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Autotaxin is released from adipocytes, catalyses lysophosphatidic acid synthesis, and activates preadipocyte proliferation: up-regulated expression with adipocyte differentiation and obesity.

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Abstract

Our group has recently demonstrated (Gesta et al. *J. Lipid. Res.*, 2002, 43:904-910) the presence, in adipocyte conditioned-medium, of a soluble lysophospholipase D-activity (LPLDact) involved in synthesis of the bioactive phospholipid, lysophosphatidic acid (LPA). In the present report, LPLDact was purified from 3T3F442A-adipocyte conditioned-medium and identified as the type II ecto-nucleotide pyrophosphatase phosphodiesterase: autotaxin (ATX). A unique ATX cDNA was cloned from 3T3F442A-adipocytes, and its recombinant expression in COS-7 cells led to extracellular release of LPLDact. ATX mRNA expression was highly up-regulated during adipocyte differentiation of 3T3F442A-preadipocytes. This up-regulation was paralleled by the ability of newly differentiated adipocytes to release LPLDact and LPA. Differentiation-dependent up-regulation of ATX expression was also observed in primary culture of mouse preadipocytes. Treatment of 3T3F442A-preadipocytes with concentrated conditioned medium from ATX expressing-COS-7 cells led to an increase in cell number as compared with concentrated conditioned medium from ATX non-expressing-COS-7 cells. The specific effect of ATX on preadipocyte proliferation was completely suppressed by co-treatment with a LPA-hydrolyzing phospholipase, phospholipase B. Finally, ATX expression was found in mature adipocytes isolated from mouse adipose tissue, and was substantially increased in genetically obese-diabetic db/db mice when compared to their lean siblings.

In conclusion, the present work shows that ATX is responsible for the LPLDact released by adipocytes, and exerts a paracrine control on preadipocyte growth via an LPA-dependent mechanism. Up-regulations of ATX expression with adipocyte differentiation and genetic obesity suggest a possible involvement of this released protein in the development of adipose tissue and obesity-associated pathologies.

Abbreviations: LPA, lysophosphatidic acid; LPLDact, lysophospholipase D activity; LPC: lysophosphatidylcholine; ATX: autotaxine; CM: conditioned medium; CCM: concentrated conditioned medium; FCS, fetal calf serum; CHCl₃, chloroform, MeOH, methanol; TLC, thin layer chromatography.

Introduction:

Because of its ability to store extra energy as triacylglycerol (lipogenesis) and to release fatty acids and glycerol (lipolysis), adipose tissue plays a crucial role in energy balance. In obesity, excessive accumulation of triacylglycerol in adipocytes (hypertrophy) results from an alteration in the balance between lipogenic and/or lipolytic activities of the adipocytes.

It is now recognized that, beside their involvement in lipid homeostasis, adipocytes also produce and secrete numerous factors. Among them are endocrine peptides (leptin, adiponectin, angiotensinogen, etc) which may play an important role in the development of morbid complications of obesity such as cardiovascular diseases, hypertension, diabetes and cancer. Other adipocyte-secreted factors (tumor necrosis factor, fatty acids, eicosanoids, lysophosphatidic acid, etc.) are produced locally and may influence adipose tissue development and/or metabolism by exerting autocrine/paracrine effects on the different cells composing adipose tissue (adipocytes, preadipocytes, endothelial cells) (1-3). Of particular interest is the ability of some adipocyte-secreted factors to exert a paracrine control on preadipocyte proliferation and differentiation, cellular processes leading the recruitment of new fat cells in adipose tissue (adipogenesis) and further increase in adipose tissue mass (3,4).

Our group has demonstrated that LPA is released from adipocytes *in vitro* and is present *in vivo* in the extracellular fluid of adipose tissue collected by microdialysis (5). In parallel, LPA is able to activate preadipocyte motility and proliferation by interacting preferentially with LPA1-R (6). Therefore LPA may participate to the paracrine control of adipogenesis.

Lysophosphatidic acid (LPA) is a bioactive phospholipid regulating a wide range of cellular responses (proliferation, survival, motility, ion flux, secretion) through the activation of G-protein-coupled receptors: LPA1-, LPA2-, and LPA3-R (7,8). Bioactive LPA was initially found in culture serum (9), and was further detected in other biological fluids such as plasma (10,11), ascitic fluid (12), follicular fluid (13), aqueous humor (14), and the extracellular fluid of adipose tissue (5). Whereas, serum-LPA mainly originates from aggregating platelets (15,16), the precise cellular origin of LPA in other biological fluids remains still unclear.

We recently showed that, in parallel to LPA, adipocytes also release a LPA-synthesizing activity that was characterized as a lysophospholipase D-activity (LPLDact) catalyzing transformation of lysophosphatidylcholine into LPA (17). Adipocyte LPLDact is soluble, and sensitive to cobalt ions (17). In addition, adipocyte lysoPLD-activity is insensitive to primary alcohol, suggesting that it could be catalyzed by a non-conventional phospholipase D that remained to be identified.

In the present study, adipocyte LPLD-activity was purified and identified as the type II ecto-nucleotide pyrophosphate phosphodiesterase, autotaxin (ATX). ATX was found to be expressed by and released from adipocytes, activates preadipocyte proliferation, and its expression was strongly up-regulated during adipocyte differentiation and in a model of genetic obesity. Because of its LPLDact, adipocyte-ATX leads to LPA synthesis and could be involved in the control of adipose tissue development as well as in obesity-associated pathologies.

Experimental Procedures:

Culture and transfection studies

The mouse preadipose cell line 3T3F442A (18) was grown and differentiated as previously described (19). Briefly, cells were grown to confluence (day 0 of differentiation) in DMEM supplemented with 10% donor calf serum, and then shifted in a differentiating medium consisting in DMEM supplemented with 10% fetal calf serum plus 50 nM insulin. In these culture conditions, quiescent preadipocytes differentiate into functional adipocytes. COS-7 monkey cells (ATCC) were grown in DMEM supplemented in 10% FCS and transfected using DEAE dextran as previously reported (20).

