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Extracellular ATP is generated by ATP synthase complex in adipocyte lipid rafts

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Abbreviations: Apo-AI, apolipoprotein AI; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CTB, cholera toxin B; EMAP II, endothelial and monocyte-activating polypeptide II; PPARγ, peroxisome proliferators-activated receptor γ; VDAC, voltage-dependent anion channels

Abstract

Mitochondrial biogenesis is known to accompany adipogenesis to complement ATP and acetyl-CoA required for lipogenesis. Here, we demonstrated that mitochondrial proteins such as ATP synthase α and β, and cytochrome c were highly expressed during the 3T3-L1 differentiation into adipocytes. Fully-differentiated adipocytes showed a significant increase of mitochondria under electron microscopy. Analysis by immunofluorescence, cellular fractionation, and surface biotinylation demonstrated the elevated levels of ATP synthase complex found not only in the mitochondria but also on the cell surface (particularly lipid rafts) of adipocytes. High rate of ATP (more than 30 μM) synthesis from the added ADP and P_i in the adipocyte media suggests the involvement of the surface ATP synthase complex for the exracellular ATP synthesis. In addition, this ATP synthesis was significantly inhibited in the presence of oligomycin, an ATP synthase inhibitor, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an ATP synthase uncoupler. Decrease of extracellular ATP synthesis in acidic but not in basic media further indicates that the surface ATP synthase may also be regulated by proton gradient through the plasma membrane.

Keywords: adipocytes; ATP synthase complex; membrane microdomains; mitochondria

Introduction

An emergence of pluralistic function of a given protein is becoming a common finding. The multifunctionality of a protein can be achieved depending on its cellular localization, secretion, expression, oligomerization, ligand/substrate concentration, binding sites and complex formation (Jeffery, 1999). Many mitochondrial proteins are found in other unexpected cellular organelles. A recent proteomic analysis of integral plasma membrane protein shows many mitochondrial proteins such as ATP synthase, NADH dehydrogenase, and cytochrome c oxidase, suggesting that ATP- generating mitochondrial proteins might have their novel functions in the plasma membrane (Zhao *et al.,* 2004).

ATP synthase complex has been reported to have ability to recruit different ligands on the cell surface. ATP synthase complex associates with angiostatin, endothelial and monocyte-activating polypeptide II (EMAPII), and apolipoprotein AI (Apo-AI) that require cell surface receptors for their functions (Moser *et al.,* 1999; Chang *et al.,* 2001; Moser *et al.,* 2001; Martinez *et al.,* 2003). Since anti-ATP synthase α and β antibodies abolish the function of angiostatin, EMAP II, and Apo-AI, the ATP synthase complex might be the receptors for these ligands. Alternatively, the surface ATP synthase complex might be involved in extracellular ATP generation because extracellular ATP generation is inhibited by treating endothelial cells with anti-ATP synthase α antibody or ATP synthase inhibitors (Moser *et al.,* 1999; Arakaki *et al.,* 2003).

Lipid rafts composed of cholesterol and sphingolipids concentrate various receptors and their downstream molecules to facilitate different cellular signal transductions on the plasma membrane (Anderson, 1998; Brown and London, 1998; Galbiati *et al.,* 2001; Anderson and Jacobson, 2002; Capanni *et al.,* 2003; Ha *et al.,* 2003; Munro, 2003). The lipid rafts have been isolated from different mammalian cells using their distinct biochemical properties such as detergent insolubility and low density (Brown, 1992). Many lipid raft proteins have been identified by subproteomic analysis using liquid chromatography and tandem mass spectrometry as well as two-dimensional elec-

trophoresis (Calvo *et al.,* 2000; Bini *et al.,* 2003; Foster *et al.,* 2003; Li *et al.,* 2003; Blonder *et al.,* 2004; Sprenger *et al.,* 2004). In addition to various receptors and signaling molecules, many mitochondrial proteins such as prohibitin, voltage-dependent anion channels (VDACs,) and ATP synthase complex have been annotated to the lipid rafts (Bini *et al.,* 2003; Foster *et al.,* 2003; Li *et al.,* 2003; Sprenger *et al.,* 2004). Since these proteins are repetitively found in the plasma membrane and lipid rafts that are highly purified, these proteins might not be contaminants from mitochondria.

Adipose tissue is not only an energy reservoir with lipid droplets composed of triacylglycerides and cholesterol but also an important endocrine organ to regulate insulin sensitivity, food intake, and whole-body energy metabolism by producing and releasing cytokines such as tumor necrosis factor (TNF), adipokine, resistin, and leptin (Evans *et al.,* 2004). Since insulin stimulates lipid biosynthesis with acetyl-CoA that is synthesized in the mitochondria, lipogenesis is accompanied with mitochondrial biogenesis (Wilson-Fritch *et al.,* 2003). Here, we showed that the expression of ATP synthase complex, and mitochondrial biogenesis were highly increased during adipogenesis. The increased ATP synthase complex was found in lipid rafts as well as in the mitochondria, suggesting that ATP synthase might have its specific function in the plasma membrane. Finally, the inhibition of high rate of extracellular ATP synthesis in the adipocytes by oligomycin, an ATP synthase inhibitor, and CCCP, an ATP synthase uncoupler suggest that the surface ATP synthase might be involved in extracellular ATP generation in adipocytes.

