Autotaxin (ATX), also known as ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP)2, is a secreted lysophospholipase D (lysoPLD) that hydrolyzes extracellular lysophospholipids into the lipid mediator lysophosphatidic acid (LPA), a lipid mediator that has been implicated in a great diversity of (patho)physiological processes, ranging from lymphocyte homing to tumor progression. Structural and functional studies have revealed that ATX produces the lipid mediator from extracellular lysophosphatidylcholine (LPC) (4–6). ATX-LPA signaling is essential for development and has been implicated in the development of drug-like inhibitors of ATX. Autotaxin (ATX), or ecto-nucleotide pyrophosphatase/phosphodiesterase-2, is a secreted lysophospholipase D that belongs to the ENPP family. The ENPP family consists of seven members ranging from lymphocyte homing to tumor progression. Structural and functional studies have revealed that ATX produces the lipid mediator from extracellular lysophosphatidylcholine (LPC) (4–6). This work was supported by the Dutch Cancer Society (KWF) and the Netherlands Organisation of Scientific Research (NWO; TOP grant to A.P. and W.H.M.).

Supplementary key words ecto-nucleotide pyrophosphatase/phosphodiesterase • lysophosphatidic acid • G protein-coupled receptors • inhibitors

The bioactive product of ATX, LPA, acts on specific G protein-coupled receptors (GPCRs) that activate multiple signaling pathways (6, 12). The biological outcome of LPA signaling depends on LPA receptor (co-)expression patterns and tissue context. The best known cellular responses to LPA include the stimulation of cell migration, proliferation, and survival, but inhibitory responses have also been documented (13, 14). However, the list of biological responses to LPA receptor stimulation is remarkably diverse, as it ranges from mitogenic and chemotactic activities to neurite remodeling and ion channel activation (6, 12). The ATX-LPA signaling axis is essential for vascular and neural development (reviewed in (15)) and has been implicated in a great variety of physiological and pathological processes, including lymphocyte homing (9, 10, 16), pulmonary fibrosis (17, 18), neuropathic pain (19), cardiovascular disease (20), cholestatic pruritus (21), and tumor progression (22–25).

ATX, named after its first discovered activity as an “autocrine motility factor” (26), is synthesized as a prepro-enzyme and, after N-glycosylation and proteolytic maturation, secreted as an active lysoPLD along the classical secretory route (27, 28). ATX acts locally rather than systemically, although ATX is also present in the circulation where it accounts for maintaining plasma LPA levels. Circulating

Abbreviations: ATX, autotaxin; ENPP, ecto-nucleotide pyrophosphatase/phosphodiesterase; GPCR, G protein-coupled receptor; HS, heparan sulfate; HSFG, heparan sulfate proteoglycan; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; lysoPLD, lysophospholipase D; NPP, nucleotide pyrophosphatase/phosphodiesterase, NUC, nucleoside-like; PA, phosphatidic acid; PDE, phosphodiesterase; PLA1, phospholipase A1; PLD, phospholipase D; S1P, sphingosine 1-phosphate; SMB, somatomedin B-like; xNPP, nucleotide pyrophosphatase/phosphodiesterase from Xanthomonas campestris.

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ATX has as a short half-life, however, as it is rapidly cleared by the liver (29). Likely sources of plasma ATX are the lymphatic high endothelial venules and adipose tissue, which express and secrete ATX at high levels (10, 30). Despite much progress made in understanding ATX-LPA receptor signaling, the inner workings of ATX have long remained elusive. Recent structural studies have changed the situation and provide new insights into ATX functioning, and what makes ATX a unique lysoPLD.

In this review, we discuss our current understanding of ATX structure-function relationships, isoforms and signaling mechanisms, and how ATX binds to its targets cells. We also briefly discuss the development of small-molecule inhibitors of ATX and their binding modes.

