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Exosomes bind to autotaxin and act as a physiological delivery mechanism to stimulate LPA receptor signalling in cells

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ABSTRACT
Autotaxin (ATX; also known as ENPP2), the lysophospholipase responsible for generating the lipid receptor agonist lysophosphatidic acid (LPA), is a secreted enzyme. Here we show that, once secreted, ATX can bind to the surface of cell-generated exosomes. Exosome-bound ATX is catalytically active and carries generated LPA. Once bound to a cell, through specific integrin interactions, ATX releases the LPA to activate cell surface G-protein-coupled receptors of LPA; inhibition of signalling by the receptor antagonist Ki1642 suggests that these receptors are LPAR1 and LPAR3. The binding stimulates downstream signalling, including phosphorylation of AKT and mitogen-activated protein kinases, the release of intracellular stored Ca2+ and cell migration. We propose that exosomal binding of LPA-loaded ATX provides a means of efficiently delivering the lipid agonist to cell surface receptors to promote signalling. We further propose that this is a means by which ATX–LPA signalling operates physiologically.

KEY WORDS: Autotaxin, Exosome, Integrin, LPA

INTRODUCTION
Autotaxin (ATX; also known as ENPP2) is a secreted lysophospholipase D, which catalyses the hydrolysis of lysophosphatidylcholine (LPC), generating lysophosphatidic acid (LPA) and choline (Moolenaar and Perrakis, 2011). Although ATX was first isolated from the culture medium of melanoma cells and implicated in motility stimulation, it is now apparent that the enzyme has a wider physiological role given that it is the predominant generator of LPA in mammalian systems. The physiological importance of this reaction is emphasised by the embryonic lethality associated with the mouse Atx knockout (van Meeteren et al., 2006). The lipid product of ATX activity, LPA, binds to members of a family of cell surface G-protein-coupled seven-transmembrane receptors and thereby stimulates a number of signalling pathways (including those comprising phosphoinositide 3-kinase, ras, phospholipase C and phospholipase D, and Rho) that activate physiological responses such as proliferation, migration, development or contraction, as well as those protecting against apoptosis, depending upon cell type (Houben and Moolenaar, 2011; Muinonen-Martin et al., 2014).

ATX is a secreted glycoprotein (Pradere et al., 2007) comprising two N-terminal cysteine-rich somatomedin-like domains, a catalytic domain and a nuclease-like domain (Hausmann et al., 2011; Nishimasu et al., 2010). The structural characterisation of ATX was used to define its substrate specificity and to identify integrin-binding sites that have been proposed to be crucial for association of the enzyme with cells to which LPA is targeted. Structural analysis has further been used to identify the existence of an extended substrate-binding hydrophobic channel that additionally exhibits high affinity for LPA and, as such, is proposed to provide a mechanism for delivery of LPA to its cognate receptors. The importance of this targeted delivery is emphasised by the rapid degradation of LPA by lipid phosphate phosphatases present on the surface of all cells, which will rapidly hydrolyse and, thus, remove free LPA, thereby reducing the effective local concentration of the lipid agonist (Reue and Brindley, 2008). The concentration of circulating ATX has been suggested to maintain the plasma LPA concentration because the blood of the heterozygous Atx-knockout mouse has been shown to contain 50% of the ATX and LPA concentrations that are found in the wild type (van Meeteren et al., 2006). The serum concentration of ATX has been proposed to be elevated in a number of cancers, and this could, in certain cases, result in an elevated LPA level, contributing to proliferation and metastasis; however, some available data contradict this proposal (Houben and Moolenaar, 2011). ATX, however, is a key enzyme in normal physiology and is present in all mammalian sera, as such LPA and ATX are important factors that contribute to the potency of sera (from fetal calves and other sources) in promoting the growth of cells in culture. The mechanism by which ATX hydrolyses LPC in serum is unclear – the lipid is present in lipoproteins or is in solution bound to albumin; thus, the interaction could simply be a random process, generating soluble or protein-bound LPA. However, because high levels of cell surface lipid phosphate phosphatases rapidly hydrolyse the LPA (Reue and Brindley, 2008), it is unclear how the serum LPA concentration is maintained.

