

**SECRETION AND LYSOPHOSPHOLIPASE D ACTIVITY OF AUTOTAXIN  
BY ADIPOCYTES ARE CONTROLLED BY N-GLYCOSYLATION AND SIGNAL  
PEPTIDASE**

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## **Abstract.**

Autotaxin (ATX) is a lysophospholipase D involved in synthesis of lysophosphatidic acid (LPA). ATX is secreted by adipocytes and is associated with adipogenesis and obesity-associated diabetes. Here we have studied the mechanisms involved in biosynthesis and secretion of ATX by mouse 3T3-F442A adipocytes. We found that inhibition of N-glycosylation with tunicamycin or by double point deletion of the amino-acids N53 and N410 of ATX inhibit its secretion. In addition, N-glycosidase treatment and point deletion of the amino-acid N410 inhibits the lysophospholipase D activity of ATX. Analysis of the amino-acid sequence of mouse ATX shows the presence of a N-terminal signal peptide. Treatment with the signal peptidase inhibitor globomycin inhibits ATX secretion by adipocytes. Transfection in Cos-7 cells of site-directed deleted ATX shows that ATX secretion is dependent on the hydrophobic core sequence of the signal peptide, not on the putative signal peptidase cleavage site sequence. Analysis of the amino-acid sequence of mouse ATX also reveals the presence of a putative cleavage site by the protein convertase furin. Treatment of adipocytes with the furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone does not modified secretion or lysophospholipase D activity of ATX. Transfection in Cos-7 cells of site-directed deleted ATX shows that the furin recognition site is not required for secretion or lysophospholipase D activity of ATX. In conclusion, the present work demonstrates the crucial role of N-glycosylation in secretion and activity of ATX. The present work also confirms the crucial role signal peptidase in secretion of ATX by adipocytes.

## Introduction.

Autotaxin (ATX), also known as ENPP2 (ectonucleotide pyrophosphatase phosphodiesterase-2), is a secreted lysophospholipase D involved in hydrolysis of lysophosphatidylcholine (LPC) into lysophosphatidic acid (LPA). LPA is a bioactive phospholipid acting via specific G-protein coupled receptors (1). ATX was initially discovered as to be secreted in the culture medium of human melanoma cells (2). It was then detected in human and bovine plasma (3). ATX expression was linked to invasiveness of breast cancer cells and was demonstrated to increase the invasive potential of transformed cells (4) and to induce strong angiogenic responses (5). Knocking-out ATX in mouse is lethal as a result of a profound alteration in blood vessel formation during early development (6).

Our group has shown that ATX is secreted by adipocytes (7, 8), and that LPA strongly influences proliferation and differentiation of preadipocytes via the activation of LPA1 receptor (9-11). The expression of ATX is up-regulated during adipogenesis (7) as well as in adipocytes from obese-diabetic db/db mice and in adipose tissue from gluco-intolerant obese women subjects (7, 12). These observations led us to propose that, beside its involvement in cancer, ATX may also be involved in adipose tissue development and/or obesity-associated pathologies such as diabetes.

ATX is N-glycosylated (13) but the role of this post-translational modification on the activity and the secretion of the protein remains unclear. Because of its homology with other members with of the ENPP family, ATX was initially thought to behave as a type-II integral membrane protein expressed at the cell surface and released into the extracellular space after proteolytic cleavage (14). Nevertheless, recent reports have shown that ATX possesses a signal peptide (15, 16) (the linear structure of ATX is illustrated in Figure 1) and does not accumulate at the cell surface (15-17) suggesting that ATX is synthesized as a secreted protein. ATX also possesses a highly conserved recognition site for the protein convertase furin, few amino-acids upstream of the signal peptide (see Figure 1). The contribution of this putative furin-cleavage site in the secretion and the activity of ATX remains unclear.

In the present work, biosynthesis and secretion of ATX was studied in mouse 3T3F442A adipocytes that endogenously express and secrete relatively high amount of ATX. In parallel, our study was also conducted in Cos-7 cells expressing various mouse ATX mutants to precise the amino-acids required for ATX maturation and secretion. The present work shows that: (i) the secretion and activity of ATX is tightly dependent on N-glycosylation; (ii) the signal peptidase activity plays a crucial role in secretion of ATX; (iii) the furin-cleavage does not contribute to secretion nor lysophospholipase D activity of ATX.

