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JB Review

Autotaxin—an LPA producing enzyme with diverse functions

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Autotaxin (ATX) is an ecto-enzyme responsible for lysophosphatidic acid (LPA) production in blood. ATX is present in various biological fluids such as cerebrospinal and seminal fluids and accounts for bulk LPA production in these fluids. ATX is a member of the nucleotide pyrophosphatase/phosphodiesterase (NPP) family and was originally isolated from conditioned medium of melanoma cells as an autocrine motility stimulating factor. LPA, a second-generation lipid mediator, binds to its cognate G protein-coupled receptors through which it exerts a number of biological functions including influencing cell motility and proliferation stimulating activity. Some of the biological roles of LPA can be mediated by ATX. However, there are other LPA-producing pathways independent of ATX. The accumulating evidences for physiological and pathological functions of ATX strongly support that ATX is an important therapeutic target. This review summarizes the historical aspects, structural basis, pathophysiological functions identified in mice studies and clinical relevance discovered by measuring the blood ATX level in human. The general features and functions of each NPP family member will be also briefly reviewed. The presence of the ATX gene in other model organisms and recently developed ATX inhibitors, both of which will be definitely useful for further functional analysis of ATX, will also be mentioned.

Keywords: autotaxin/G protein-coupled receptor/lysophosphatidic acid/nucleotide pyrophosphatase/phosphodiesterase.

Abbreviations: ATX, autotaxin; GPCR, G-protein-coupled receptor; LPA, lysophosphatidic acid; LPP, lipid phosphate phosphatase; lysoPLD, lysophospholipase D; PA, phosphatidic acid; PAP, prostatic acid phosphatase; PLA, phospholipase A.

Lysophosphatidic acid (LPA) is the simplest phospholipid but induces many kinds of cellular responses including cellular proliferation, prevention of apoptosis, cell migration, cytokine and chemokine secretion, platelet aggregation, smooth muscle contraction, transformation of smooth muscle cells and neurite retraction (*1*, *2*). In addition, LPA has been implicated in certain human diseases such as arteriosclerosis (*3*) and cancer cell invasion (*4*). It is likely that most of the LPA actions are explained by G-protein-coupled receptors (GPCR) specific to LPA, although LPA was reported to activate the nuclear-type receptor, PPARγ (*5*). Currently, there are at least six identified GPCRs for LPA, LPA_{1-6} (*6*–*13*). From studies of knockout mice and genetic disorders in humans, much has been learned about the physiological roles of LPA through a series of studies on LPA actions and its receptors (*14*).

In contrast to the mechanisms of LPA action through LPA receptors, the molecular mechanisms of LPA production are poorly understood. LPA is produced under various conditions both in cells and in biological fluids, where multiple synthetic reactions occur. Recent studies identified that LPA is produced via at least two routes (*15*, *16*). In both routes LPA is produced by a degradative reaction from phospholipids (PLs). In biological fluids such as serum and plasma, LPA is converted from lysophospholipids (LPLs). In contrast, in cells such as platelets and cancer cells, LPA is converted from phosphatidic acid (PA). In the former route, PLs are first converted to LPLs such as lysophosphatidylcholine (LPC) and then to LPA (Fig. 1). In the latter route, phosphatidic acid (PA) is first generated and then PA is converted to LPA (Fig. 1). Recent studies revealed that a secreted enzyme named PA-selective phospholipsae A_1 α ($PA\text{-}PLA_1α$/LIPH) has a critical role by hydrolysing PA to generate LPA (*17*) (Fig. 1). Previous studies have indicated that the former route explains the bulk LPA production *in vivo*, especially in blood and that autotaxin (ATX), a multifunctional ecto-phosphodiesterase, is responsible for the LPA production (Fig. 1). In this review, we will mainly focus on the functions as well as structural and biochemical properties of ATX. We also review recent clinical studies of ATX and small molecules that modulate the functions of ATX.

Brief history

ATX was originally identified as a tumour cell autocrine motility factor towards malignant cancer cells and then purified from the conditioned medium of A2058 melanoma cells, where it elicits chemotactic and chemokinetic cellular responses at picomolar to nanomolar concentrations in a pertussis toxin sensitive manner (*18*). Sequencing the cDNA clone of ATX revealed that ATX has high homology with a rat brain nucleotide pyrophosphatase (PD-1α) (*19*) and to a