In examining extracellular LPA generation, we have found that a significant proportion of ATX is bound in vesicles. In particular, we have found that ATX binds to exosomes – small multivesicular-body-derived vesicles that have been found to transport signalling molecules, mRNA and microRNAs (Ono et al., 2014; Skog et al., 2008; Valadi et al., 2007). Identification of the vesicular population as exosomes has provided a mechanism to explain how ATX is transported and, thus, how the active generation of LPA adjacent to cell surface receptors is regulated.

RESULTS
ATX is bound in vesicles
Western blotting of ultracentrifuged fetal calf serum and cell culture medium demonstrated that a substantial proportion of ATX...
was bound in vesicles (Fig. 1). A similar distribution was also observed in serum from bovine, murine and human sources (data not shown); this analysis also highlighted the differences in the glycosylation of the proteins between the different organisms. Cells release distinct populations of vesicles – microparticles bud-off from the plasma membrane, whereas exosomes are released from the multivesicular body (MVB). Exosomes have a characteristic size (50–100 nm) and are sedimented at centrifugal forces greater than 100,000 g, they have been reported to carry microRNAs and characteristic proteins such as HSP70 (Ono et al., 2014; Skog et al., 2008; Valadi et al., 2007). Transmission electron microscopy examination of vesicles that had been sedimented from cell culture medium showed the vesicles to be a fairly homogenous population, primarily elliptical in shape and less than 100 nm in diameter, thus exhibiting the characteristics of exosomes (Fig. 1C). Scanning-densitometry analysis of immunoblots of serum and cell culture media ATX showed the proportion of total immunodetectable ATX that was exosomal ranged from 5 to 30%, depending upon the experiment (see Fig. 1B for example). Sucrose density gradient separation of the ATX-positive vesicles from tissue-culture medium and serum demonstrated that the vesicles exhibited the characteristic density of exosomes; ATX was located in the same fraction as HSP70 and major histocompatibility complex I (MHC-1), and in the fraction that showed greatest acetylcholinesterase
enzyme activity (Fig. 2). Notably, however, ATX was detected in a limited portion of the exosome fraction, sedimenting at a density of 1.08–1.11 g/ml (**P ≤ 0.01, significance relates to activity comparison between density of 1.08 g/ml and 1.05 g/ml sucrose), distinct from portions containing LDLs, which sediment at a density between 1.006 and 1.06 g/ml. Incubation of exosomes with trypsin resulted in hydrolysis of the ATX with no effect on HSP70, in contrast, in the presence of Triton X-100, HSP70 was also degraded. These experiments suggest that ATX binds to the external face of the exosome, whereas HSP70 is characteristically enclosed within the exosome (Fig. 3).

It has been previously documented that ATX is secreted through the classic secretory pathway, pointing to an extracellular association of the enzyme with the exosome; this would be in keeping with exosomes being released from cells through the MVB pathway. Incubation of cells with brefeldin A, to suppress overall cellular secretion, reduced both detectable soluble and exosomal ATX (P ≤ 0.01), with minimal effect upon HSP70 and, thus,
exosome levels ($P \leq 0.05$) (Fig. 4A). Incubation of the cells with GW4869, a neutral sphingomyelinase inhibitor that suppresses exosome generation (Trajkovic et al., 2008), reduced both the HSP70 level ($P \leq 0.05$) and the amount of detectable ATX that was associated with exosomes, without affecting the amount of soluble ATX ($P \leq 0.05$) (Fig. 4A). To confirm the extracellular interaction of exosomes with ATX, secreted His–ATX was purified from the medium of transfected HEK293 cells and added to a culture of control and GW4869-treated HEK293 cells; Fig. 4B shows that tagged ATX bound to the newly secreted exosomes within 1 h, which was abrogated when exosome biogenesis was limited through treatment with GW4869 ($P \leq 0.001$). In common with many secreted proteins, ATX has been reported to be N-glycosylated at residues N53, N410 and N524, among which, only the N524-linked glycan is essential for the catalytic activity (Jansen et al., 2007). Glycosylation at N411 has also reported to be required for ATX activity (Pradere et al., 2007), and glycosylation of both N53 and N410 are required for ATX secretion (Pradere et al., 2007). We mapped the N-linked glycosylation of purified His–ATX using mass spectrometry to four sites – N54, N411, N525 and N807. Glycosylation at N807, which has not previously been reported, was only detected in 10% of the ATX molecules analysed, but this percentage was similar to that found for glycosylation of N54 residues (15%); in contrast, almost all N411 and N525 residues were glycosylated. No difference was detected between the glycosylation patterns of exosomal and non-exosomal ATX.