## Material and Methods

### *Materials*

Globomycin was obtained from Dr. Shinichi Miyakoshi (Sankyo Co, Japan); furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK) was purchased from Alexis Biochemicals; concanavalin-A (ConA) sepharose was purchased to Amersham Biosciences (ref: 17-0440-01); ATX-peptide (573-KNKLEELNKRRLHTKGS-588) antibody was purchased from Cayman Chemical, USA; Amicon Ultra 10,000 centrifugation columns were from Millipore; FITC anti-rabbit antibody was from TEBU Bio; IGFI-receptor  $\beta$  antibody (C-20) was from Santa Cruz Biotechnology; N-glycosidase (3U) and O-glycosydase (3.6 mU) were purchased from Roche Applied Science; chemiluminescence detection system (ECL) was from Amersham Biosciences; anti rabbit-HRP antibody was purchased from Signam Aldrich; .Gel Nu-PAGE 4-12% was from Invitrogen; Quick Change TM Site-directed

Mutagenesis kit was from Stratagene; [ $^{14}\text{C}$ ]palmitoyl-lysophosphatidylcholine (2035 MBq/mmol) was from NEN; silica gel 60 TLC glass plate were purchased from Merck; Biomax-MS film were from Kodak; tunicamycin and brefeldin A were purchased from Sigma Aldrich.

**Cell lines and culture:** The mouse preadipose cell line 3T3F442A (18) was grown and differentiated as previously described (19). Briefly, cells were grown to confluence in DMEM supplemented with 10% donor calf serum, and differentiated into adipocytes by culturing them in a adipogenic medium consisting in DMEM supplemented with 10% fetal calf serum plus 50 nM insulin for 7 days. COS-7 monkey cells (ATCC) were grown in DMEM supplemented in 5% FCS and transfected using DEAE dextran as previously reported (7). Transfection were performed with either empty pcDNA3 vector or with wild type or mutated pcDNA-mATX vector encoding for mouse ATX. Details about the construction and sequence of these vectors have previously been described (7).

**Preparation of conditioned media:** 3T3F442A adipocytes and COS-7-cells, were washed twice with PBS 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 18h. Conditioned medium (CM) was collected and centrifuged to eliminate cell debris, and concentrated (about 50 fold) using an Amicon Ultra 10,000 (Millipore) and stored at  $-20^{\circ}\text{C}$  before analysis. In parallel, cells were homogenized in RIPA buffer (0.01 M Tris-HCl (pH 7.0), 0.15 M NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 0.1% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, and 2 mM phenylmethylsulfonylfluoride), centrifuged (15 min, 13000 rpm,  $4^{\circ}\text{C}$ ), and supernatant were stored at  $-20^{\circ}\text{C}$  before analysis.

**Detection of ATX by immunocyto fluorescence:** Cells were cultured on glass cover-slips in the same conditions than described above. Cells were washed twice with PBS and fixed with methanol (100%) for 8 min at  $-20^{\circ}\text{C}$ , dried and washed once with PBS. After 30 min saturation with PBS containing 0.1% Tween and 5% fetal calf serum (PBST-FCS), cells were incubated with ATX-peptide antibody (5.2 mg/ml) in PBST-FCS for 1h at room temperature. Cells were then incubated in PBST-FCS with 0.5 mg/ml FITC anti-rabbit antibody for 30 min at room temperature. The cells were finally rinsed twice in PBS and once in water, and then dried and mounted before analysis of the fluorescence under microscope.