**LPA AND ITS RECEPTORS**

LPA acts on six distinct GPCRs (termed LPA₁–₆ or LPAR1–6), which are differentially expressed and show both overlapping and distinct signaling properties (12). LPA receptors can be divided into two subfamilies. The classical LPA₁–₃ receptors belong to the so-called “endothelial differentiation gene” (Edg) family, which includes five GPCRs for the lipid mediator sphingosine 1-phosphate (S1P) (31). Three additional LPA receptors (LPA₄–₆) are more closely related to the purinergic receptor (P2Y) family of GPCRs (32).

LPA and its major precursor LPC comprises various molecular species that vary in the length and degree of saturation of their fatty acid chain, which is esterified at the sn-1 (or, less common, sn-2) position of the glycerol backbone. Ether-linked 1-alkyl-LPA and 1-alkenyl-LPA species also exist, but are much less abundant. All six LPA receptors can be stimulated by 1-acyl-LPA, albeit with different potencies. Of note, some LPA receptors (LPA₁ and LPA₂) prefer unsaturated 2-acyl-LPA as a ligand, while LPA₃ exhibits a strong preference for ether-linked 1-alkyl-LPA species (13, 33). Detailed accounts of LPA receptor signaling pathways, their impact on gene expression, and biological outcomes can be found elsewhere (6, 12, 34).

**LPA PRODUCTION AND BIOAVAILABILITY**

All LPA species examined can be produced by ATX-mediated choline release from the corresponding LPC substrate. LPC is by far the most abundant lysophospholipid in plasma and serum (concentration 100–200 μM), where it is bound mainly to albumin, but also to lipoproteins and α-1-acid glycoprotein (35–37). Lysophospholipids such as LPC and lysophosphatidylethanolamine can exist as free monomers in aqueous solutions, with critical micelle concentrations in the low micromolar range, while they bind to albumin with relatively low affinity [Kᵦ~2–5 μM (38, 39)]. Therefore, free monomeric LPC exists in dynamic equilibrium with the large carrier-bound pool(s) and most likely serves as the physiological substrate for ATX. Consistent with this, a few micromoles of free LPC is sufficient for ATX to mediate LPA receptor activation (5, 13).

While ATX is the primary LPA-producing phospholipase, a second but less common route of LPA production involves the hydrolysis of phosphatidic acid (PA) in the outer leaflet of the plasma membrane by membrane-associated phospholipase A₁ (PA-PLA₁) (7, 40). The resulting unsaturated 2-acyl-LPA acts preferentially on the LPA₆ receptor. Studies in mice show that the PA-PLA₁-LPA₆ signaling axis regulates hair follicle development (40), and that ATX is dispensable for this process (41). It is further of note that bioactive LPA can also be produced by exogenous PLDs, notably those from *Streptomyces chromofuscus* (42) and the toxic PLDs from spider (*Loxosceles*) and certain pathogenic corynebacteria (43, 44). The latter PLDs hydrolyze both sphingomyelin and various lysocephospholipids to produce ceramide-1-phosphate and bioactive LPA, respectively. It is currently unclear to what extent excessive LPA production contributes to the toxicity of these exogenous PLDs.

In a cellular context, newly produced LPA is rapidly degraded by membrane-associated lipid phosphatase phosphatases that dephosphorylate LPA into biologically inactive mono-acylglycerol (45–48). In cell-free seminal plasma, on the other hand, LPA is degraded by soluble prostatic acid phosphatase (49). When injected intravenously, LPA is rapidly cleared from the circulation by hepatic uptake, most likely in the liver sinusoidal endothelial cells (50), as is radio-labeled ATX (29). The bioavailability of LPA may also be regulated by LPA-binding proteins. For example, in the presence of albumin, LPA activates LPA₁ and LPA₂ receptors, but not LPA₃ receptors (51). High-affinity binding to plasma gelsolin may also influence LPA’s bioavailability (52, 53). In summary then, the local level of receptor-active LPA is determined by a complex interplay between ATX, free lysophospholipid substrates, lipid phosphatases, and LPA-binding proteins.