Mode of binding of ATX to exosomes

Because the ATX structure contains no known lipid-binding motifs, it is probable that the enzyme associates with the exosome through protein–protein interactions. Deletion of the ATX somatomedin-B and linker-I regions did not alter association with exosomes; further, removal of the catalytic domain also did not prevent the truncated enzyme from associating with exosomes, indicating that the C-terminus of the protein is important in the interaction. In addition, mutation of residue H119, previously shown to be crucial for integrin association, had no effect upon ATX–exosome binding (data not shown).

To determine the proteins involved in ATX–exosome binding, transfected 6×His-tagged ATX was purified from exosome and non-exosome fractions with Co2+ Sepharose beads, and the associated proteins were identified by performing mass spectrometry. Nine proteins were detected exclusively in the exosome fraction: nidogen-2, agrin, the laminin subunits $\alpha_1$, $\alpha_3$, $\alpha_4$, $\beta_1$, $\beta_2$ and $\gamma_1$, and perlecan was additionally identified as being bound to ATX both in its exosome-bound and soluble forms. The majority of the identified proteins have important roles in the functioning of the extracellular matrix, including the laminin subunits, perlecan, nidogen-2 and agrin. Because laminin proteins exist as heterotrimers, the laminin $\gamma_1$ subunit was used to immunopurify laminin complexes from exosomes, and indeed, this co-purified ATX (Fig. 5A). However, the binding of ATX to laminin was indirect given that immobilised His–ATX was unable
to bind to human recombinant laminin proteins [comprising laminin subunits α2, β1 and γ1 (α2,β1,γ1), or subunits α5, β1 and γ1 (α5,β1, γ1)], indicating an indirect binding mechanism (Fig. 5B) that potentially involves agrin. Nevertheless, the addition of soluble α2, β1,γ1 or α2,β1,γ1 laminin proteins disrupted the ability of part-purified soluble His–ATX to bind to isolated exosomes (Fig. 5C). This suggests that, in the presence of free rather than exosomal laminins, additional soluble components, perhaps agrin, prevent from ATX interacting with the exosome.

**Exosomal ATX generates and binds to LPA**

Mass spectrometry analysis of lipids determined that exosomes contain both LPA and LPC, with the relative levels being affected by the magnitude of ATX content: higher levels of LPA were observed with greater levels of exosomal ATX (Fig. 6A). Analysis of the molecular species showed that ATX demonstrates substrate specificity given that the predominant molecular LPA species detected were C16:0, C18:0 and C18:1, whereas the exosome contained a range of LPC species, with C16:1 being the major form present (Fig. 6B). Higher levels of ATX expression resulted in the increased generation of C18:1 LPA in particular, and of longer-chain species in exosomes – C18:2 LPA, C20:4 LPA and C22:6 LPA – although these were present at lower levels (Fig. 6A). LPA is a hydrophilic lipid and is thus protein-bound in the exosomes, indeed, mass spectrometry analysis of immunopurified His–ATX detected bound C16:0, C18:0, C18:1 and C18:2 LPA, pointing to ATX as the source of exosomal LPA (data not shown). Surprisingly, incubation of exosomes with the ATX inhibitor HA-130 had minimal effect upon LPA content (Fig. 6C). In contrast, HA-130 inhibited the in vitro generation of LPA from LPC by purified His–ATX (Fig. 6D). This suggests that LPA is either retained within the active site of exosomal ATX, or binding of LPA to the second non-catalytic site of ATX inhibits further enzyme activity. In contrast to the stability of ATX-bound LPA, C17-containing LPA that had been added to cells was rapidly degraded, presumably by the activity of cell surface phosphatases, with 50% degraded in 15 min and the added LPA being undetectable within 24 h, despite the presence of serum albumin, which is known to bind to the lipid.

