging of glutamate has also been used to observe feedback behavior in dendritic spines shaping synaptic response (Ngo-Anh et al. 2005).

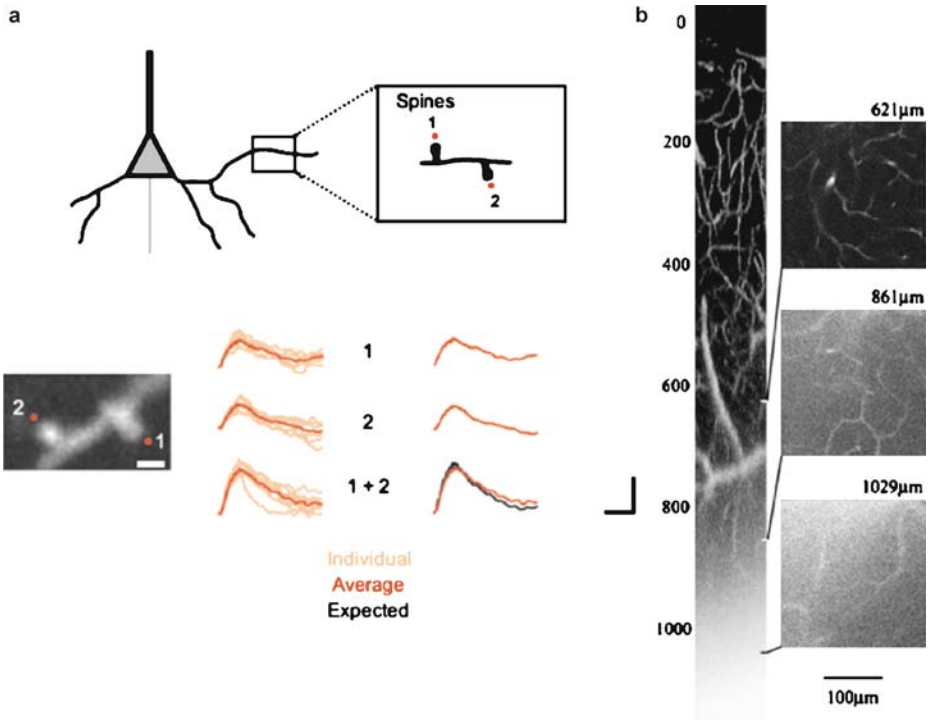

Fig. 2 a Data from a study showing how glutamate-induced depolarizations in dendritic spines combine through linear summation. Glutamate is uncaged at points *1* and *2* adjacent to dendritic spines, with the resultant membrane depolarization being monitored through electrophysiology. The depolarizations generated at spines *1* and *2* sum linearly. (Data reproduced from Araya et al. 2006a, copyright 2006, National Academy of Sciences, USA). **b** Image of stained cerebral blood vessels demonstrating that two-photon microscopy can allow imaging depths greater than 1 mm in neuronal tissue. An x–z projection and several x–y planes are shown. (Reproduced from Theer et al. 2003)

There are numerous other examples of utilizing two-photon microscopy for highly localized uncaging of bioactive compounds, for studying neural activity and networking. This review intends to highlight recent physiological studies performed in other biological systems following the increased proliferation in understanding of two-photon microscopy. The reader is directed to reviews such as Rubart (2004) and Svoboda and Yasuda (2006) covering the extensive application of two-photon uncaging in neurosciences.

Imaging electrical activity in deep tissue

A principal advantage of two-photon microscopy is increased imaging depths for deep tissue imaging. As discussed in the introduction this is principally because two-photon microscopy generates no fluorescence from out-of-focus scattered excitation. This compares with confocal microscopy where scattered excitation light can excite fluorescence that, at moderate imaging depths (<100 μm), scatters back through the pinhole and "washes out" image contrast (Centonze and White 1998). As such, intra-vital two-photon microscopy has allowed researchers unprecedented access to imaging depths of many hundreds of micrometers in excised organs and whole animals in order to study molecular and cellular structure and function with high spatial resolution in their native environment.

One initial application of two-photon microscopy in neuroscience was performed by Denk and Yuste for mapping neuronal electrical activity in brain slices, through imaging of cellular calcium dynamics (Yuste and Denk 1995). This tool has now become invaluable for studying neuronal electrical activity and mapping neuronal architecture in brain slices as well as the brains of live animal. Imaging depths greater than 1 mm have been achieved in live mouse brains (Theer et al. 2003), as shown in Fig. 2b, allowing access to large parts of the neuronal processing regions of the brain. Again the reader is directed to reviews such as Helmchen and Denk (2005) Rubart (2004) and Svoboda and Yasuda (2006) for the extensive applications of two-photon microscopy to deep-tissue neurobiological studies.

As well as for mapping network organization of neuronal electrical activity, intra-vital two-photon microscopy has been used to map the 3D network organization of pituitary growth hormone (GH)-secreting cells (Bonnefont et al. 2005). Utilizing GFP-GH transgenic mice, GH cells were observed to be highly networked; it was shown that the interconnected network organizes during the pubescent stage of mouse development. Observing this development would have been significantly more challenging through conventional imaging of serial sections. It was also possible to measure the electrical activity in individual GH cells through $[Ca^{2+}]$ levels and the team discovered extensive inter-cellular electrical coupling coordinating in vivo GH secretion. Observing this 3D network activity would have been possible from serial sections alone; furthermore, two-photon microscopy was necessary to resolve subcellular morphology and electrical activity that was at a depth of hundreds of micrometers.

Two-photon microscopy has more recently been applied to study electrical activity in other biological systems, where increased tissue density means that deep-tissue imaging is more challenging. Studies of electrical activity through $[Ca^{2+}]$ imaging have been performed in excised hearts for the study of electrical synchronization (Rubart et al. 2003). Additionally, electrical activity, through calcium imaging, has been studied in the cardiomyocytes of excised hearts originating from diabetic mice to elucidate the mechanism of cardiac function breakdown (Pereira et al. 2006). Mitochondrial function has also been studied in excised hearts with two-photon microscopy. Using a fluorescent indicator of mitochondrial membrane potential ($\Delta\Psi$), the spatiotemporal changes in $\Delta\Psi$ in response to ischemia at a subcellular level could be studied (Matsumoto-Ida et al. 2006).

Quantitative imaging of blood flow

Functional intra-vital two-photon microscopy has also been used in the brain for studying blood flow. The precise coupling between neuronal electrical activity and changes in blood flow still remains unclear. Studies have shown that functional magnetic resonance imaging (fMRI) can report on electrical activity via localized increases in blood oxygenation levels (Logothetis et al. 2001; Smith et al. 2002). fMRI, however, does not have sufficient temporal and spatial resolution to resolve individual neuronal/blood vessel coupling that participates in cellular events. Charpak and coworkers showed how two-photon microscopy has sufficient temporal and spatial resolution to resolve individual capillaries in live mouse brains, thus allowing the noninvasive study of blood flow (Chaigneau et al. 2003). They were able to quantitatively measure flow velocity through imaging fluorophore-dextran labeled blood plasma, as described in Fig. 3a. This allowed them to study the highly localized blood flow variation in the olfactory bulb glomeruli as different smells activated specific neuronal units.

Kleinfeld and coworkers utilized two-photon excitation to perturb individual blood vessels, through targeted ablation, for mapping the 3D blood flow architecture in the brain (Nishimura et al. 2006). For similar reasons to the advantages brought about by using photoactivatable bio-active compounds, it is highly desirable to use two-photon microscopy for targeted photo-ablations. The intrinsic optical confinement of the two-photon excitation to the femtoliter focal volume allows localized ablation of single capillaries with minimal collateral damage. In a series of papers, they quantitatively studied the downstream blood flow response to targeted ablations at the cortical surface (Schaffer et al. 2006), in both penetrating arterioles (Nishimura et al. 2007) and subsurface cortical vessels (Nishimura et al. 2006). They were thus able to determine the vascular architecture of the brain from observation of downstream flow disruption after the occlusion of individual vessels, as highlighted in Fig. 3b, c. The cortical surface had minimal disruption in flow upon vessel occlusion, suggesting a highly interconnected network architecture (Schaffer et al. 2006). Subsurface occlusion or blood clots, however, caused substantial flow reduction downstream in one to three branches as well as resulting in localized neurological damage (Nishimura et al. 2006). Finally, it was discovered that penetrating arterioles are the major bottleneck in blood flow through the brain: occlusions to single vessels disrupted flow over a large cortical area: more than ten branches (Nishimura et al. 2007). These findings compare well with clinical observations that micro-strokes received in patients are largely due to obstructions in penetrating arterioles. They were also able to observe the reintroduction of downstream flow after treatment with hemo-diluting agents (Nishimura et al. 2006).

Two-photon microscopy is also starting to be utilized for the simultaneous measurement of neural activity and cerebral blood flow to determine the mechanisms of neurovascular coupling. Following from their initial work highlighted above, Charpak and coworkers studied the relationship between blood flow and neural activity in the olfactory bulb glomeruli (Chaigneau et al. 2007). They studied odor-induced changes in calcium activity and flow velocity simultaneously, and concluded that postsynaptic glutamate receptor activation is a major step in neurovascular coupling. Using a different strategy, Takano and coworkers studied the role of astrocytes in controlling in vivo local microcirculation (Takano et al. 2006). Astrocyte activity was monitored through calcium imaging, and an associated vasodilation was observed through imaging the vessel cross-section with micrometer spatial resolution and millisecond-scale temporal resolution. Additionally, photoactivatable calcium was utilized to modulate astrocyte activity and associated vasodilation. Isolating the role of astrocytes in controlling local microcirculation required the precise targeting of stimulation

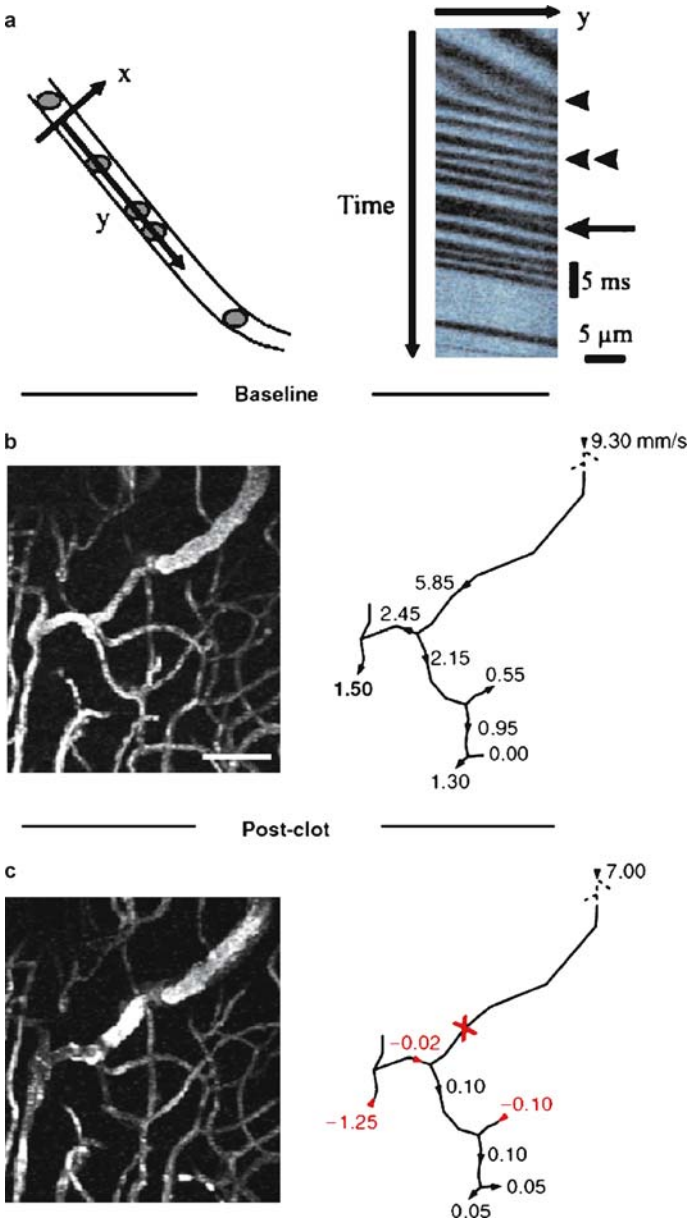

Fig. 3 a In vivo red blood cell (RBC) flow measurements (Chaigneau et al. 2003; Nishimura et al. 2006). Line scanning is performed along the capillary, where RBCs are seen as *dark areas* in the fluorescent plasma. RBC velocity is calculated from the resultant kymograph as v=dy/dx. (Image and schematic reproduced from Chaigneau et al. 2003, copyright 2003, National Academy of Sciences, USA). **b** Measurement of RBC flow velocity in the subcortical vasculature before and **c** after a clot is photoinduced in the vessel (indicated with an *X*). After the clot is formed, significant modulation in flow downstream of the clot is measured. (Images and schematic reprinted by permission from Macmillan Publishers Ltd: *Nature Methods*, Nishimura et al. 2006, copyright 2006)

as well as the high 3D spatial resolution to resolve vasodilatation, afforded by two-photon microscopy through the femtoliter-sized confinement of the nonlinear excitation.

Utilizing two-photon microscopy for flow measurements has also been extended into imaging renal tissues by Peti-Peterdi and coworkers (reviewed in Peti-Peterdi 2005) as well as by Molitoris and coworkers (reviewed in Molitoris and Sandoval 2005). The kidney also contains a complex network of vasculature, particularly in the glomerulus in which filtration occurs. As well as for quantifying blood flow through red blood cell velocity, two-photon microscopy can also be used in conjunction with fluorophores of different size and net charge to quantify glomerular permeability, especially important in studying renal disease (Molitoris and Sandoval 2005). Recently, renal blood flow was studied in vivo to demonstrate the ability of activating protein C in protecting renal microvasculature from injury (Gupta et al. 2007).

Quantitative imaging of immune-cell motility and morphology

The enhanced imaging depth provided by two-photon microscopy also allows for substantial improvements in 4D imaging (x,y,z,t) for observing in vivo dynamic process. There has been a large body of work using two-photon microscopy for dynamically imaging immune cell motility, interactions and clustering in vivo, as reviewed by Cahalan and Gutman (2006). These studies, many involving intra-vital imaging of lymph nodes and bone marrow, have all required high temporal and spatial resolution, hundreds of micrometers in imaging depth, along with the minimal invasiveness to image for periods of hours, all afforded by two-photon microscopy.

The in vivo interaction of T cells with dendritic cells (DCs) has been resolved using two-photon microscopy and related to immunological tolerance in lymph nodes (Shakhar et al. 2005). In this study, imaging depth is a critical parameter to reach the region of the lymph nodes at which the majority of these interactions occurs. Through observation of T cell dynamics such as the speed, directionality, and confinement of motility, as well as arrest in motility, it was possible to differentiate the behavior of antigen- and nonantigen-specific T-cells. This required sufficient imaging speeds to resolve T cell movement of tens of micrometers per second over three dimensions. Two-photon microscopy has also been used to observe T cell motility in order to differentiate phases of T cell behavior as they interact with antigen-presenting DCs (Mempel et al. 2004). In addition, the in vivo 3D dynamics of T cell motility were imaged in lymph nodes over many hours such that three distinct phases of behavior could be resolved, defined by mobility, relative immobility, and recovered mobility. Figure 4 presents some data from this study (Mempel et al. 2004) showing T cell motility and interaction. Dustin, Nussenzweig, and coworkers provided one of the first in vivo studies of B cell dynamics in lymph node germinal centers, enabled by two-photon microscopy (Schwickert et al. 2007). They observed reduced motility and increased size for antigen-specific B cells as well as significant confinement. Within the germinal center structure, the antigen-specific B cells were highly transient in their interactions with DCs, rarely stopping for prolonged period in contrast to naive B and T cells. Similarly, in vivo B cell dynamics were imaged in lymph nodes to study the transport of immune complexes (Phan et al. 2007). Individual B cells were tracked in 3D as they interacted with macrophages and bound to and transported labeled immune complexes.