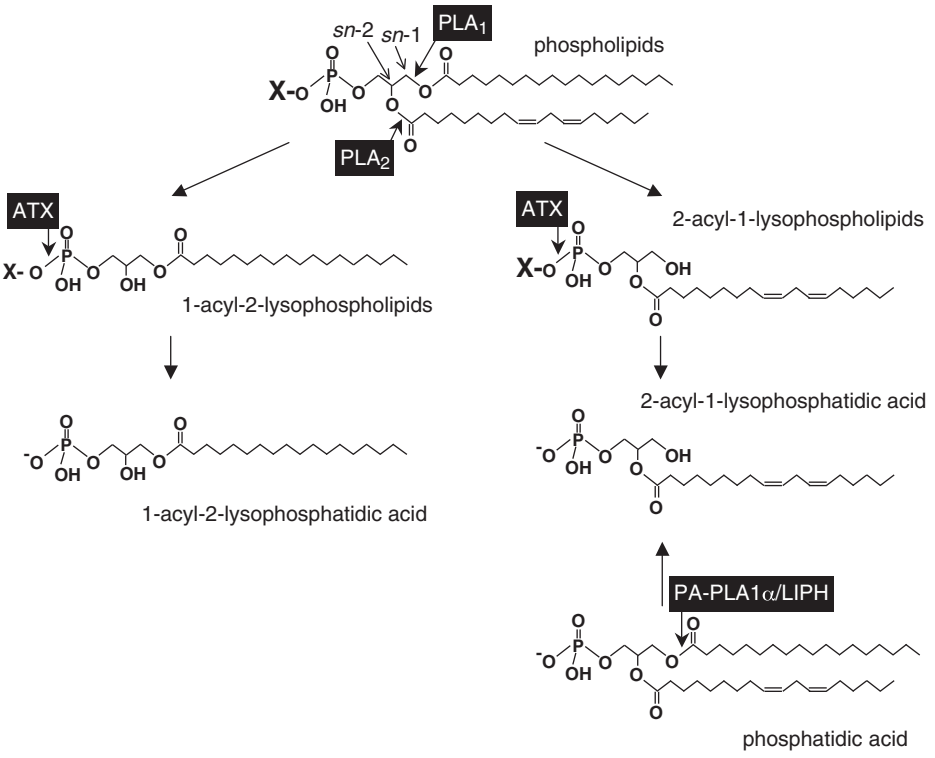

Fig. 1 ATX produces LPA through hydrolysing other lysophospholipids. ATX hydrolyses the phosphodiester bonds in lysophospholipids. Two types of lysophospholipids can be observed in biological fluids. One is the lysophospholipids with a saturated fatty acid, and the other is the one with an unsaturated fatty acid. Since the majority of fatty acids at the sn-1 or sn-2 position consist of saturated and unsaturated fatty acids, respectively, it is predicted that ATX produces LPA with a saturated fatty acid or an unsaturated fatty acid at *sn*-1 or *sn*-2 position, respectively.

lesser extent with a rat neural differentiation antigen (gp130) (*20*) and the plasma cell glycoprotein-1 (PC-1) (*21*, *22*), which has been demonstrated to have nucleotide pyrophosphatase/phosphodiesterase (NPP) activities. Like PC-1, ATX hydrolyses nucleotide pyrophosphatase substrates and its catalytic activity was shown to be essential for stimulating effects on cellular motility (*23*, *24*). However, it was still not clear how extracellular nucleotide hydrolysis can explain the motility-stimulating capacity of ATX. This question was resolved by the identification of ATX as a lipid mediator-producing enzyme.

The molecular basis of ATX function was not uncovered until the identification of ATX as an enzyme which produced a lipid mediator, LPA (*25*, *26*). LPA is one of the smallest and structurally simplest PLs (Fig. 1A) but has numerous biological activities. LPA stimulates proliferation and motility of many cell types (*1*, *2*). It also induces retraction of neurite outgrowth, calcium ion mobilization, elevation and depression of cAMP level and activates inositol phosphate metabolism mainly through GPCRs specific to LPA (*27*). LPA was originally identified as a hypertensive factor in a soybean PL fraction (*28*). Later LPA was found to be present in blood, especially in incubated plasma, and is responsible for the hypertensive activity of the plasma (*29*). Because freshly prepared plasma does not have hypertensive activity, it is postulated that LPA is produced in the plasma itself (*29*). Further studies indicated that LPA is produced from LPC and that lysophospholipase D (lysoPLD) activity, which converts LPC to LPA, is responsible for the LPA production (*29*) (Fig. 1B). In 2002, two groups including ours purified this enzyme from serum samples and identified it as ATX (*25*, *26*). LysoPLD activity was also detected in the conditioned-medium of adipocytes and later identified as ATX (*30*, *31*). The following experiments supported that LPA is a real product of ATX. First, affinity of ATX for LPC is much higher than that for nucleotides, suggesting that LPC is likely to serve as a physiological substrate for ATX (*25*). Second, the cell migration-stimulating activity of ATX was dramatically enhanced by the presence of LPC in the culture media (*25*). Third, the cell motility-stimulating activity of ATX was absent in LPA receptor (LPA_1)-deficient fibroblasts (*32*). Taken together, this data indicated that ATX exerts its activity through endogenous LPA production by its lysoPLD activity. Today ATX is recognized as a major LPA-producing enzyme in serum based on the following observations: in human serum samples the LPA level strongly correlates with the ATX level (*33*), in ATX heterozygote mice both ATX protein and LPA levels are just half of wild type animals (*34*, *35*), and both lysoPLD activity and LPA production were completely absent in ATX-depleted serum (*34*, *36*). It is believed that many biological roles of LPA will be clarified through the studies of ATX as well as through the studies of LPA receptors.