Materials and Methods

Antibodies

Anti-ATP synthase α and β antibodies, and rhodamine conjugated-cholera toxin B (CTB) were obtained from molecular probes. Anti-insulin receptor β, caveolin-1, and flotillin-1 antibodies were from BD Biosciences. Anti-cytochrome c, and PPARγ antibodies were purchased from Santa Cruz.

Adipocyte differentiation

3T3-L1 fibroblasts were purchased from ATCC, and grown in DMEM supplemented with 1% penicillin/streptomycin (Jeil Biosciences Inc.), and 10% calf serum (Jeil Biosciences Inc.) in 5% CO_2 incubator at 37°C. Three-day-postconfluent 3T3-L1 fibroblasts were differentiated into adipocytes by incubating with DMEM supplemented with the same antibiotics, 10% FBS, 0.25 mM 3-isobutyl-1-methylxanthine (Sigma), 0.25 μM dexamethasone (Sigma), and 200 nM insulin (Sigma). After 48 h, the adipocytes were continuously incubated with fresh DMEM containing 10% fetal bovine serum (FBS) and 200 nM insulin. The adipocytes were re-fed with DMEM containing 10% FBS every 48 h.

Preparation of human adipocyte

Dissected fat pads from human were immersed in Krebs Ringer Hepes (KRH) buffer containing 120 mM NaCl, 4 mM KH_2PO_4, 17 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM $NaHCO_3$, 30 mM Hepes, 2.5 mM D-glucose, 200 nM adenosine, and 2% albumin. Adipose tissues were minced and digested with 2.5 mg/ml collagenase (Sigma) for 1 h at 37°C in shaking water bath. The suspension was centrifuged and the floating adipocytes were collected and filtered through 400-μm nylon mesh to remove tissue pieces. The cell suspension was then centrifuged at 400 *g* for 1 min. Adipocytes were collected from the supernatant of the suspension and resuspended in cold PBS. The centrifugation was repeated 3 times.

Isolation of mitochondria and plasma membrane

Plasma membrane was isolated according to the method of Hubbard *et al.* (1983) with small modifications. Differentiated 3T3-L1 adipocytes were washed twice with TES buffer (20 mM Tris HCl, 1mM EDTA, 8.7% sucrose; pH 7.4) and scraped. Cells were homogenized in TES buffer in a tight Dounce homogenizer (Kontes). The homogenized cells were centrifuged at 12,000 rpm in a SW55Ti rotor (Beckman) for 30 min. The pellet was resuspended in TES buffer, loaded on 50% sucrose and 38.5% sucrose cushion, and centrifuged at 100,000 *g* in a SW55Ti rotor for 60 min, 4°C. Plasma membrane was collected from the top of the sucrose cushion, resuspended in TES buffer, and repelleted by centrifugation at 31,000 *g* for 60 min, 4°C. Mitochondria were collected from the bottom of the sucrose cushion, resuspended in TES buffer, and repelleted by centrifugation at 5,000 *g* for 60 min, 4°C.

Immunoblotting and immunofluorescence

Preadipocytes and adipocytes were lysed with SDS lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodim deoxy cholate, 0.1% SDS, 1 mM EDTA, 1 mM PMSF, and protease cocktail (Roche)) for 20 min at 4°C. After microcentrifugation at 14,000 rpm, 10 min, 4°C, the whole cell lysates (supernatant) were separated by SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. Antigens were visualized by sequential treatment with specific antibodies, HRP-conjugated secon-

dary antibodies, and an enhanced chemiluminescence substrate kit.

For immunofluorescence, cells were fixed with 10% formaldehyde in PBS for 20 min. The cells were incubated with blocking buffer (5% BSA in PBS), and primary antibodies. The primary antibodies were detected with fluorescein-conjugated secondary antibodies. Also cells were incubated with rhodamine-conjugated CTB. Cells were observed with a Bio-Rad confocal microscope (MRC 1024).

Electron microscopy

3T3-L1 preadipocytes and adipocytes were fixed for 1 h at 4°C in 0.1 M sodium phosphate buffer (pH 7.4) containing 2% paraformaldehyde-2.5% glutaraldehyde mixture. Cells were harvested with cell lifter and washed with same buffer, centrifuged at 3,000 rpm for 5 mins. The pellets were post-fixed for 1 h in the same buffer containing 1% OsO_4, subsequently dehydrated using graded acetone, and embedded in Epon-Araldite. Thick and thin sections were prepared on RMC MT-X ultramicrotome. The sectioned thick tissues were stained with 1% toluidin blue-borax solution, mounted on copper grids, and double-stained with uranyl acetate. The grids were examined in JEM 100 CX-II electron microscope.

Surface biotinylation

Cell monolayers were biotinylated with 0.2 mg/ml EZ-link-sulfo-NHS-LC-biotin (Pierce) prepared in 10 mM Hepes, pH 8.0, 150 mM NaCl, 0.2 mM $MgCl_2$, and 0.2 mM $CaCl_2$ at 4°C for 30 min. The reaction was terminated by washes with 10 mM Tris · HCl (pH 7.5). The biotinylated cells were lysed with octylglucoside lysis buffer containing 1% Triton X-100, 60 mM *n*-octyl β-D-glucopyranoside, 150 mM NaCl, 25 mM Tris (pH 7.4), 1 mM EDTA, and protease cocktail at 4°C for 30 min. After microcentrifugation at 4°C for 20 min, the supernatant with 700 μg of protein was reacted with 50 ml of avidin-conjugated agarose beads at 4°C for 12 h. The beads were washed four times with octylglucoside lysis buffer. The precipitated proteins were resolved on 10% SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by immunoblotting with ATP synthase α and β antibodies.