Intra-vital two-photon microscopy has also been used to observe in vivo T cell and DC dynamics in cranial bone marrow as the cells interact (Cavanagh et al. 2005). In this study it was possible to differentiate the interaction of T cells with mature and immature DCs (DCs

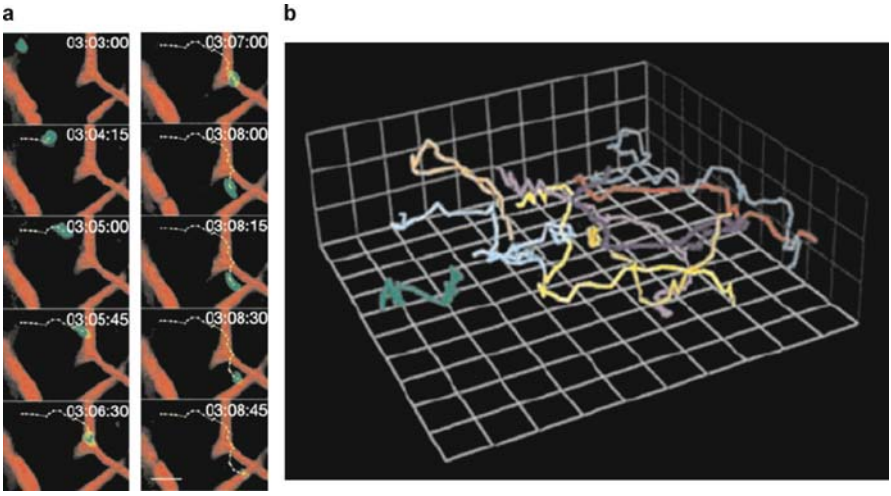

Fig. 4a, b Imaging in vivo T cell motility in lymph nodes. **a** High resolution imaging of a T cell migrating and transiently interacting with topical magnetic resonance (TMR)-labeled capillaries in the absence of transferred dendritic cells. **b** Plot of the motility of multiple T cells. Through high-resolution 4D imaging provided by two-photon microscopy information such as confinement, speed, and directionality can be obtained from tracking a population of T cells. (Images and diagram reprinted by permission from Macmillan Publishers Ltd: *Nature*, Mempel et al. 2004, copyright 2004)

presenting and not presenting antigens). T cell interaction with mature DCs was characterized by a reduced directional velocity, long contact times, and significant morphological changes, whereas the interaction with immature DCs were characterized by random movement, brief contact times, and no morphological change. As well as tracking the T cell dynamics at sufficient speeds in 3D, two-photon microscopy also allowed sufficient 3D spatial resolution to resolve the differences in T cell morphology during interactions with mature and immature DCs.

T cell dynamics have also been imaged in tumors using intra-vital two-photon microscopy. In one study T cell motility was observed to transiently drop during tumor rejection, as T cells interacted with antigen-presenting tumor cells (Boissonnas et al. 2007). Additionally, T cells were seen to migrate along collagen fibrils or blood vessels with a morphological change through the late phase of tumor rejection. In another study T cell motility was observed in tumors consisting of nonantigen-presenting cells (Mrass et al. 2006). T cells were not observed to interact with tumor cells, but there was significant interaction of T cells with macrophages. Again, these studies both required high temporal and spatial resolution to track characteristics of T cell motility and morphology deep within the tumor.

Finally, tracking immature T cell (thymocyte) dynamics and morphology has also been combined with imaging the thymocyte calcium dynamics (Bhakta et al. 2005). Positive selection of thymocytes for maturation was correlated with an arrest of motility—a characteristic of a prolonged interaction—along with increased oscillatory intracellular calcium levels.

Minimally invasive imaging of embryo development

Two-photon microscopy has also been found to have many advantages when imaging live embryo development. As well as improvements of imaging depths, the noninvasiveness of two-photon microscopy at low excitation powers is critical. Since fluorescence is generated only in the femtoliter focal volume, at any one point in time the phototoxic effects associated with fluorescence are also confined to this focal volume. In contrast, using confocal microscopy, the fluorescence and associated phototoxicity are generated throughout the sample. To demonstrate the substantial improvements two-photon microscopy can yield over confocal microscopy, Squirrell and coworkers carried out time-lapse 3D imaging of hamster embryo development (Squirrell et al. 1999). Mammalian embryos are highly sensitive to culture conditions as well as being adversely affected by exposure to visible light, such that these embryos are ideal to show the noninvasiveness of two-photon microscopy. After imaging mitochondrial-stained embryos for 24+ hours, embryo viability was not significantly altered when using two-photon microscopy, shown in Fig. 5. One of the stained two-photon imaged embryos subsequently developed into a healthy full-grown adult hamster able to produce a full litter of healthy pups. Confocal microscopy with visible light, however, impaired development significantly, even in unstained embryos, to the point where many embryos were unable to undergo a further cell division step after irradiation. Voiculescu, Stern and coworkers also recently utilized this noninvasiveness by studying cellular dynamics in the early stages of chick embryo development (Voiculescu et al. 2007). The high resolution afforded by two-photon microscopy allowed for the morphology, movement, and organization of multiple cells in the developing embryo. Cells were seen to migrate and intercalate, which leads to an important developmental step in birds and mammals that differs from fish and amphibians. Carroll and coworkers also used two-photon microscopy for noninvasively studying the role calcium plays in cell division during early stages of mouse embryo development (FitzHarris et al. 2005).

Piston and coworkers utilized two-photon microscopy for lineage tracing in sea urchin embryo development (Piston et al. 1998; Summers et al. 1996). Caged fluorescein was microinjected into the single cell zygote, then at a later stage of development fluorescein was photoactivated in a specific cell from which to trace its lineage. Fluorescence imaging of the embryo at subsequent stages of development could then be used to reveal which cells were derived from the uncaged cell. As already discussed, two-photon excitation allows the photoactivation to be well localized in an individual cell within the embryo, without causing uncaging nor significant phototoxicity elsewhere in the embryo. Through this technique, they were able to show how the mechanism of cell determination is independent of the early cleavage planes (Summers et al. 1996). Further work examined later stages in the embryo development, tracing the origin of organogenesis, and further suggesting positional rather than lineage determination in regulating embryo development in urchins (Piston et al. 1998). This method of photoactivation of caged fluorescein is preferable to traditional microinjection methods since groups of cells can be marked with less potential for cellular damage.

Beaurepaire and coworkers utilized the ability to cause targeted ablations with the two-photon microscope in *Drosophila* embryos (Supatto et al. 2005). Utilizing substantially higher excitation powers, specific subregions of *Drosophila* embryos could be photo-disrupted and the impact on the overall development could be studied. Again, since two-photon excitation is confined to the femtoliter focal volume, this photo-disruption can be highly localized, allowing targeted deformation of specific tissue patterns. Using this methodology they were able to modulate and subsequently observe and quantify

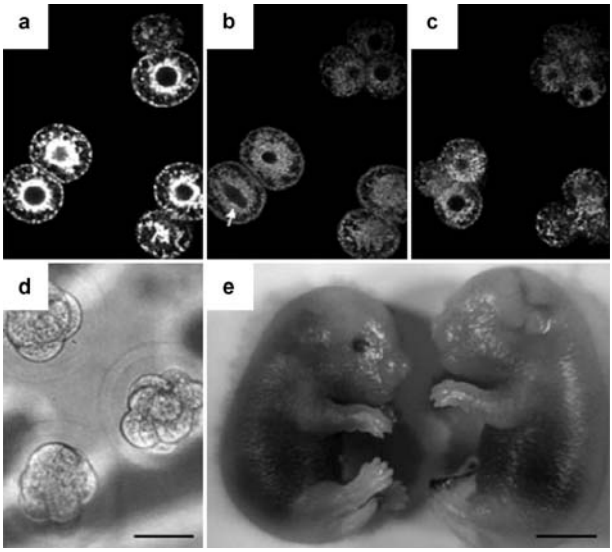

Fig. 5a–e Long term two-photon imaging of mammalian embryo development. Mitochondrial labeled embryos at **a** initiation of two-photon imaging, **b** after an 8-h imaging sequence (note mitotic spindle highlighted by an *arrow*) and **c** after a 24-h imaging sequence. After imaging, embryos were further cultured for **d** 82 h and **e** (*left*) transferred to a recipient female. (Figure reprinted by permission from Macmillan Publishers Ltd: *Nature Biotechnology*, Squirrell et al. 1999, copyright 1999)

morphology, thereby yielding insight into the mechanisms of mechano-sensitive gene expression in embryo development.

In studying embryo development, the main motivation for using two-photon microscopy is that it is minimally invasive in comparison to using confocal microscopy. This is especially important when studying factors involving cell division such as in developmental biology. Additionally, we have highlighted how two-photon microscopy has also been used for inducing well-controlled dye-tracer uncaging or perturbations at highly specific spatial and temporal positions in the development cycle.

Imaging structure and function of cancer

Intra-vital two-photon microscopy has also been used to provide new insights into tumor pathology and physiology. Jain and coworkers first showed the potential for two-photon microscopy in studying gene expression and physiological function in the deep internal regions of tumors, not accessible with confocal microscopy techniques (Brown et al. 2001). Taking advantage of the high spatial resolution and imaging depths, they were able to quantitatively resolve the vascular architecture, giving an insight into the mechanisms of angiogenesis in tumors. Additionally, they were able to study the growth and localization of mutations in the tumor cells, such as those resistant to hypoxia-induced apoptosis. Quantitative measurements of RBC velocity and vascular permeability could also be imaged in a similar fashion to those studies described earlier, to gain further mechanistic insight into tumor function. Finally they showed that it is possible to visualize the location of therapeutic drug delivery in the tumor architecture. These studies showed the wide-ranging possibilities of utilizing two-

photon microscopy for studying tumor function, enabled by the high 3D spatial resolution and imaging depths.

Recent work in imaging tumors has also utilized fluorescent semiconductor nanocrystals, called quantum dots (QDs), for labeling tumor cells. QDs are very bright and resistant to photodamage compared to organic or genetically encoded fluorophores and have been shown, in combination with two-photon microscopy, to enhance imaging through highly scattering tissue such as skin and adipose tissue at hundreds of micrometers of depth (Larson et al. 2003). QDs and two-photon microscopy have been used to resolve multiple populations of cells throughout the tumor, allowing single cell study of early stages of metastasis (Voura et al. 2004). Jain and coworkers also showed that two-photon microscopy and QDs could allow high-resolution angiography-like imaging of tumor vasculature without significant extravasation often found with dextran-conjugated organic fluorophores (Stroh et al. 2005). Furthermore, they were able to track multiple subpopulations of cells as they recruited to the tumor vasculature. Thus, the use of two-photon microscopy and quantum dot nanocrystals is especially promising in studying tumor development and function.

Imaging UV fluorophores, such as NADH, for metabolic activity

Another principal advantage of two-photon microscopy is as a convenient alternative to UV excitation. Since absorption occurs from two photons of half the energy used in single-photon absorption, single-photon excitation in the 340–400 nm range can be replaced by two-photon excitation in the 680–800 nm range: conveniently provided by the commercial turnkey Ti:sapphire systems. There are many fluorescent probes that require UV excitation that can cause rapid photobleaching and high phototoxicity; this is especially problematic in live samples where relatively low fluorophore concentrations are necessary. Additionally, the current dearth of UV lasers means that using UV-excitable probes in conjunction with confocal microscopy is challenging. Two-photon excitation is thus the most convenient method for achieving high 3D spatial resolution with UV-excitable probes.

Using this advantage, one of the most successful applications of two-photon microscopy outside of the neurosciences has been for imaging metabolic activity via the endogenous fluorescent cofactor β-nicotinamide adenine dinucleotide (phosphate), abbreviated to NAD(P)H. In its oxidized form, NAD(P)H can be conveniently excited at approx. 710 nm with much reduced phototoxicity compared with 355 nm UV excitation. The reduced form $NAD(P)^+$ is nonfluorescent, hence imaging of NAD(P)H levels provides a method for imaging the in situ redox state. This was initially applied by Piston and coworkers to elucidate the metabolic pathways involved in glucose stimulated insulin secretion (GSIS) in pancreatic islets (Bennett et al. 1996). NAD(P)H is produced from NAD^+ in many metabolic processes such as glycolysis and citric acid cycle metabolism, such that as glucose is metabolized in the islet cells the level of metabolic activity can be observed through NAD(P)H fluorescence, as highlighted in Fig. 6. Further related work involved quantitatively determining NAD(P)H levels (Patterson et al. 2000) and separating NADH and NAD(P)H responses in GSIS (Rocheleau et al. 2004a).

Applications of two-photon microscopy to imaging NADH levels have also been applied in other areas. Imaging of endogenous autofluorescent species such as NAD(P)H has been used to classify tissues as normal, pre-cancerous, or invasive cancer (Skala et al. 2005). Goodman and coworkers imaged NAD(P)H to observe how the redox state can regulate certain transcription regulatory elements such the repressor carboxyl-terminal binding protein (CtBP) (Zhang et al. 2002). They further showed that CtBP played a significant role

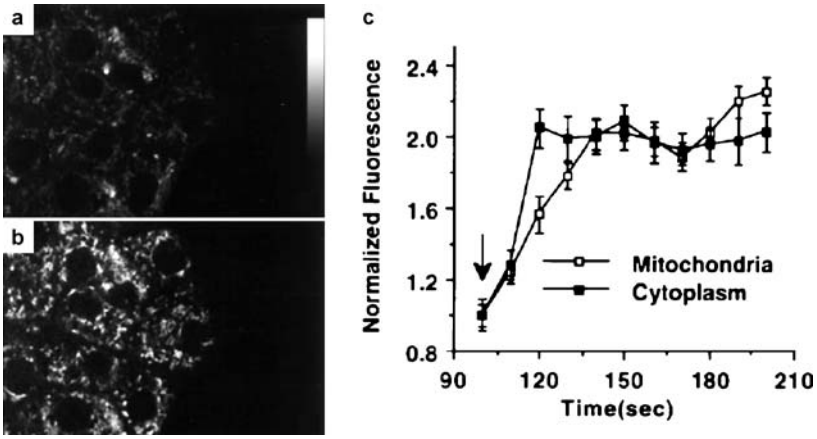

Fig. 6a–c Imaging NAD(P)H in cultured pancreatic islet beta cells at **a** basal 1 mM glucose and **b** after 20 mM glucose stimulation. **c** The cytoplasmic and mitochondrial NAD(P)H can be quantified representing cytoplasmic glycolysis metabolism and mitochondrial citric acid cycle metabolism, respectively. (Reproduced from Patterson et al. 2000, copyright 2000, National Academy of Sciences, USA)

in controlling tumor cell metastasis, via hypoxia-induced NAD(P)H changes (Zhang et al. 2006). In aggressive rapidly growing tumors poor vascularization results in hypoxia and increased NAD(P)H levels, as observed with two-photon microscopy. NAD(P)H increases subsequently promote CtBP recruitment, leading to a repression of cell adhesion protein expression and rendering the tumor highly liable to metastasize. This important mechanism of tumor malignancy required two-photon microscopy to probe redox levels in the tumor without perturbing the cellular environment, as occurs with UV excitation wavelengths.