**ATX AND ITS ISOFORMS**

ATX and its closest relatives, ENPP1 and ENPP3, are multi-domain proteins consisting of two N-terminal Cy-rich somatomedin B-like (SMB) domains, a central catalytic phosphodiesterase (PDE) domain (approximately 400 amino acids), and a C-terminal nuclease-like (NUC) domain that is catalytically inactive (Fig. 1). Despite their structural similarities, the three ENPPs have very different physiological and catalytic activities. ATX functions as a lysoPLD, ENPP1 is a membrane-bound nucleotide pyrophosphatase (2), and ENPP3 may preferentially hydrolyze nucleotide sugars (34). Although ATX is capable of hydrolyzing nucleotides in vitro, the apparent affinity of ATX for LPA is some 10-fold higher than for nucleotides and, furthermore, extracellular nucleotide levels are normally very low. In fact, all known biological effects of ATX are attributable to LPA production and subsequent receptor stimulation. However, additional noncatalytic functions of ATX cannot be excluded, as will be discussed below.
The intricacy of ATX-LPA signaling is further enhanced by alternative splicing of ATX mRNA, which gives rise to distinct isoforms (Fig. 1). The three best known splice variants of ATX, termed α, β, and γ, differ by the presence or absence of sequences encoded by exons 12 and 21 (6, 55). A recently identified fourth isoform (ATXδ) is nearly identical to ATXβ, as it lacks four residues in the "lasso loop" or L2 linker region that connects the PDE and NUC domains (56) (Fig. 1). However, isoform-specific functions of ATX have long remained uncharacterized. The canonical and most predominant isoform, termed ATXα, was originally cloned from teratocarcinoma cells and later found to be identical to plasma lysoPLD (4, 5), accounting for LPA production in the circulation, although ATXδ may contribute as well. Virtually all of our current understanding of ATX is derived from studies on ATXβ.

The "long" isoform, termed ATXα, is the original melanoma-derived "autocrine motility factor" (26). ATXα is less widely expressed and is characterized by a 52-residue polybasic insertion (encoded by exon 12) in the heart of the catalytic domain. The "brain-specific" ATXγ isoform contains a 25-aa insertion (encoded by exon 21) close to the catalytic domain (55, 57, 58). It is currently unclear whether any of the ATX isoforms are specifically associated with a distinct (patho)physiological condition.

All ATX isoforms display similar lysoPLD activities and substrate hydrolysis follows simple Michaelis-Menten kinetics, with no evidence for an interfacial activation mechanism and consistent with the notion that ATX acts on free lysophospholipids in the aqueous phase. However, there are significant differences in kinetic parameters between natural and artificial substrates, such as LPC versus fluorescent analogs (60, 61).

Because the insertions in ATXα and ATXγ appear not to affect catalytic activity, they could serve to confer distinct cellular localization, processing, or binding partner preference. On the basis of the ATXβ structure (see below), it is safe to conclude that those insertions do not perturb the structure of the mature ATXα and ATXγ proteins. We recently showed that ATXα binds specifically to negatively charged heparin with high affinity ($K_r \sim 10^{-8} M$), most likely through the Arg/Lys-rich clusters in the insertion, whereas ATXδ does not (59). Heparin enhanced the lysoPLD activity of ATXα toward LPC up to 2-fold, but it had no detectable effect on the activity of ATXβ (59).

Heparin is structurally very similar to heparan sulfate (HS), which is present as HS proteoglycans (HSPGs) at the cell surface and in extracellular matrices, where it recruits growth factors, chemokines, and other molecules to fine tune signaling events (62). Indeed, ATXα, but not ATXβ, was found to bind abundantly to cultured mammalian cells in a manner strictly dependent on HS (59). Thus, by mediating bindings to HSPGs, the ATXα insertion loop likely serves to target LPA production close to the LPA receptors. The insertion loop also contains a conserved furin cleavage site, but the functional significance of ATXα cleavage at this site is unclear (59). As heparin stimulates ATXα activity in vitro, binding of ATXα to HSPGs may not only target LPA production to the plasma membrane, but also increase the catalytic efficiency of this particular isoform locally. Cocrystallization studies using defined heparin fragments should help determine the precise heparin/HS-binding mode of ATXα.