**Concanavalin-A-mediated purification of ATX from cell homogenates:** Preliminary experiments allowed us to observed that specific detection of ATX by western blot in cell homogenates required a pre-purification with concanavalin A-sepharose as previously described (20). Thirty  $\mu\text{l}$  of Concanavalin-A (ConA) sepharose beads suspension was first washed once in 1 ml Binding Buffer (20 mM Tris base, 0.3 M NaCl, pH 7.4) followed by centrifugation (5 min 14000 rpm). The ConA beads pellet was then suspended in 1 ml of binding buffer supplemented with cell homogenate (500  $\mu\text{g}$  protein). After overnight incubation at  $4^{\circ}\text{C}$  under shaking, the suspension was centrifuged (5 min 14000 rpm) and the beads pellet was washed twice with 1 ml of binding buffer. For western, ConA beads pellet was suspended in loading buffer and directly loaded in the gel. For lysoPLD activity measurement, ATX was first eluted by suspending ConA beads pellet in 0.5 ml alphaD mannopyranoside 0.3 M, and incubate 2h at  $37^{\circ}\text{C}$  under shaking. After centrifugation (3 min, 14 000 rpm) lysoPLD activity was measured on the supernatant.

**Subcellular fractioning by ultracentrifugation:** 3T3F442A adipocytes were homogenized by 3 cycles of freezing-sonication in buffer H (Hepes 50 mM, NaCl 150 mM,

EDTA 10 mM, Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> 10 mM, NaF 100 mM, sodium orthovanadate 2 mM, protease inhibitor cocktail (Sigma) 1/100), and ultracentrifuged at 100,000 g for 10 minutes. Total homogenate, pellet and supernatant were then concanavalin A-purified before western blot analysis.

**Treatment with glycosidases:** Concentrated CM (30 µg protein) were incubated for 18 h at 37°C with N-glycosidase (3U) and O-glycosydase (3.6 mU) in a final volume of 30 µl of DMEM before western blot analysis or measurement of lysophospholipase D activity.

**Detection of ATX secretion by western blot:** Concentrated CM (30 µg) or ConA beads pellet, were loaded and separated on a Gel Nu-PAGE 4-12% and transferred on nitrocellulose membrane. The blot was pre-incubated for 1 hour at room temperature in TBS/Tween 0.05% containing 3% dry-milk, and overnight at 4°C in the same solution supplemented with 0.7 µg/ml ATX-antibody. After washing in PBS/Tween 0.05%, ATX was visualized by enhanced chemiluminescence detection system using an anti rabbit-HRP antibody.

**Site directed Mutagenesis:** Deleted mutants were generated from plasmid pcDNA-mATX, by using site-directed mutagenesis kit according to manufacturer instructions. The positions of mutated amino acid residues are listed in Table 1.

**Measurement of lysophospholipase D activity:** Lysophospholipase D activity of ATX was measured by conversion of radiolabeled LPC into radiolabeled LPA as previously described (7). A solution of [<sup>14</sup>C]palmitoyl-lysophosphatidylcholine at 0.092 kBq/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 conditioned medium 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 TLC glass plate, and separated using CHCl<sub>3</sub>/MeOH/NH<sub>4</sub>OH (60/35/8) as migration solvent. The thin layers were autoradiographed overnight at -80°C using a Biomax-MS film in order to localize radiolabelled LPA spots, which were scraped and counted with 3ml of scintillation cocktail.

## RESULTS

We reported that ATX is expressed and secreted by adipocytes as a differentiation-dependent protein (7). As shown in Figure 2, ATX protein was detected by western blot in conditioned media (CM) from 3T3F442A adipocytes. In contrast, ATX was undetectable in CM from undifferentiated 3T3F442A preadipocytes (Figure 2A). The apparent molecular weight of ATX was estimated to 120 kDa (Figure 2A). Immunocyto-fluorescence microscopy revealed that ATX was detected in 3T3F442A adipocytes (Figure 3, B and C), and was almost undetectable in 3T3F442A preadipocytes (Figure 3, A). The fluorescent signal was considered to be specific to ATX since it was detected in Cos-7 cells transfected with mouse ATX expression vector (Figure 3, F and G), not in Cos-7 cells transfected with empty expression vector (Figure 3, E). Comparing visible and fluorescent images (Figure 3, C and D) revealed that the fluorescent signal was located in the cytoplasm of adipocytes with no detection at the plasma membrane. Same observation was made in Cos-7 cells transfected with mouse ATX expression vector (Figure 3, G and H). In order to precise the sub-cellular localization of ATX in adipocytes, the presence of ATX was analyzed by western blot of crude (pellet) and soluble (supernatant) fractions obtained after ultracentrifugation of 3T3F442A adipocyte homogenate

in detergent-free buffer. As shown in Figure 2B most of ATX was detected in the soluble fraction with only traces detected in the crude fraction. These results suggested that cell ATX behaves mainly as a soluble protein.