Several groups have utilized two-photon imaging to observe the coupling of metabolic activity with electrical activity. NAD(P)H imaging has been used in excised pancreatic islets to observe the connection between glucose metabolism and coupled electrical activity (Rocheleau et al. 2004b). NAD(P)H imaging in excised pancreatic islets has also been used to compare the stimulatory effects of glucose and the mitochondrial substrate methyl succinate in metabolic and calcium activity as well as insulin secretion (Heart et al. 2007). Neuron mitochondrial signaling has been observed through imaging NAD(P)H, specifically to determine the role of small Ca signals in activating the malate-aspartate shuttle (Pardo et al. 2006). Webb and coworkers have imaged NADH in vivo to connect astrocyte metabolic activity and electrical activity (Kasischke et al. 2004). Through the high 3D spatial resolution afforded by two-photon microscopy they were able to localize neuronal electrical activity in initially activating neuronal oxidative metabolism, subsequently followed by astrocyte glycolysis. The authors note that techniques such as positron emission tomography (PET) or nuclear magnetic resonance (NMR) would not have sufficient temporal resolution to resolve the early oxidative metabolism from astrocyte glycolysis.

Finally, we note that in a similar vain to imaging NAD(P)H, two-photon microscopy can also be used to image other UV-excitable fluorophores with 3D spatial resolution and overall reduced phototoxicity. Another UV-excitable fluorophore is Laurdan, which is often used as a marker for spatially resolving membrane fluidity (Gaus et al. 2003). Recent applications of Laurdan utilizing two-photon excitation include investigating the importance of membrane order in the cellular adhesion to the extracellular matrix (Gaus et al. 2006) or how membrane order promoted acetylcholine receptor clustering in the postsynaptic membrane (Stetzkowski-Marden et al. 2006).

SHG imaging of endogenous collagen

Second harmonic generation (SHG) is another related nonlinear microscopy technique that has the ability to generate signal from endogenous biological structures such as collagen, myosin, and tubulin without the use of exogenous labels. Whereas two-photon excited fluorescence results from the simultaneous absorption of two photons at high incident intensities, SHG results from the nonlinear combination of two infrared photons to form one visible photon of half the wavelength at high incident intensities, as shown in Fig. 7a. The process of SHG does not undergo any absorption and only occurs with significant efficiency in structures where there is no center of symmetry. While SHG imaging was demonstrated long before two-photon fluorescence microscopy (Gannaway and Sheppard 1978), it is only recently that physiological studies have been performed through its use. The molecular structure of fibrillar collagen in particular is such that it is very efficient at producing a strong SHG signal with minimal background from nonfibrillar components, as shown in Fig. 7b. Since collagen is such an important structural protein in the mammalian body, SHG imaging is a very useful technique for imaging the structural organization of this protein (Cox and Kable 2006; Zipfel et al. 2003).

Several studies have utilized SHG collagen imaging in relation to tumor-induced changes in matrix organization. These include changes to extracellular matrix organization (1) during carcinoma invasion and proliferation (Estrada et al. 2006), (2) during macrophage-assisted tumor intravasation (Wyckoff et al. 2007), and (3) for imaging how collagen in the matrix is reorganized by tumors to facilitate their migration and invasion throughout the tissue

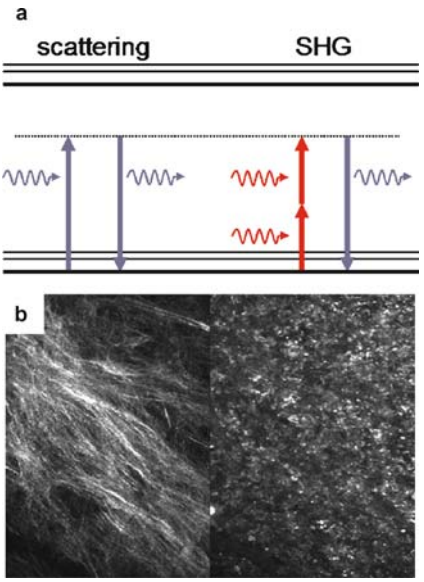

Fig. 7 a Energy level (Jabłoński) diagram representing elastic scattering and second harmonic generation (SHG). No absorption occurs in either interaction. Elastic scattering results in an outgoing photon of equal energy to the incoming photon, whereas SHG results in a single photon of energy twice that of the two incoming photons. **b** Due to the structures required to produce SHG, fibrillar collagen can be imaged using the SHG signal with a high signal compared to the background (*left*), where as imaging the autofluorescence (*right*) results in a high background signal from heterogeneously distributed nonfibrillar molecules. (Images reprinted by permission from Macmillan Publishers Ltd: *Nature Medicine*, Brown et al. 2003, copyright 2003)

(Provenzano et al. 2006). This latter study, through the inherent optical section associated with SHG imaging, allowed the 3D collagen organization to be studied during tumor invasion that would not have been possible with conventional histological techniques. SHG imaging has also been used to dynamically resolve the in vivo 3D collagen structure and organization inside tumors. Jain and coworkers showed how drug accessibility in tumors could be observed, as well as noninvasively imaging how certain hormones altered tumor matrix (Brown et al. 2003). They also utilized SHG collagen imaging for assaying the ability of semiconducting nanocrystals to access the tumor vasculature (Stroh et al. 2005).

Imaging of fibrillar collagen with SHG has also been used in other studies involving extracellular matrix; for example, SHG imaging of collagen has been used for understanding matrix homeostasis (Thompson et al. 2006). Local increases in collagen were observed in response to epithelial injury revealing one of the regulatory mechanisms required for normal collagen homeostasis. SHG imaging of collagen has also been used to study the 3D structure and organization of the collagen matrix surrounding mammary gland buds during their development (Ingman et al. 2006). Macrophages were found to be necessary to promote the assembly of the collagen into long fibrous structures that can be imaged with SHG, which subsequently modulates the development of the end bud structure. Finally, SHG imaging of collagen has also allowed the study of how interstitial matrix reorganizes during angiogenesis (Kirkpatrick et al. 2007), where it was observed that angiogenic sprouts and new vessels actively remodel existing collagen fibrils.

Several studies of the 3D collagen microorganization using SHG imaging have also been made in the cornea, for example, revealing important information for the role that collagen structure plays in biomechanical support (Morishige et al. 2006), and allowing visualization of the disorganization in corneal collagen under genetic mutations (Lyubovitsky et al. 2006). Imaging collagen through its intrinsic SHG signal can also be combined with imaging elastin through its endogenous two-photon excited fluorescent signal, both of which are major components of the arterial wall. This has allowed the study of how the arterial microstructure affects mechanical properties of the vessel as well as the effects of chemicals on the arterial structure (Boulesteix et al. 2006).

Third harmonic generation (THG), a three-photon analog of SHG, also provides structurally based contrast. In particular, lipid bodies have been shown to provide a major source of THG, allowing lipoid metabolism to be studied without the use of exogenous probes (Debarre et al. 2006). This was also used by Beaurepaire and coworkers in their two-photon imaging study of embryo development, where THG imaging gave them additional information on the dynamics of lipid-rich tissue and yolk structures (Supatto et al. 2005).

The future

This review has introduced the concepts behind two-photon microscopy and covered recent developments in its application to physiological studies. It is important to understand that two-photon microscopy is not a magical do-all replacement to the confocal microscope. This misconception has led to many redundant systems in laboratories where complaints such as “low signal”, “it’s blowing up my sample” and “I can’t get the laser mode-locked” have occurred. In many instances, 1 μm z resolution is not required, thus, for moderate 3D image resolution in thin samples the confocal microscope is still king. Two-photon microscopy does enable many important studies involving deep 3D tissue structures; for resolving 3D structure and morphology as well as enabling high-resolution subcellular access to functional

information such as 3D tracking/motility, electrical activity, blood flow rates, and metabolic activity. Additionally, two-photon microscopy has allowed well-defined perturbations to be introduced into systems through using targeted photo-uncaging or photo-ablations. Finally, endogenous substances such as NAD(P)H, collagen, and elastin can also be imaged with two-photon fluorescence or second harmonic generation.

The outlook for two-photon microscopy is very promising for further in vivo physiological studies. Many important advances are currently underway that will further extend the utility of this imaging modality. In particular, work by Schnitzer and coworkers has focused on developing a portable two-photon micro-endoscope for 3D imaging of mouse brains. This endoscope has a footprint of less than 4 mm and is sufficiently light to enable free movement of the mouse once cranially attached (Flusberg et al. 2005). They demonstrated that lateral resolutions of approx. 1 μm and axial resolutions of approx. 10 μm can be obtained. High-resolution 3D optical imaging is thus no longer limited to accessible features 1 mm within surface tissue; rather, two-photon imaging in any internal organ can be possible by introducing the micro-endoscope via the esophagus, blood vessels, ear, or other entry points.

Additional developments in fluorescent probe design have also yielded brighter and more red-shifted fluorophores, including genetically encoded red fluorescent proteins (Shaner et al. 2004; Shu et al. 2006). Red shifting the excitation wavelength will result in substantially reduced absorption of the excitation and fluorescence emission, allowing greater imaging depths to be achieved. The development of ultra-fast lasers in the 1,000- to 1,300-nm wavelength range will allow the use of these red-shifted probes in two-photon microscopy (Tsai et al. 2006). For further improved intra-vital two-photon microscopy, adaptive wavefront correction techniques have been developed and optimized to compensate for the image distortion caused by heterogeneous biological material (Neil et al. 2000; Rueckel et al. 2006). More advanced quantitative imaging and spectroscopy techniques such as fluorescence lifetime imaging and fluorescence correlation spectroscopy have also taken advantage of two-photon microscopy to resolve in vivo binding and diffusional transport (e.g., Alexandrakis et al. 2004; Calleja et al. 2007). Finally we can look forward to the possibility of increased two-photon imaging rates as line scanning techniques (Brakenhoff et al. 1996; Zipfel et al. 1996) are introduced, employing sophisticated optics and charge-coupled device (CCD) technology[1]. Additionally, other innovative techniques for improving signal and speed have been employed (e.g., Benninger et al. 2005; Tal et al. 2005).

To summarize, as two-photon microscopy has become more accessible to nonoptics experts, it has been successfully applied to a wide range of challenging physiological studies. The range of applications covered in this review from neuroscience to immunology, cancer, developmental biology, and endocrinology shows the power two-photon microscopy can now bring to these and other fields of study. With the currently available turnkey systems, the breadth and depth of two-photon microscopy applications will continue to increase. We fully expect further discoveries that would not have been possible to achieve with any other existing techniques.

Acknowledgements. We are grateful to Gert-Jan Kremers for helpful comments on the manuscript. The authors acknowledge financial support from National Institute of Health grants R01-DK53434, P20-GM72048 and the Medical Free-Electron Laser Program of the United States Department of Defense.

[1]Technology such as that used on the LSM5Live line scanning microscope: www.zeiss.com/micro.

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Rev Physiol Biochem Pharmacol (2008) 160:93 –125
DOI 10.1007/112_2008_802

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Lipid homeostasis in macrophages – Implications for atherosclerosis

Published online: 19 April 2008

Abstract In industrialized societies with excess food supply, obesity is an expanding problem. As a result of metabolic overload, besides obesity, insulin resistance, type-2 diabetes, dyslipidemia, hypertension, and atherosclerosis develop, which together make up the metabolic syndrome. The imbalance of lipid uptake, metabolism, and removal in many organs such as the liver, muscle, adipose tissue, vessel wall, and macrophages triggers organ transdifferentiation toward lipid storage phenotypes. Macrophages, foam cells, and osteoclasts in calcifying lesions are a hallmark of atherosclerosis and the metabolic syndrome, and must be regarded as an important therapeutic target. In this review, pathways regulating lipid homeostasis in macrophages are updated. These include lipid influx through different receptor entry pathways, the role of membrane microdomains, endolysosomal and cytosolic lipid storage leading to *phospholipidosis*, and lipid droplet accumulation or activation of lipid efflux either through the Golgi system or bypassing this organelle on the way to the plasma membrane. The interdependence of these pathways and pharmacological modifications are described. The monocyte innate immunity receptor complex in defining monocyte subpopulations and their role in cardiovascular disease is taken into account. The composition of certain molecular lipid species in membrane microdomains and other organelles is essential for cellular functions affecting raft dynamics, signal transduction, and membrane and organelle trafficking. It is very likely that the underlying defects in lipid-associated rare genetic diseases such as *ABCA1* deficiency, Niemann–Pick disease type C, as well as the more frequent complex disorders associated with atherosclerosis and phospholipidosis are related to disturbances in membrane homeostasis, signal transduction, and cellular lipid metabolism.

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Abbreviations

ABCA1	ATP binding cassette transporter A1
ABCG1	ATP binding cassette transporter G1
ACAT1	Acyl-coenzyme A:cholesterol acyltransferase 1
AGE	Advanced glycation end product
Apo	Apolipoprotein
ARF	ADP-ribosylation factor (ARF)-like
CAD	Coronary artery disease
CETP	Cholesteryl ester transfer protein
CR	Complement receptor
CRP	C-reactive protein
CSF-1	Colony-stimulating factor-1
DRM	Detergent resistant membrane
E-LDL	Enzymatically degraded LDL
ER	Endoplasmatic reticulum
FADD	Fas-associated death domain
FCDR-assay	Flow cytometric differential detergent resistance assay
FRET	Fluorescence resonance energy transfer
FcγR	Fcγ receptor
GPI	Glycosylphosphatidylinositol
HDL	High-density lipoproteins
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A
ITAM	Immunoreceptor tyrosine-based activation motifs
ITIM	Immunoreceptor tyrosine-based inhibition motifs
LDL	Low-density lipoproteins
LOX-1	Lectin-like Ox-LDL receptor
LPS	Lipopolysaccharide
LXR	Liver X receptor
MARCO	Macrophage scavenger receptor with collagenous structure
MPO-LDL	Myeloperoxidase oxidized LDL
mTOR	Mammalian target of rapamycin
NPC1	Niemann–Pick disease C1
Ox-LDL	Oxidized LDL
PPAR	Peroxisome proliferators-activated receptor
PPRE	Peroxisome proliferation response element
RAGE	Receptor for advanced glycation end-products
RXR	Retinoid X receptor
SCAP	SREBP-cleavage activation protein
SIRS	Systemic inflammatory response syndrome
SR-BI	Scavenger receptor BI
SREBP	Sterol regulatory element binding protein
SREC	Scavenger receptor expressed by endothelial cells
SR-PSOX	Scavenger receptor for phosphatidylserine and oxidized lipoprotein