**Exosomes mediate LPA signalling**

Because exosomes contain bound LPA, their role in cellular LPA delivery was examined. The addition of isolated exosomes to either HEK293 or NIH3T3 cells stimulated acute phosphorylation of MAPK1 and MAPK3 (p42/44 ERK; Fig. 7A), and of AKT (Fig. 7B), as well as a rise in intracellular free-Ca2+ concentration (Fig. 7C); these responses were sensitive to the presence of the LPA receptor antagonist Ki16425 (Fig. 7A,B). These increases were modest ‘bumps’ in the Fura2 signal (Fig. 7C), which equated to an increase in cytosolic [Ca2+] of ~100 nM. This increase in Ca2+ was similar to that for previously described elementary Ca2+ signals that were observed upon low levels of cell stimulation (Bootman et al., 1997). The exosome-induced Ca2+ mobilisation was observed as a series of low-amplitude transient Ca2+ rises that apparently de-sensitised cells, in keeping with the proposal that these are due to activation of the LPA receptor. Indeed, those cells that responded to exosomes had a reduced response to subsequent addition of a threshold LPA concentration, highlighting activation and subsequent de-sensitisation of the LPA receptor. The
ability of exosomes to stimulate MAPK phosphorylation was dependent upon ATX–integrin binding given that mutation of residue H119, the RGD motif within the second somatomedin-B (SMB) domain, the LDV motif or deletion of both SMB domains–each involved in ATX–integrin binding (Fulkerson et al., 2011; Hausmann et al., 2011; Kanda et al., 2008)–severely reduced phosphorylation (Fig. 7D). Incubation of serum-starved quiescent NIH3T3 cells with exosomes in the absence of serum for 24 h stimulated DNA synthesis, as determined by examining [3H] thymidine incorporation; however, this response was not sensitive to the inclusion of Ki16425 (Fig. 8A). In contrast, exosomes were able to stimulate cell migration in a wound healing scratch assay in a Ki16425- and HA-130-sensitive manner (Fig. 8B,C; ****P ≤ 0.0001). (Student’s t-test). Data are mean±s.d.

DISCUSSION
The data in this paper point to an important role for exosome-bound ATX as a mechanism for delivering LPA to cell surface receptors. Previous studies have suggested that the integrin-binding motifs in ATX are crucial for its interaction with cells and that this facilitates targeting of the ATX-generated LPA to the cell surface receptor (Moolenaar and Perrakis, 2011). The binding of ATX to platelet integrins has been shown to increase ATX catalytic activity (Fulkerson et al., 2011) and the directional migration of breast cancer cells (Wu et al., 2014). The data in this report suggest that exosome-surface-bound ATX is primed to activate target cells, once it binds to cell surface integrins, by first releasing LPA and then generating more.