Lipid raft isolation

Four 100-mm dishes of differentiated 3T3-L1 adipocytes, or 500 μl cell volume of human adipocytes were mixed with in 1 ml of lysis buffer (1% Brij 35, 25 mM Hepes, pH 6.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and protease cocktail), homogenized 20 times with a tight Dounce homogenizer (Kontes), and incubated at 4°C for 30 min. The extract was mixed with 1 ml of 2.5 M sucrose, transferred to an SW41Ti centrifuge tube, and overlaid with 6 ml of 30% sucrose solution and 4 ml of 5% sucrose solution containing 25 mM Hepes, pH 6.5, and 150 mM NaCl. The discontinuous sucrose gradients were centrifuged for 18 h at 4°C in an SW41Ti rotor at 39,000 rpm. The gradient was fractionated into 12 fractions from the bottom to the top.

Quantification of ATP by bioluminescent luciferase assay

Extracellular ATP was measured following the protocol described previously (Arakaki *et al.,* 2003). 3T3-L1 preadipocytes were seeded and differentiated in 24-well plates and confluent 3T3-L1 adipocytes were used for cell surface ATP synthesis. Cells were washed with Hepes buffer (10 mM Hepes, pH 7.4, 150 mM NaCl) three times and were incubated with buffer only or 0.3 ml of Hepes buffer containing 200 μM ADP, 20 mM potassium phosphate (P_i), 2 mM $MgCl_2$ at room temperature. After 10-480 s, the extracellular media were removed and supernatants (20 μl) were used for the determination of extracellular ATP content. ATP levels were measured by the bioluminescence assay according to the protocol provided with an ATP determination kit (Molecular probes).

Results

ATP synthase complex is highly expressed during adipogenesis

Recent proteomic analysis reveals that adipogenesis is accompanied with mitochondrial biogenesis as evidenced by significant increase of mitochondrial proteins in the differentiated 3T3-L1 cells. After post-adipocyte differentiation, there is a significant increase of known mitochondrial proteins involved in the lipid biosynthesis: carnitine aceyltransferase, succinyl CoA ligase, pyruvate decarboxylase, and malate dehydrogenase (Wilson-Fritch *et al.,* 2003). To better understand an effect on the ATP synthase complex, an essential mitochondrial machinery for ATP synthesis during adipogenesis, 3T3-L1 preadipocytes were differentiated into adipocytes for 0, 2, 4, 6, and 8 days, and the cell lysates were analyzed by immunoblotting with anti-ATP synthase α and β, cytochrome c, peroxisome proliferators-activated receptor γ (PPAR γ), and β-actin antibodies. As shown in Figure 1, mitochondrial proteins such as ATP synthase α and β and cytochrome c were highly expressed in fully-differentiated adipocytes with high level of PPARγ, an adipocyte differentiation marker, suggesting that mi-

tochondrial proteins are highly expressed during adipocyte differentiation.

For additional evidence of the increased mitochondrial biogenesis during adipogenesis, the morphological features of preadipocytes and adipocytes were examined under electron microscopy. Mitochondria in preadipocyte were rare, irregular, and small-sized (Figure 2). On the contrary, adipocyte mitochondria were densely packed, elongated, and large-sized. In addition, abundant lamellar cristae, which were rare in preadipocytes, were observed in fully-differentiated adipocytes, suggesting that mitochodria in adipocytes appear to be functional. The number of mitochondria was estimated by counting in seven images containing whole-cell profiles sectioned through the middle of the nucleus. A preadipocyte had 7 mitochondria while an adipocyte 132 in average (data not shown).

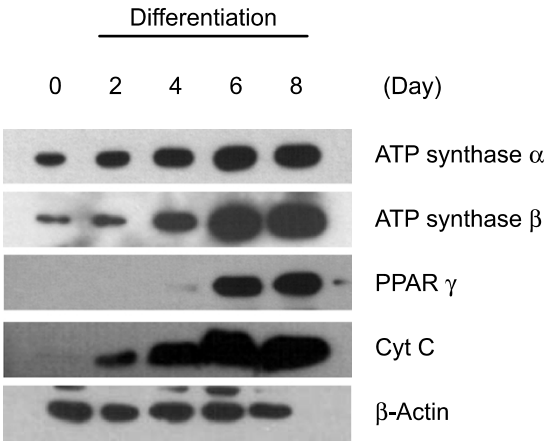

Figure 1. The expression of ATP synthase complex is highly induced during 3T3-L1 adipogenesis. 3T3-L1 preadipocytes were differentiated to adipocytes for 0, 2, 4, 6, and 8 days. The differentiated adipocytes were lysed with SDS lysis buffer, and the lysates were analyzed by immunoblotting with anti-ATP synthase α and β, cytochrome c, PPARγ, and insulin receptor antibodies.