***N-glycosylation influences the secretion and the activity of ATX.*** CM from 3T3F442A adipocytes were treated with N-glycosidase or O-glycosidase, and the apparent molecular weight of ATX protein was analyzed by western blot. Whereas, N-glycosidase led to a consistent reduction of the apparent molecular weight of ATX, O-glycosidase was without influence (Figure 4A). The magnitude of the shift generated by N-glycosidase treatment was  $9.6 \pm 0.4$  kDa (n=3). N-glycosidase treatment was accompanied by a strong inhibition of lysophospholipase D (lysoPLD) activity of ATX (Figure 4B). O-glycosidase treatment has no influence on lysoPLD-activity (Figure 4B). Treatment of 3T3F442A adipocytes with tunicamycin (glycosylation inhibitor) dramatically inhibited ATX secretion (Figure 4C). Similar observation was made after treatment with brefeldin-A (inhibitor of trans-golgi transport) (Figure 4C) suggesting that ATX secretion requires an active trans-golgi network. We concluded that N-glycosylation of ATX strongly influences its secretion and its lysoPLD activity.

Computerized analysis of the amino acid sequence deduced from mouse ATX coding sequence with NetNGlyc 1.0 and NetOGly 3.1 software (<http://www.cbs.dtu.dk/biotools/>) revealed the presence of two significant putative N-glycosylation sites (53-NTSG-56 and 410-NLTC-413). To determine the specific contribution of the N-glycosylation on secretion of ATX, the amino acids N53 and N410 were deleted either separately (mutants 118 and 119) or together (mutant 120) (Figure 5A and Table 1). Wild type (construct 81) and mutant ATX constructs were transfected in Cos-7 cells and ATX secretion and lysophospholipase D-activity were analyzed. When compared to wild type ATX, deletion of N53 did not modify the secretion and slightly reduced (25%) the activity of ATX (Figure 5B CM and Figure 5C). Deletion N410 did not modified the secretion of ATX (Figure 5B CM), but strongly inhibited its activity (Figure 5C). Double deletion of N53 and N410 strongly inhibited ATX secretion, without altering the amount of ATX in cell homogenate (Figure 5B). It is noticeable that deletions of the amino-acids N53 and N410 led to a reduction of the apparent molecular weight of ATX (Figure 5B). We concluded that: (i) secretion of ATX requires the simultaneous glycosylation of the two asparagine residue (N53 or N410); (ii) glycosylation of the asparagine residue N410 plays a preferential role in lysophospholipase D-activity of ATX.

***A signal peptidase activity is required for ATX secretion.*** Analysis of mouse ATX sequence with ProP and Signal-P software predicts the existence of a signal peptide at the N-terminus with a signal peptidase site located between amino-acids G27 and F28 (Figure 1 and 8A). Cleavage by a signal peptidase is also in agreement with previously established feature of signal peptide sequences *ie* a hydrophobic core of 10 amino acids plus the presence of a small residue at position -1 (G27) and an uncharged residue at position -3 (C25) (21).

The involvement of a signal peptidase in ATX secretion by adipocytes was tested by a pharmacological approach using the inhibitor globomycin (22). ATX secretion by 3T3F442A adipocytes was inhibited by 6h pre-treatment with globomycin (Figure 6). Inhibitory effect of globomycin is efficient at concentrations higher than 80  $\mu$ g/ml and complete inhibition at 320  $\mu$ g/ml (Figure 6). We concluded that ATX secretion by adipocytes required a signal peptidase activity.

To test whether a proteolytic cleavage at the N-terminus of ATX was indeed required for ATX secretion, Cos-7 cells were transfected with expression vectors encoding either N- or C-terminal-M2 tagged mouse-ATX protein. The presence of ATX protein in CM was analyzed by western blot using either an ATX-peptide antibody or an anti-M2 antibody. Both

N- and C-terminal-M2 tagged ATX were detected with the anti-ATX-antibody (Figure 7). In contrast, when using the anti-M2-antibody, the C-terminal M2-tag-ATX is detected but the N-terminal-M2 tagged ATX is not (figure 7). We concluded that N-terminal cleavage precedes ATX secretion.