Preparation of conditioned media

3T3F442A-cells or COS-7-cells, were washed twice with PBS in order to remove serum, and incubated (5 ml for a 10 cm diameter plate; 1 ml for a 3 cm diameter plate) in serum-free DMEM supplemented (for LPA release) or not (for LPLDact release) with 1% free-fatty acid BSA at 37°C in a humidified atmosphere containing 7% CO₂. Conditioned media were also prepared from adipose tissue by incubating 300 to 500 mg of finely cut out subcutaneous adipose tissue from db/+ or db/db mice in 3 ml of serum-free DMEM in order to measure released LPLDact. After various time of incubation (0 to 5 hours for measurement of LPLD-activity; 7 hours for quantification of LPA and lysophosphatidylcholine (LPC)), conditioned-medium (CM) was separated from the cells or tissue, centrifuged to eliminate cell debris and stored at -20°C before measurement of LPLDact or quantification of LPA. In some experiments, conditioned media were concentrated (about 50 fold) using an Amicon Ultra 10,000 (Millipore). After adjustment of the protein concentration concentrated conditioned media (CCM) were aliquoted and stored at -20°C before use.

Measurement of lysophospholipase D activity

LPLDact was measured by conversion of radiolabeled LPC into radiolabeled LPA as previously described (21) with minor modifications. A solution of [¹⁴C]palmitoyl-lysophosphatidylcholine (NEN, 55.8 mCi/mole) at 0.0025 μCi/μl in DMEM supplemented with 1% free fatty acid BSA was first prepared, and 20 μl of this solution was incubated with 500 μl thawed CM plus 1 μl of sodium orthovanadate 0.5 mM for 90 min at 37°C. At the end of the incubation period phospholipids were extracted with 500 μl of 1-butanol, evaporated, spotted on a silica gel 60 TLC glass plate (Merck), and separated using CHCl₃/MeOH/NH₄OH (60/35/8) as the migration solvent. The plate was autoradiographed overnight at -80°C using a Biomax-MS film (Kodak) in order to localize radiolabelled LPA spots, which were scraped and counted with 3ml of scintillation cocktail.

Purification of lysophospholipase D-activity

All the procedures were performed at 0-4° C. One hundred twenty ml of 3T3F442A adipocyte conditioned medium containing LPLDact was centrifuged 1h at 100,000 g to remove cells debris and concentrated 8 fold on polyethylene glycol 20,000 (PEG 20,000). After overnight dialysis against 10 liters of purification buffer (PB) using Spectra-Por 1.7 ml/cm tubing (Pierce Chemicals, Interchim, Montluçon, France) to remove salts and small proteins, 16 ml of the concentrated medium was applied (60 ml/h) onto a heparin-sepharose column (CL+6B, Amersham-Pharmacia,

15 ml packed volume), equilibrated with 100 ml PB. The column was washed with 150 ml of PB and eluted with 125 ml of PB containing 0.5 M NaCl. The elution fraction containing LPLDact was dialyzed overnight against 10 liters of PB using Spectra-Por 1.7 ml/cm tubing (Pierce Chemicals, Interchim, Montluçon, France) to remove NaCl, and concentrated on polyethylene glycol 20,000 to obtain a volume of 40 ml. This concentrate was applied (60ml/h) on a phosphocellulose column (P11, Whatman, 15 ml packed volume) equilibrated with 100 ml PB. The column was washed with 150 ml of PB, and eluted with 125 ml of PB containing 0.5 M NaCl. The elution fraction containing LPLDact was dialyzed overnight against 10 liters of purification buffer using Spectra-Por 1.7 ml/cm tubing (Pierce Chemicals, Interchim, Montluçon, France) to remove NaCl, and concentrated on PEG 20,000 to obtain a final volume of 5 ml. This concentrate was separated (30 ml/h) over a gel filtration column (HiLoad 16/60, Superdex 200 PrepGrade, Amersham-Pharmacia) on a FPLC Amersham-Pharmacia system previously equilibrated with PB. The column was eluted with the same buffer. Aliquots of the collected fractions were assayed for LPLDact. All the fractions containing LPLDact were pooled and concentrated 10 times on dialysis tubing (0.5ml/cm) in PEG 20000. The protein concentration was determined by the Bradford assay (Protassay, BioRad, Ivry s/Seine, France) with bovine serum albumin as standard.

SDS-PAGE separation

SDS-PAGE 4-20% was performed according to Laemmli (22) followed by Sypro Ruby or colloidal blue staining. After addition of sample buffer (Novex, Invitrogen...) concentrated fractions from gel filtration were boiled at 100°C for 5min. Electrophoretic separation of proteins was carried out on a 1mm-thick 8 x 6-cm gel 10% acrylamide. A 40 µg portion of total protein in sample buffer was loaded into a 4-mm well of the gel and separated at 40mA. A total of 30µg of standards (Mark12, Invitrogen) migrated in a neighboring lane. After coloration with colloidal Coomassie blue (Biosafe, Bio-Rad), the 110kDa protein was cut, reduced and alkylated using DTT and iodoacetamide, respectively, and subjected to digestion with trypsin overnight following the protocol of Shevchenko et al. (23). Gel pieces were successively washed with ammonium bicarbonate and dehydrated with acetonitrile. After drying in a Speedvac (Heto, Denmark) they were incubated at 56°C with 10 mM DTT and then at room temperature with 55mM iodoacetamide. After successive washing steps with ammonium bicarbonate and acetonitrile, gel pieces were completely dried in the speed-vac. Trypsin solution (Promega, USA) was added and digestion was performed overnight at 37°C. Peptide digests were extracted successively with acetonitrile and 5% formic acid. Before analysis peptides were dried in the speed-vac, diluted in 5% formic acid and desalted on Poros R-2 resin. Peptides were eluted with 2µl 60% methanol/formic acid 1% directly into nanospray capillary needles (Micromass, UK).

Tandem mass spectrometry

Peptides were analysed on a Q-TOF 2 mass spectrometer (Micromass, UK). MS acquisitions were performed within the mass range of 550-1300 m/z and MS/MS within 50-2000 m/z.

Database searching and sequence analysis

Amino acid sequences were used to search SWISS-PROT and TREMBL with BLAST interface (US National Center for Biotechnology Information). Partial

amino acid sequences (sequence TAG) were used to search database with PeptideSearch interface (EMBL, Heidelberg, Germany). Hits were confirmed by performing a theoretical trypsin digestion with GPMW (Lighthouse data, Denmark) and comparing the resulting peptide mass and sequence to those obtained experimentally.