Surface expression of ATP synthase complex in adipocytes

In previous reports, ATP synthase complex has been found in the surface of endothelial cells and hepatocyte and associated with different ligands such as angiostatin, EMAPII, and Apo-AI. Such findings of ATP synthase complex in both the plasma membrane and in mitochondria presented yet undefined interrelationship of two ATP synthase complexes. In order to address this issue, preadipocyte and adipocyte were immunostained with anti-ATP synthase α and β antibodies. It should be noted that permeabilization step was omitted to see only immunofluorescent

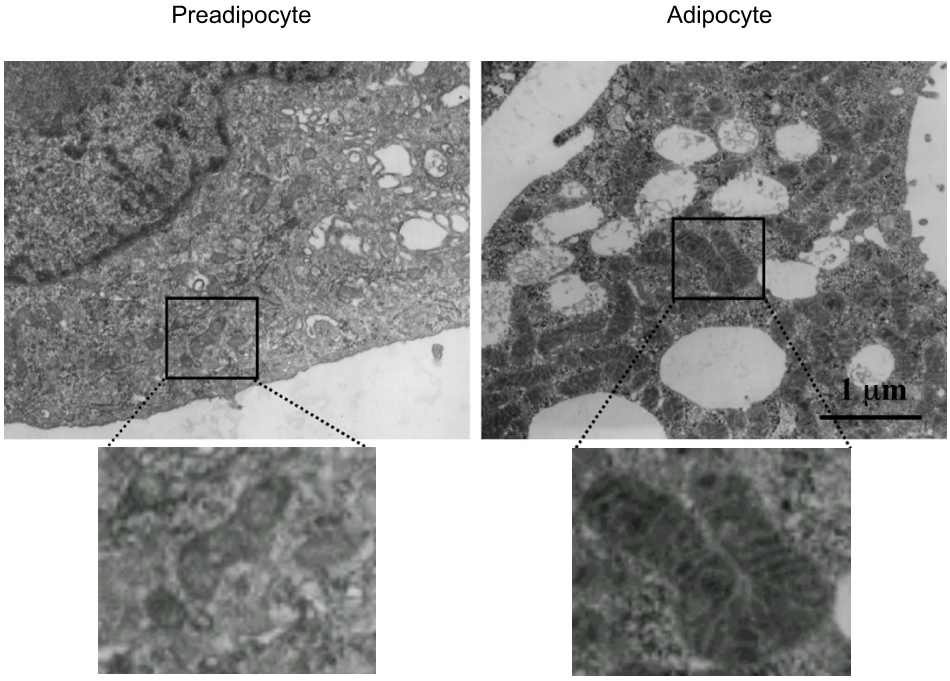

Figure 2. Mitochondria are packed full in fully-differentiated adipocytes. On day 8 of differentiation, 3T3-L1 cells were used as fully-differentiated adipocytes. 3T3-L1 preadipocytes and adipocytes were prepared for electron microscopy (magnification, ×25,000). Insets in upper panels were enlarged in bottom panels to see mitochondrial morphology in detail.

signal from the cell surface. Figure 3A showed that ATP synthase α and β were expressed in the cell surface of preadipocytes and adipocytes. However, the immunostaining signal was stronger in adipocytes than in preadipocytes, suggesting that more ATP synthase complex is expressed in the cell surface of adipocytes than that of preadipocytes.

In order to confirm the surface expression of ATP synthase complex in preadipocytes and adipocytes, both cells were labeled with membrane impermeable biotin. The biotin-labeled surface proteins were precipitated with avidin-conjugated agarose beads. The precipitates were immunoblotted with anti-ATP synthase α and β antibodies. Both ATP synthase α and β were biotinylated (Figure 3B), demonstrating that they are indeed expressed on the cellular surface. However, since more ATP synthase α and β were appeared to be biotinylated in adipocytes than in preadipocytes, additional experiments were carried out to clarify that ATP synthase complex is highly expressed on the cell surface during adipogenesis. Plasma membrane and mitochondria proteins in preadipocytes and adipocytes were isolated and analyzed by immunoblotting with anti-ATP synthase α and β, cytochrome c, and insulin receptor antibodies. Cytochrome c was mainly found in mitochondria fraction whereas insulin receptor only in the plasma membrane, assuring that there was no cross-contamination between plasma membrane and mitochondria proteins during their preparation. As shown in Figure