To define the N-terminal amino-acids that are required for ATX secretion, several mutations were generated in the 5'-end of its coding sequence. Mutants 116 and 117 target the hydrophobic core of the signal peptide. Mutants 117, 115, 106, 109, 110, 111 and 112 target the putative signal peptidase cleavage site (Figure 8A and Table 1). Mutants were transfected in Cos-7 cells and ATX secretion was analyzed by western blot. ATX secretion remains unchanged with mutants 115, 106, 109, 110, 111 and 112 (Figure 8B CM). Conversely, ATX secretion is completely suppressed with mutants 116 and 117 (Figure 8B CM). Interestingly, with mutants 116 and 117 ATX also disappeared in the homogenate (Figure 8B H). One possible interpretation of this result could be that, because of the suppression of the hydrophobic domain in the signal peptide pre-ATX was not be addressed properly to the reticulum leading to its degradation by the proteasome. In order to test this hypothesis treatments with proteasome inhibitor were performed. As shown in the Figure 8C, treatment with 25  $\mu$ M of lactacystin restored the detection of ATX in cell homogenate of the mutants 116 and 117. These results show that synthesis and secretion of ATX are highly dependent on the hydrophobic core of the signal peptide, but not on the amino acid composition the putative signal peptidase cleavage site.

***Furin is not required for secretion and activity of ATX.*** ProP analysis of ATX sequence reveals the presence of a highly conserved recognition site (32RIKR35) for the protein convertase furin, with a putative cleavage site located after the amino acid R35 (Figure 1 and 10A). The possible involvement of furin in secretion of ATX by adipocytes was tested by a pharmacological approach using the inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK) (23). The efficiency of CMK to inhibit furin activity in 3T3F442A, was determined by its ability to inhibit furin-dependent processing of pro-IGFI-receptor as previously described (24). In agreement with what has previously been reported in 3T3-L1 adipocytes (24), 24 h treatment with 200  $\mu$ M CMK led to an increase in the amount of pro-IGFI-receptor (Figure 9A). In contrast, 24h treatment with 200  $\mu$ M CMK had no influence on ATX secretion by 3T3F442A adipocytes (Figure 9B~~A~~). We also tested whether furin could influence the lysophospholipase D activity of ATX secreted by adipocytes. For that, lysophospholipase D activity was measured in CM from 3T3F442A adipocytes treated with increasing concentrations of CMK. Whatever the dose of CMK, lysoPLD activity remained unchanged when compared to control (Figure 9B). We concluded that furin convertase is not required for the secretion or the lysoPLD activity of adipocyte-ATX.

Mutants of mouse ATX (constructs 96, 98, 109, 102 and 107a) (Figure 10A and Table 1) bearing amino-acid deletions encompassing the potential furin cleavage site were generated. Each construct was transfected in Cos-7 cells and ATX secretion was analyzed by western blot. As shown in Figure 10B, none of ATX mutants modified ATX secretion. Secreted lysoPLD-activity from wild type ATX ( $1.6 \pm 0.5$  pmoles LPA/ $\mu$ g protein) was not significantly different from that of mutant 96 ( $1.0 \pm 0.2$  pmoles LPA/ $\mu$ g protein;  $p < 0.253$ , unpaired t-test,  $n=7$ ). We concluded that deletions of the furin-cleavage site is not involved in secretion and activity of ATX.

## DISCUSSION

The present work shows that: (i) the secretion and activity of ATX is tightly dependent on its N-glycosylation; (ii) the signal peptidase activity plays a crucial role in secretion of ATX; (iii) the furin-cleavage does not contribute to secretion nor lysophospholipase D activity of ATX.