### ATX Structure-Function

Structural studies have revealed how the different domains of ATX are organized and interact, and what makes ATX a unique lysoPLD (63, 64). The crystal structure of ATXβ (Fig. 2) shows that the central catalytic PDE domain interacts extensively with the SMB domains on one side and with the NUC domain on the other. This interaction...
is strengthened by an N-linked glycan between both domains and by an inter-domain disulfide bridge. In addition, a long “lasso” loop, starting at the end of the PDE domain, wraps tightly around the NUC domain and enters the NUC fold from the opposite side. All these features serve to maintain the structural rigidity of the ATX catalytic domain (63, 64). The NUC domain tightly binds Ca²⁺ via an EF hand-like motif, the precise function of which remains to be determined (63).

**A shallow groove and a deep hydrophobic pocket**

The catalytic domain of ATX and that of ENPP1, whose structure was also recently determined (65, 66), is structurally similar to that of the prototypic nucleotide pyrophosphatase/phosphodiesterase (NPP) from *Xanthomonas axonopodis* (XaNPP), an evolutionary relative of alkaline phosphatases (67). The catalytic sites in ATX, ENPP1, and XaNPP are almost identical, with the nucleophile Thr in proximity to two zinc ions, coordinated by conserved His and Asp residues. All three enzymes share a shallow groove, capable of binding the substrate nucleotides and directing the phosphodiester bond for catalysis, as revealed by the structures of XaNPP and ENPP1 in complex with various nucleotides. The same shallow groove also accommodates the glycerol moiety of lysophospholipid substrates (64). Yet the glycerol moiety is not critical for recognition by ATX because a nonglycerol-lysophospholipid, notably sphingosylphosphorylcholine, also can serve as a good substrate for ATX (68).

The ATX catalytic domain is unique in that it has evolved a deep hydrophobic pocket that is not found in ENPP1 (65, 66) or, to the best of our knowledge, in any other phospholipase. This pocket has an estimated volume of about 800 Å³, is about 15 Å deep, and is located down from the shallow groove, sculpted inside the hydrophobic core of the catalytic domain (Fig. 2 and Fig. 3). Formation of this pocket has been made possible by the deletion of a 18-aa stretch, unique to ATX. This was apparently the crucial evolutionary event that allowed ATX to function specifically as a lysoPLD. The pocket can readily accommodate lysospholipid as shown by cocrystal structures of ATX and various LPA species, and confirmed by mutational and biochemical studies (64). It appears that the acyl chains of LPA adopt distinct conformations in the pocket, while the pocket itself shows some structural plasticity (64). The short-chain, saturated species, 14:0 and 16:0, are readily accommodated in the pocket, whereas 18:0 does not seem to fit. In contrast, 18:1 and 18:3 bend at the unsaturated bonds, while unsaturated 22:6 adopts a U-shaped conformation in the pocket (64). It remains to be seen if the observed conformations of bound LPA are identical to those adopted by the respective LPC substrates, or whether they represent an intermediate conformation prior to LPA release.

The lipid-binding pocket and active site in ATX appear freely accessible to solvent and lipid substrates (63, 64). This contrasts to the canonical phospholipases that function as interfacial enzymes, where the active site is occluded by a lid or an auto-inhibitory loop that is removed upon interaction with membranes, thereby allowing the lipid substrate to bind. Typical examples of interfacial enzymes are the secreted PLA2s (69), where catalysis involves facilitated phospholipid diffusion from the membrane bilayer directly into the catalytic site (70). But ATX would not need a deep lipid-binding pocket if catalysis would take place at the membrane-water interface (or carrier surface, for that matter). Once free LPC is bound in the ATX pocket, catalysis may be driven through “substrate destabilization.” In this process, the choline head group in close proximity to the zinc ions destabilizes the complex and lowers the activation energy barrier of the PDE reaction (71).