Structural features of ATX

ATX is a member of the NPPs [or ectonucleotide pyrophosphatase/phosphodiesterases (ENPPs)] family

and is also referred as NPP2 (or ENPP2). The NPP family consists of seven members (NPP1-7). Each contains a catalytic domain, which is responsible for catalytic activity (Fig. 2). In addition, like its closest family members, NPP1 and NPP3, ATX has two additional domains, a somatomedin-B-like domain and a nuclease-like domain, which are located at the N-terminus and C-terminus of the protein, respectively. The somatomedin-B-like domain is rich in cysteine residues and contains a RGD tripeptide motif that is possibly involved in cell-extracellular matrix interactions (*37*). The nuclease-like domain contains an EF hand-like motif and is structurally similar to the DNA- or RNA-non-specific endonucleases. However it may lack catalytic activity because the amino acid residues essential for the nuclease activity are mutated (*37*). Recent mutagenesis studies have suggested that all three domains are essential for catalytic activity, although the precise role of the two non-catalytic domains remains to be determined. It was shown that the catalytic domain and nuclease-like domains are covalently linked via a disulfide bond between C413 and C805 (in human ATX), which is shown to be essential for catalytic activity (*38*). It is also suggested that the C-terminus of ATX has some role in the secretion of ATX because mutant ATX lacking C-terminal 12 amino acid residues is not secreted (*38*). It was once proposed that, like NPP1 and NPP3, ATX is a type II membrane protein and is cleaved to form soluble protein. However, recent studies have suggested that hydrophobic residues at the N-terminus of ATX functions as a signal sequence and thus ATX is a secreted protein (*39*, *40*). ATX has four possible *N*-glycosylation sites and N542-linked glycan was suggested to be essential for catalytic activity (*41*). Finally, three alternative splicing variants of human ATX have been reported. Tetracarcinoma- and melanoma-derived ATX is referred as ATXt and ATXm, respectively (*24*, *23*). PD-1α is known as a 'brain-specific' isoform (*42*, *43*). The differences in the catalytic activity of biological significance between each isoform are still elusive.

Substrate specificities of ATX

ATX exhibits phospholipase D activity against LPLs, thus once was named lysoPLD. No diacyl phospholipids are hydrolysed by ATX. ATX hydrolyses various LPLs including lysophosphatidylcholine (LPC) (*25*), lysophosphatidylethanolamine (LPE) and lysophosphatidylserine (LPS) (*44*). ATX also acts on sphingosylphosphorylcholine (SPC) to produce sphingosine 1-phosphate (S1P) (*45*), a similar bioactive LPL to LPA. Thus, it is unlikely that ATX recognizes the head group of a LPL. LPC is present in plasma at a concentration of several hundred micromolar and is

