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Mild mitochondrial uncoupling does not affect mitochondrial biogenesis but downregulates pyruvate carboxylase in adipocytes: role for triglyceride content reduction

Aurélia De Pauw,¹ Stéphane Demine,¹ Silvia Tejerina,¹ Marc Dieu,¹ Edouard Delaive,¹ Alexander Kel,² Patricia Renard,¹ Martine Raes,¹ and Thierry Arnould¹

¹Laboratory of Biochemistry and Cellular Biology, Namur Research Institute for Life Sciences, University of Namur, Namur, Belgium; and ²GeneXplain, Wolfenbuettel, Germany

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De Pauw A, Demine S, Tejerina S, Dieu M, Delaive E, Kel A, Renard P, Raes M, Arnould T. Mild mitochondrial uncoupling does not affect mitochondrial biogenesis but downregulates pyruvate carboxylase in adipocytes: role for triglyceride content reduction. *Am J Physiol Endocrinol Metab* 302: E1123–E1141, 2012. First published February 21, 2012; doi:10.1152/ajpendo.00117.2011.—In adipocytes, mitochondrial uncoupling is known to trigger a triglyceride loss comparable with the one induced by TNF α , a proinflammatory cytokine. However, the impact of a mitochondrial uncoupling on the abundance/composition of mitochondria and its connection with triglyceride content in adipocytes is largely unknown. In this work, the effects of a mild mitochondrial uncoupling triggered by FCCP were investigated on the mitochondrial population of 3T3-L1 adipocytes by both quantitative and qualitative approaches. We found that mild mitochondrial uncoupling does not stimulate mitochondrial biogenesis in adipocytes but induces an adaptive cell response characterized by quantitative modifications of mitochondrial protein content. Superoxide anion radical level was increased in mitochondria of both TNF α - and FCCP-treated adipocytes, whereas mitochondrial DNA copy number was significantly higher only in TNF α -treated cells. Subproteomic analysis revealed that the abundance of pyruvate carboxylase was reduced significantly in mitochondria of TNF α - and FCCP-treated adipocytes. Functional study showed that overexpression of this major enzyme of lipid metabolism is able to prevent the triglyceride content reduction in adipocytes exposed to mitochondrial uncoupling or TNF α . These results suggest a new mechanism by which the effects of mitochondrial uncoupling might limit triglyceride accumulation in adipocytes.

adipocytes; carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; tumor necrosis factor- α ; mitoproteome

OBESITY RESULTS LARGELY FROM A CHRONICALLY POSITIVE energy balance. Therefore, reducing body energy intake and increasing energy expenditure are two approaches that have been translated into strategies to limit fat accumulation. Because mitochondria play a critical role in the process of energy expenditure, the alteration of oxidative phosphorylation (OXPHOS) efficiency through OXPHOS uncoupling from ATP production in white adipocytes has been considered as an attractive approach to fight obesity (7, 69). In other words, uncoupling increases cellular metabolic demand since uncoupling OXPHOS lowers ATP production and increases the demand for reducing equivalents to restore/maintain mitochondrial membrane potential, thereby increasing substrate catabo-

lism, which would theoretically decrease triglyceride (TG) stores in adipocytes. Indeed, although mitochondrial dysfunction could represent a major cause of lipid metabolism disorders and pathological triglyceride accumulation in several cell lines (50, 75), we and others have shown that mitochondrial uncoupling in adipocytes triggers lipolysis, limits fatty acid synthesis, and leads to a reduction in TG content (40, 61, 65, 67, 73), a characteristic also found during the “dedifferentiation” of adipocytes (86).

Mature adipocyte dedifferentiation has been defined as the acquisition of a more primitive phenotype and gain of cell proliferative ability (48) as well as a reduction in TG content in adipocytes (29, 52). In this study, we will refer to adipocyte dedifferentiation as a reduction of TG content and downregulation of adipogenic markers and effectors. As long as the free fatty acids (FFAs) released by adipocytes in these conditions can be oxidized by other tissues (such as heart, kidney, liver, and activated brown fat) and do not accumulate in the blood stream, mild and controlled mitochondrial uncoupling could maintain white adipocyte TG content to a lower amount than maximal charge, limiting the dysfunction of adipose tissue. By limiting overaccumulation of TG, endoplasmic reticulum stress and alterations of adipokine secretion are prevented, conditions that could thus be beneficial for limiting low-grade inflammation and systemic insulin resistance (60).

The dedifferentiation program of adipocytes has been studied mainly in 3T3-L1 adipocytes challenged with TNF α , a proinflammatory cytokine secreted by macrophages and white adipocytes abundantly present in obese individuals and known to alter mitochondrial function (by inducing an oxidative stress) (64, 81). The loss of TG content triggered by TNF α during adipocyte dedifferentiation is mediated mainly by a stimulation of lipolysis (62, 86). Recent evidence has shown that TNF α also triggers mitochondrial dysfunction resulting from a modification in the abundance of mitochondrial proteins associated with energy production such as effectors of fatty acid β -oxidation, Krebs cycle, and OXPHOS (10), as well as changes in mitochondrial morphology and dynamic, in treated adipocytes (9). Additionally, because lipogenesis in adipocytes is highly dependent on ATP production, a decrease in ATP production resulting from mitochondrial membrane potential dissipation induced by mitochondrial uncoupling might also contribute to limit TG synthesis in adipose cells exposed to mitochondrial uncouplers and could then prevent excessive accumulation of body fat. Indeed, expression of uncoupling protein-1 (UCP1) in white and brown adipose cells of aP2-*Ucp1* mice has been reported to lower body weight, to change

Address for reprint requests and other correspondence: T. Arnould, Univ. of Namur (FUNDP), rue de Bruxelles, 61, 5000 Namur, Belgium (e-mail: thierry.arnould@fundp.ac.be).

fat distribution in the body (enlarged gonadal fat and decreased subcutaneous fat), and to prevent development of genetic or dietary obesity (38, 39, 41). Mitochondrial uncoupling induced by pharmacological molecules such as FCCP or ectopic UCP1 expression also leads to 3T3-L1 adipocyte dedifferentiation controlled by modifications in the expression of many genes (65, 67, 73).