**An open tunnel**

An unexpected and intriguing feature of the ATX catalytic domain is the presence of an open tunnel (or...
“channel”), which forms a sort of “T-junction” with the shallow groove (63, 64). This tunnel is absent in ENPP1 and the bacterial NPP. The walls of this tunnel are formed partially by the catalytic domain, and partially by the first SMB domain. The deletion of the 18-aa stretch in ATX, responsible for the formation of the lipid-binding pocket, may also have facilitated the formation of this tunnel, allowing the interaction with the SMB1 domain. The ATX tunnel is partially hydrophobic in nature, but at least one of its walls is hydrophilic (Fig. 3). It has been suggested that the tunnel functions as an LPA exit channel, as inferred from the observed electron density maps that allowed modeling of LPA acyl chains (64, 72). This could allow delivery of LPA to its cognate GPCRs. Although attractive, this model remains to be validated experimentally. Delivery of LPA from the pocket into the tunnel involves a relatively long path, in which case significant structural rearrangements must occur. Given the nature and the size of the tunnel, a variety of molecules may also be accommodated, at least transiently. Its proximity to the active site suggests implications for catalysis and/or product uptake and delivery.

The SMB domains and integrin binding

SMB domains are known to mediate protein-protein interactions. The two N-terminal SMB domains in ATX are of special interest as they interact extensively with the catalytic domain. Both SMB domains are structurally similar, but have differential intra- and inter-molecular interactions. While SMB1 is involved in the formation of the tunnel, the SMB2 surface is in the best position to engage with other binding partners (63, 75). Indeed, ATX binds to activated platelets and other cells through β1 and β3 integrins via its N-terminal SMB2 domain (73, 74). ATX-integrin binding provides one mechanism to localize LPA production to the cell surface. The SMB2 domain contains a canonical RGD integrin-binding motif, but mutagenesis studies revealed that ATX-integrin interaction is RGD-independent (63). In fact, the structural context of the RGD motif lacks the required flexibility to bind integrins. Precisely how SMB2 interacts with integrin β subunits remains to be explored.

ATX also binds to activated lymphocytes via integrins, an interaction that leads to enhanced lymphocyte motility in response to localized LPA production (9, 10, 16). The binding site on ATX is unknown, but it is of note that a known integrin-binding motif (LDV) on the surface of the PDE domain is exposed and probably accessible for integrin interaction (72). In conclusion, the available evidence supports a model in which distinct domains mediate ATX binding to integrins, thus serving to localize ATX to the plasma membrane (73).

Integrins not only function as cell-matrix adhesion sites, but also mediate outside-in signaling events. Recent evidence suggests that ATX may exert noncatalytic signaling functions via cell-surface integrins. Specifically, ATX-mediated directional migration of MDA-MB-231 carcinoma cells was not completely abrogated upon full inhibition of lysoPLD activity (75). Furthermore, the isolated SMB domains of ATX were still capable of stimulating cell migration, although the effect was rather modest (75). Collectively, the data support the notion that ATX may exert LPA-independent functions in an integrin-dependent manner, a scenario that warrants further investigation.

ATX AS A DRUG TARGET: SMALL-MOLECULE INHIBITORS

Given the involvement of ATX-LPA receptor signaling in several pathologies, it is logical that much effort has been spent in developing specific ATX inhibitors both in academia and in industry (76). As an extracellular PDE, ATX is an attractive and highly druggable target. Some first-generation ATX inhibitors have been based on the finding that LPA and S1P can inhibit ATX activity against very low LPC concentrations, nucleotides, and artificial substrates (K, about 100 nM) (77). However, as we discussed previously, there is no evidence that ATX is subject to product inhibition by LPA under physiological conditions (72). Nevertheless, a number of nonhydrolyzable LPA analogs have been developed as ATX inhibitors, but their potency is very poor when tested in ATX-mediated LPC hydrolysis assays. It therefore seems unlikely that the reported in vivo effects of those LPA analogs are attributable to ATX inhibition. In fact, LPA analogs are rapidly cleared from the circulation and have as yet not been shown to lower plasma LPA levels (50).