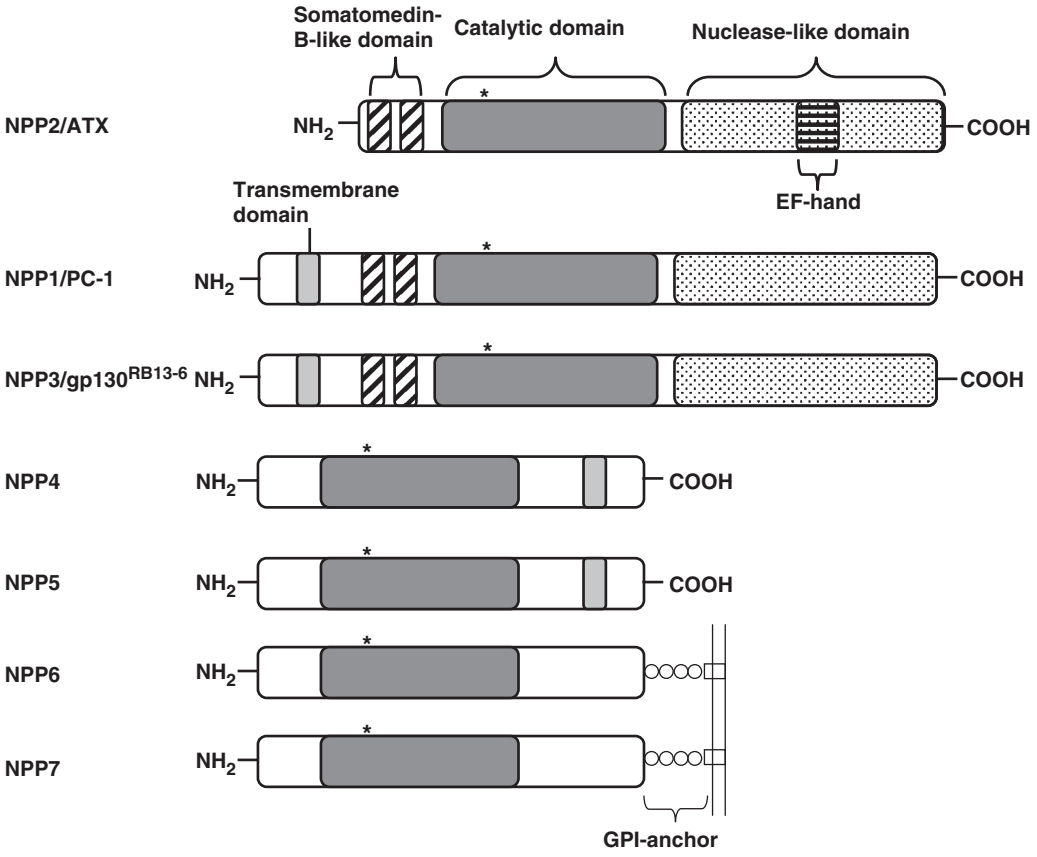

Fig. 2 The primary structures of NPP family protein. NPP family proteins can be classified into two groups. NPP1-3 contains multidomains including somatomedin-B-like domain, catalytic domain and nuclease-like domain. In contrast, NPP4-7 contains only catalytic domain. Asterisk indicates the catalytic centre responsible for the catalytic activity in each NPP family protein. Note that NPP6 and NPP7 are covalently attached to plasma membrane by GPI anchor.

mainly produced from PC on lipoprotein. In addition to LPC, LPE and LPS are produced in serum in a blood coagulation-dependent manner and are converted to LPA by ATX (*44*). Thus, these LPLs are also physiological substrates of ATX. In contrast SPC concentration in plasma is too low (~μM) to consider ATX as S1P producing enzyme. The catalytic activity of ATX is significantly affected by the fatty acid moiety of LPLs. Among saturated LPCs, ATX preferably hydrolyses LPC with lauroyl-, myristoyl- or palmitoyl-fatty acids (*26*). In addition, ATX hydrolyses LPC with unsaturated fatty acids more efficiently than LPC with saturated fatty acids. For instance, LPC with oleoyl-, linoleoyl- or arachidonoyl-fatty acid is hydrolysed more efficiently than LPC with stearic- or arachidic-fatty acid (*26* and Aoki J., unpublished data). Unsaturated LPC is probably a physiological substrate because unsaturated LPA species (18 : 2, 20 : 4 and 22 : 6-LPA) consist mainly of LPA produced in incubated plasma (Aoki J., unpublished data). Interestingly, the substrate preference of ATX is significantly altered in the presence of some divalent cations such as Co^{2+} or Mn^{2+}(*46*, *47*). The recent study of bacterial NPP (*48*) indicates that two Zn^{2+} ions bind in the vicinity of the catalytic site, indicating divalent cations (Co^{2+} and Mn^{2+}) modify the substrate specificity of ATX by replacing the Zn^{2+} with either Co^{2+} or Mn^{2+}. Finally, under anhydrous conditions such as within an ether/H_2O interphase, ATX produces cyclic PA (cPA), an analog of LPA, from LPC (*36*). cPA is detected as a physiological constituent of human serum (*49*), and displays antiproliferative and inhibitory activities towards cancer cell invasion (*50*). The physiological significance of cPA produced by ATX needs to be further determined.