Macrophages and atherosclerosis

Atherosclerosis is a chronic vascular disease characterized by lipid retention and inflammation. As a result of disturbed lipid homeostasis, chronic remodeling, inflammation, and focal calcification or fibrosis accelerate atherogenesis. Innate immune recognition receptors such as charge and motif receptors or opsonin receptors such as Fcγ and complement receptors which bind either antibodies and pentraxins, or complement, are involved in inflammatory activation during disease progression. In response to modified low-density lipoproteins (LDL), endothelial cells and vascular smooth muscle cells release cytokines and chemoattractants. This leads to the adherence of monocytes to the endothelium and migration to the subendothelial space where they differentiate into macrophages. Macrophages accumulate modified LDL such as *advanced glycation end product LDL (AGE-LDL)* (Sano et al. 1999), enzymatically degraded LDL (E-LDL) (Bhakdi et al. 1995), and oxidized LDL (Ox-LDL) (Kita et al. 1990). Following internalization, modified LDL are degraded and fatty acids and cholesterol are either stored in lipid droplets as cholesteryl esters and triglycerides upon E-LDL loading leading to foam cell formation (Bhakdi et al. 1995) or they accumulate in the endolysosomal compartment upon Ox-LDL uptake (Jessup et al. 2002). Foam cells are a characteristic feature of atherosclerosis and are typical for fatty streak lesions in early events of atherosclerosis. The large cholesterol-rich core of advanced lesions appears to be substantially derived from necrosis and/or apoptosis of foam cells (Tabas 2000). Foam cells also contain small but significant amounts of various lipid oxidation products, which are potent mediators of macrophage function (Jessup et al. 2004). Genetic and environmental risk factors contribute to the progression of early fatty streak lesions into complex lesions characterized by lipid-rich cores covered by fibrous caps and a large number of activated inflammatory cells particularly macrophages and T cells. These inflammatory processes may ultimately lead to plaque rupture and acute coronary syndromes. Vascular calcification is a strong indicator of chronic inflammation in atherosclerosis, and osteoclast-like cells are found in calcified atherosclerotic plaques (Abedin et al. 2004). Osteoclasts derived from blood monocytes after extravasation and macrophages exposed to particulate calcium mineral have been reported to undergo osteoclastic differentiation (Merkel et al. 1999). In addition, colony-stimulating fator-1 (CSF-1) synergizes with the receptor activator of NF-κ B ligand (RANKL) to regulate the differentiation of mononuclear phagocytes to osteoclasts (Teitelbaum and Ross 2003; Pixley and Stanley 2004). Macrophages also may contribute to mineral formation indirectly through the release of inflammatory cytokines and lipid oxidation products that promote vascular cell mineralization. CD163, a member of the macrophage cysteine-rich scavenger receptor family, is responsible for the uptake of hemoglobin/haptoglobin complexes which are formed for the removal of increased heme/iron levels from damaged red blood cells (Fabriek et al. 2005; Schaer et al. 2007). The secretion of proinflammatory cytokines upon CD163–ligand interaction promotes an inflammatory response and macrophage proliferation characteristic of progressing atherosclerotic lesions (Li et al. 2004). This heme/haptoglobin/CD163 pathway seems to be significantly involved in rapid calcified lesion progression and plaque rupture during atherosclerosis. Dependent on exogenous stimuli, monocytes may be targeted either toward phagocytic macrophages leading to lipid uptake and foam cell formation or osteoclastic macrophages leading to lesion calcification.

The efflux process for the removal of lipids from macrophage foam cells is critical for the maintenance of lipid homeostasis and the development of atherosclerosis. Cholesterol is removed from macrophages through various transporters to extracellular high-density lipopro-

tein (HDL) as acceptors. ATP-binding cassette transporter *ABCA1*, ABCG1, and scavenger receptor BI (SR-BI) are involved in cholesterol efflux from macrophages via HDL leading to reverse cholesterol transport (Jessup et al. 2006). Various lipid-associated disorders show defects in either HDL-mediated cholesterol efflux from macrophages and other cells including *ABCA1* deficiency (*Tangier disease)* (Bodzioch et al. 1999) or endolysosomal lipid storage such as acid lipase deficiency (Panchagnula et al. 2000), *Niemann–Pick disease* type A, B, and C (Schuchman 2007; Sevin et al. 2007), Fabry's disease (Clarke 2007), and Gaucher's disease (Butters 2007).

Another important aspect of macrophage function in atherosclerosis is that macrophages are necessary for removal of apoptotic cells and cell debris from plaques and they also exert strong pro- and antiatherogenic properties upon receptor mediated uptake and *phagocytosis* of erythrocytes, platelets, or other cells. Apoptotic clearance in principle is a beneficial process and might help to prevent the progression of advanced atherosclerotic lesions (Schrijvers et al. 2007). In addition to phagocytosis, *autophagy* is an important mechanism for various cell types including macrophages in atherosclerosis (Verheye et al. 2007; Jia et al. 2007; Schrijvers et al. 2007) that is necessary for intracellular clearance of surplus organelles from the cytoplasm and is directly connected to apoptosis and survival. During autophagy, cytoplasmic components and organelles are sequestered either selectively or nonselectively in double membrane vesicles and are degraded upon fusion with lysosomal compartments. Degradation of intracellular macromolecules through autophagy provides the energy required for minimal cell function when nutrients are scarce, and it secures organelle homeostasis. Specific toxins and pathogens are sequestered and degraded through autophagy-mediated elimination of altered cytosolic constituents, such as aggregated proteins or damaged organelles to preserve cells from further damage. Activation of autophagy may play a protective role in early stages of atherosclerosis and other diseases (Kundu and Thompson 2007) including neurodegenerative diseases (Mizushima et al. 2002), myopathy (Mizushima et al. 2002), and invasion by microbial pathogens (Dorn et al. 2002). In addition, autophagy is linked to Alzheimer's disease, different forms of cancer, muscular disorders, and cardiomyopathies (Cuervo 2004). Failure of autophagy might be directly related to chronic metabolic overload of all cellular entry and storage pathways. Two connected signaling pathways encompassing class-I and class-III phosphatidylinositol 3-kinase and mammalian target of rapamycin (mTOR) play a central role in controlling macroautophagy in response to starvation (Codogno and Meijer 2005).

For pharmacological treatment of autophagic processes, glucocorticoids are known to stimulate phagocytic clearance of apoptotic cells; however, the serious adverse effects of long-term glucocorticoid therapy render this approach impractical (Maderna and Godson 2003). Examples of other pharmaceuticals that promote phagocytic clearance of apoptotic cells are the cholesterol-lowering agent lovastatin (Morimoto et al. 2006), the macrolide antibiotic azithromycin (Hodge et al. 2006), and members of the lipoxin family (Godson et al. 2000). Future studies are necessary to determine whether these drugs may provide the basis for a novel therapeutic strategy to prevent the progression of advanced atherosclerotic plaques via interference with the process of *phagocytosis/autophagy*. It is assumed that macrophage removal stabilizes plaques (Boyle 2005) because they play a pivotal role in the destabilization process, whereas smooth muscle cells contribute to plaque stability (Libby 2002). Systemic therapy with statins, which are inhibitors of cholesterol biosynthesis, reduce but do not eliminate macrophages from atherosclerotic lesions (Crisby et al. 2001). Manipulation of mTOR, the master regulator of autophagy, provides new insights into therapeutic interventions of autophagic processes. Recently it has been reported that stent-based delivery of the mTOR inhibitor rapamycin had a profound effect on inflammatory cell activity and

cytokine release in nonatherosclerotic porcine arteries, further supporting the importance of autophagy (Suzuki et al. 2001). Stent-based delivery of everolimus, a rapamycin derivative, selectively cleared macrophages in rabbit atherosclerotic plaques by autophagy (Verheye et al. 2007). This provides an mTOR inhibition-dependent novel mechanism to modulate autophagy and to induce cell death in mammalian cells. 7-Ketocholesterol, a byproduct of Ox-LDL, induces a complex mode of cell death in human vascular smooth muscle cells associated with myelin figure formation and light chain 3 (LC3) processing, inducing autophagic processes (Martinet et al. 2004). These data indicate that the autophagic activity of macrophages is important for the balance of apoptosis and survival and thereby may modulate vascular remodeling.

Differential expression of the monocyte innate immunity receptor complex defining monocyte subpopulations

Following adherence to endothelial cells, defined subpopulations of circulating monocytes that express the *lipopolysaccharide (LPS)* receptor CD14, the Fcγ receptor IIa (FcγRIIa)/CD32, and the FcγRIIIa/CD16a ($CD14^{bright}CD16^{bright}$) extravasate into the subendothelial space (Rothe et al. 1996, 1999; Stohr et al. 1998). In addition, the $CD14^{bright}CD16^{bright}CD32^{bright}$monocytes are associated with higher amounts of MHC class II molecules and it was suggested that these cells resemble mature tissue macrophages (Ziegler-Heitbrock et al. 1993). Distinct chemokine-receptor expression profiles were also among the phenotypic differences that were recognized between these subsets: for example, $CD14^{bright}CD16^{bright}$ monocytes expressed C-C-chemokine receptor 5 (CCR5), whereas $CD14^{bright}CD16^{dim}$ monocytes expressed CCR2 (Gordon and Taylor 2005). The $CD14^{bright}CD16^{dim}$ phenotype is identical to the FcγRI $CD64^{bright}$ population, resembling the classical monocytes (Passlick et al. 1989; Grage-Griebenow et al. 2001). These cells have a typical monocytic phenotype and function such as high cytokine production (Szabo et al. 1990; Schinkel et al. 1998), high chemotactic (Ohura et al. 1987) and phagocytic activity (Grage-Griebenow et al. 1993, 2000), high cytotoxicity for tumor cells, and suppressive activities in lymphocyte proliferation (Zembala et al. 1984, 1986).

The heterogeneous expression of CD14, CD16, and CD32 suggested a different capacity for IgG-dependent *phagocytosis* (Rothe et al. 1996). Moreover, the population size of $CD14^{dim}CD16^{bright}$ monocytes correlates positively with low HDL cholesterol and the apoE4 allele supporting a link between peripheral blood monocyte heterogeneity and cardiovascular risk factors (Rothe et al. 1996). Expression of CD14 on monocytes is reduced in systemic inflammation and during treatment of hypercholesterolemic patients with the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor fluvastatin (Rothe et al. 1999). Moreover, a functional genetic polymorphism of CD14 is associated with myocardial infarction (Hubacek et al. 1999), suggesting a role of CD14 in inflammation and atherogenesis.

Interestingly, ceramide as a constituent of atherogenic lipoproteins competes with exogenous LPS for ligand-specific CD14/CD11b complement receptor clustering in lipid membrane microdomains (Pfeiffer et al. 2001) (Fig. 1). On monocytes/macrophages these lipid membrane microdomains provide a dynamic microenvironment for integrated CD14-dependent clustering of a set of receptors involved in innate immunity and clearance of atherogenic lipoproteins (Schmitz and Orso 2002). In resting cells, CD14 was associated with CD55, the FcγRIIA/CD32 and FcγRI/CD64, and the pentaspan CD47. Ceramide

further recruited *complement receptor 3 (CD11b/CD18)* and the scavenger receptor CD36 in the proximity of CD14. In addition, LPS induced coclustering of CD14 with Toll-like receptor 4, FcγRIIIa/CD16a, and the tetraspanin CD81 while CD47 was dissociated. Thus clustering of signaling competent receptors to a common recognition platform in lipid membrane microdomains may provide an interesting mechanism by which different ligands induce distinct cellular processes in systemic inflammation [systemic inflammatory response syndrome (SIRS)/sepsis] and cardiovascular disease. With fluorescence resonance energy transfer (FRET) the sterical coassociation between the proteins can be determined upon ligand stimulation (Pfeiffer et al. 2001) (Fig. 1).

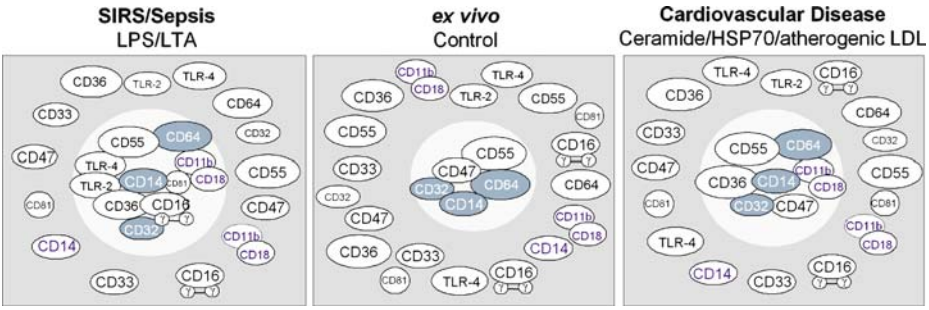

Fig. 1 The innate immunity receptor complexes in inflammation (SIRS/Sepsis) and cardiovascular disease. The basic cluster includes specific and nonspecific opsonin recognition sites (ex vivo, control) whereas ligand-induced clustering is dependent on ligand surface composition (*LPS*/LTA or ceramide/HSP70 and atherogenic LDL). (Adapted from Pfeiffer et al. 2001)

Interestingly Niemann–Pick disease type C (NPC) monocytes with impaired cholesterol influx compared to control monocytes show an increased detergent resistant membrane (DRM) association of the surface antigens CD16, CD32, CD64, CD36, CD91, CD14, CD55, and CD47 as determined by a novel detergent-based flow cytometric assay (FCDR assay; flow cytometric differential detergent resistance assay) (Wolf et al. 2007; Gombos et al. 2004) (Fig. 2).

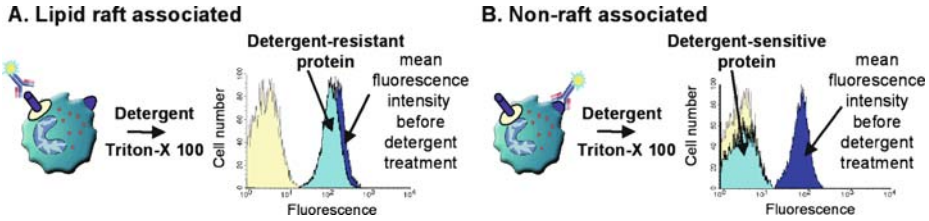

Fig. 2 Flow cytometric differential detergent resistance (FCDR) assay strategy for the detection of DRM association of membrane proteins. **A** Lipid raft-associated plasma membrane proteins show high resistance to detergent treatment. **B** Nonraft-associated proteins are rapidly dissolved by even low concentrations of non-ionic detergents in a short time. These processes can be followed by measuring fluorescence means before and after 5 min of detergent treatment by flow cytometry. (Adapted from Gombos et al. 2004; Wolf et al. 2006)

In contrast, surface antigens of *ABCA1*-mutant monocytes with impaired cholesterol efflux show no alterations in their DRM-association compared to control monocytes (Wolf et al. 2007). Increased DRM-association of the glycosylphosphatidylinositol (GPI)-anchored proteins CD14, CD55, the FcγRI CD64, the scavenger receptors CD36, CD91

and CD163, the integrin CD11a, and the *complement receptor 3 complex CD11b/CD18* are also observed in monocytes from patients with SIRS/sepsis or coronary artery disease (CAD)/myocardial infarction (Wolf et al. 2007). In addition the tetraspan CD81 shows increased DRM-association in SIRS/sepsis patients, but not in CAD patients, while the pentaspan CD47 and the FcγRIII CD16 show increased DRM partition in CAD patients but are disassembled from DRMs in SIRS/sepsis patients. This detergent-based flow cytometric assay provides a novel tool for rapid screening of blood monocyte DRMs to preselect patients with potential raft/microdomain abnormalities for more detailed analysis (Wolf et al. 2006, 2007).

In summary, the FRET and FCDR assays are two methods for screening of patients for abnormalities in the protein and surface receptor composition of lipid membrane microdomains, respectively.