Real time RT-PCR analysis

Total RNAs were isolated from using RNA Easy mini kit (Qiagen). Total RNA (1 µg) were reverse transcribed for 60 min at 37°C using Superscript II reverse transcriptase (Life Technology) in the presence of random hexamer. A minus RT reaction was performed in parallel to ensure the absence of genomic DNA contamination. Real time RT-PCR was performed starting with 25 ng cDNA and 900 nM (human Atx and mouse Atx) or 900 nM (mouse ap2) concentration of both sense and antisense primers in a final volume of 25 µl using the SYBR green TaqMan Universal PCR Master Mix (Applied Biosystem). Fluorescence was monitored and analyzed in a GeneAmp 5700 detection system instrument (Applied Biosystems). Analysis of the 18S ribosomal RNA was performed in parallel using the Ribosomal RNA control Taqman Assay Kit (Applied Biosystem) in order to normalize gene expression. Results are expressed as following: $2^{-(Ct^{18S}-Ct^{gene})}$ where Ct corresponds to the number of cycles needed to generate a fluorescent signal above a predefined threshold.

Oligonucleotide primers were designed using the Primer Express software (Perkin Elmer Life Sciences). Oligonucleotides used were:

Mouse ap2: sense 5'-TTCGATGAAATCACCGCAGA-3', antisense: 5'-GGTCGACTTTCCATCCCCTT-3';

Mouse ATX: sense: 5'-GACCCTAAAGCCATTATTGCTAA-3', antisense: 5'-GGGAAGGTGCTGTTTCATGT-3'

Mouse LHS: sense 5'-GGCTTACTGGGCACAGATACCT-3', antisense: 5'-CTGAAGGCTCTGAGTTGCTCAA-3'

Cloning of autotaxin from 3T3F442A adipocytes

Total RNA from 15 days post-confluent 3T3F442A adipocytes was reversed transcribed using random hexamers and Superscript II reverse transcriptase (Invitrogen, France). First strand cDNA (corresponding to 1 µg of total RNA) was amplified using a program consisting of 30 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 3 min with a pre- and postincubation of 95°C for 2 min and 72 for 10 min, respectively. PCR amplification utilized oligonucleotide primers based on GenBank entry for mouse autotaxin (forward 59-64, reverse 2630-2651, accession number BC003264). The PCR fragment was isolated and ligated into pcDNA3 (Invitrogen) down-stream of the FLAG peptide sequence (Sigma). The recombinant plasmid, designated pcDNA-mATX-FLAG, was sequenced on both strands by automated sequencing.

Quantification of LPA and LPC

LPA was butanol-extracted from conditioned medium and quantified using a radioenzymatic assay as previously described (11). The amount of lysophosphatidylcholine present in conditioned media was determined as previously described (17), after 30 min treatment with bacterial phospholipase D followed by LPA quantification.

Housing and treatment of animals

Male C57BL/KsJ db/db and db/+ mice (Jackson Laboratory) and were housed in an animal room maintained at 22°C, on a 12:12 h light/dark cycle, with ad libitum access to food and water. Animals were handled in accordance with the principles and guidelines established by the National Institute of Medical Research (INSERM). On the day of sacrifice, mouse blood was collected on heparin and glucose was immediately measured with a glucose meter (Glucometer 4; Bayer Diagnosis, Puteaux, France). Perigonadic and inguinal white adipose tissues were removed and adipocytes were isolated as described above and immediately processed for RNA extraction using RNA Easy mini (Qiagen). The other tissues (interscapular brown adipose tissue, brain, kidney and liver) were removed and snap-frozen in liquid nitrogen before RNA isolation using RNA STAT (Ams Biotechnology Ltd, Oxon, UK) kit.

Isolation and culture of preadipocytes and adipocytes from mouse adipose tissue

Adipose tissue was dissected out from mice, and digested in 5 ml DMEM supplemented with 1 mg/ml collagenase, 1% BSA, and 2 µg/ml gentamycin, for 45 to 60 min at 37°C under shaking. Digestion was followed by filtration through a 100 µm screen and centrifugation at 800 g for 10 min at room temperature. This phase allow separating floating adipocytes from the pellet containing the stromal-vascular fraction. Adipocytes were washed twice in DMEM and further processed for RNA extraction using RNA Easy mini kit (Qiagen). The stromal-vascular pellet was washed twice in DMEM and resuspended in 1 ml lysis buffer (Tris 17 mM, NH₄Cl 0.83% pH 7.6) for 3 min in order to get rid of erythrocytes, and seeded in 12 well plates (150 000 cells per plate) in DMEM/HamF12 supplemented with 10% FCS. After overnight culture the medium was replaced by a serum-free differentiating medium consisting in DMEM/HamF12 supplemented with transferrin (10 µg/ml), biotine (33 µM), insulin (66 nM), T3 (1 nM) and panthotenate (17 µM). That time point is considered as day 0. In these conditions adipocyte differentiation occurs few days after (24). Total RNAs were prepared at different time point after induction of the differentiation using RNA Easy mini kit (Qiagen).

Results

1/ Co-purification of adipocyte lysophospholipase D-activity with autotaxin:

In order to identify the enzyme responsible for LPLDact activity present in adipocyte-CM (17), its purification from 3T3F442A adipocyte conditioned-media (CM) was undertaken. LPLDact hydrolyzes the phospho-choline bound in lysophosphatidylcholine (LPC) to generate lysophosphatidic acid (LPA) and free choline. In the present work, LPLDact was measured using [¹⁴C]lysopalmitoyl-phosphatidylcholine as substrate, followed by TLC separation of synthesized [¹⁴C]LPA (see Experimental Procedures). Initially the release of LPLDact in adipocyte-CM was observed in a Hepes Buffer incubation medium (17). As shown in Figure 1, a stronger (3.3 fold after 5h incubation) LPLDact was measured when using DMEM as the incubation medium. In both incubation media LPLDact was detectable after 30-min incubation and reached a maximum after 5h. Based upon these results purification of LPLDact was undertaken from 3T3F442A adipocyte-CM prepared after 5h incubation in DMEM.

After 10 fold concentration CM was first applied onto a heparin affinity chromatography (Figure 2, 1), washed and eluted with 0.5 M NaCl at once (lane e) and concentrated again. This allowed recovering LPLDact but no specific band was identified by SDS-PAGE (lane f). As a second purification step, concentrated fraction eluted from heparin column was applied onto a phosphocellulose column (Figure 2), eluted at 0.2M NaCl (lane i), concentrated 25 times (lane j), applied on gel filtration column (Figure 1, 3), and eluted (fractions 52 to 82). LPLDact was detected in fraction 68 to 76. SDS-PAGE analysis revealed the presence of a band (molecular weight between 116 and 97 kDa) in the fractions exhibiting maximal lysoPLD activity (70, 72, and 74). The fraction 70 was concentrated 100 times, separated on 4-20% SDS-polyacrylamide gel electrophoresis, stained with Coomassie blue, and cut out from the gel. Tryptic cleavage followed by tandem mass spectrometry of the specific band of fraction 70 led to the generation of 27 peptides exhibiting 100% homology (Table 1) with mouse ecto-nucleotide pyrophosphatase phosphodiesterase (ENPP2), also called autotaxin (25,26). These results showed that adipocyte-LPLDact was co-purified with ATX.