Newly synthesised ATX is released from cells through the classic secretory pathway; however, it is apparent from data in Fig. 1 that a large proportion of the enzyme in cell culture medium is present in a vesicle-bound form rather than in a soluble form; that this is also observed in mouse, human and fetal calf serum points to vesicular ATX as the physiologically relevant form of the enzyme. The vesicles that bind to ATX are exosomes, as shown by presence of known exosome markers – such as HSP-70 and MHC-1 – by their morphology (observed with electron microscopy; Fig. 1C) and by their density (sucrose density gradient centrifugation) (Fig. 2). The narrow distribution of ATX in the sucrose gradient in Fig. 2 points to a distinct population of exosomes that act as carriers of the lysophospholipase rather than to this being a property of all exosomes.
Exosomes have been proposed to function as intercellular signalosomes carrying, for example, microRNAs and prostaglandins (Record et al., 2011). In contrast to the data presented here, proteomic analyses of exosomes have not previously reported the presence of ATX; however, this might reflect less-stable cell-surface binding, demonstrated by trypsin sensitivity (Fig. 3), rather than incorporation or inclusion of the enzyme into the exosome. The labile nature of exosome–ATX binding is further suggested by the exosomal adsorption of purified His-tagged ATX when added to culture medium (Fig. 4). Truncation and site-directed mutagenesis of ATX demonstrated that it is not itself binding to integrins and that the interaction with the exosome is through the C-terminal nuclease-like or lasso 2 regions. Mass spectrometry analysis of immuno-isolated ATX complexes from both the soluble and exosomal fractions of enriched tissue culture medium identified perlecan, a multidomain extracellular matrix proteoglycan, as an ATX binding partner. The mass spectrometry analysis of ATX complexes that had been isolated from exosomes additionally identified nidogen-2, agrin and seven laminin subunits, these together with perlecan are components of the extracellular matrix and the basement membrane. Agrin and laminin subunits have previously been detected using a proteomic approach to analyse exosomes, such as those produced by prostate cancer cells (Hosseini-Beheshti et al., 2012).

Studies of basement membrane assembly have led to the proposal that a complex laminin network is generated—made up of α, β and γ subunits—that is bound to integrins. Nidogen and agrin associate with the basement membrane by binding lamin, and perlecan is present in the basement membrane due to binding to nidogen.

Fig. 7. Vesicular ATX stimulation of signalling is dependent of LPA receptors. Exosomes were added to serum-starved HEK293 cells that had been pre-treated with either EtOH or 10 μM Ki16425. His ATX T210A is a catalytically inactive mutant. (A) Phosphorylated (pERK) and ERK2 in lysates were detected by immunoblotting. (B) Phosphorylated (pAKT) and total AKT in lysates were detected by immunoblotting. Blots were quantified by using scanning densitometry analysis. (C) Exosomes induce Ca\(^{2+}\) mobilisation in NIH3T3 cells in an LPA-like manner. Quiescent cells were seeded onto glass coverslips and loaded with Fura-2 AM, which was alternately excited at 340 nm and 380 nm wavelengths, and the Fura-2 emission at >450 nm was sampled at 2 Hz. The y-axis represents the ratio of the Fura-2 emission, and is proportional to cytosolic Ca\(^{2+}\) concentration. Cells were stimulated with exosomes followed by a threshold and a maximal dose of LPA. The cytosolic Ca\(^{2+}\) concentration in four individual cells is shown. The arrows indicate the series of low-amplitude exosome-induced transient Ca\(^{2+}\) elevations, which were not seen in untreated cells, or in cells that had been co-incubated with exosomes and Ki16425 (n=40 cells imaged on four separate days). (D) HEK293 cells were transfected with His–ATX, His–ATX-RGE, His–ATX-LNV, His–ATX-RGE–LNV, His–ATX-H119A or His–ATX-ΔSMB and cultured in OptiMEM for 36 h to condition media. Exosomes were isolated by differential centrifugation and added to serum-starved NIH3T3 cells. Phosphorylated and total ERK were detected by immunoblotting of lysates. Bands were quantified by performing scanning densitometry analysis and normalised to levels of total ERK. The shown immunoblot is representative of three experiments, and quantification is mean±s.d. of three experiments. The immunoblots shown in A,B,D are from the same membranes. *P≤0.05, **P≤0.01, ***P≤0.0001 (Student’s t-test). Control, OptiMEM without LPA or exosomes.
that LPAR4, LPAR5 and LPAR6, which also activate a range of G-
synthesis was insensitive to Ki16425. Although it remains possible
of LPAR1, LPAR2 and LPAR3 because the promotion of DNA
stimulation of cells by exosomes was not solely through signalling
to the selective receptor antagonist Ki16425. Importantly, the
LPAR1, LPAR2 and LPAR3, given that signalling was sensitive
with LPA activated LPA receptor signalling through, at least,
the LPA remains within or occludes the active site.