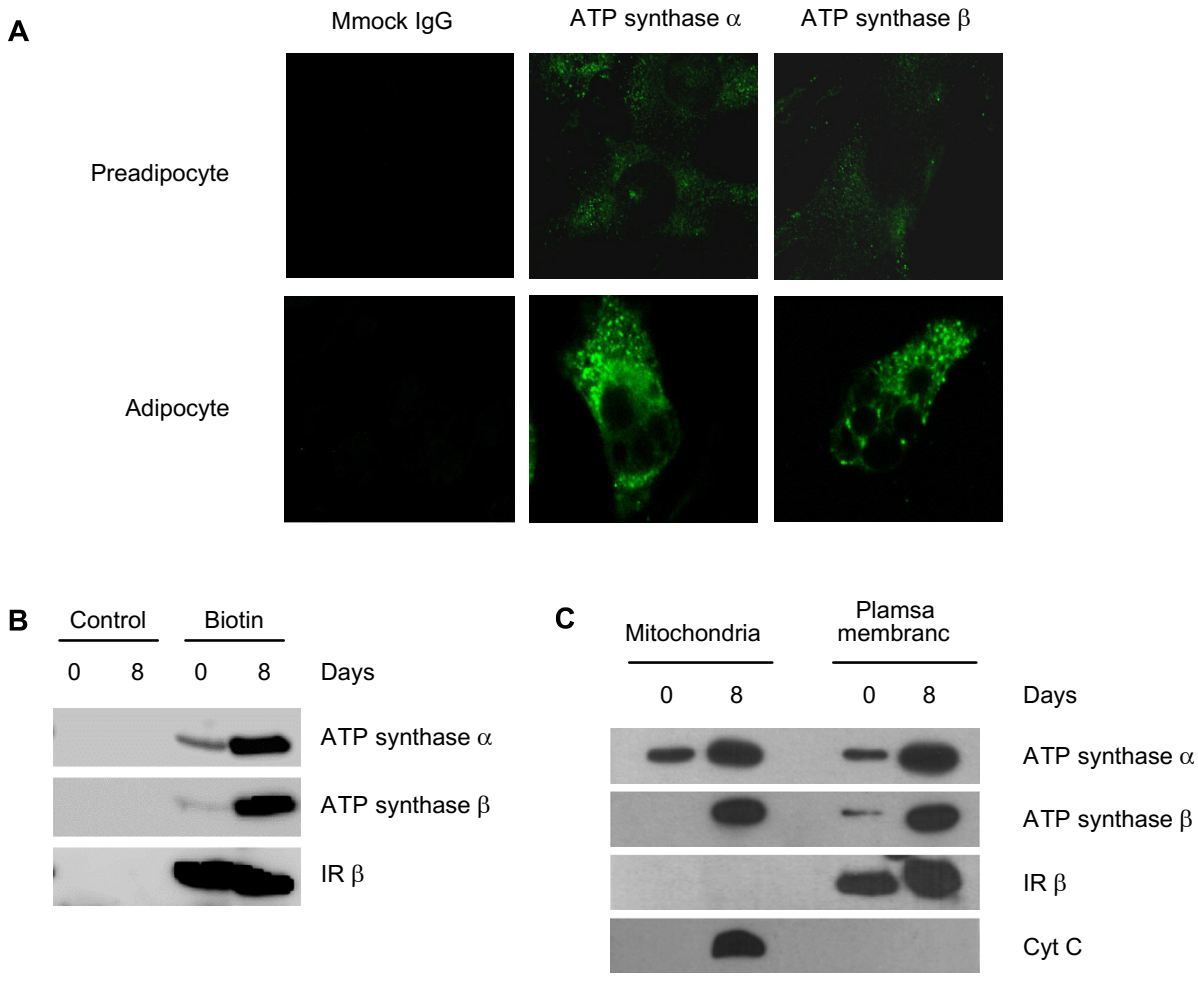

Figure 3. ATP synthase complex is expressed on the outer leaflet of the plasma membrane. On day 8 of differentiation, 3T3-L1 cells were used as fully-diffentiated adipocytes. (A) 3T3-L1 preadipocytes and adipocytes were immunostained with anti-ATP synthase α and β antibodies, and mock IgG. It should be noted that permeabilization step was skipped out for visualizing the surface expression of ATP synthase complex. Scale bar = 10 μm. (B) 3T3-L1 preadipocytes and adipocytes were labeled with membrane-impermeable biotin and lysed with lysis buffer. The lysates were precipitated with avidin-conjugated agarose beads and the precipitates were analyzed with anti-ATP synthase α and β antibodies. (C) Plasma membrane, and mitochondria were prepared from 3T3-L1 preadipocytes and adipocytes. Each protein (5 μg) from plasma membrane, and mitochondria were analyzed by immunoblotting with anti-ATP synthase α and β antibodies. IR, insulin receptor.

3C, more ATP synthase α and β were found in the plasma membrane and mitochondria in adipocytes than preadipocytes, supporting that adipogenesis is accompanied with high surface expression of ATP synthase complex.

ATP synthase complex is found in lipid rafts

ATP synthase α and β might be in the plasma membrane rafts because of its patchy and punctate staining pattern that is typically shown in raft proteins. We observed the co-localization of ATP synthase α or β with cholera toxin B (CTB) that is a ganglioside-binding protein used as lipid raft landmarker. Figure 4A showed that ATP synthase α and β were indeed co-localized with CTB, indicating that ATP synthase complex is localized in lipid rafts. It should be noted that the cells were not permeabilized for immunofluorescence.

In order to make certain that ATP complex is a lipid raft protein, we isolated detergent-resistant lipid rafts from the differentiated 3T3-L1, and human adipocytes, and analyzed them by immunoblotting with anti-ATP synthase α and β, cytochrome c, insulin receptor, caveolin-1, and flotillin-1 antibodies. Since cytochrome c was found only in the bottom fractions whereas insulin receptor, caveolin-1, and flotillin-1 were present in raft fractions, there was little mitochondrial contamination in lipid raft fractions (Figure 4B). As shown in Figure 4B, lipid raft fractions