General aspects of NPP family members

As stated earlier, ATX is a member of the NPP family. NPP family members are classified into two sub-groups depending on their primary structure. ATX/NPP2, NPP1 and NPP3 form a sub-family and consist of three domains, somatomedin-B-like, catalytic and nuclease-like domains. In contrast NPP4, 5, 6 and 7 consist of only a single catalytic domain. In spite of their structurally related catalytic domains, each NPP shows distinct substrate specificity and, thus, functions (Table I). The genetic study on 'tiptoe walking' mice revealed that NPP1 regulates skeletal remodelling and calcification (*51*). NPP1 is involved in catalysing nucleotides and generating pyrophosphate (PPi), which inhibits bone calcification through blocking the growth of hydroxyapatite crystals (*37*). The bone in NPP1 deficient-mice shows hypercalcification (*51*), and conversely, the stable over-expression of NPP1 is associated with severely reduced bone calcification (*52*). In cohort studies, it was shown that the prevalence of subjects carrying the polymorphic K173Q of the human NPP1 allele was increased in the diabetic group (*53*). In addition, mice over expressing human NPP1 in liver cells showed insulin resistance and glucose tolerance (*54*). *In vitro* studies showed that NPP1 interacts with insulin receptor (*55*, *56*), and that insulin signalling changes the sub-cellular localization of NPP1 (*57*). These results suggest an intriguing function for NPP1 in insulin resistance and type-2 diabetes, but further studies will be necessary to elucidate the molecular basis for these observations. NPP3 is the only molecularly defined white blood cell marker that is exclusively expressed on resting and activated basophils but not on any other peripheral blood cells (*58*). NPP6 is highly expressed in kidney and brain, and was recently shown to have lysophospholipase C activity towards choline-containing glycerophosphodiester including lysophosphatidylcholine, SPC and glycerophosphorylcholine (*59*). NPP7 was identified to have intestinal alkaline sphingomyelinase activity (*60*). NPP7 can also catalyse LPC, and is thought to be important for the digestion of dietary choline-containing phosphodiesters (*60*). NPP4 and NPP5 (*61*) were identified by database search, but little is known about their specific substrates or functions (*62*).

Roles of ATX in developmental stages

During the developmental stage, ATX expression is first observed immediately rostral to the midbrain-hindbrain boundary on embryonic day (E) 8.5, and in floor plate and neural tube on E9.5 (*63*, *64*). In later stages, ATX is highly expressed in multiple tissues

Table I. Substrates and functions of NPP family protein.

Protein	Substrate	Functions	References
NPP1	Nucleotides	Inhibition of excessive bone calcification by producing pyrophosphate	*(37, 51, 52)*
NPP2/ATX	Lysophospholipids	Vasculature and neural tube formation in embryos	*(34, 35, 65, 66)*
		Lymphocyte migration	*(74, 75)*
		Tumour progression	*(32, 76–78)*
		Development of neuropathic pain	*(92)*
NPP3	Unknown	Basophil marker	*(58)*
NPP4	Unknown	Unknown	*(62)*
NPP5	Unknown	Unknown	*(61, 62)*
NPP6	Glycerophosphorylcholine Lysophosphatidylcholine Sphingosylphosphorylcholine	Unknown	*(59)*
NPP7	Sphingomyelin Lysophosphatidylcholine	Unknown	*(60)*

and organs such as choroids plexus and kidney (*63*, *64*). ATX deficient mice are lethal around E9.5∼10.5 with a number of defects including profound vascular defects in the yolk sac and embryo, abnormal lysosome formation in the visceral endoderm cells of the mouse yolk sac, aberrant neural tube formation and developmental delay (Table II) (*34*, *35*, *65*, *66*). *In vitro* studies have indicated that these ATX actions are mediated by LPA. Tanaka *et al*. (*34*) showed that ATX and LPA are involved in stabilizing blood vessels in mice allantois explants, while Im *et al*. (*67*) showed ATX and LPA promote the regression of blood vessels in the tube forming assay using bovine and human endothelial cells. It was also shown that ATX and LPA stimulate neurite outgrowth in tissues isolated from ATX-null mice (*66*). However, because none of the LPA receptor knockout mice reported so far showed similar phenotypes to that of ATX-null mice (see below), further analysis using model organisms, such as zebrafish (see below), are needed to know the precise modes of ATX actions *in vivo*. It can be safely to be said that ATX exerts its biological roles through its catalytic activity, because the embryonic lethality similar to ATX-null mice was also observed in mutated ATX knock-in mice, in which the single amino acid responsible for the catalytic activity of ATX was modified (*68*). These results show that LPA produced by ATX evokes the signalling of its cognate receptors, and thereby exerts multiple effects in embryonic development. However, the phenotypes observed in ATX deficient mice are much more severe than the ones in LPA receptor deficient mice reported so far; any single ($lpa_1^{-/-}$, $lpa_2^{-/-}$, $lpa_3^{-/-}$ or $lpa_4^{-/-}$), double ($lpa_1^{-/-}lpa_2^{-/-}$, $lpa_2^{-/-}lpa_3^{-/-}$ or $lpa_1^{-/-}lpa_3^{-/-}$) and triple ($lpa_1^{-/-}lpa_2^{-/-}lpa_3^{-/-}$) KO mice are viable with different phenotypes depending on their genotype (Table II) (*69*–*73*). Thus, it is possible that the developmental functions of ATX can be mediated through recently identified LPA receptors including LPA_5 (*11*) and LPA_6/p2y5 (*13*) or other unknown LPA receptors.