Utilisation of C18:0 LPC as a substrate. The lack of effect of the catalytic
brings about a conformational change in the protein, promoting the
species are not strongly bound to the active site of ATX (Nishimasu
suggested from structural analysis that the longer-chain saturated LPA
species present in the exosome. However, because the lipid product
remains bound to ATX, it is probable that the enzyme hydrolyses an
LPC species before association with the exosome. The presence of
C16:0 and, in particular, C18:0 LPA is perhaps surprising as it has been
suggested from structural analysis that the longer-chain saturated LPC
species are not strongly bound to the active site of ATX (Nishimasu
et al., 2010). Thus, it is possible that the binding of ATX to the exosome
brings about a conformational change in the protein, promoting the
utilisation of C18:0 LPC as a substrate. The lack of effect of the catalytic
inhibitor HA-130 upon exosome LPA generation demonstrated that retention of the product prevented further ATX activity, suggesting that the LPA remains within or occludes the active site.

Addition to cultured cells of exosomes containing ATX loaded
with LPA activated LPA receptor signalling through, at least,
LPAR1, LPAR2 and LPAR3, given that signalling was sensitive to the selective receptor antagonist Ki16425. Importantly, the stimulation of cells by exosomes was not solely through signalling of LPAR1, LPAR2 and LPAR3 because the promotion of DNA synthesis was insensitive to Ki16425. Although it remains possible that LPAR4, LPAR5 and LPAR6, which also activate a range of G-protein-mediated signalling pathways (Yung et al., 2014), could be responsible for stimulating DNA synthesis in cells, it is more likely to be due to growth factors, such as EGF, that are known to be carried by exosomes (Record et al., 2011). In contrast, exosome-stimulated cell migration in a scratch wound assay was absolutely dependent upon LPAR1, LPAR2 and LPAR3.

The stimulation of LPAR-dependent signalling by exosomes was dependent upon the integrin-binding motifs of ATX. This is in keeping with the demonstration that integrin binding recruits ATX to the cell surface and with the proposal that this delivers LPA to its cognate receptor (Hausmann et al., 2011; Perrakis and Moolenaar, 2014; Wu et al., 2014). However, the exosome itself might play a role in binding to target cells — the integrin-binding capability of the laminins, for example, could stabilise interaction with the cell before interaction between ATX and the cell, and LPA delivery. The clear advantage brought about by this mechanism is that LPA is protected from the hydrolytic activity of the cell surface lipid phosphate phosphatases, and it is likely that once the LPA transfers from ATX to the receptor, the readily available LPC in the exosome can be hydrolysed by ATX to generate more receptor agonist. However, further experiments will be required to definitively demonstrate that LPA bound to ATX is protected from the activity of cell surface LPA phosphatases. Thus, the system can be exquisitely controlled through changes in the cell surface integrin conformation releasing the bound exosome and ATX, thereby terminating the signalling. This process of intercellular signalling confers signalling specificity and regulation, and it is possible that activation of LPA signalling in this way could promote exosome internalisation, thereby delivering content such as microRNAs into the cell. Additionally, the exosome intercellular signalling could be a mechanism for tumour stroma interaction, a process known to involve LPA receptors (Lee et al., 2015) and tumour metastasis, which we have previously shown, at least for melanoma, to be LPA dependent (Muinonen-Martin et al., 2014).
MATERIALS AND METHODS

Reagents, plasmids and antibodies

Reagents were from Cayman Chemical, Sigma-Aldrich, Clontech or Addgene. Mutations of ATX were introduced by performing PCR-site-directed mutagenesis to produce catalytically inactive and integrin-binding mutants. All constructs were confirmed by sequencing.