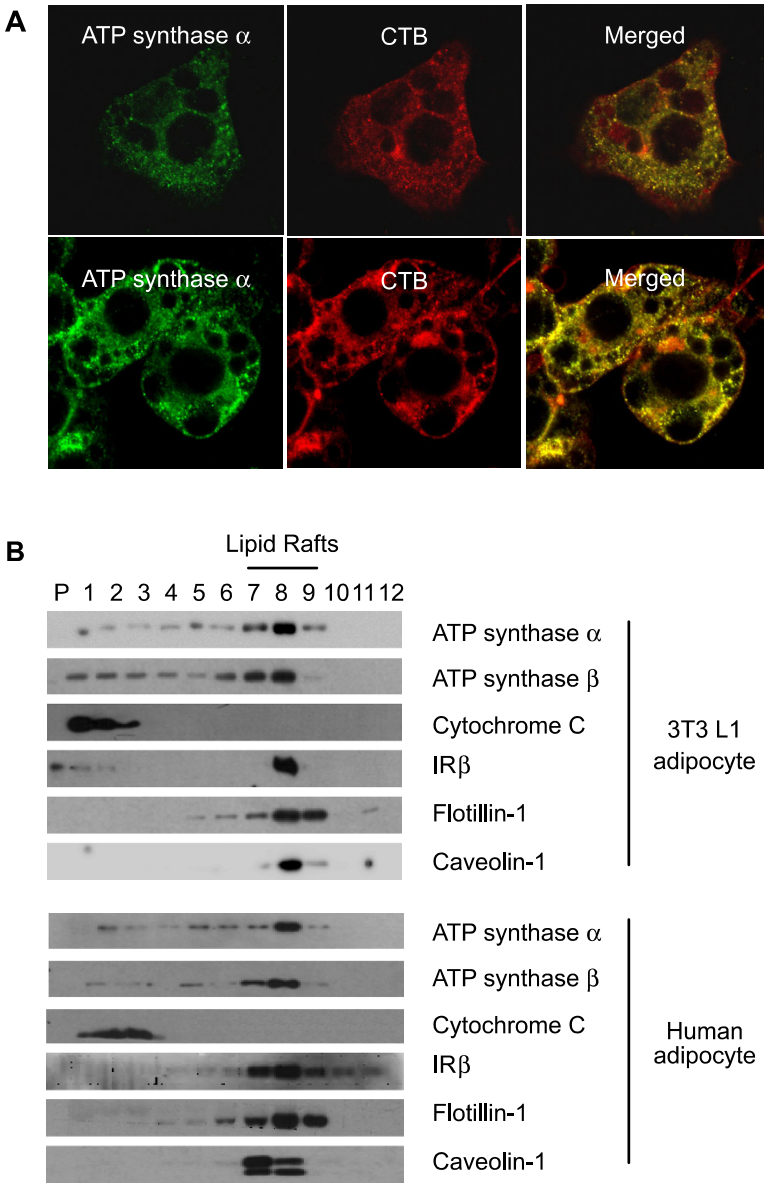

Figure 4. ATP synthase α and β are enriched in lipid rafts of adipocytes. (A) ATP synthase α and β are co-localized with CTB in fully-differentiated adipocytes, indicating that the complex is present in the plasma membrane rafts. 3T3-L1 adipocytes were immunostained with anti-ATP synthase α or β antibody and rhodamine-conjugated CTB. It should be noted that permeabilization step was skipped out for immunofluorescence. Scale bar = 10 μm. (B) Detergent-resistant lipid rafts were isolated from 3T3-L1 adipocytes and human adipocytes as outlined in Materials and Methods. Each fraction from the sucrose gradient was blotted with anti-ATP synthase α, and β, cytochrome c, insulin receptor (IR), flotillin-1, and caveolin-1 antibodies.

contained large portions of ATP synthase α and β, indicating that ATP synthase complex is present in the detergent-resistant lipid rafts of mouse and human adipocytes.

ATP synthase is required for the generation of extracellular ATP

In order to explore the function of surface ATP synthase, ATP content was determined from adipocyte media after treating the cells with ADP and inorganic phosphate (P_i).

Extracellular ATP content had greatly increased with a biphasic velocity with an inflection point at 30 seconds after ADP and P_i addition to adipocyte media and reached a plateau level (30 μM) at 8 min that was continuously maintained for longer time (Figure 5A). The result of the rapid generation of the extracellular ATP upon addition of substrates, ADP and P_i and no lag period strongly suggest a direct catalysis by the exposed surface ATP synthase. The extracellular ATP level was extremely low (less than 10 nM) in the absence of substrate ADP and P_i.

The efficiency of ATP synthase catalysis was further examined by rapid response of ATP synthase inhibition. Adipocytes were pretreated with oligomycin (10 μg/ml) or CCCP (0.4 μg/ml) for 5 min. Extracellular ATP content from media was measured 1 minute after adding ADP and P_i. As shown in Figure 5B, extracellular ATP synthesis was inhibited by about 53% in the presence of oligomycin, presumably supporting that catalysis by the exposed cellular ATP synthase is affected. Since ATP synthase complex uses proton gradient to generate ATP in mitochondria, it is tempting to speculate that surface ATP synthase also requires proton gradient for synthesizing extracellular ATP. Thus, the content of extracellular ATP was measured in the presence of carbonyl cyanide m-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler or proton gradient destroyer. CCCP decreased extracellular ATP content by about 46%, suggesting that proton gradient is required for extracellular ATP generation. Figure 5B showed that extracellular ATP generation was not affected by DMSO that is a carrier solution solubilizing oligomycin and CCCP.