Our work shows that ATX secreted by adipocytes is N-glycosylated and lacks O-glycosylation, as previously reported for ATX secreted by A2058 melanoma cell line (13). Our conclusion is in agreement with computerized analysis of ATX amino acid sequence that predicts the presence of two significant N-glycosylation sites (N53 and 410) and the absence of O-glycosylation sites. Glycosylation is a post-translational event occurring in endoplasmic reticulum and that plays a crucial role in sorting and targeting of numerous proteins at the appropriate cell compartment (25). It was therefore expected that altering glycosylation of ATX could strongly influence its secretion. We indeed observed that pharmacological inhibition of N-glycosylation by tunicamycin blocks ATX secretion by adipocytes. In addition, we found that double deletion of the two asparagines residues N53 and N410 also inhibited ATX secretion. Apart from its influence on correct protein targeting, glycosylation is also involved in the protein stability and folding (25). It was therefore expected that altering glycosylation of ATX could influence its enzyme activity. We indeed found that inhibition of N-glycosylation by N-glycosydase strongly inhibited the lysoPLD activity of ATX by adipocytes. In addition, we found that among the two N-glycosylation sites of ATX, the residue N410 was mainly involved in the lysophospholipase D-activity of ATX. It is interesting to notice that the amino acid N410 belongs to the catalytic domain (amino acids 163 to 518). One can therefore propose that glycosylation of N410 is required to get the optimal stabilization of the conformation of the catalytic domain of ATX.

ATX possesses a N-terminal signal peptide with a putative signal peptidase cleavage site located between amino-acids G27 and F28 (15, 16). We demonstrate that treatment by a signal peptidase inhibitor inhibits ATX secretion by adipocytes. These results confirm the involvement of a signal peptidase in ATX secretion. The signal peptide of ATX is composed of a hydrophobic core of 10 amino acids followed by the signal peptidase cleavage site preceded by a small residue at position -1 (G27) and an uncharged residue at position -3 (C25). By using site directed mutagenesis of mouse ATX N-terminal sequence we confirmed the crucial role of the hydrophobic core since its deletion completely abolished ATX secretion. Therefore, anchoring of the signal peptide of pre-ATX in membrane via its hydrophobic core appears to be crucial for ATX secretion. Cell fractioning experiments reveals that intra-adipocyte ATX behaves as a cyto-soluble protein rather than a membrane-anchored protein. This suggests that signal peptidase cleavage occurs early during ATX maturation, very likely in endoplasmic reticulum. Conversely to the hydrophobic core, none of the deletion in the putative signal peptidase cleavage site modified ATX secretion. For example, in the mutant 109 despite a complete change in the carboxy-terminus for the signal peptide, ATX is still normally secreted. This is in agreement with the concept that signal peptidases do not act on a precise consensus amino-acid sequence but rather behave as positional proteolytic enzymes (21).

#### ***Role of furin:***

Analysis of ATX sequence reveals the presence of a highly conserved recognition site for the protein convertase furin, few amino-acids upstream of the signal peptide. We demonstrate that treatment with a furin inhibitor do not alter ATX secretion by adipocytes. In addition, site directed deletion of furin recognition site did not modified ATX secretion. These results strongly support that furin-cleavage is not required for ATX secretion. The same conclusions was recently drawn by Jansen and colleagues using Flag-insertion experiments and N-terminal sequencing (15). In parallel to ATX secretion we also examined the possible



influence of furin on the lysoPLD activity of ATX. We observed that treatment with the furin inhibitor or site directed deletion of furin recognition site did not significantly altered the lysoPLD activity of ATX. We therefore conclude that furin has no influence on the lysoPLD of ATX. Our conclusion is not in complete agreement with that of Jansen and colleagues who showed that lysoPLD activity of furin-cleaved ATX was 30% higher than that of un-cleaved ATX (15). Maturation of ATX by furin was therefore proposed to be necessary to reach its full enzymatic activity. This apparent discrepancy may be explained by the fact that, whereas Jansen and colleagues measured lysoPLD-activity on purified ATX (furin-cleaved or not), our measure was performed on non-purified CM. As reported by Koike and colleagues, only a part of total secretion of ATX is cleaved by furin (16). In adipocytes, furin-cleaved may represents a minor portion of the total amount of secreted ATX, and its presence may consequently be difficult to detect with our assay.