Receptor entry pathways of modified LDL

Cellular internalization of LDL takes place via receptor-mediated endocytosis in a clathrin-dependent transport process (Fig. 3). This process begins at the cytosolic leaflet of the plasma membrane with the sequential assembly of coat components to form clathrin-coated pits. Lipid membrane microdomains and membrane lipid composition, especially membrane cholesterol content, are important in receptor-mediated endocytosis, and severe cholesterol depletion can inhibit clathrin-coated pit endocytosis (Subtil et al. 1999). Depending on the degree of oxidation, in minimally oxidized LDL apoB is still able to bind to the LDL receptor and can be taken up via the clathrin-coated pit pathway (Jessup et al. 2002). In heavily oxidized LDL, apoB is recognized by scavenger receptors such as SR-A or CD36 (Jessup et al. 2002). CD36 has been reported to be localized in lipid rafts but not in caveolae, and that binding of Ox-LDL to CD36 leads to endocytosis through a lipid raft pathway that is distinct from the clathrin-mediated or caveolin internalization pathways (Zeng et al. 2003). After uptake, Ox-LDL shows prolonged residence time in endosomes because of impaired cholesteryl-ester hydrolysis before it is released to lipid droplets (Jessup et al. 2002; Schmitz and Grandl 2007).

In contrast to the LDL receptor clathrin-coated pit and coated vesicle-dependent pathway which is feedback inhibited by cholesterol accumulation inside the cell, the scavenger receptor-dependent uptake of modified LDL is determined by unregulated lipid uptake resulting in macrophage foam cell formation (Steinbrecher et al. 1989). Scavenger receptors expressed by macrophages in atherosclerotic lesions include scavenger receptors class A consisting of SR-AI, SR-AII, SR-AIII, and macrophage receptor with collagenous structure (MARCO). Class B scavenger receptors are CD36 and SR-BI. CD36 is also referred to as fatty acid translocase due to its role in transportation of long-chain fatty acids. It could be shown that E-LDL preferentially binds to $CD14^{bright}CD16^{bright}CD32^{bright}$ monocytes and induces foam cell formation mediated only in part by the class B scavenger receptor CD36 (Kapinsky et al. 2001) in contrast to Ox-LDL, which is mainly taken up by CD36 (Nicholson et al. 1995). Class D includes the macrosialin receptor CD68. Lectin-like Ox-LDL receptor-1 (LOX-1) belongs to scavenger receptor class E, scavenger receptor expressed by endothelial cells (SREC) belongs to class F scavenger receptors, and scavenger receptor for phosphatidylserine and oxidized lipoprotein/CXC chemokine ligand 16 (SR-PSOX) is a class G scavenger receptor. FEEL-1/2, a class H scavenger receptor, facilitates cell

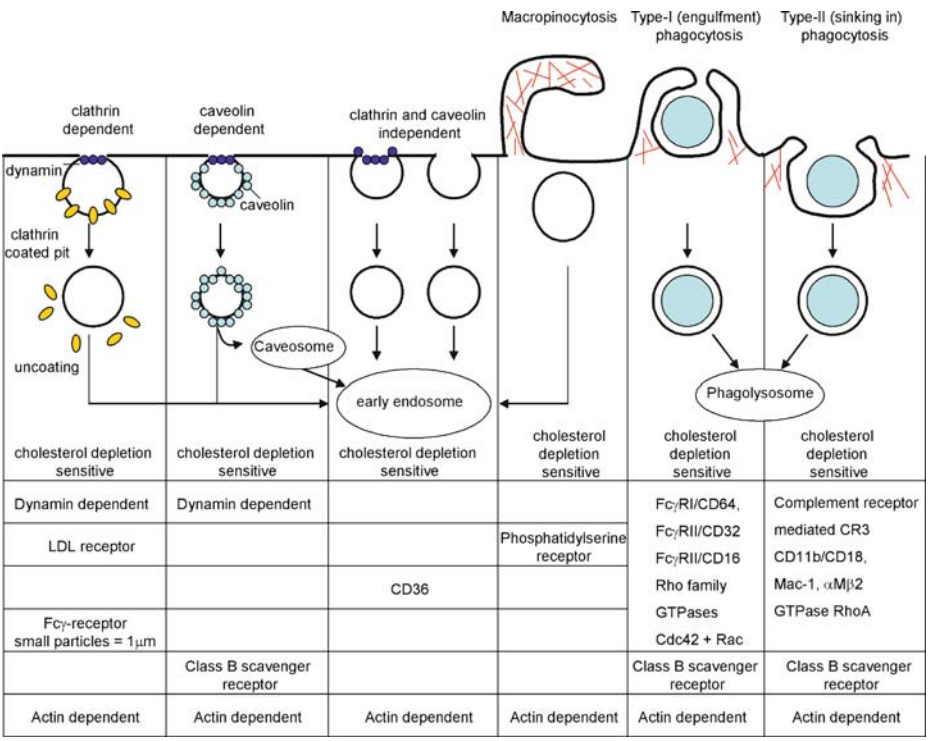

Fig. 3 Different entry pathways into the cell. Lipid particles can be taken up either through clathrin or caveolin dependent or independent pathways. Another possibility of lipid particle uptake is phagocytosis

adhesion properties and transmigration and might therefore influence lesion formation. The class I cysteine-rich CD163 scavenger receptor is responsible for the removal of hemoglobin/haptoglobin complexes from atherosclerotic lesions as mentioned before, and the class J scavenger receptor might be involved in the clearance of apoptotic residual bodies from atherosclerotic lesions (for more detailed review of scavenger receptors see van Berkel et al. 2005). The *receptor for advanced glycation end-products (RAGE)* also binds *AGEs* like SRs class AI and AII, LOX-1, CD36, and SR-BI. RAGE is known to be involved in microvascular complications in diabetes (Koyama et al. 2007). RAGE is also profoundly associated with macrovascular complications in diabetes through regulation of atherogenesis, angiogenic response, vascular injury, and inflammatory response. Therefore RAGE and also soluble RAGE may be useful as therapeutic targets and biomarkers for those diseases.

Caveolae are alternative endocytic carriers and are only present in mouse but not in human macrophages (Fig. 3). They are uncoated flask-shaped invaginations (55–65 nm diameter) at the cell surface of mammalian cells like vascular endothelium, smooth muscle cells, and adipocytes and are well-characterized subdomains of glycolipid rafts (Nabi and Le 2003). A third pathway, which is both clathrin- and caveolae-independent, may constitute a specialized high-capacity endocytic pathway for lipids and fluids (Fig. 3). Many markers for clathrin-independent endocytosis are found in DRM fractions (Nichols and Lippincott-Schwartz 2001). A general depletion of sphingolipids (*glycosphingolipids* and sphingomyelin) also blocks clathrin-independent pathways, including caveolae-mediated

endocytosis as well as the RhoA- and Cdc42-regulated pathways. In contrast, depletion of *glycosphingolipids* alone selectively blocks caveolar endocytosis (Cheng et al. 2006).

Macropinocytosis is another endocytic pathway, which involves the internalization of large areas of the plasma membrane together with significant amounts of fluid (Fig. 3). In this process membrane protrusions fuse back with the plasma membrane to generate large irregular vesicles called macropinosomes. Lipid membrane microdomains are involved in macropinosome formation, and cholesterol depletion inhibits both membrane ruffling and macropinocytosis (Grimmer et al. 2002).

Particles can also be engulfed by type I and type II *phagocytosis*. E-LDL particles are internalized predominantly by opsonin-driven type I and type II phagocytosis, leading to rapid formation of lipid droplets associated with the cytosolic membrane of the endoplasmatic reticulum (ER) (Schmitz and Grandl 2007). Type-I phagocytosis (engulfment phagocytosis) is mediated by the Fcγ receptors (FcγRI/CD64, FcγRII/CD32, and FcγRIII/CD16) which bind to IgG-coated particles. It goes along with membrane ruffling and pseudopodia extension by a zippering mechanism and uses the Rho family GTP-ases Cdc42 and Rac. FcRs that are responsible for FcR-mediated endocytosis recognize the Fc portion of antibodies and deliver signals when they are aggregated at the cell surface. FcRs can be functionally divided into two major types: FcRs that can trigger cell activation and FcRs that cannot. The aggregation of FcRs having immunoreceptor tyrosine-based activation motifs (ITAMs) activates sequentially src family tyrosine kinases and syk family tyrosine kinases that connect transduced signals to common activation pathways shared with other receptors. FcRs with ITAMs elicit cell activation, endocytosis, and phagocytosis. There are four multichain FcRs with ITAMs: FcγRI, FcγRIIIA, FcεRI, and FcαRI. FcRs having immunoreceptor tyrosine-based inhibition motifs (ITIMs) do not trigger cell activation. FcγRIIB (CD32B) is a single-chain low-affinity IgG receptor not containing ITAMs. FcγRIIB contains ITIMs and is a negative coreceptor of all receptors with ITAMs, and coaggregation with antigen receptors or FcRs having ITAMs negatively regulates cell activation (Daeron 1997). Type-II phagocytosis (sinking in phagocytosis) is complement receptor (CR)-mediated *(CR3: CD11b/CD18, Mac-1, αMβ2)* which binds C3bi-opsonized targets and particles sink into the cell. This mechanism uses only the GTP-ase RhoA. Beyond type-II phagocytosis, CR3 also mediates type-I phagocytosis in conjunction with FcγRs when ligands are opsonized by both antibodies and complement (Le V et al. 2002). This suggests that CR3 triggers different intracellular signals that mediate distinct phagocytic processes. Interaction between receptors and ligands on the particle results in signal transduction events that lead to actin polymerization, particle engulfment, and formation of phagosomes. During a maturation process, phagosomes interact and fuse with early and late endosomes as well as with lysosomes transforming them into phagolysosomes where degradation takes place. The fusion processes are regulated by small GTP-binding proteins of the Rab family.

In all these different endocytotic pathways the role of lipid–lipid and lipid–protein interactions with their associated signal transduction pathways need additional studies to further elucidate the importance of lipid membrane microdomains in these endocytotic processes and their relation to diseases.

Specific characteristics of E-LDL and Ox-LDL

Various modifications of LDL are known, including LDL modified by advanced glycation end-products *(AGE-LDL),* myeloperoxidase-oxidized LDL (MPO-LDL), oxidized LDL

(Ox-LDL), and enzymatically modified LDL (E-LDL). They differ in their uptake/receptor-mediated recognition and endocytosis mechanisms and are delivered to lysosomes. In this review, especially E-LDL and Ox-LDL as two principal chemical modifications of LDL are described.

Lipoprotein particles which are converted to proatherogenic particles with proteolytic cleavage of apoB and hydrolysis of core cholesteryl esters by extracellular cholesteryl ester-hydrolase are similar to lesion-derived LDL as related to particle size, biological properties, and composition (Bhakdi et al. 1995; Seifert et al. 1990). E-LDL shows the same morphology as extracellular matrix-associated lipid droplets that have been isolated from atherosclerotic lesions (Seifert et al. 1990), and direct evidence for the presence of complement-opsonized E-LDL in early human atherosclerotic lesions is based on the use of specific monoclonal antibodies that recognize epitopes on E-LDL, but not on native or Ox-LDL (Torzewski et al. 1998). Using trypsin in combination with cholesteryl ester-hydrolase allows in vitro generation of E-LDL (Bhakdi et al. 1995). Through this treatment solid 20–25-nm spherical LDL particles containing esterified cholesterol are transformed to unesterified cholesterol-rich lipid particles with a multilamellar, liposomal-like core structure and an irregular shape with mean diameters of 110 nm (Chao et al. 1992). LDL particles isolated from atherosclerotic lesions have a similar content of free cholesterol and also a liposome-like structure (Chao et al. 1992; Kruth 1984; Simionescu et al. 1986). In accordance with the response to retention hypothesis (Williams and Tabas 1995), which describes the subendothelial retention of cholesterol-rich, atherogenic lipoproteins, both neutral and acid cholesteryl ester-hydrolase have been demonstrated to be present in the human and rabbit arterial wall (Hajjar et al. 1982; Sakurada et al. 1976) and in atherosclerotic lesions (Hakala et al. 2003). The pentraxin acute phase opsonin C-reactive protein (CRP) and activated complement components colocalize with E-LDL (Torzewski et al. 1998; Bhakdi et al. 1999), which is important for the development and progression of atherosclerotic lesions. CRP also binds to E-LDL generated in vitro and enhances complement activation (Bhakdi et al. 1999). In monocytes (Kapinsky et al. 2001), macrophages (Bhakdi et al. 1995), and smooth muscle cells (Klouche et al. 2000) E-LDL induces foam cell formation, which is further enhanced in the presence of CRP and complement. E-LDL also stimulates monocyte chemotactic protein 1 (MCP-1) production (Klouche et al. 1998) and directly promotes adhesion and transmigration of monocytes through endothelial cell monolayers (Klouche et al. 1999).

In contrast to E-LDL, Ox-LDL is only sparsely present in early lesions, does not activate complement, and differs morphologically from E-LDL and LDL derivatives isolated from atheromas (Bhakdi et al. 1995). Lipoprotein fractions isolated from atherosclerotic lesions include lipoprotein particles with characteristic properties of Ox-LDL, such as increased electrophoretic mobility and reactivity with antibodies directed against Ox-LDL that do not react with LDL (Yla-Herttuala et al. 1989; Palinski et al. 1989). Ox-LDL was principally found in hypercholesterolemic patients. ApoE-deficient mice, which are hyperlipidemic and spontaneously develop atherosclerosis, have extensively high autoantibody titers to various epitopes of Ox-LDL. Therefore a series of monoclonal IgM autoantibodies which bind to epitopes of Ox-LDL from apoE-deficient mice (EO autoantibodies) have been cloned (Itabe et al. 1996). These EO autoantibodies were selected for binding to copper-oxidized LDL but they also bind to oxidized phospholipids (e.g., EO6) and prevent the binding of copper-oxidized LDL by macrophage scavenger receptors (Horkko et al. 1999). EO autoantibodies have been shown to immunostain atherosclerotic lesions and recognize specific epitopes on circulating LDL (Itabe et al. 1994, 1996; Palinski et al. 1996; Tamai et al. 1997). In LDL receptor-deficient mice circulating autoantibodies to Ox-LDL epitopes reflect the progres-

sion and regression of experimental atherosclerosis (Tsimikas et al. 2001). This was assessed by anatomic measurements of atherosclerosis and determination of Ox-LDL content (Tsimikas et al. 2001). Interleukin (IL)-5 may link adaptive and natural immunity specific to epitopes of Ox-LDL and protects from atherosclerosis, in part by stimulating the expansion of atheroprotective natural IgM specific for Ox-LDL (Binder et al. 2004). The presence of circulating IgM antibodies against Ox-LDL certainly suggests that LDL oxidation occurs in vivo (Horkko et al. 2000). A number of studies have shown that humans have significant titers of autoantibodies directed against Ox-LDL, and these tend to be higher in patients at higher risk of developing coronary heart disease (Horkko et al. 2000; Salonen et al. 1992).