Since ATP synthase complex was exposed to extracellular space (Figure 3), there could be a proton gradient from cytoplasm to extracellular space for generating extracellular ATP. Cell media should be more basic than cytosol to form a proton gradient through the plasma membrane. In order to address

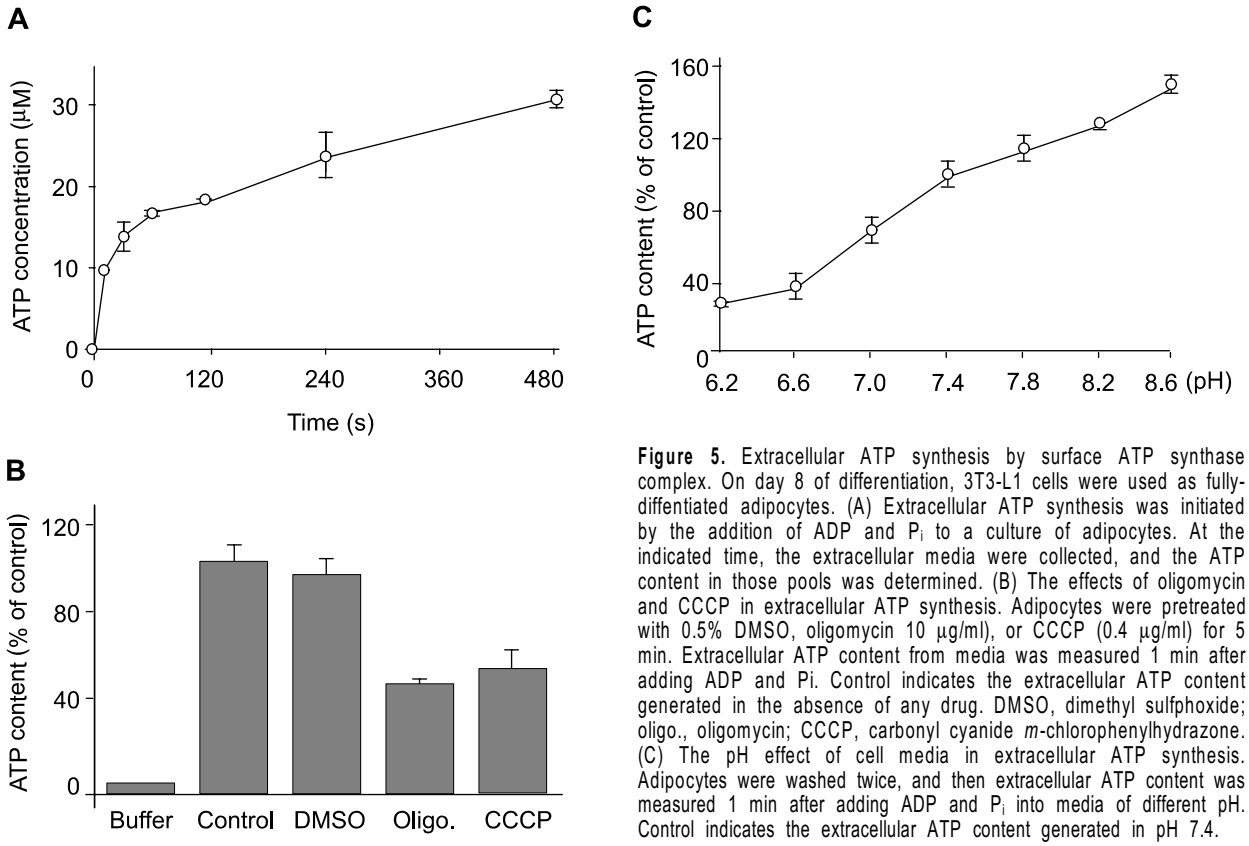

Figure 5. Extracellular ATP synthesis by surface ATP synthase complex. On day 8 of differentiation, 3T3-L1 cells were used as fully-diffentiated adipocytes. (A) Extracellular ATP synthesis was initiated by the addition of ADP and P_i to a culture of adipocytes. At the indicated time, the extracellular media were collected, and the ATP content in those pools was determined. (B) The effects of oligomycin and CCCP in extracellular ATP synthesis. Adipocytes were pretreated with 0.5% DMSO, oligomycin 10 μg/ml), or CCCP (0.4 μg/ml) for 5 min. Extracellular ATP content from media was measured 1 min after adding ADP and Pi. Control indicates the extracellular ATP content generated in the absence of any drug. DMSO, dimethyl sulfphoxide; oligo., oligomycin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone. (C) The pH effect of cell media in extracellular ATP synthesis. Adipocytes were washed twice, and then extracellular ATP content was measured 1 min after adding ADP and P_i into media of different pH. Control indicates the extracellular ATP content generated in pH 7.4.

the issue, we incubated adipocytes in acidic, neutral, or basic condition and then measured extracellular ATP. As shown in Figure 5C, less extracellular ATP was generated in less basic condition. Meanwhile, more extracellular ATP was synthesized in more basic condition. Taken together all these data in Figure 5, we can conclude that surface ATP synthase is required for extracellular ATP synthesis.