### ***Conclusion:***

Our group has shown that ATX is secreted by adipocytes (7, 8) and its expression was found was up-regulated in adipocytes from obese-diabetic db/db mice as well as in massively obese women subjects (7, 12). ATX may therefore constitute an attractive target in the treatment of obesity and/or diabetes. Whereas inhibiting the catalytic site of ATX appears to be the most direct way to develop efficient ATX inhibitor, the results of the present study show that targeting glycosylation pathways or signal peptidase activity may also constitute interesting ways to block activity or secretion of ATX.

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## FOOTNOTES

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## Legends of the Figures:

**Figure 1: Linear structure of pre-autotaxin protein.** NG: N-glycosylation site; SP: signal peptide; SMD: somatomedin-like domain; CAT: catalytic domain; YPTKT: catalytic site; NUCL: nuclease-like domain; *SPse*: Signal peptidase cleavage site; FRS: furine recognition site; *Fr*: furin cleavage site; H2N-: N-terminus; -COOH: carboxy terminus.

**Figure 2: Secretion and sub-cellular localization of ATX in 3T3F442A adipocytes.** (A) Conditioned media were prepared (see Methods) from 3T3FF42 preadipocytes (pread) or adipocytes (adip) or from Cos-7 cells transfected with empty (empty) or mouse ATX expression (mATX) vector. The presence of ATX in conditioned media was analyzed by western blot analysis using an ATX-peptide antibody (see Methods). (B) 3T3F442A adipocyte homogenate was ultracentrifuged to separate crude and soluble proteins in pellet and supernatant respectively. Total homogenate (H), pellet (P) and supernatant (S) were concanavalin-purified (see Methods) and the presence of ATX was analyzed by western blot.

**Figure 3: Subcellular localization of ATX in 3T3F442A adipocytes.** Fixed cells were first stained with ATX-peptide antibody and with an FITC labeled secondary antibody and fluorescence was analyzed under microscope (see Methods). (A) 3T3F442A preadipocytes; (B, C, D) 3T3F442A adipocytes; (E) Cos-7 cells transfected with empty vector; (F, G, H) Cos-7 cells transfected with mouse ATX expression vector. C and D correspond respectively to the fluorescent and light transmission images of B insert. G and H correspond respectively to the fluorescent and light transmission images of E insert.

**Figure 4: Influence of N-glycosylation in maturation, activity and secretion of ATX in 3T3F442A adipocytes.** (A and B) conditioned media from 3T3F442A adipocytes were treated or not (cont) with 0- or N-glycosidases (see Methods), and the apparent molecular weight of ATX (A) and its lysophospholipase D activity (B) were analyzed. Values are mean  $\pm$  SE from 4 separate experiments. (C) Conditioned media were prepared from 3T3FF42 preadipocytes adipocytes previously treated or not (cont) with 15  $\mu$ g/ml tunicamycin (tun) or 10  $\mu$ g/ml brefeldin-A (bref) for 24h. The presence of ATX in conditioned media was analyzed by western blot analysis using an ATX-peptide antibody.

**Figure 5: Influence of glycosylation of N53 and N410 on maturation, secretion and activity of recombinant mouse ATX.** (A) location of mutant 118 and 119 related to the N-glycosylation sites (NG). The presence of ATX protein (B) and its lysophospholipase D activity (C) were analyzed in conditioned media (CM) from Cos-7 cells transfected with empty pcDNA vector (71) or pcDNA vector encoding wild type (81) or deleted ATX mutants 118, 119 and 120. Values are mean  $\pm$  SE from 3 separate experiments. Comparisons were performed using Student's *t* test (\*:  $P < 0.05$ ; \*\*\*:  $P < 0.001$ ).

**Figure 6: Influence of signal peptidase inhibition on ATX secretion by 3T3F442A adipocytes.** Conditioned media were prepared from 3T3FF42 preadipocytes adipocytes previously treated for 6h with increasing concentrations of globomycin. The presence of ATX in conditioned media (CM) and cell homogenate (H) was analyzed by western blot analysis using an ATX-peptide antibody. Autoradiogram is representative of 2 separate experiments.