Physiological and pathological aspects of ATX

In the process of lymphocyte recirculation, lymphocytes interact with and transmigrate through high endothelial venules (HEVs) in lymph nodes and other secondary lymphoid tissue. It was recently shown that ATX is abundantly expressed in HEVs and promotes the lymphocyte-endothelial cell interaction (*74*, *75*). The lymphocyte trafficking into the lymphnodes was moderately inhibited in mice injected with enzymatically inactive ATX (*74*). Controversially, the trafficking was not affected by the systemic depletion of circulating ATX with anti-ATX monoclonal antibodies (*75*). Thus, further analysis including conditional expression or deletion of ATX in HEVs in mice will be required to uncover the precise roles of ATX signalling in the lymphocyte movement in lymphoid tissues.

The pathological functions of ATX in tumour progression are also being clarified in the light of LPA producing enzyme. *In vitro* ATX was shown to promote tumour cell migration in a LPA-specific receptor (LPA_1)-dependent manner (*32*), and stimulate human endothelial cells grown on Matrigel to form tubules (*76*). In addition, a recent *in vivo* study showed that over expression of ATX in mammary glands resulted in breast cancer initiation and progression in mice (*77*). Furthermore, over expression of ATX promoted the bone metastasis of breast cancer cells (*78*). ATX is highly expressed in malignant tumour tissues or cells in Hodgkin lymphoma (*79*), glioblastoma (*80*, *81*), non-small-cell lung cancer (*82*), renal cell carcinoma (*83*), hepatocellular carcinoma (*84*), breast cancer (*85*) and thyroid carcinomas (*86*). In addition, ATX concentration and activity were found to be elevated in sera from follicular lymphoma (*87*). Taken together, these results indicate that ATX signalling possibly through LPA receptors is involved in the progression of tumour malignancy as well as in angiogenesis and ATX is a potent therapeutic target for cancer treatment.

Table II. Phenotypes of LPA receptor deficient mice.

Receptors	Lethality	Phenotypes of KO mice	References
LPA_1	Semi-lethality	Impaired suckling behaviour	(*69*)
		Decreased postnatal growth rate, reduced size	(*69*)
		Craniofacial dysmorphism	(*69*)
		Frontal hematoma	(*69*)
		Increased apoptosis in sciatic nerve Schwann cells	(*69*)
		Changes in neurotransmitters (schizophrenic-like pathology)	(*111*)
		Inhibition of the neuropathic pain (inhibition of demyelination)	(*88*)
		Proliferation of preadipocyte and inhibition of adipocyte differentiation	(*112*, *113*)
		Cerebral cortex growth and folding *ex vivo* (LPA_1/LPA_2-deficient mice)	(*114*)
		Inhibition of renal tubulointerstitial fibrosis	(*115*)
		Inhibition of pulmonary fibrosis	(*116*)
LPA_2	Viable	Inhibition of cholera toxin-induced secretory diarrhea	(*117*)
		Inhibition of tumour formation in an colitis-associated cancer	(*118*)
LPA_3	Viable	Disruption of spacio-temporal blastocyst implantation in uterus	(*71*)
LPA_4	Viable	Enhanced migratory response stimulated with LPA in fibroblasts	(*72*)
LPA_5	N.D.	N.D.	
LPA_6/P2Y5	N.D.	Hypotrichosis (human)	(*119*, *120*)

The neuropathological roles of the ATX-LPA axis have also been extensively investigated. LPC is originally known to induce neuropathic pain, such as behavioural allodynia, thermal hyperalgesia and demyelination (*88*). Recently it was shown that nerve injury causes *de novo* LPA production (*89*), and that LPA is responsible for the neuropathic pain through the activation of a LPA-specific receptor (LPA_1) and the small G protein, Rho (*90*, *91*). Recent analysis also showed that the LPA production and neuropathic pain induced by nerve injury were significantly attenuated in ATX hetero knockout mice, suggesting that ATX promotes neuropathic pain by converting from LPC to LPA and activating LPA_1 signalling (*92*). ATX is highly expressed in choroids plexus and present in cerebrospinal fluids, while the concentration of LPC in cerebrospinal fluids is almost undetectable (J. Aoki unpublished data). During the occurrence of pain, the source of LPLs as substrates for ATX is still unclear. A possible candidate would be the blood stream, which contains abundant LPC (several hundred micro molar). Moreover, LPL such as LPS are synthesized in activated platelets through the action of phospholipase A_2 and released into blood stream (*15*). Taken together, it is possible that LPL are supplied from the blood stream along with increased vascular permeability caused by multiple stresses such as inflammatory response and injury.