Non-lipid small-molecule inhibitors obviously hold more promise. High-throughput screening has identified thiazolidinedione-based compounds as a new class of ATX inhibitors (78). Their potency was increased dramatically by introduction of a boronic acid moiety, designed to target the catalytic Thr residue in ATX. Similar results were obtained with compounds from an independent screen (79). Injection of a boronic acid-based ATX inhibitor into mice results in a rapid fall in plasma LPA levels (78), consistent with LPA being rapidly cleared from the circulation (50). The crystal structure of ATX in complex with a boronic acid-based inhibitor (HA155) revealed that it forms a reversibly covalent bond with the Thr nucleophile in the lipid-binding pocket of ATX (63). Another small-molecule inhibitor of ATX, termed PF-8380, shows adequate oral bioavailability and potency in reducing LPA levels in plasma and at sites of inflammation (80), but precisely how the compound binds to ATX is unknown.

The tripartite ATX binding site, featuring the groove, pocket, and surface, offers much potential for selective and specific inhibitors. While both the groove and pocket of ATX are well characterized for their binding of substrates and inhibitors, the open tunnel may be an attractive target for inhibitor design as well. Figure 4 shows characteristic bindings poses for lipids and inhibitors in ATX, and for nucleotides in ENPP1. The lipid
CONCLUDING REMARKS

ATX is arguably the most fascinating member of the ENPP family, as it is the major LPA-producing enzyme being involved in a great diversity of physiological and pathological processes. Crystal structures of ATX have answered many outstanding questions but, naturally, also have raised many new ones. On the basis of current evidence, Fig. 5 summarizes the domain structure, substrate preference, and cell-binding modes of ATX and its closest relatives, ENPP1 and ENNP3. Binding of ATX to activated integrins via its N-terminal SMB2 domain provides one mechanism for localized production of LPA close to its cognate receptors. Binding of ATX to cell-surface HSPGs, via its polybasic insertion loop, represents an additional isoform-specific mechanism for spatially and temporally restricted LPA production. Precisely how ATX activity is regulated, what the structural determinants of and nucleotide products bound to ATX and ENPP1 suggest that many additional cores can be envisaged in the further development of inhibitors [see also (81)]. The ATX inhibitors structurally characterized until now share common characteristics and explore the hydrophobic pocket with fluoro-benzene and chloro-benzene moieties. The reversible covalent bond of the boron atom with the Thr nucleophile is a special case that has not been explored in drug development, with the notable exception of the proteasome inhibitor Bortezomib (82). As ATX is an extracellular target, boron-based chemistry might be well tolerated in lead compounds and drug candidates in vivo. Novel small-molecule inhibitors with adequate pharmacokinetics should serve as useful tools for elucidating the role of ATX in normal physiology and pathophysiology in mice, and hopefully may be applied in future clinical studies.

Fig. 4. Structures of various substrates and inhibitors bound to ATX/ENPP2 and ENPP1. Top row, structures of distinct LPA species bound to ATX (64). Middle row, structures of small-molecule inhibitors bound to ATX (78, 79). Bottom row, structures of nucleotides bound to ENPP1 (66). The structures shown are available in the PDB. The structure of 3BoA (79) has been re-refined and manually adjusted to impose the correct geometry of the ligand (84) (A. Perrakis, unpublished observations communicated to the authors of the original publication).

Fig. 5. Cartoon summarizing the domain structure and cell-surface localization of ENPP1, ATX/ENPP2 (α and β isoforms), and ENPP3. PPi, pyrophosphate. For details and discussion see text.
ATX-integrin binding are, and how the LPA product is released are questions that remain to be addressed. And, last but not least, the function of the open tunnel still remains enigmatic. Future structure-function analysis will undoubtedly shed more light on these issues.  

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