**Figure 7: Requirement of N-terminal cleavage on secretion of recombinant mouse ATX.** Conditioned media from Cos-7 cells transfected with empty pcDNA vector (71) or pcDNA vector encoding mouse ATX bearing an M2 epitope at its C- (C-term) or at its N- (N-term) terminus. The presence of ATX in conditioned media was analyzed by western blot using either the ATX-peptide antibody (ATX antibody) or an M2-tag antibody (M2 antibody). Autoradiogram is representative of 2 separate experiments.

**Figure 8: Influence of signal peptide deletions on secretion of recombinant mouse ATX.** (A) location of ATX deleted mutants (SP: signal peptide; *SPse*: Signal peptidase cleavage site; FRS: furine recognition site; *Fr*: furin cleavage site). (B) The presence of ATX protein was analyzed by western blot in conditioned media (CM) and cell homogenate (H) from Cos-7 cells transfected with empty pcDNA vector (71) or pcDNA vector encoding wild type (81) or deleted ATX mutants 116, 117, 109, 110, 111, 112, 115. (C) Cos-7 cells transfected with deleted ATX mutants 116, 117 were treated or not with 25  $\mu$ M lactacystin for 18 h and the presence of ATX protein in cell homogenate (H) was analyzed by western blot. Autoradiograms are representative of at least 2 separate experiments.

**Figure 9: Influence of inhibition of furin activity on secretion and activity of ATX in 3T3F442A adipocytes.** (A) Influence of 24h treatment with 200  $\mu$ M CMK on the amount of pro-IGFI-receptor present in homogenate from 3T3F442A adipocytes. The presence of ATX protein (B) and its lysophospholipase D activity (C) were measured in conditioned media prepared from 3T3F442A adipocytes previously treated or not with CMK. Autoradiograms are representative of 3 separate experiments. Values are mean  $\pm$  SE from 3 separate experiments.

**Figure 10: Influence of furin recognition site deletions on secretion of recombinant mouse ATX.** (A) location of ATX deleted mutants. The presence of ATX protein (B) was analyzed by western blot in conditioned media from Cos-7 cells transfected with empty pcDNA vector (71) or pcDNA vector encoding wild type (81) or deleted ATX mutants 96, 98, 109, 102, 107a. Autoradiogram is representative of at least 2 separate experiments.

**Table 1: Mouse ATX deletion mutants.**

<b>Clone number</b>	<b>Amino acid deletion</b>	<b>Characteristic</b>	<b>Secretion</b>
<b>71</b>		Empty pcDNA vector	
<b>81</b>		Wild type	+
<b>96</b>	<b>R32-R35</b>	Furin site	+
<b>106</b>	<b>G27-A30</b>	Contains potential signal peptidase cleavage site	+
<b>115</b>	<b>N23-G27</b>	Encompasses -1 and -3 amino acids referring to the potential signal peptidase cleavage site	+
<b>116</b>	<b>V12-V22</b>	Hydrophobic domain of signal peptide	-
<b>117</b>	<b>V12-G27</b>	Hydrophobic domain of signal peptide + signal peptidase cleavage site	-
<b>110</b>	<b>N23</b>	Amino acid -5 referring to the potential signal peptidase cleavage site	+
<b>111</b>	<b>C25</b>	Amino acid -3 referring to the potential signal peptidase cleavage site	+
<b>112</b>	<b>G27</b>	Amino acid -1 referring to the potential signal peptidase cleavage site	+
<b>109</b>	<b>G27-R35</b>	Encompasses both furin and signal peptidase cleavage sites	+
<b>98</b>	<b>A30-I33</b>	Between signal peptidase and furin sites	+
<b>101</b>	<b>P43-L46</b>		+
<b>102</b>	<b>A36-E40</b>		+
<b>103</b>	<b>L46-S49</b>		+
<b>104</b>	<b>E40-P43</b>		+
<b>105</b>	<b>S49-N53</b>	Contains N-glycosylation site	+
<b>107a</b>	<b>A30-G41</b>	Encompasses furin site	+
<b>118</b>	<b>N53</b>	Contains a N-glycosylation site	+
<b>119</b>	<b>N410</b>	Contains a N-glycosylation site	+
<b>120</b>	<b>N53 + N410</b>	Two N-glycosylation sites	-