LDL oxidation activates a platelet-activating factor (PAF) acetylhydrolase-like activity of the denatured apoB molecule with a phospholipase A_2-like activity which strips phosphatidylcholine from the Ox-LDL surface (Steinbrecher et al. 1984; Parthasarathy et al. 1985; Tjoelker et al. 1995; Parthasarathy and Barnett 1990). This leads to core aggregation of Ox-LDL particles forming a polar surface with the remaining phospholipids on the aggregated particles. The extent to which LDL is oxidized varies considerably and depends on the original LDL particle composition, on the type and concentration of oxidant used, and the time of exposure (Stocker 1994; Berliner and Heinecke 1996). Oxidative agents present in Ox-LDL that may be responsible for its proatherogenic effect are aldehyde end products of lipid peroxidation of polyunsaturated fatty acids like 4-hydroxynonenal, derived from phospholipids, mono-, di-, and triacylglycerols, or cholesteryl esters, as well as cholesterol oxidation products (Schmitz and Grandl 2007). Oxysterols can contribute to foam cell formation by inhibiting reverse cholesterol transport (Kilsdonk et al. 1995; Kritharides et al. 1995). Macrophages enriched with 7-ketocholesterol, one of the major oxysterols found in plaques, have impaired cholesterol efflux to lipid-poor *apoA-I* (Gelissen et al. 1996) but not to phospholipids containing *apoA-I* disks (Gelissen et al. 1999). The potentially proatherogenic effects of Ox-LDL are activation and damage of the endothelium, promoting lipoprotein extravasation and release of microsomal enzymes (Steinberg et al. 1989; Cathcart et al. 1985). Ox-LDL attract monocytes, enhance monocyte adhesion to endothelium, promote foam cell formation, induce necrosis and apoptosis as well as migration and proliferation of smooth muscle cells, impede endothelial cell migration, and promote procoagulant properties of vascular cells (Navab et al. 1995; Steinberg 1997). Minimally modified LDL and its oxidized phospholipids have been found to bind to CD14 or activate Toll-like receptors on macrophages (Miller et al. 2003b). In turn, various biological activities have been induced, including the stimulation of cytoskeletal rearrangements that alter phagocytic activity and the stimulation of cytokine secretion, such as IL-8. These findings link modified LDL with innate pattern recognition receptors, such as those involved in *LPS* signaling. Human epidemiological studies support the involvement of CD14 and Toll-like receptor 4 in cardiovascular diseases (Miller et al. 2003a). It has been proposed that E-LDL might be more important for the initiation of atherosclerosis, while Ox-LDL might be more helpful for diagnosis and prognosis of the disease (Torzewski and Lackner 2006). In addition, Ox-LDL loading of human macrophages increases ceramide and lactosylceramide expression and induces ceramide-enriched membrane microdomains and *phospholipidosis*, whereas loading with E-LDL induces cholesterol-rich microdomains (Grandl et al. 2006). The formation of different lipid membrane microdomains upon Ox-LDL and E-LDL uptake may have consequences for lipid membrane microdomain-associated signaling in cholesterol homeostasis and apoptosis in human macrophages.

Abnormalities of cholesterol trafficking

Phospholipidosis

Intracellular lipid accumulation, either caused by genetic or pharmacological factors, is a serious problem for human health (Anderson and Borlak 2006). Drug-induced phospholipidosis is characterized by endolysosomal accumulation of phospholipids and lamellar bodies originating most likely from impaired phospholipid hydrolysis in lysosomes (Anderson and Borlak 2006; Schmitz and Muller 1991). Several cationic amphiphilic drugs (CADs) cause local or systemic endolysosomal phospholipidosis after chronic exposure in preclinical studies. Alveolar (McCloud et al. 1995), liver, lung (Kannan et al. 1991), and splenic macrophages (Reasor 1989; Halliwell 1997; Kodavanti and Mehendale 1990) are common cellular targets of CAD-induced phospholipidosis. In addition, oxidation of LDL render Ox-LDL resistant to lysosomal hydrolysis and trap Ox-LDL within the endolysosomal compartment (Jessup et al. 2002) with an accumulation of lamellar bodies resembling the characteristics of phospholipidosis. Niemann–Pick disease C1 (NPC1) and acid sphingomyelinase (ASMase), or acid lipase deficiency syndromes like Wolman disease (WD: zero acid lipase activity) and cholesteryl ester storage disease (CESD: <5% residual acid lipase activity), also show symptoms of phospholipidosis (Schmitz et al. 1996). Experiments from our laboratory show that this endolysosomal phospholipidosis directly relates to ceramide lipid membrane microdomain formation at the plasma membrane connected to a strong activation of the *ABCA1*/ABCG1/AP-3 pathway. The formation of ceramide-rich membrane microdomains may originate from the release of lysosomal enzymes from luminal membranes in multivesicular bodies, which are only partially degraded by a higher cathepsin-mediated luminal degradation of lysosomal enzymes (Kolter and Sandhoff 2005), and also induces plasma membrane release of acid and neutral sphingomyelinase to generate ceramide rafts.

Niemann–Pick disease type C—A cholesterol storage disease

NPC1 and 2 are involved in the exit of cholesterol and/or other lipids from late endosomes. The mobilization of cholesterol from late endosomes and lysosomes requires functional NPC1 and NPC2 proteins, originally identified as an important secreted protein from human epididymis (HE1) (Kirchhoff et al. 1996). NPC1 may function as a permease to allow hydrolyzed cholesterol to exit the endosomal system. This activity may depend on NPC2, which may function as a chaperone for sterol insertion into the endolysosomal membrane by acting as a bridge to allow free cholesterol, released from its fatty-acid moiety by the action of lysosomal acid lipase, to insert into the inner membrane of the organelle. MLN64, a protein which was initially identified as an upregulated transcript in malignant cells, has been colocalized with NPC1 in the membrane of late endosomes, indicating that it might play a role in cholesterol egress together with NPC1. MLN64, however, could also act independently of NPC1 to facilitate shuttling of cholesterol between the endosomal membrane and an acceptor (Ioannou 2001). NPC is caused by mutations in the NPC1 or NPC2 gene. NPC is a rare autosomal-recessive lipidosis, characterized by the accumulation of unesterified cholesterol in late endosomes, lysosomes, and the Golgi apparatus (Pentchev et al. 1994; Vanier et al. 1991). Cholesterol relocation to and from the plasma membrane is delayed. In NPC cells in contrast to other storage diseases caused by defective metabolic enzymes, accumulation of cholesterol in degradative compartments of the endocytic pathway is due

to a failure in the mechanism responsible for redistribution of cholesterol taken up by endocytosis of LDL. Patients show progressive neurodegeneration and hepatosplenomegaly, which leads to death during early childhood. Several Rab GTPases are also involved in the regulation of sterol-dependent endosomal trafficking. Rab8 and Rab11 play a role in the recycling endosome, while Rab7 and Rab9 are found in late endosomes. A role for Rab7 and Rab9 in the Golgi targeting of *glycosphingolipids* was demonstrated and suggested as a new therapeutic approach for restoring normal lipid trafficking in NPC cells (Choudhury et al. 2002).

Although it has been assumed that NPC is primarily a cholesterol-storage disorder, *glycosphingolipids* such as lactosylceramide (LacCer) or globotriaosylceramide (Gb_3Cer) also accumulate to a similar extent in peripheral organs (Sillence and Platt 2003). It is suggested that the accumulation of *glycosphingolipids* is part of a mislocalized membrane microdomain and is responsible for the deficit in endocytic trafficking found in NPC disease (te Vruchte D. et al. 2004). It has been proposed that late endosome accumulation of cholesterol and sphingolipids may lead to an overload of cholesterol-rich raft-like membrane domains (i.e., an increase in raft-to-nonraft membrane ratio) causing a general traffic jam in the endosome compartment (Simons and Gruenberg 2000). In NPC1 protein-deficient cells, the association of LDL cholesterol with DRMs was enhanced and its transport to the plasma membrane was inhibited (Lusa et al. 2001). In addition, the NPC1 protein was normally recovered in detergent-soluble membranes and its association with DRMs was enhanced by lysosomal cholesterol loading (Lusa et al. 2001). These results suggest that endocytosed LDL cholesterol is efficiently recycled to the plasma membrane in an NPC1-dependent process. The cholesterol-sphingolipid accumulation characteristic of NPC disease, and potentially of other sphingolipidoses, causes an overcrowding of rafts forming lamellar bodies in the degradative compartments. In contrast to this, cholesterol accumulation did not lead to raft overload and the raft-to-nonraft membrane ratio was not altered in late endosomes (Sobo et al. 2007). However, stabilization of raft-like domains and decreased trafficking between the intraluminal vesicles and the limiting membrane of the compartment was demonstrated associated with a drastic enlargement of the compartment (Sobo et al. 2007).

In NPC mouse hepatocytes, a lipid imbalance was found, which increases lipid ordering in the plasma membrane, alters the properties of lipid rafts, and leads to defective function of the raft-associated plasma membrane insulin receptor (Vainio et al. 2005). This mechanism may participate in the pathogenesis of NPC disease and contribute to insulin resistance (Fonseca 2007) in other disorders of lipid metabolism.

Cellular cholesterol metabolism and lipid storage

Modified LDL taken up by the cell are delivered to the endolysosome pathway, where enzymes hydrolyze cholesteryl esters to free cholesterol and fatty acids. The levels of free cholesterol and cholesteryl esters are regulated with the help of neutral cholesteryl ester hydrolase, which converts cholesteryl ester to free cholesterol. After cholesterol leaves the lysosome, it is transported to the ER and to the plasma membrane by means of an intermediate step through the Golgi apparatus. Recycling compartments, especially multivesicular endosomes, harbor most of the cholesterol in the endocytic pathway. The intra-endosomal membranes of multivesicular late endosomes that are enriched in the phospholipids lysobisphosphatidic acid/bismonoacylglycerophosphate serve as important regulators of cholesterol transport (Kobayashi et al. 1999; Ikonen 2008). Lysobisphosphatidic acid is structurally

important for formation of the highly curved internal membranes of multivesicular bodies (Matsuo et al. 2004) and is implicated in cholesterol mobilization (Kobayashi et al. 1999), possibly by controlling the back-fusion of internal vesicles with the limiting late endosomal membrane (Le I et al. 2005).

Excess free cholesterol is esterified by acyl-coenzyme A:cholesterol acyltransferase 1 (ACAT1/SOAT1), an enzyme that localizes to the ER, and is stored as cytosolic lipid droplets of cholesteryl esters. The accumulation of lipid droplets in macrophages is associated with adipophilin also known as adipose differentiation-related protein (ADFP). Adipophilin is the most abundant lipid droplet-associated protein in these cells and Ox-LDL upregulates adipophilin expression in macrophages in vitro (Wang et al. 1999). This suggests a potentially proatherosclerotic role of adipophilin. E-LDL also upregulates adipophilin in monocytes and this induction of adipophilin is accompanied by a significant increase of free fatty acids (Buechler et al. 2001). Lipid droplets emerge from the ER lipid bilayer or from a subset of ER membranes as a lens of neutral lipid that then buds off from the cytoplasmic face of the bilayer to form a discrete nascent droplet within the cytoplasm (Brasaemle 2007). A recent re-evaluation by freeze-fracture electron microscopy revealed the association of lipid droplets with ER-like membranes, like "an egg cup (the ER) holding an egg (the lipid droplet)" (Robenek et al. 2006). Interestingly, adipophilin is concentrated in the ER membrane leaflet most closely adjacent to the monolayer of the lipid droplet, strategically placed to transport fatty acids from the ER to the droplet surface. The coordination of lipid storage and utilization is regulated by the PAT family of lipid droplet coat proteins (perilipin, adipophilin/ADFP, S3–12, TIP47, and MLDP/OXPAT/LSDP5) (Brasaemle 2007), caveolins, Rab proteins, CGI58 and associated lipases, lipid and protein kinases, and phosphatases and lipid transport proteins. Recently lipid droplets are considered as dynamic organelles, and the stored lipid can be used as substrate for synthesis of other important cellular molecules, such as membrane phospholipids and eicosanoids. Hormone-sensitive lipase initially described as an intracellular adipocyte-specific triacylglycerol lipase plays a role as neutral cholesteryl ester hydrolase associated with movement of HDL from the cytosol to the surface of lipid droplets (Brasaemle et al. 2000). The possible presence of hormone-sensitive lipase in macrophages remains controversial (Yeaman 2004). Interestingly, two types of lipid droplets exist: small and large lipid droplets. Small lipid droplets (mediator lipid droplets) are enriched preferentially with polyunsaturated fatty acid (PUFA) containing acylglycerols and *glycerophospholipids*. They contain all enzymes and regulators involved in eicosanoid and lipid mediator synthesis (Bozza and Bandeira-Melo 2005). Large lipid droplets are enriched with saturated and monounsaturated fatty acids as fuel for energy generation (burner lipid droplets). Lipid agonists (*cis*-PUFA, PAF, *LPS* , PKC) and protein stimuli (chemokines, cytokines) induce lipid droplets in macrophages, neutrophils, and eosinophils.

Pharmacological treatment of atherosclerosis through the use of ACAT1 inhibitors and inhibition of foam cell formation paradoxically fail to retard atherosclerosis (Fazio et al. 2001; Nissen et al. 2006). Inhibition of ACAT1 may reduce the cholesterol burden in plaque macrophages through increased efflux of free cholesterol into the HDL pathway, but inefficient efflux or compartmentalization of free cholesterol to sites disconnected from the efflux machinery may induce macrophage apoptosis owing to the toxic effects of free cholesterol (Fazio and Linton 2006). In addition, increased generation of apoptotic cells may stimulate atherogenesis.

Cholesterol efflux from macrophages to prevent foam cell formation

The initial steps of reverse cholesterol transport involve export of cholesterol from peripheral cells to plasma lipoproteins for subsequent delivery to the liver. In vivo, HDL or its apolipoproteins act as acceptors of cholesterol from peripheral cells, carrying it to the liver for degradation. When cholesterol acceptors such as HDL are present, cholesterol efflux from macrophages is accelerated, which prevents foam cell formation. To produce this efflux, neutral cholesteryl ester hydrolase catalyzes intracellular hydrolysis of cholesteryl esters into free cholesterol in the lysosome (Avart et al. 1999).

Free cholesterol or unesterified cholesterol/phospholipid complexes are transported via *ABCA1* and ABCG1 through cell membranes and are accepted from the membranes by *apoA-I*, which generates preβ (nascent)-HDL particles. In humans and mice, *apoA-I* is produced primarily in the liver and intestine. Extracellular sources of *apoA-I* have been shown to increase cholesterol efflux from macrophages in vitro (Hara and Yokoyama 1991), and the presence of *apoA-I* in the extracellular space is considered to be necessary for the activation of cholesterol efflux through the *ABCA1* pathway (Takahashi et al. 2002). Mutations in the *ABCA1* transporter gene cause familial HDL-deficiency syndromes (Tangier disease) associated with impaired cholesterol efflux and the development of either CAD or splenomegaly dependent on the location of *ABCA1* mutations (Brooks-Wilson et al. 1999). Decreased efflux in *ABCA1* deficiency is connected to enhanced phagocytosis (Bared et al. 2004).

Two possibilities exist for the source of cholesterol utilized by *ABCA1*: plasma membrane or intracellular sources. *ABCA1* may utilize plasma membrane cholesterol from isolated lipid rafts (Drobnik et al. 2002) or caveolae (Sviridov et al. 2001; Arakawa et al. 2000), although one study showed that *ABCA1* does not utilize detergent (Triton)-isolated raft cholesterol (Mendez et al. 2001). Alternatively, *apoA-I* can obtain cholesterol from intracellular sources (Oram et al. 1991; Walter et al. 1994; Rogler et al. 1995) and it was demonstrated that an endolysosomal pool is a preferred source of *ABCA1*-mediated cholesterol efflux (Chen et al. 2001). A kinetic model suggests that cholesterol efflux to *apoA-I* is a two-step process (Gaus et al. 2001). In the first step, some of the plasma membrane cholesterol contributes to a fast initial efflux (possibly from lipid rafts) and leads to a second pathway that mobilizes intracellular cholesterol mobilization. It is demonstrated that in macrophages and fibroblasts, the secretory vesicular transport from the Golgi to the plasma membrane increases in response to *apoA-I*-mediated cholesterol efflux (Zha et al. 2003). This increase was observed in raft-poor and raft-rich vesicle populations originating from the Golgi. In Tangier fibroblasts enhanced vesicular transport in response to *apoA-I* is absent. Therefore *ABCA1* may influence cholesterol efflux in part by enhancing vesicular trafficking from the Golgi to the plasma membrane (Zha et al. 2003).