2/ Cloning of 3T3F442A adipocyte-autotaxin.

A unique cDNA encoding ATX was cloned from 3T3F442A adipocytes and subcloned in pcDNA-FLAG vector. This cDNA exhibited 100% identity with previously identified mouse-ATX cDNA present in Genbank data-base (BC003264, NM_015744, AF123542) (Table 1). The corresponding protein is composed of 863 amino acids (molecular mass of approximately 100 kDa) which, by analogy with human, corresponds to the previously described β -form (27-29). In human ATX the site of cleavage has been located between S-48 and D-49 in the amino acid sequence EGPPTVLS/DSPWNT which is entirely conserved in mouse (BC003264, NM_015744, AF123542, and herein 3T3F442A-cDNA sequence). Based upon mouse-ATX sequence, the expected cleavage site of mouse-ATX should be between S-47 and D-48. This should lead to a secreted protein of 816 amino acids (molecular mass of approximately 94 kDa). Purification of lysoPLD-activity from 3T3F442A adipocytes led to identification of a protein with a molecular mass between 97 and 116 kDa (Figure 1). This mass is slightly higher than that calculated for the secreted form of mouse-ATX. Such a discrepancy could be attributed to the presence, in

mouse sequence, of at least 4 glycosylation sites (Net Nglyc 1.0 Prediction analysis) that would increase the apparent molecular weight of the secreted protein.

3/ ATX behaves as secreted a lysophospholipase D.

In order to determine whether ATX indeed behaved as a secreted lysophospholipase D, 3T3F442A adipocyte-ATX cDNA was transiently transfected in COS-7 cells. After transfection with empty pcDNA vector, COS-7 cells did not release LPLDact in culture medium (Figure 3). Conversely, transfection with pcDNA-mATX-FLAG vector led to the release of LPLDact in the culture medium (Figure 3). Similar results were obtained when transfecting COS-7 cells with a pcDNA vector expressing human ATX (30,31).

4/ Up-regulation of ATX with adipocyte differentiation.

We previously observed that adipocytes release more LPLDact than preadipocytes (17). We therefore tested the existence of an adipocyte differentiation-dependent regulation of ATX. Adipocyte differentiation of 3T3F442A cells was generated by treating confluent preadipocytes (day 0) with a differentiation medium (see Experimental Procedures). This treatment led to a rapid (4 days after confluence) emergence of specific genetic markers of differentiation such as adipocyte fatty acid binding protein (aP2) and hormone sensitive lipase (HSL) mRNAs (Figure 4B), followed (7 days after confluence) by the accumulation of triglycerides (Figure 4A), the final adipocyte phenotype. Whereas aP2 and HSL mRNA level reached a plateau after 10 days (Figure 4B), triglyceride content continued to increase regularly thereafter (Figure 4A).

In these conditions, ATX mRNAs were detected 7 days after confluence and their level increased regularly thereafter (Figure 4B). Interestingly, the emergence of ATX mRNAs was delayed by 3-4 days as compared to that of aP2 and HSL mRNAs.

Up-regulation of ATX mRNA was tightly associated with the increase in LPLDact (Figure 4C) and in LPA concentration (Figure 4D) in the culture medium. In parallel, the culture medium also contained LPC (the substrate for LPLDact) which was present at higher concentration than LPA, but which was not significantly modified during adipocyte differentiation (Figure 4D).

Up-regulation of ATX was also observed after adipocyte-differentiation of primary culture of preadipocytes. As previously described (24), the preadipocytes present in the stroma vascular fraction of adipose tissue can be differentiated into adipocytes when cultured for 7 to 10 days in the presence of a differentiating cocktail (see Experimental procedure). As shown in Figure 5, after 10 days the expression of ATX mRNA was increased 10 fold as compared to the first day of culture. These results clearly show the existence of a regulation of ATX with adipocyte differentiation. ATX mRNA were also present in mature adipocytes isolated from mouse adipose tissue and their expression was lower than in 10 days-differentiated adipocytes (Figure 5).

5/ Recombinant ATX allowed the release of LPA by preadipocytes

Our group has previously demonstrated that LPA is able to increase preadipocyte proliferation (5,6). Conversely to adipocytes, preadipocytes did not release LPLDact (see Figure 4C) nor LPA (Figure 4D), but released LPC (see Figure 4D), the substrate of the LPLDact of ATX. Therefore, we tested whether ATX could exert an LPA-dependent increase in preadipocyte proliferation.

In order to test this hypothesis, conditioned media from COS-7 cells transfected with empty pcDNA or with pcDNA-mATX-FLAG were concentrated and used to treat 3T3F442A preadipocytes. As shown in Figure 6A, 5 h treatment with concentrated conditioned medium from pcDNA-mATX-FLAG transfected COS-7 cells (ATX-CCM), led to a dilution-dependent increase in LPA concentration in 3T3F442A preadipocyte culture medium. LPA was significantly detected with 10 μ l/ml dilution, and reached a maximum at 100 μ l/ml with a range of LPA concentration varying from 13 to 130 nM. Conversely no significant concentration LPA was generated after treatment with concentrated conditioned medium from empty-pcDNA transfected COS-7 cells (sham-CCM) (Figure 6A).

In order to test the influence of recombinant ATX on preadipocyte proliferation, 3T3F442A preadipocytes were exposed to 50 μ l/ml sham-CCM or ATX-CCM and cell number was determined after 72h treatment. As shown in Figure 6B, sham-CCM led to an increase in cell number suggesting the presence of a growth factor different from ATX in sham-CCM. Nevertheless, treatment with ATX-CCM led to an additional increase in cell number (Figure 6B), which was completely abolished by co-treatment phospholipase B, a lysophospholipase that we and others have previously demonstrated to hydrolyze and inactivate LPA (5,6,9,32). These results strongly supported that recombinant ATX was able to increase preadipocyte proliferation according to an LPA-dependent mechanism.