ATX in model organisms

Genes belonging to the NPP family are distributed not only in vertebrates but also in invertebrates such as yeast, nematodes and plants (Fig. 3). Even in bacteria, NPP homologues exist and the crystal structure of NPP protein from *Xanthomonas axonopodis pv. citri* (Xac) was recently resolved (*48*). However, the functions of each NPP family member in invertebrates are totally unclear, since significant phenotypes cannot be observed in invertebrates in which NPP family genes are mutated or knocked down in our database search (WormBase and *Saccharomyces* genome database for nematode and yeast, respectively). In addition, it is not expected that the functions and structures of NPP genes in vertebrates are conserved in invertebrates due to the following reasons. First, the sequence of NPP is not well conserved at the amino acid level between in vertebrates and invertebrates. For example, the amino acid similarity between human and nematode NPPs varies between 18% (human NPP3 versus NPPδ) and 33% (human NPP1 versus *Caenorhabditis elegans* NPPα). Secondly, the number of NPP family members in invertebrates is smaller than in vertebrates and each NPP gene in invertebrates does not match the one in vertebrates. In contrast, longer forms of NPP family members such as NPP1-3 exist only in vertebrates. For example, zebrafish contains all NPP family members except for NPP3. The amino acid similarity between human and zebrafish ATX/NPP2 is close to 70%. In addition, lysoPLD activity was observed in zebrafish NPP2 (Hama K. and Aoki J., unpublished data). Notably, all LPA receptors are highly conserved in zebrafish. These results suggest that LPA signalling is functional in lower vertebrate model organisms such as fish, which could be useful tools to elucidate the molecular basis of the ATX functions *in vivo*.

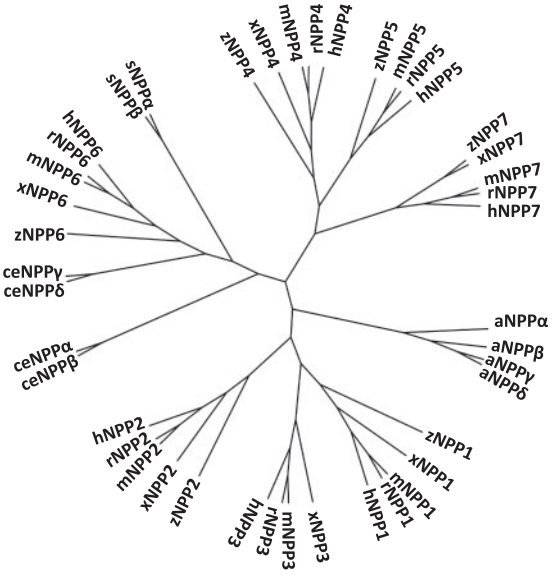

Fig. 3 NPP family genes in model organisms. Based on the genomic and EST database for model organisms, we identified NPP genes in human (h), rat (r), mouse (m), flog (x), fish (z), nematode (ce), yeast (s) and plant (a). Note that each NPP genes in vertebrate model organisms were clustered well.

Clinical aspects of ATX

Studies on ATX in clinical samples have provided important clues to the patho-physiological functions of ATX. ATX is abundantly expressed in various biological fluids such as blood plasma, serum, urine, seminal fluids and cerebrospinal fluids (*93*). As mentioned earlier, accumulating evidences have identified a number of patho-physiological functions of ATX. This indicates that the levels of ATX concentration and activity in clinical samples can be used for the diagnosis of several diseases. In addition, ATX has ideal features as a diagnosis marker; ATX is highly stable and its level varies within a relatively small range among healthy subjects (*33*). Recently high-throughput assays for determination of ATX concentration and activity for clinical laboratory testing were developed (*33*). These studies have revealed a correlation between the level of ATX and several patho-physiological conditions (*33*, *94*). ATX concentration was significantly increased in the serum from patients with chronic liver disease (*33*). This may reflect the delayed metabolism of ATX in the liver based on the following observations: serum ATX activity was significantly elevated in liver injury in rats (*94*) and ATX in circulating plasma was cleared by the scavenger receptors of liver sinusoidal endothelial cells in mice (*95*). Interestingly, it was found that the serum ATX level is higher in women than men (*33*), and ATX activity becomes even higher in women who are pregnant (*96*). In contrast, the serum ATX level was decreased after operation in prostate cancer patients,

which seemed to reflect post-operative damage or nutritional status (*33*). ATX expression is increased in the cerebrospinal fluid of multiple sclerosis patients at the protein level (*97*), and ATX mRNA was observed in synoviocytes from rheumatoid arthritis patients (*98*). In adipose tissues, ATX mRNA increased during the adipocyte differentiation and was up-regulated in genetically obese diabetic mice (*db*/*db*) (*31*). These results indicate that ATX has some role in the progression of tumours, liver dysfunction, pregnancy and obesity.