The antibody against ATX was produced from 4F1 hybridoma cells, as previously described (Baumforth et al., 2005), and conjugated to FITC by cross-linking. Other antibodies used were against: GFP (cat. no. 11 814 460 011, Roche), CD63 (clone MEM-259, Abcam; 1:2500 dilution), LAMC1 (HPA001908, Sigma; 1:2500 dilution) and HSP70 (ab8219, Abcam; 1:10000 dilution), His (27-4710-01, GE Healthcare; 1:5000 dilution), MAPK3 (610031, BD Laboratories; 1:10000 dilution), phosphorylated MAPK1 and MAPK3 (at T202 and Y204; cat. no. 05-797R, Millipore; 1:5000 dilution), phosphorylated AKT (at S473; cat. no. 4058, Cell Signaling Technology; 1:1000 dilution), AKT (cat. no. 9272, Cell Signaling Technology; 1:1000 dilution), MHC-1 (kindly donated by Cancer Research UK; 1:500 dilution).

Cell culture and transfection

HEK293 and NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (PAA) and 100 µg/ml penicillin-streptomycin and 200 µg/ml G418. Cells were transiently transfected with ATX or ATX T210A plasmids (plasmid 17839: pSecTag-ATX, https://www.addgene.org/17839/ or plasmid 17840: pSecTag-T210A-ATX, https://www.addgene.org/17840/, respectively). Using FuGENE 6 reagent (Roche).

Cell conditioning and isolation of exosomes

Cells were cultured in OptiMEM serum-free medium (Invitrogen) at a density of 3×10⁶ cells per ml for between 24 and 72 h to generate conditioned media. Conditioned media were pre-cleared of cells and debris by centrifugation at 15,000 g for 10 min and 1500 g for 15 min. Microparticles were pelleted by ultracentrifugation at 4°C of the pre-cleared media for 30 min at 15,000 g. Conditioned media were pre-cleared of cells and debris by centrifugation at 150,000 g for 30 min at 4°C of 3×10⁶ cells per ml for between 24 and 72 h to generate conditioned media. Lipids were extracted into N-butanol or chloroform:methanol:H2O (2:5:1) and injected for LC-MS/MS analysis into a Shimadzu ion-trap Perkin Elmer system, as previously described (Peppiatt et al., 2004).

Phospholipid extraction and mass spectrometry analysis

Lipids were extracted into N-butanol, and the levels of C17 LPC and C17 LPA were determined by performing mass spectrometry.

Immunoblotting

Cells were lysed in TG lysis buffer (20 mM Tris-HCl pH 7.5, 137 mM NaCl, 1 mM EGTA, 1% Triton X-100, 10% Glycerol, 1.5 mM MgCl₂, 1 mM NaVO₄, 50 mM NaF and protease inhibitors) and insoluble proteins were pelleted by centrifugation at 16,000 g for 10 min at 4°C. Exosomes, microparticles, concentrated soluble proteins and cell lysates were fractionated through 6–12% Bis-Tris-acrylamide gels by performing reducing or non-reducing SDS-PAGE. Proteins were transferred to PVDF membranes and immunoblotted overnight with indicated antibodies.

Ca²⁺ mobilisation microscopy

Quiescent NIH3T3 cells were loaded with 1 µM Fura2 AM for 30 min, followed by a 30-min de-esterification period in 121 mM NaCl, 5.4 mM KCl, 800 µM MgCl₂, 6 mM NaHCO₃, 5.5 mM glucose, 5 mM HEPES pH 7.3, 5.5 mM CaCl₂. Single-cell Fura2 imaging was performed using a Perkin Elmer system, as previously described (Peppiatt et al., 2004).

Thymidine incorporation

NIH3T3 cells were seeded into 24-well plates at a density of 2.5×10⁵ cells per well, grown to 70% confluence and then cultured in serum-free DMEM for 24 h to quiesce and synchronise the cells. Where stated, cells were pretreated for 10 min with the LPA receptor antagonist Ki16425 (10 µM) and then stimulated for 24 h with either 10 µM LPA or 5 µg/ml exosomes. 5 µM unlabelled thymidine and 0.5 µCi [³H] thymidine was added, and the cells incubated for a further 6 h. Cells were fixed with 5% ice-cold trichloroacetic acid, solubilised with 0.1 M NaOH, and was radioactivity determined by scintillation counting.