6/ Up-regulation of adipocyte ATX in db/db mice

In order to test the existence of a regulation of ATX expression with obesity, adipocyte ATX expression was measured in genetically obese diabetic mice: db/db. As expected 12 weeks old db/db mice were obese as attested by higher body weight (1.7 fold) and higher adipose tissue weight (9.5 and 10 fold in perigonadic and inguinal depot respectively) when compared to their lean siblings db/+ mice (Table 2). As also expected, db/db mice were diabetic as attested by their higher (3.6 fold) non-fasting glycemia when compared with db/+ mice (Table 2). As shown in Figure 7A, ATX mRNA expression was significantly higher in isolated adipocyte from db/db (3.8 and 4.8 fold in perigonadic and inguinal adipocytes respectively) as compared to db/+. A significant increase in ATX mRNA expression was also observed in interscapular brown adipose tissue (2.4 fold) and kidney (2.2 fold) (Figure 7B). Conversely no changes were observed in ATX mRNA expression in liver and brain (Figure 7B). In a separate experiment, small explants of subcutaneous adipose tissue from db/+ and db/db mice were incubated in serum-free DMEM and released LPLDact was measured. After 5 h incubation, LPLDact released in the incubation medium was 18.2 ± 2.0 and 29.3 ± 4.0 pmoles LPA/mg protein in db/+ (n=5) and db/db (n=5) adipose tissue explants ($P < 0.01$). This result showed that up-regulation of ATX mRNA in adipose tissue of db/db mice was accompanied by up-regulation of its activity. Taken together above results showed that db/db genetic obesity was associated with up-regulation of ATX expression in some tissues including adipose tissue.

Discussion

The initial objective of the present study was to identify the enzyme responsible for LPLDact released in the culture medium of adipocytes. Our results show: (i) that adipocyte LPLDact is co-purified with ATX; (ii) that ATX is expressed in adipocytes; (iii) that recombinant expression of ATX in COS-7 cells permits the release of LPLDact in their culture medium; (iv) that ATX expression and activity are strongly increased during adipocyte differentiation; (v) that recombinant ATX is able to increase preadipocyte proliferation via a LPA-dependent mechanism; (vi) that adipocyte-ATX is substantially over-expressed in a genetic model of obesity.

ATX is an ecto-nucleotide pyrophosphatase phosphodiesterase initially discovered as a motility-stimulating protein in the culture medium of A2058 melanoma cells (25). ATX was then found to be expressed in several cell lines and tissues (28). Motility-stimulating action of ATX is blocked by pretreatment with pertussis toxin, suggesting the involvement of a G-protein coupled receptor in its action (31). ATX is an ecto-protein anchored to the plasma membrane by a short N-terminal sequence, and released in the extracellular medium likely after proteolytic cleavage. In its extracellular domain, ATX contains a large phosphodiesterase catalytic site involved in hydrolysis of phosphodiester and pyrophosphate bonds present in classical substrate such as ATP or ADP (26,30,33).

Our results strongly support that ATX is responsible for the LPLDact released from 3T3F442A adipocytes. LPLDact was indeed co-purified with a protein exhibiting 100% homology with previously cloned mouse-ATX. In addition, a release of LPLDact in culture medium was obtained after recombinant expression of mouse ATX cDNA in COS-7, a cell line that does not normally release LPLDact. Finally, ATX expression was clearly demonstrated in adipocytes, and was tightly co-regulated with the release of LPLDact and LPA in culture medium during differentiation of 3T3F442A preadipocytes. Our conclusions are in agreement with recent publications, which appeared during the completion of the present study, reporting that ATX was responsible for a LPLDact present in bovine and human sera (34,35).

ATX expression is strongly up-regulated during adipocyte differentiation. Terminal differentiation of adipocytes is a complex process that requires the emergence of specific genes such as aP2 and HSL (analyzed in the present work), necessary for the adipocytes to ensure its specific metabolic functions such as triglyceride accumulation. When comparing with aP2 and HSL, ATX expression is rather late during adipose conversion, and is more closely associated with the accumulation of triglycerides. These observations suggest that ATX expression is a consequence of, rather than a requirement, for adipocyte differentiation. Further experiments will be necessary to precisely identify the factor(s) involved in the regulation of ATX expression with adipocyte differentiation.

ATX expression in adipocyte is substantially increased in genetically obese diabetic db/db mice. These mice bear a mutation in leptin receptor resulting in hyperphagia and massive accumulation of adipose tissue associated with type II diabetes and ultimately type I diabetes. Up-regulation of adipocyte ATX in db/db suggests that this protein could either be associated with obesity, or diabetes, or both. ATX belongs to the ecto-nucleotide pyrophosphate phosphodiesterase (ENPP) family. This family encompasses the membrane glycoprotein plasma cell 1 (PC-1), a protein with a high structure homology with ATX (36). It is interesting to notice that PC-1 expression was found to be increased in adipose tissue of obese diabetic

Zucker fatty rats (37) which, like db/db mice bear a mutation on leptin receptor. Alterations in PC-1 expression have been associated with alterations in whole body insulin sensitivity as well as insulin receptor-tyrosine kinase activity (38,39). Further experiments will be necessary to identify the factor(s) involved in regulation of ATX in obesity and/or diabetes.

What could be the role of adipocyte-ATX? In adipose tissue, adipocytes and preadipocytes are present in the same environment and communicate with each other via paracrine mediators. As demonstrated in the present study, ATX catalyses a LPLDact which is responsible for the presence of LPA in adipocyte-culture medium. We previously demonstrated that LPA is present in adipocyte culture medium and can activate preadipocyte growth via the activation of LPA1-receptor (6). In contrast to adipocytes, preadipocytes do not release ATX but release high concentration of LPC (see Figure 4), the substrate of ATX for LPA synthesis. It can be speculated that, in intact adipose tissue, one of the possible roles of adipocyte ATX would be to exert a paracrine control on preadipocyte growth due to the generation of LPA at the extracellular face of the preadipocytes in close contact with adipocytes. This hypothesis is supported by our results showing that recombinant ATX is able to activate preadipocyte proliferation. In addition, the blocking effect of phospholipase B strongly suggests that the action of ATX on preadipocytes is very likely to be mediated by synthesis of LPA and the activation of LPA-receptor present at the surface of the preadipocytes. This hypothesis is in agreement with previous reports showing that ATX activates cell motility and cell proliferation in several cell lines via a G-protein coupled receptor which could be a LPA-receptor (31,34).

What could be the consequence of the proliferative activity of ATX on preadipocytes? Preadipocyte proliferation is a key step in adipose tissue development since it conditions the number of potential new adipocytes in the adipose tissue. In most obesity models, such as db/db mice (where we observed an over-expression in adipocyte-ATX), the increase in adipose tissue mass results from an excess accumulation of triglycerides in existing adipocytes (hypertrophy) followed, above a certain level of hypertrophy, by the recruitment of new adipocytes (hyperplasia). It could be proposed that adipocyte-ATX plays a role in the recruitment of new adipocytes by exerting a paracrine control on preadipocyte growth. ATX could therefore play a role in obesity-associated hyperplasia. This hypothesis is reinforced by very recent report (40) showing that LPA behaves as an agonist of the Peroxisome Proliferator Activated Receptor γ , a crucial transcriptional factor involved in adipocyte differentiation program.