A newly characterized *apoA-I* high-affinity binding site in the plasma membrane was the β-chain of human ATP-synthase, a major protein complex of the mitochondrial inner membrane also present in the plasma membrane, involved in ATP synthesis (Martinez et al. 2003). It has two major domains, F_0 and F_1, the latter containing five different subunits among which the β-chain interacts with *apoA-I*. It has also been reported that both, the β-chain and α-chain of ATP synthase, are receptors for apoE-enriched HDL in the plasma membrane (Beisiegel et al. 1988). The possible involvement of the F_0/F_1-ATPase in the lipid influx/efflux rheostat together with *ABCA1* and the participating receptors is shown in Fig. 4.

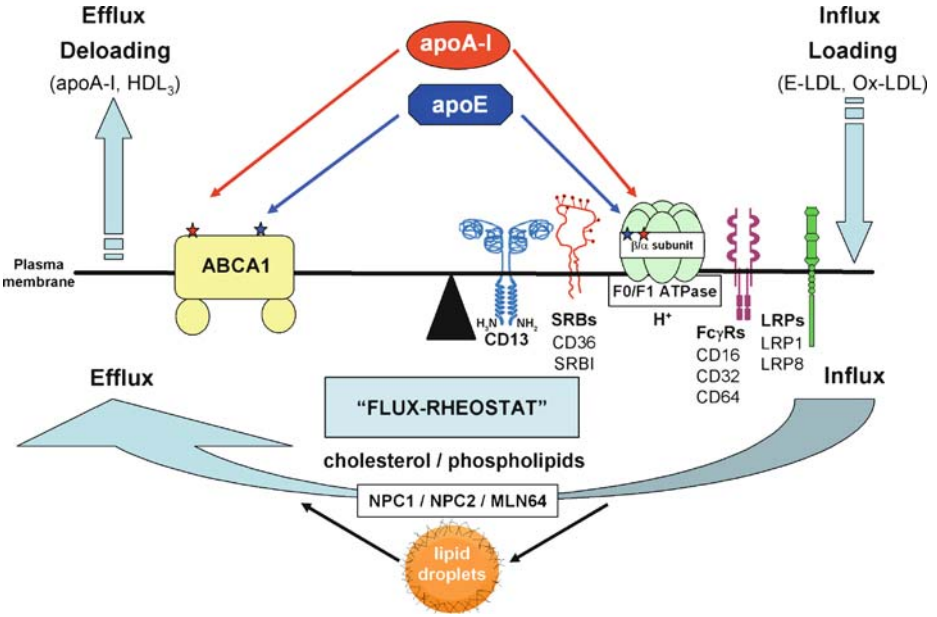

Fig. 4 The lipid influx/efflux rheostat model maintains lipid uptake and export mechanisms in a balance. ATP synthase is regulated by *apoA-I* or apoE leading to enhanced conversion of ATP to ADP. The absence of *apoA-I* would lead to enhanced sinking in phagocytosis since actin can bind ATP, polymerize, and form F-actin which is essential for type II phagocytosis. Hence *apoA-I* could lead to increased influx. On the other hand, *apoA-I* binds to ABCA1 leading to enhanced lipid efflux. Dysfunction of this equilibrium may lead to severe disturbances of cellular lipid traffic. This is obvious in Tangier disease patients where ABCA1 is inoperative and *apoA-I*-dependent cholesterol is absent. Cholesterol influx, however, is enhanced due to *apoA-I*-dependent stimulation of ATP synthase B leading to cholesteryl ester formation and enhanced foam cell formation

Glycosphingolipids and lipid efflux

Glycosphingolipid accumulation inhibits cholesterol efflux in the *ABCA1*/*apoA-I* pathway in fibroblasts treated with lactosylceramide or the glucocerebrosidase inhibitor conduritol B epoxide (Glaros et al. 2005). In addition, *apoA-I*-mediated cholesterol efflux from fibroblasts derived from patients with genetic *glycosphingolipid* storage diseases (Fabry's disease, Sandhoff's disease, and GM1 gangliosidosis) is impaired compared with control cells (Glaros et al. 2005). The accumulation of both *glycosphingolipids* and cholesterol in lipid membrane microdomains may contribute directly to lysosomal accumulation by altering endosomal transport pathways (te et al. 2004). This is consistent with previous proposals that alterations to membrane lipid microdomain structure could contribute to sphingolipidosis (Kobayashi et al. 1999; Simons and Gruenberg 2000; Lusa et al. 2001).

Puri et al. provided evidence supporting a "molecular trap" hypothesis, whereby increased *glycosphingolipid* levels lead to increased cholesterol sequestration in late endosomes/lysosomes (Puri et al. 1999, 2003). Previous work has suggested that cholesterol that accumulates as a result of *glycosphingolipid* storage may not be available to the sensing pathways that normally control intracellular cholesterol balance. Specifically, it has been shown that macrophages induced to store cholesterol by treatment with lactosylceramide or conduritol B epoxide downregulate their apoE secretion (Garner et al. 2002). This is in

contrast to the predicted upregulation of apoE secretion in response to cholesterol loading (Laffitte et al. 2001).

Ceramide and lipid efflux

There is evidence that cholesterol maintains a preferential contact with sphingomyelin in cellular membranes (Patton 1970; Porn et al. 1993; Ohvo et al. 1997). It was found that lipid domain/raft formation is dependent on the sterol component having a structure that allows tight packing with lipids having saturated acyl chains (Xu and London 2000), and small amounts of ceramide significantly stabilize domain/raft formation (Xu et al. 2001). The ability of ceramide to strongly promote domain formation could mean that it can partially substitute for cholesterol under some conditions in vivo. In addition, it could be shown that ceramide selectively displaces cholesterol from ordered lipid domains probably resulting in marked effects on raft structure and function (Megha and London 2004). Sphingomyelinases can modulate sphingomyelin content and cholesterol could either be made available to or sequestered from *ABCA1*. The action of sphingomyelinases on sphingomyelin results in ceramide, a potent lipid-signaling molecule shown to potentiate a variety of cellular events including cell growth, apoptosis, cell differentiation, and the stress response (Kolesnick 1991; Perry and Hannun 1998; Kolesnick et al. 2000; Huwiler et al. 2000). Moreover, cell surface sphingomyelin hydrolysis leads to rapid translocation of plasma membrane cholesterol to the ACAT compartment for cholesterol esterification in fibroblasts (Slotte and Bierman 1988). This confirms that the membrane sphingomyelin content and the interaction with cholesterol is an important determinant of the overall distribution of cholesterol within intact cells. Endogenous ceramide enhances cholesterol efflux to *apoA-I* by increasing the cell surface presence of *ABCA1* (Witting et al. 2003).

ABCA1-interacting proteins and lipid membrane microdomains

The Rho family GTPase Cdc42 directly interacts with *ABCA1* to control filopodia formation, actin organization, and intracellular lipid transport (Diederich et al. 2001; Tsukamoto et al. 2001). We have previously shown (Drobnik et al. 2002) that *ABCA1* and Cdc42 were partially localized in Lubrol- but not in Triton-X DRMs and that *apoA-I* preferentially depleted unesterified cholesterol/phospholipids from Lubrol DRMs, whereas HDL_3 additionally decreased the cholesterol content of Triton-X DRMs.

The relationship of *ABCA1* function to vesicular transport pathways revealed that *ABCA1* rapidly recycles between the cell surface and intracellular compartments (Neufeld et al. 2001). Vesicular traffic pathways are regulated by molecular mechanisms that enable fusion of incoming vesicles as well as formation of distinct transport carriers. Components of these molecular machineries include members of the Rab-family, v-SNAREs, and t-SNAREs, like the syntaxins. An association of *ABCA1* with syntaxin 13 and flotillin-1 and enhanced phagocytosis in *ABCA1*-deficient cells was found, which is reversible upon *ABCA1* transfection (Bared et al. 2004). Syntaxin 13 is preferentially associated with early endosomes, suggesting that specific endosomal compartments may be directly involved in *ABCA1*-mediated lipid efflux. In addition, syntaxin 13 partitions together with *ABCA1* and flotillin-1 into Lubrol DRMs. Syntaxin 13, flotillin-1, and *ABCA1* were also identified as phagosomal proteins associated with the AP-3 pathway indicating the involvement of the phagosomal compart-

ment in *ABCA1*-mediated lipid efflux. It is concluded that *ABCA1* forms a complex with syntaxin 13 and flotillin-1, residing at the plasma membrane and in phagosomes that are partially located in raft microdomains (Bared et al. 2004). Moreover, the interaction with syntaxin 13 and syntaxin 13-interacting protein (pallidin) (Huang L et al. 1999) associates *ABCA1* and ABCG1 to the AP-3 pathway defective in the Hermansky–Pudlack syndrome complex (Wei 2006) and leads to lower HDL levels in pallidin knockout mice as shown in experiments in our laboratory.

ADP-ribosylation factor (ARF)-like 7, ARL7, also contributes to a vesicular transport step between the late endosome and *ABCA1*-accessible cholesterol pools at the plasma membrane apparently linked to the *ABCA1*-mediated Golgi-dependent cholesterol secretion pathway (Engel et al. 2004). The fact that ARL7-mediated stimulation of cholesterol efflux is dependent on the presence of *apoA-I* makes it likely that ARL7 acts in concert with *ABCA1* either by modulating *apoA-I* and/or *ABCA1* endocytic transport or altering the half-life of *ABCA1*.

Fas-associated death domain protein (FADD) was identified as another protein that interacts with the C-terminus of *ABCA1* (Buechler et al. 2002a), and a dominant negative form of FADD or a C-terminal peptide of *ABCA1* markedly reduced the transfer of phospholipids to *apoA-I* in HepG2 cells. FADD was described to redistribute to raft domains after stimulation of T cells with CD95L. The antiapoptotic gene *TOSO* was described to regulate apoptosis by directly binding to FADD through its C-terminal domain (Sigruener et al. 2007), and E-LDL and phagobead internalization upregulates *TOSO* expression enhancing the survival of human macrophages. These data indicate that *ABCA1* function directly relates to the regulation of apoptosis and that both *ABCA1* and E-LDL may promote prolonged survival of macrophages.

In order to identify proteins associated to the *ABCA1* C-terminus, human liver and placenta yeast two-hybrid libraries were screened with the carboxyterminal 144 amino acids of *ABCA1*, which includes a perfect binding domain for PDZ proteins. Using this approach a number of PDZ proteins were identified, including β2-syntrophin. By coimmunoprecipitation, utrophin was found associated with the *ABCA1*/β2-syntrophin complex and we suggest that β2-syntrophin might function as an adaptor protein coupling *ABCA1* via utrophin to the cytoskeleton (Buechler et al. 2002b) similar to the interaction of ICA512 with β2-syntrophin in the insulin secretion pathway. *ABCA1* in the plasma membrane of human macrophages was found to be partially associated with Lubrol rafts, and effluxed choline-phospholipids involve these microdomains. β2-Synthrophin does not colocalize in these rafts, indicating that β2-syntrophin may participate in the retaining of *ABCA1* in cytoplasmic vesicles and in the targeting of *ABCA1* to plasma membrane microdomains when *ABCA1* is released from β2-syntrophin.

DRMs associated with the *ABCA1* pathway using different detergents and density gradient separations were characterized by our laboratory. Various cell types including macrophages and fibroblasts from controls and monogenetic human diseases (*ABCA1*-, NPC1 deficiency) were analyzed. *ABCA1* copurifies only with Lubrol- but not with Triton- or Brij-DRMs. Lubrol-DRM association of *ABCA1* is modulated by cholesterol loading and *apoA-I* stimulated lipid efflux upon *apoA-I* interaction. *ABCA1* may act as a raft disrupter, and this function is disturbed in *ABCA1*-deficient cells as assessed in patient macrophages and fibroblasts. To analyze cell type-dependent dynamic differences of the lipid species composition in DRMs and shedded membrane vesicles from filipodia upon HDL_3/*apoA-I* mediated *ABCA1* activation, quantitative tandem mass spectrometry methods with stable isotope labeling for the analysis of various lipid species from total and fractionated cellular extracts were developed (Binder et al. 2006; Liebisch et al. 2004, 2006). Using this method it was shown that modified lipoprotein incubation of fibroblasts has different effects

on glycerophospholipid synthesis (Binder et al. 2006). In addition, it was demonstrated that *apoA-I* induces a preferential efflux of monounsaturated phosphatidylcholine and medium chain sphingomyelin species from a cellular pool distinct from HDL_3-mediated phospholipid efflux (Schifferer et al. 2007).

Regulation of cellular cholesterol levels through transcription factors

Sterol regulatory element-binding protein

Many genes involved in cholesterol and fatty acid synthesis are regulated by sterol regulatory element-binding protein (SREBP) (Brown and Goldstein 1999). SREBPs are a family of basic helix loop helix leucine zipper transcription factors consisting of three isoforms. SREBP-1a and SREBP-1c are produced from the single gene SREBP-1. SREBP-2 is derived from a separate gene (Brown and Goldstein 1997). In mammalian cells SREBP-2-responsive targets include genes of the cholesterol metabolism such as HMG-CoA synthase and reductase, and SREBP-1c-responsive targets include genes of fatty acid metabolism such as fatty acid synthase and stearoyl-CoA desaturase (Shimano 2001).

The C-terminal domain of SREBP interacts with the C-terminal domain of SREBP-cleavage-activation protein (SCAP). In response to low cellular cholesterol this complex exits the ER and transits to the Golgi apparatus, where two distinct proteases cleave the SREBP precursor to release the transcriptionally active N-terminus (Goldstein et al. 2006). In the presence of cholesterol, the integral membrane proteins, insig-1 and insig-2, are required to retain the SREBP-SCAP complex in the ER. In contrast to most n-3 and n-6 fatty acids, which inhibit SREBP-1 expression, *trans*-9, *trans*-11-conjugated linoleic acid induces SREBP-1 expression (Schmitz and Ecker 2007) and activates ABCG1 by a SREBP-1c-dependent mechanism (Ecker et al. 2007). SCAP ligands are a potent new class of compounds that represent a promising new form of hypolipidemic drugs by upregulation of the LDL receptor and activity (Grand-Perret et al. 2001).

PPARs, RXR, and LXRs

The nuclear receptors peroxisome proliferators-activated receptors (PPARs) and liver X receptors (LXRs) are ligand-activated transcription factors that have been implicated to play an important role in obesity-related metabolic diseases such as hyperlipidemia, insulin resistance, and CAD. These orphan receptors are activated by fatty acids and derivatives and are proposed to control lipid homeostasis in vascular endothelial cells, smooth muscle cells, monocytes, and monocyte-derived macrophages by regulating transcriptional networks involved in lipid metabolism, transport, storage, and elimination (Chawla et al. 2001). Downregulation of several atherogenic genes by PPAR activation suggests that stimulation of PPAR expression and/or activation may have beneficial effects on the progress of atherogenesis.