Wound healing assay

HEK293 cells were serum-starved for 24 h in DMEM, and then a scratch across the monolayer was made using a sterile pipette tip. Exosomes in OptiMEM±DMSO±10 µM Ki16425 were added to the scratched cell monolayer, and images were acquired using an Olympus IX81 microscope equipped with a 4×0.13 NA lens, Mäzhäuser motorised stage, Hamamatsu ORCA ER CCD camera and a Solent Scientific incubation chamber. For each experiment, two fields of view per well were imaged at 1×1 binning with a 50-ms exposure every 1 h for a total of 24 h. Images were analysed to identify the progression of the wound healing. Briefly, raw tiff images were processed using the ‘Find Edges’ algorithm in ImageJ (NIH), with the resultant image data exported to Velocity (PerkinElmer) for segmentation. The pixel area covered by cells was calculated as a proportion of the total image, and the change in this value over time was calculated.

Phospholipid extraction and mass spectrometry analysis

PBS-solubilised exosome pellets were incubated at 37°C for 30 min. Lipids were extracted into N-butanol or chloroform:methanol:ethanol (2:1:1 containing (50 ng each of C2₄:0 phosphatidic acid, C17:0 LPA and 100 ng each of C24:0 phosphatidylincholine and C17:0 LPC). Dried samples were resuspended in 50 µl of chloroform:methanol:ethanol (2:5:1) and injected for LC-MS/MS analysis into a Shimadzu ion-trap-time-of-flight mass spectrometer (IT-TOF-MS) hyphenated with a five-channel on-line degasser, four pumps, column oven and autosampler with cooler (Prominence HPLC, Shimadzu) was used for lipid analysis. The lipid classes were separated on a normal-phase silica gel column (2.1×150 mm, 4 µm, MicroSolv Technology) with different mixtures comprising hexane, dichloromethane, chloroform, methanol, acetonitrile, water or ethylamine solvent gradients based on the polarity of the head group. In detail, different classes of lipids were separated on a normal-phase silica gel column (2.1 mm×150 mm, particle size 4 µm; MicroSolv Technology, Eastontown, NJ) with a ternary gradient of solvents. The column was equilibrated with a 3:1 mixture of hexane and chloroform (volume ratio) and eluted with a gradient of solvent B comprising...
dichloromethane:chloroform:methanol (45:45:10, by volume) containing 0.08% ethylamine (v/v), followed by a gradient from solvent B to solvent C, comprising chloroform:methanol:acetonitrile:water (30:30:30:10, by volume) containing 0.12% ethylamine (v/v). With post-column addition of ~10% (v/v) 20 mM ammonium formate in 50% aqueous methanol during the gradient from solvents B to C, the column eluent was infused into the Shimadzu IT-TOF instrument for analysis. Accurate mass (mass accuracy of <5 ppm) and tandem mass spectrometry were used for molecular species identification and quantification. The identity of lipids was further confirmed with appropriate lipid standards. The IT-TOF MS operating conditions were: electrospray ionisation (ESI) interface voltage, +4.5 kV for positive ESI and -4 kV for negative ESI; heat-block temperature, 230°C; nebulizing gas flow, 1.4 l/min; curved desolvation line temperature, 210°C with drying gas on at a pressure of 100 kilopascals. All the solvents used for lipid extraction and LC-MS/MS analysis were of LC-MS/MS grade (Fisher Scientific).

Statistical analysis

Results were representative of at least three independent experiments and are expressed as mean± s.d. Differences were analysed with a Student’s t-test, assuming equal variances (Microsoft Excel), and GraphPad Prism 6 version 6.0d (GraphPad Software)

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Competing interests

The authors declare no competing or financial interests.

Author contributions


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