Obesity-associated increase in fat mass is also dependent on its vasculature and therefore requires angiogenesis (41). ATX has previously been demonstrated to behave as a pro-angiogenic mediator (42). Adipocyte-ATX could then play a role in the paracrine control of angiogenesis in adipose tissue. Finally, an endocrine action of adipocyte-ATX can also be proposed. As pointed out earlier, ATX has recently been identified in blood (serum and plasma) (34,35) but, despite the presence of ATX mRNA in different tissues (28), the origin of circulating ATX remains unknown. When comparing with other tissues, ATX mRNA appears to be fairly abundant in adipose tissue. In addition, considering the high mass of adipose tissue in the body, particularly in obese individuals, its significant contribution in circulating ATX can be proposed. Future development of transgenic animals exhibiting altered expression of adipocyte-ATX will help to verify these different hypotheses.

In conclusion, the present work identifies ATX as a new adipocyte-mediator exerting its biological activities via neo-synthesis of the bioactive phospholipid LPA. Its close association with adipocyte differentiation and obesity and/or diabetes suggests a possible role of ATX in normal or pathological development of adipose tissue.

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Tables

Table 1: Sequence of the peptides derived from the 97-116 kDa protein co-purified with adipocyte LPLDact . The specific band of 97-116 kDa in fraction 70 of the Figure 2 was submitted to tryptic cleavage and analyzed by tandem mass spectrometry allowing the identification of 27 peptides A exhibiting 100 % homology with mouse ATX (Accession number BC003264) (B). Underlined and bold characters indicates the peptides identified by mass spectrometry. <> indicates potential proteolytic cleave site.

1. List of the peptides positively recognised by mass spectrometry

SYSSCCHDFDELCLK	TSYDILYHTDFESGYSEIFLMPLWTSYTISKQAE
VPECPAGFVRPPLIIFSVDGFR	VSSIPEHLTNCVRPDVR
SCGTHAPYMRPVYPTK	VSPGF SQNCLAYK
WWGGKPLWITATK	QMSYGF LFPY LSSSPEAK
WWGGKPLWITATKQGVRA	YDAFLVTNMVPMYPAFKR
GTF FWSVSIPHER	NGVNVISGPIFDYNYDGLR
ILTILQWLSLPDNERP	YASERNGVNVISGPIFDYNYDGLR
SVYAFYSE QPDFSGHKYGP F	VSPGF SQNCLAYKNDK
GPEMTNPLR	QYVEGSSIPVPTHYYSIITSCLDFTQPADK
WWGGKPLWITATKQGV R	VRDIEHLTGLDFYR
TEFLSNYLTNVDDITLVPGLGR	DIEHLTGLDFYR
KPDQHFKPYMKQHLPK	DIEHLTGLDFYRK
VNSMQTVFVGYGPTFK	SYSEILTLKTYLHTYESEI
	TYLHTYESEI

2. Mouse autotaxin sequence*

MARQGC FGSYQVISLFTFAIGVNLCLGFTASRIKRAEWDEGPPTVLS<>DSPWTNTSGSCKG
RCFELQEVGPPDCRCDNLCK**SYSSCCHDFDELCLK**TARGWECTKDRCGEVRNEENACHCS
EDCLSRGDCCTNYQVVCKGESHVWDDDCEEIR**VPECPAGFVRPPLIIFSVDGFR**ASYMKKGS
KVMPNIEK**LRSCGTHAPYMRPVYPTK**TFPNLYTLATGLYPESHGIVGNSMYDPVFDATFHRLR
GREKFNHR**WWGGKPLWITATKQGV RAGTFFWSVSIPHERILTILQWLSLPDNERPSVYAF**
YSEQPDFSGHKYGPFGPEMTNPLREIDKTVGKLM DGLKQLKLHRCVNVIFVGDHGMEDVTC
DR**TEFLSNYLTNVDDITLVPGLGR**IRPKIPNNLKYDPKAIANLTCK**KPDQHFKPYMKQHLPK**
RLHYANNRRIEDLHLLVERRWHVARKPLDVYKKPSGKCFQGDHGF DNK**VNSMQTVFVGYG**
PTFKYRTKVPPFENIELYNVMCDLLGLKPAPNNGTHGSLNHLLRTNTFRPTLPEEVS RPNYPG
IMYLQSDFDLGCTCDDKVEPKNKLEELNKRLHTKGS TEERHLLYGRPAVLRYR**TSYDILYHTDF**
ESGYSEIFLMPLWTSYTISKQAEVSSIPEHLTNCVRPDVRVSPGF SQNCLAYKNDKQMSYGF
LFPY LSSSPEAKYDAFLVTNMVPMYPAFKRRVWTFYQRVLVKKY**YASERNGVNVISGPIFDYN**

**YDGLRDIEDEIKQYVEGSSIPVPTHYYSIITSCLDFTQPADKCDGPLSVSSFILPHRPDNDESC
NSEDESKWVEELMKMHTARVRDIEHLTGLDFYRKTSRSYSEILTLKTYLHTYESEI**

Table 2: Weights and glycemia of 3-month-old male db/db (+/-) and db/db (-/-) mice. Values are means \pm SE of 4 animals for each group.

Mice	Glycemia (g/l)	Body weight (g)	Adipose tissue weight	
			Perigonadic (g)	Inguinal (g)
db/db (+/-)	1.4 \pm 0.1	23.6 \pm 1.9	0.20 \pm 0.04	0.21 \pm 0.03
db/db (-/-)	5.2 \pm 0.7	40.8 \pm 2.4	1.89 \pm 0.15	2.22 \pm 0.29
P<	0.01	0.001	0.001	0.01

Legend of the figures:

Figure 1: Release of LPLDact from 3T3F442A adipocytes. Differentiated 3T3F442A adipocytes were incubated in serum-free DMEM and LPLDact present in the culture medium after various time of incubation (0 to 5 hours) was measured as described in Experimental Procedures using [¹⁴C]palmitoyl-lysophosphatidylcholine ([¹⁴C]LPC) as the substrate and [¹⁴C]lysophosphatidic acid ([¹⁴C]LPA) as the product. Representative of three separated experiments.