Non-enzymatic functions of ATX

It is likely that most of the ATX actions are mediated through LPA production and consequent activation of LPA receptors. It is intriguingly reported that ATX facilitates morphological change in oligodendrocytes in a catalytic activity-independent manner (*99*). A novel functionally active domain referred to as the modulator of oligodendrocyte remodelling and focal adhesion organization (MORFO), mainly consists of the nuclease-like domain but not the catalytic domain of ATX (*100*). The MORFO domain contains an EF hand-like motif, which was found to mediate a reorganized assembly of focal adhesions and promotes process outgrowth in post-migratory, premyelinating oligodendrocytes (*101*). A cell surface 'receptor' can be involved in this process to mediate the MORFO domain function. Further studies will be necessary to show whether the MORFO domain and/or other unknown functional domains are involved in the ATX functions *in vivo*.

Small molecule compounds for ATX study

Given the functions of ATX in a number of physiological and pathological processes, potent inhibitors of ATX are desired not only as feasible tools for ATX studies but also as novel therapeutic leads. The structures of ATX inhibitors published so far are summarized in the previous report (*102*). Among these inhibitors, several compounds such as palmitoyl α-bromomethylene phosphonate (BrP-LPA) and NSC 48300 were shown to inhibit the growth of tumour xenografts in mice (*103*) or the tumour cell motility *in vitro* (*104*). These compounds inhibit ATX activity in the micromolar range, and thus, other compounds that can inhibit ATX-induced biological processes much more efficiently are desired. An approach to optimize ATX inhibitors utilizing the information from the crystal structure of the bacterial enzyme Xac (*48*), which shares 35% identity with the central catalytic domain of ATX was undertaken (*105*). Studies on crystal structures of ATX are definitely helpful for the optimization of the current ATX inhibitors and even *in silico* screening of ATX inhibitors.

A simple and highly sensitive assay for ATX activity is necessary in order to employ high throughput screening methods for diagnosis and drug discovery. Fluorescence resonance energy transfer (FRET)-based reporters such as CPF4 and FS-3 were recently developed to detect ATX activity (*106*, *107*). ATX hydrolyses these fluorescent substrates very efficiently compared with the conventional substrates such as pNP-TMP; the K_m values for CPR4 and FS-3 are within the 1–10 μM range, whereas the value for pNP–TMP is close to 1 mM. Using CPF4, it was successfully shown that ATX is specifically inhibited by LPA and sphingosine-1-phosphate that can be produced by lysoPLD activity of ATX itself (*106*), demonstrating the feasibility of the reporter in the study of ATX.

Conclusions

The accumulating clinical and experimental evidences for the crucial roles of ATX in pathological and physiological conditions substantiate the role of ATX as an essential therapeutic target. Moreover, the identification of ATX as the LPA producing enzyme in plasma greatly promoted the understanding the molecular basis of ATX functions in a number of physiological and pathological processes. Given the crucial roles of ATX in LPA signalling, further studies on ATX may reveal unexpected functions of LPA signalling, which would be mediated through known and/or unknown LPA receptors, and vice versa. However, a number of questions still remain. One of the most fundamental issues is the identification of the signalling pathway mediated by a specific LPA receptor involved in ATX function in live animals. For example, the phenotype of the abnormal angiogenesis observed in ATX deficient null mice is quite similar to the one in $G\alpha_{13}$ deficient mice (*108*), but not to the one in the other LPA receptor deficient mice generated to date (Tables I and II). Since $G\alpha_{13}$ is a known downstream LPA receptor, it is expected that ATX produces LPA and activates its cognate receptors leading to the promotion of angiogenesis. Considering the broad substrate specificity of ATX *in vitro*, it is also an intriguing issue to identify genuine substrates of ATX *in vivo* as well as the molecular basis for producing the substrates. In addition, mechanisms responsible for producing a number of LPC species with different fatty acids should also be clarified to understand the potency of ATX to produce 'highly active' LPA. The acyl chain moiety of LPA produced by ATX directly reflects the one of substrate LPC and biological effects of each LPA molecule dramatically vary according to its fatty acid chain. For instance, LPA with an unsaturated long-chain fatty acid preferably activates LPA receptors such as LPA_3 and LPA_6/P2Y5 (*13*, *109*). In addition, the importance of LPA with an unsaturated long-chain fatty acid is also implicated in the neointimal formation in the vasculature (*110*). LPC in blood plasma is mainly produced from lipoprotein, indicating the contribution of the enzymes involved in lipoprotein metabolism in producing a variety of LPC species as substrates for ATX. There have not been convenient assays to detect each LPA species with different fatty acid chains. Recent advances in liquid chromatography and mass spectrometry may help to provide the means to measure each LPA species directly leading to clarifying the upstream, lysophospholipids-producing machinery or

downstream, functions of each LPA species, aspects of ATX.

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