Discussion

After cellular exposure to insulin, lipogenesis is activated in adipocytes. Thus, many enzymes for lipid biosynthesis are highly up-regulated and activated in adipocytes. The lipid biosynthesis requires mitochondrial and cytoplasmic enzymes. Mitochondria are necessary for the formation of acetyl-CoA and NADH that are essential components for lipid biosynthesis. Thus, adipogenesis should be accompanied with mitochondrial biogenesis. Here, we demonstrate that mitochondria are extravagantly generated during adipogenesis by immunoblotting and electron microscopy. Compared to preadipocyte mitochondria, adipocyte mitochondria have more elongated and electron-dense morphology (Figure 2). In addition, there is a tremendous increase of mitochondria number during adipogenesis; adipocyte has 19 times more mitochondria than preadipocyte. With their large number and elongated morphology, adipocyte mitochondria could be active for synthesizing ATP and NADH, and for producing and transferring more acetyl-CoA to cytosol to synthesize lipid. Thus, many mitochondrial proteins are highly up-regulated during adipogenesis. We also demonstrated that cytochrome c and ATP synthase complex are highly expressed in adipocytes (Figure 1), suggesting that the adipocytes are active for oxidative respiration to generate NADH and ATP.

Some of mitochondrial proteins such as prohibitin, VDACs, ATP/ADP exchanger, and ATP synthase complex have been described in association with the plasma membrane or lipid rafts. Among these proteins, ATP synthase complex has been proven to be localized in the plasma membrane of human umbilical vein endothelial cells (HUVECs), and hepatocytic HepG2 cells (Moser et al., 1999; Moser et al., 2001; Martinez et al., 2003). We also found that the ATP synthase complex was highly expressed on the cell surface of C2C12 myocytes (data not shown) as well as 3T3-L1 adipocytes (Figure 3). Interestingly, endothelial cells, hepatocytes, and myocytes, which express ATP synthase complex on their cell surface, respond with angiostatin to inhibit cellular proliferation (Moser *et al.,* 2001; Wajih *et al.,* 2003). Thus, angiostatin might bind to the cell surface through ATP synthase complex because its binding to the cell surface was abolished by competing with anti-ATP synthase antibody.

Martinez *et al.* advocated that Apo-AI binds to surface ATP synthase complex in hepatocytes to induce the uptake of high density lipoprotein (HDL) (Martinez *et al.,* 2003). Since the uptake of Apo-AI-containing HDL is active in adipocytes (Dagher *et al.,* 2003), and ATP synthase complex is expressed on the surface of adipocytes (Figure 3), we tested the HDL uptake after neutralizing ATP synthase complex with anti-ATP synthase β antibody that previously used for preventing surface binding of Apo-AI (Martinez *et al.,* 2003). From the experiments, HDL uptake was significantly inhibited by anti-scavenger receptor I (SR-BI) antibody but not by anti-ATP synthase β antibody (data not shown), suggesting that the surface ATP synthase of adipocytes is not involved in HDL uptake.

The major function of surface ATP synthase might be to synthesize extracellular ATP. Indeed, extracellular ATP generation is abolished in human endothelial cells when neutralized with its antibody (Moser *et al.,* 2001). Since the extracellular ATP generation is dramatically decreased by treating human endothelial cells with ATP synthase inhibitors such as efrapeptins, piceatannol, and resveratrol (Arakaki *et al.,* 2003), surface ATP synthase is necessary for ATP synthesis on the cell surface. We also measured ATP content in culture media of adipocytes after adding ADP plus P_i. Eight minutes after treating adipocytes with ADP plus P_i, more than 30 μM extracellular ATP was found. Since the extracellular ATP synthesis was prevented by oligomycin, the extracellular ATP generation might require surface ATP synthase complex. Interestingly, the extracellular ATP generation was abolished when proton gradient was disrupted by treating adipocytes with CCCP or in acidic condition, suggesting that proton gradient through the plasma membrane is necessary for activating surface ATP synthase complex.

The extracellular ATP and nucleotides recognize and activate their receptors called P2 receptors (Schwiebert and Zsembery, 2003). P2 receptors are divided into two classes, ionotropic P2X ($P2X_{1-7}$), and G protein-coupled P2Y ($P2Y_{1-11}$) receptors. ATP and other nucleotides modulate Ca^{2+} influx through P2X receptors. Unlike P2X receptors, P2Y receptors activates G_q, phospholipase C, the generation of diacylglycerol and inositol triphosphate, increase of intracellular Ca^{2+}, and protein kinase C or modulates G_s or G_i, adenylyl cyclase, and resultant cAMP formation. Depending on the cell types, extracellular ATP ignites cellular responses such as cellular proliferation, muscle contraction and differentiation, inflammation, and neuronal communication (Bae and Ryu, 2001; Schand Zsembery, 2003).

The extracellular ATP is rapidly dephosphorylated into ADP, AMP, and adenosine at the cell surface (Schwiebert and Zsembery, 2003). For example, ecto-apyrases, ecto-ATPases, ecto-ADPases, b-5'-nucleotidase, and alkaline phosphatase are released from cells or bind to the outer leaflet of plasma membrane with glycosylphosphatidylinositol (GPI) anchor to hydrolyze the extracellular ATP. Interestingly, many ATP-hydrolyzing enzymes are found in lipid rafts (Strohmeier *et al.,* 1997; Kittel *et al.,* 1999; Ko *et al.,* 1999). In addition, we demonstrate that lipid rafts concentrate ATP synthase complex, suggesting that lipid rafts are major plasma membrane compartments regulating ATP synthesis and hydrolysis.

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