Figure 2: Purification of LPLDact from 3T3F442A adipocyte conditioned medium. Conditioned media from 3T3F442A adipocytes (a) was concentrated 10 times on PEG 20000 (b) and applied on a Heparin agarose column (1), washed with Tris buffer (c, d), eluted with 0.5M NaCl (e), concentrated 10 times on polyethylene glycol (f) and washed with Tris buffer (g, h). Fraction f was applied on a phosphocellulose agarose column (2), and eluted in NaCl gradient at 0.2M NaCl (i), concentrated 25 times (j) on polyethylene glycol, applied on a gel filtration column (3) and fractionated in Tris buffer: fraction 52 to 82 (fractions 52-56 are flow-through fractions). The protein content of each fraction was analyzed on SDS-PAGE (A) in parallel to the measurement of LPLDact (B). Wm: weight marker. Arrow corresponds to the band which was analyzed by mass spectrometry (Table 1) in fraction 70 and 72 (**).

Figure 3: Recombinant expression of ATX in COS-7 cells. COS-7 cells were transiently transfected with empty pcDNA (lane 2) or with pcDNA-mATX-FLAG (lane 3), or with pcDNA-human ATX (lane 4). Twenty-four hours after transfection, COS-7 cells were incubated for 5 h in serum-free DMEM before measuring the LPLDact in the culture medium. Lane 1 corresponds to LPLDact measured in 3T3F442A adipocyte conditioned medium as described in Figure 1. The figure is representative of at least 3 separate experiments.

Figure 4: Up-regulation of ATX expression with adipose differentiation of 3T3F442A preadipocytes. Confluent 3T3F442A preadipocytes (day 0) were induced to differentiation as described in Experimental Procedures. At different time after induction cells were either collected to measure triglyceride content ($\mu\text{g}/\text{mg}$ cell protein) (A) and mRNA expression ($/18\text{S RNA} \times 10^4$) (B), or placed in serum-free medium to measure LPLDact (pmoles LPA/ mg cell protein) (C), and the amount of LPA and LPC (pmoles/mg cell protein) (D) in the culture medium as described in Experimental Procedures. Values are means \pm SE of 3 experiments.

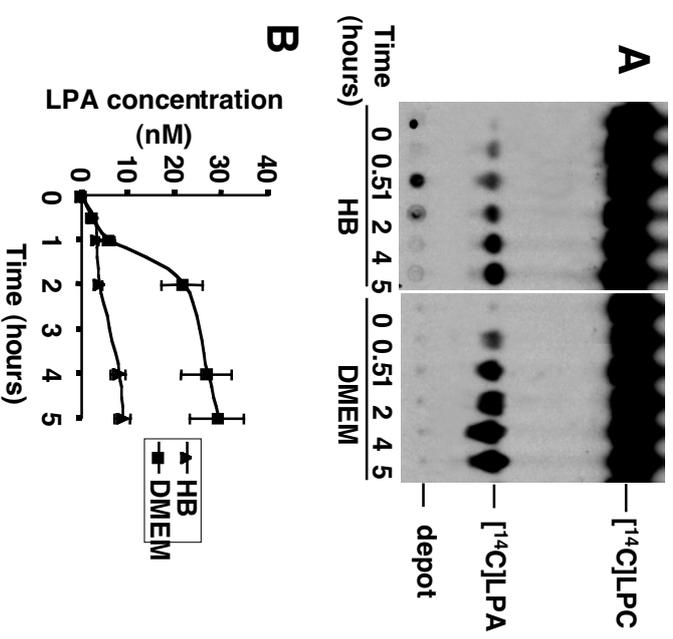
Figure 5: Differentiation-dependent regulation of ATX in primary mouse preadipocytes. Preadipocytes were isolated from the stroma vascular fraction of the adipose tissue of 4 weeks old C57BL6J mouse as described in Experiment Procedures. ATX mRNA expression was measured after overnight attachment (day 0) cells, and after 10 days culture (day 10) in a differentiating-medium (see Experimental Procedures). ATX mRNA expression was also measured in mature adipocytes freshly isolated from mouse adipose tissue. Values are means \pm SE of 3 separate experiments.

Figure 6: Influence of recombinant ATX on preadipocytes. (A) Serum starved 3T3F442A preadipocytes were treated with decreasing dilutions of

concentrated conditioned medium (CCM) from COS-7 cells transiently transfected with either empty pcDNA (sham-CCM) or pcDNA-mATX-FLAG (ATX-CCM) in DMEM supplemented with 1% BSA. After 5h treatment LPA was quantified in preadipocyte culture medium using a radioenzymatic assay. (B) Serum starved 3T3F442A preadipocytes were treated with 50 μ l/ml sham-CCM or ATX-CCM in DMEM supplemented with 1% BSA. Treatments were performed in the presence or absence of 0.1 U/ml phospholipase B (PLB). After 72h treatment preadipocyte number was measured using a cell counter.

Figure 7: ATX expression in obese diabetic db/db mice. Total RNA were extracted from different tissues of lean db/+ and obese db/db mice: (A) adipocytes isolated from white adipose tissues (PG: perigonadic; ING: inguinal); (B) whole inguinal white adipose tissue (WAT), brown adipose tissue (BAT), liver, kidney, and brain. ATX mRNA level (/18S RNA x 10⁴) was measured by real time RT-PCR. Values are mean \pm SE of 4 animals.