PPAR and LXR bind, upon heterodimerization with retinoid X receptor (RXR), to specific peroxisome proliferator response elements (PPRE) in the promoter of target genes, thus regulating the transcription of these genes. Both the formation of the PPAR/RXR heterodimer and the subsequent transcriptional activation of the target genes are ligand-dependent. Ligand binding may evoke conformational changes within the DNA-binding

domain, thereby altering the potential to stimulate transcription of target genes. The LXR subfamily is composed of two receptor isoforms, LXRα and LXRβ, and regulates cellular cholesterol level (Kalaany and Mangelsdorf 2006). In addition, LXR activates the transcription of ABC cholesterol transporters. By combining lipid species analysis with transcriptomic profiling (Affymetrix gene array), retinoids as potent inducers of macrophage lipid efflux were identified (Langmann et al. 2005). A strong up-regulation of a cluster of genes involved in cholesterol trafficking including apolipoproteins (apoC-I, apoC-II, apoC-IV, apoE), the scavenger receptor CD36, steroid-27-hydroxylase (CYP27A1), LXRα, *ABCA1*, and ABCG1 was found. As a functional consequence of the retinoid effect, a strong induction of *apoA-I*-dependent lipid efflux was observed indicative of an important role of retinoids in macrophage lipid homeostasis. In addition, LXR inhibit inflammation by antagonizing NF-κ B signaling (Joseph et al. 2003).

The nuclear receptor subfamily of PPARs consists of isoforms α, γ, and δ with distinct expression patterns and biological activities. Although these different members are encoded by separate genes, they have a similar protein structure.

PPARα is expressed in liver, heart, muscle, and kidney where it regulates fatty acid catabolism (Peters et al. 1997) and is the molecular target of the lipid-lowering fibrates (e.g., fenofibrate and gemfibrozil). It was suggested that PPARα may enhance *apoA-I* synthesis in the liver and intestine and thereby may promote *apoA-I*-dependent cholesterol efflux from macrophages and also have anti-inflammatory effects with the vascular wall (Chinetti et al. 2001; Plutzky 2001). Recently it has been reported that Ox-LDL activates PPARα and PPARγ through MAPK-dependent COX-2 expression in macrophages (Taketa et al. 2008).

PPARγ is highly enriched in adipocytes and macrophages and is involved in adipocyte differentiation, lipid storage, and glucose homeostasis (Kubota et al. 1999). It mediates the activity of the insulin-sensitizing thiazolidinediones (e.g., rosiglitazone and pioglitazone). PPARγ is activated by arachidonic acid metabolites derived from the cyclooxygenase and lipoxygenase pathways, e.g., 15-deoxy-Δ-12, 14-prostaglandin J2 (PG-J2), and 15-hydroxyeicosatetraenoic acid (15-HETE) (Nagy et al. 1998; Kliewer et al. 1995; Forman et al. 1995). In addition, fatty acid-derived compounds of Ox-LDL, such as 9- and 13-hydroxyoctadecadienoic acid (9- and 13-HODE), activate PPARγ. Free cholesterol can be converted by the mitochondrial enzyme Cyp27 into 27-hydroxycholesterol, which also activates changes in macrophage foam cell transcription via PPARγ.

Treatment of macrophages with Ox-LDL in vitro induces expression of both PPARγ and LXRα mRNAs (Tontonoz et al. 1998). Internalization of Ox-LDL provides the cell with activators of PPARγ, such as oxidized fatty acids as well as with activators of LXRs such as 27- and 25-hydroxycholesterol (Nagy et al. 1998; Fu et al. 2001). PPARγ ligands can also be produced locally in atherosclerotic lesions through the oxidation of fatty acids by 12/15 lipoxygenase (Huang JT et al. 1999). PPARγ activators inhibit the expression of matrix metalloproteinase 9 (MMP-9) of human macrophages (Marx et al. 1998b) and vascular smooth muscle cells, thus interfering with vascular smooth muscle cell proliferation (Marx et al. 1998a). The production of the inflammatory cytokines tumor necrosis factor (TNF)-α, IL-6, and IL-1β by activated monocytes is inhibited by PPARγ (Jiang et al. 1998) and the transcription of monocyte chemoattractant protein-1 (MCP-1) is decreased. The expression of inducible nitric oxide synthase (iNOS) and the scavenger receptor A in interferon-γ-stimulated mouse macrophages (Ricote et al. 1998) is inhibited by PPARγ. Activation of PPARγ has been shown to enhance CD36 expression of macrophages, which may indicate that PPARγ could stimulate uptake of Ox-LDL and contribute to foam cell formation (Nagy et al. 1998; Tontonoz et al. 1998). These CD36 effects may be compensated by increased expression of *ABCA1* and apoE in macrophages (Chinetti et al. 2001) through the activa-

tion of LXRα. Inhibition of the expression of the adhesion molecules, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), by troglitazone in human endothelial cells (Pasceri et al. 2000) and the induction of the scavenger receptor CLA-1/SR-BI by PPARγ in human macrophages (Chinetti et al. 2000) also suggest that PPARγ may influence monocyte recruitment and cholesterol efflux from foam cells. Earlier results suggested that PPARγ is expressed at high levels in circulating human monocytes and its activation increases the expression of macrophage-specific markers, such as CD14 and CD11b (Tontonoz et al. 1998).

PPARδ is expressed ubiquitously with a less defined function. It has been implicated in keratinocyte differentiation and wound healing and, more recently, in mediating very-low-density lipoprotein (VLDL) signaling of the macrophage (Michalik et al. 2001; Tan et al. 2001; Chawla et al. 2003). Recent studies have identified PPARδ as an important regulator of lipid metabolism, energy expenditure, and atherosclerosis (Evans et al. 2004). In the first human study using the highly specific PPAR-δ agonist GW 501516 it is suggested that up-regulated enzymes are critical to fatty acid oxidation in human cells and enhanced fatty acid and beta-oxidation in skeletal muscle (Sprecher 2007).

Niacin, statins, CETP inhibitors, *apoA-I* mimetics, and ezetimibe as pharmacological modulators of lipid homeostasis

Niacin, also known as nicotinic acid, as a drug for prevention of atherosclerosis is used to lower LDL cholesterol and triglycerides. It acts via the human orphan receptors HM74A (GPR109A) as well as the mouse homolog PUMA-G (protein upregulated in macrophages by interferon-γ). Niacin inhibits lipolysis in adipocytes, an effect which is linked to GPR109A.

It also inhibits the uptake of HDL-*apoA-I* by HepG2 cells without affecting the uptake of cholesteryl ester from HDL (Jin et al. 1999). This is a potential mechanism by which niacin raises HDL levels very effectively. The increase in HDL-*apoA-I* may augment reverse cholesterol transport, allowing removal of greater amounts of cholesterol from the vascular wall. Recently, niacin was shown to activate PPARγ in a monocytoid cell line, presumably via a metabolite of prostaglandin D2. Both cholesterol efflux and the transcription of efflux-related receptors, namely, CD36 and *ABCA1*, were increased by niacin. The effect on CD36 was cyclooxygenase dependent, whereas the effect on *ABCA1* was blocked by an inhibitor of protein kinase A downstream from adenylyl cyclase (Rubic et al. 2004). The most prominent side effects of niacin are flushing effect in skin and lowering of blood pressure. Retard formation and usage in the evening lower these effects. Hyperglycemic responses are minor and manageable, liver toxicity is very infrequent when appropriately dosed, and myopathic potential is absent or minimal even in combination with statins (Guyton 2007). In addition, lipid-lowering therapies such as statins and niacin have demonstrated an ability to reduce serum markers of inflammation (Devaraj et al. 2007). Treatment of patients with rheumatoid arthritis with the TNF-α blockers etanercept, infliximab, and adalimumab has resulted in significant increases of total and HDL cholesterol levels (Seriolo et al. 2006).

Different therapeutic HDL-raising approaches are currently under investigation including cholesteryl ester transfer protein (CETP) inhibitors and infusions of reconstituted HDL and mimetic peptides. Although the CETP inhibitor torcetrapib failed in clinical trials because of increased mortality due to blood pressure raising, HDL-based treatments are promising and worth further exploration. HDL mimetics including *apoA-I* mutants and peptide mimet-

ics of *apoA-I* facilitate reverse cholesterol transport and are thought to be "fast acting" (Pal and Pillarisetti 2007). In addition, when developing new strategies to raise HDL the quality of the resulting HDL has to be considered because HDL quality is more important than HDL cholesterol levels (Navab et al. 2004b). The *apoA-I* mimetic peptide 4F synthesized from D-amino acids (D-4F), administered orally to mice, did not raise HDL-cholesterol concentrations but promoted the formation of pre-β HDL containing increased paraoxonase activity, resulting in significant improvements in HDLs anti-inflammatory properties and their ability to promote cholesterol efflux from macrophages in vitro (Navab et al. 2004a). Oral D-4F also promoted reverse cholesterol efflux from macrophages in vivo. Early human clinical trials are now being carried out on 4F.

Hypercholesterolemia can be treated by the use of ezetimibe, a cholesterol-lowering drug that acts at the brush border of the small intestine. It blocks the absorption of dietary and biliary cholesterol and plant sterols resulting in intracellular cholesterol depletion. Ezetimibe was identified as a molecular target of the NPC1-like transporter. It was demonstrated that ezetimibe can eliminate aminopeptidase N (CD13) from DRM fractions (Orso et al. 2006). The lowering effect of ezetimibe on total and LDL cholesterol can be improved by combination with other lipid-lowering drugs, particularly HMG-CoA reductase inhibitors (statins) (Farnier 2007). The combination of statins with niacin shows enhanced cholesterol-lowering effects and in addition reduces plasma triglycerides and elevates HDL cholesterol (Guyton and Capuzzi 1998).

Conclusion

Depletion of macrophages or suppression of lipid overload may be targets to interrupt perpetuation of vascular remodeling during atherogenesis. Lowering LDL cholesterol by statins is a successful strategy. In addition, improved lipid processing through different receptor entry pathways as well as endolysosomal and cytosolic lipid storage and lipid efflux should be taken into account as further drug targets.

Ezetimibe blocks the absorption of dietary and biliary cholesterol and plant sterols and in combination with cholesterol synthesis inhibitors increases the efficacy of reaching cholesterol target levels and reducing the risk of statin adverse reactions. In addition, nonglucuronidated ezetimibe may lead to impaired lipid influx through disruption of lipid membrane microdomains. Modulation of membrane microdomains that are involved in membrane trafficking, cell polarization, and signaling could be a promising target which needs further investigation to regulate lipid homeostasis either through nutrition or pharmacological agents.

Some combinations of lipid-altering drugs are currently available such as extended-release niacin/lovastatin and ezetimibe/simvastatin, which are more promising for lipid-lowering therapy with stronger effects and decreased adverse reactions.

Fibrates such as PPARα agonists and glitazones such as PPARγ agonists are prominent in affecting lipid metabolism but may have significant side effects. There is still a need for HDL-elevating and reverse cholesterol transport-promoting agents. HDL mimetics including *apoA-I* mutants and peptide mimetics of *apoA-I* that facilitate reverse cholesterol transport could be more promising agents for cholesterol-lowering therapy but need approval in clinical trials.

More research is necessary to develop new drugs to treat high triglyceride/low HDL syndromes and compounds that regulate lipid homeostasis at the different stages of lipid influx

and efflux in order to interrupt chronic remodeling, storage, adhesion, foam cell formation, and calcification.

However, lifestyle change (e.g., smoking cessation, weight reduction, increased physical activity, and heart-healthy diet modification) and blood pressure control should be the first approach to reduce the risk of cardiovascular complications and metabolic problems.

Glossary

Advanced glycation end products (AGEs): AGEs are the result of a chain of chemical reactions after an initial nonenzymatic glycosylation. AGEs can be formed exogenously by heating sugars with fats or proteins, or endogenously through normal metabolism and aging. AGEs are thought to be major factors in aging and age-related chronic diseases.

Apolipoprotein A-I (ApoA-I): ApoA-I is the major protein component of HDL in plasma. The protein helps to clear cholesterol from arteries and promotes cholesterol efflux form tissues to the liver for excretion. It is a cofactor for lecithin cholesterol acyltransferase (LCAT), which is responsible for the formation of most plasma cholesteryl esters.

ATP-binding cassette transporter ABCA1 (member 1 of human transporter subfamily ABCA): ABCA1 is a human protein and gene. This transporter is a major regulator of cellular cholesterol and phospholipids homeostasis. ABCA1 functions as a cholesterol efflux pump in the cellular lipid removal pathway.

Autophagy: Autophagy, or autophagocytosis, is a catabolic process involving the degradation of a cell's own components through the lysosomal machinery. It is a tightly regulated process that plays a normal part in cell growth, development, and homeostasis, helping to maintain a balance between the synthesis, degradation, and subsequent recycling of cellular products. It is a major mechanism by which a starving cell reallocates nutrients from unnecessary processes to more essential processes.

Complement receptor 3 (CD11b/CD18): Integrin alpha M (ITGAM, CR3A or CD11b) and integrin β_2 (ITGB2 or CD18) form the heterodimeric integrin alpha-M beta-2 ($\alpha_M\beta_2$) molecule, also known as macrophage-1 antigen (Mac-1) or complement receptor 3 (CR3). Integrins are integral cell-surface proteins composed of an alpha chain and a beta chain. Integrins are known to participate in cell adhesion and migration as well as cell-surface-mediated signaling. $\alpha_M\beta_2$ is expressed on the surface of many leukocytes involved in the innate immune system. Furthermore, it plays a role in the complement system due to its capacity to bind inactivated complement component 3b (iC3b).

Glycerophospholipids: Glycerophospholipids or phosphoglycerides are glycerol-based phospholipids. They are the main component of biological membranes.

Glycosphingolipids (GSLs): GSLs are ceramide derivatives containing more than one sugar residue and are constituents of lipid membrane microdomains.

Lipopolysaccharide (LPS): LPS is a large molecule consisting of a lipid and a polysaccharide (carbohydrate). LPS acts as the prototypical endotoxin, because it binds the CD14/TLR4/MD2 receptor complex, which promotes the secretion of proinflammatory cytokines in many cell types, but especially in macrophages.

Niemann–Pick disease: Niemann–Pick disease is an autosomal recessive disorder affecting lipid metabolism resulting in an accumulation of harmful amounts of lipids in the spleen, liver, lungs, bone marrow, and brain. There are three variants (A, B, and C) of Niemann–Pick disease based on the genetic cause and the symptoms exhibited by the patient.

Phagocytosis: Phagocytosis is the cellular process of engulfing solid particles by the cell membrane to form an internal phagosome (vacuole formed around a particle). The phagosome is usually delivered to the lysosome where it fuses with the lysosome and forms a phagolysosome.

Phospholipidosis: Phospholipidosis is a lipid storage disorder which is characterized by lamellar body formation and excess phospholipid accumulation within cells. Drug-induced phospholipidosis is an adverse drug reaction that occurs with many cationic amphiphilic drugs.

Receptor for advanced glycation end products (RAGE): RAGE is expressed by many cells in the body (e.g., endothelial cells, smooth muscle cells, or cells of the immune system). When binding AGEs, RAGE contributes to age- and diabetes-related chronic inflammatory diseases such as atherosclerosis, asthma, arthritis, and myocardial infarction.

Tangier disease: Tangier disease is a rare autosomal recessive disorder characterized by a severe reduction in the amount of HDL in the bloodstream, which is a risk factor for CAD. People with Tangier disease have defective ABCA1 transporters resulting in a greatly reduced ability to transport cholesterol out of their cells, leading to an accumulation of cholesterol in many body tissues.

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