Figure 1



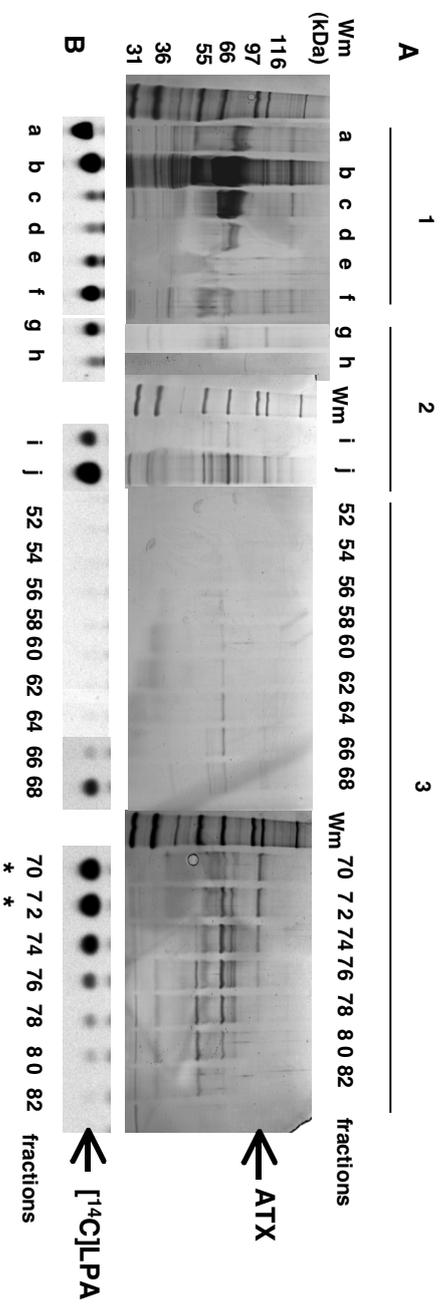


Fig. 2

Figure 3

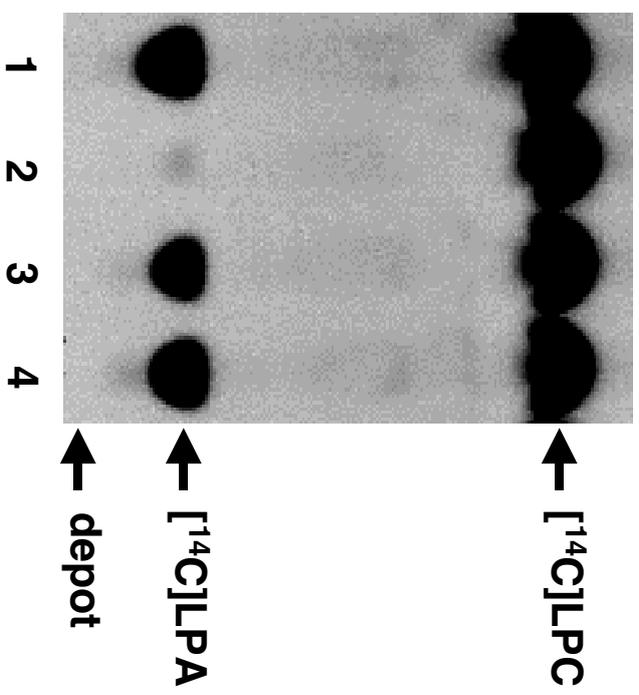


Figure 4

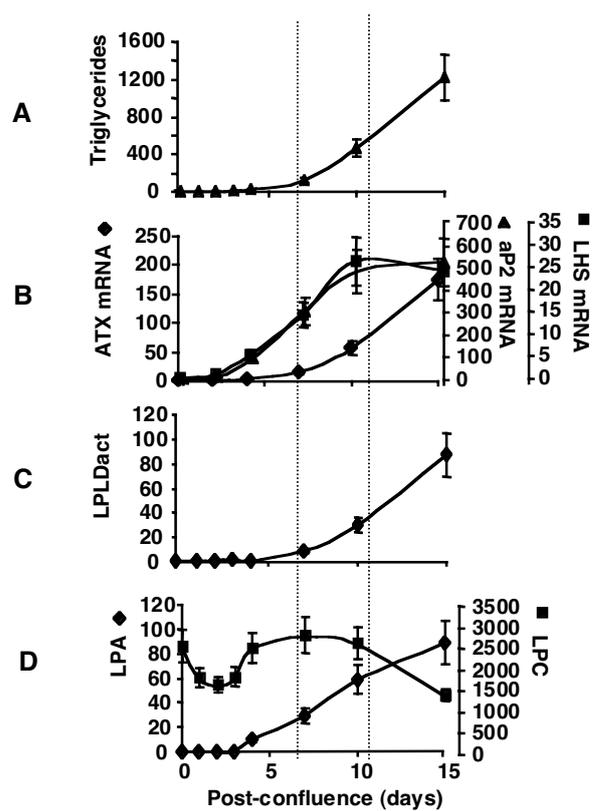


Figure 5

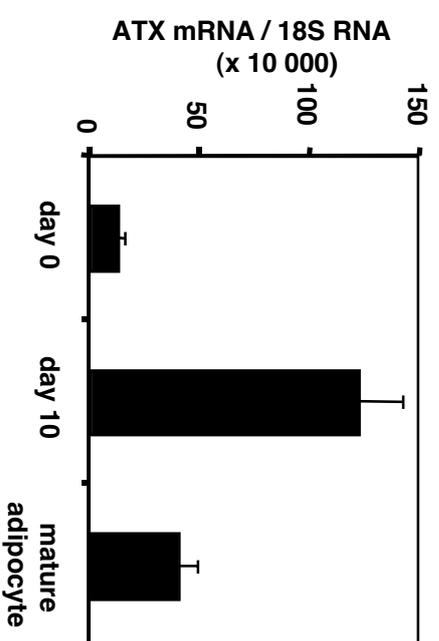


Figure 6

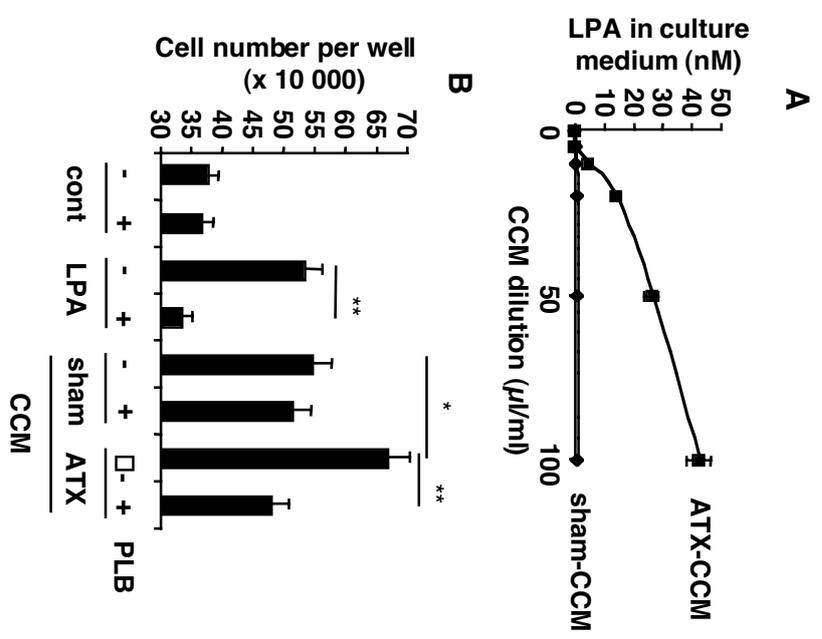


Figure 7