Currently, several lines of evidence clearly show that mitochondrial dysfunction leads to a compensatory response reflected by an enhanced mitochondrial biogenesis in muscle cells of patients affected by the Myoclonic Epilepsy with Ragged Red Fibers syndrome (78) or in muscle cells of healthy individuals trained for prolonged physical exercise and muscle contractile activity (30). Finally, in vitro, 3T3-L1 adipocytes treated with 2,4-dinitrophenol (DNP), another chemical uncoupler, also exhibit an increased mitochondrial biogenesis reflected by the increase in COXIV and nuclear respiratory factor 1 (NRF1) expression (59).

Nevertheless, whereas the biogenesis of mitochondria is enhanced and stimulated during adipogenesis (80), the effect of mitochondrial uncoupling on the abundance, plasticity, and protein content of the mitochondrial population in adipocytes as well as molecular mechanisms leading to a reduction of TG content in adipocytes exposed to mitochondrial uncoupling (73) are still largely unknown.

In this study, we assessed the impact of a mild mitochondrial uncoupling on mitochondrial population and the putative link with TG content decrease in 3T3-L1 adipocytes treated with 0.5 μ M FCCP for 6 days. This treatment with molecules known to dedifferentiate the cells is maintained for 6 days, especially for testing the putative impact of these molecules on the biogenesis of mitochondria, a process that takes between 4 and 6 days in response to strong inducers such as nitric oxide donors (54). We showed that even if no quantitative modification of mitochondrial population is observed in FCCP-treated cells, some qualitative alterations can be seen in mitochondrial proteomes of these cells. Among the various candidates found to be differentially abundant in the mitochondria of FCCP-treated adipocytes, the downregulation of pyruvate carboxylase (PCx), a major mitochondrial effector of de novo fatty acid synthesis, seems to play a crucial role in the reduction of TG content observed in adipocytes exposed to mild and chronic mitochondrial uncoupling.

MATERIALS AND METHODS

Cell culture conditions and experimental models. Mouse 3T3-L1 preadipocytes, purchased from American Type Culture Collection, were maintained in DHG-L1 medium (Dulbecco's modified Eagle's medium) containing 4.5 g/l glucose (Gibco-BRL) and 1.5 g/l NaHCO₃ and supplemented with 10% FCS (Gibco-BRL). 3T3-L1 preadipocytes, 1 day postconfluent, were differentiated into adipocytes for 12 days and subsequently dedifferentiated in the presence of 10 ng/ml TNF α (R & D Systems) or 0.5 μ M FCCP (Sigma-Aldrich), as described previously (73). Because different incubation periods were used according to the assay performed, incubation time is indicated in the figure legends.

Fluorescence confocal microscopy and transmission electron microscopy. Mitochondria morphology and structure were analyzed by fluorescence confocal microscopy of cells stained for 40 min with 70 nM MitoTracker Green FM (Molecular Probes), as described by Wilson-Fritch et al. (80), of cells incubated in the presence of either 10 ng/ml TNF α or 0.5 μ M FCCP, or by transmission electron

microscopy, as described by Mercy et al. (49). Quantification of mitochondrial population fragmentation was performed. After staining, two-dimensional micrographs of the mitochondrial networks were taken using a confocal microscope (Leica). Branching and reticulation status of the mitochondrial networks were then evaluated using the Image J software and the protocol described by De Vos and Sheetz (14). First, the aspect ratio (AR), the ratio between the bigger (major) and the smaller (minor) side for each mitochondrial fragment, has been determined using the Image J software. The images were processed to maximize the signal/background ratio using the automatic adjustment of brightness/contrast of the Image J software. After smoothing of the image, images were filtered using the hat 7×7 kernel. Finally, the major and the minor of each fragment were determined using the "analyze particles" function of Image J. Second, the end points (EP)/branch points (BP) ratio was determined. Mitochondrial networks were skeletonized, and the number of EPs and BPs was determined using the BinaryConnectivity plugin.

ATP content determination. ATP content was measured using a luciferin-luciferase reaction assay. Cells were permeabilized for 10 s with 500 μ l of ATP-releasing agent (Sigma-Aldrich). The solution was recovered and diluted 400 times in pure water before being incubated with an ATP assay mix solution (Sigma-Aldrich) at a 1:1 ratio (vol/vol). Relative light units, based on emitted photons quantified using a luminometer (FB 12 Luminometer from Berthold Detection Systems), were normalized for DNA content determined by propidium iodide staining (Sigma-Aldrich), as described previously (75).

Mitochondrial matrix Ca²⁺ assay. Mitochondrial matrix Ca²⁺ was assessed using 2 μ M X-rhod-5F fluorescent probe (Molecular Probes) according to the manufacturer's instructions. When indicated, ruthenium red (Sigma-Aldrich) was added at 10 μ M for 30 min after cells were loaded with X-rhod-5F. Fluorescent signals were normalized for cell DNA content (75).

Flow cytometry. Mitochondrial mass/abundance from 3T3-L1 fibroblasts, differentiated adipocytes, and adipocytes treated with either 10 ng/ml TNF α or 0.5 μ M FCCP for 6 days was assessed using 70 nM MitoTracker Green FM (Molecular Probes), as reported previously (80). A total time of 50 min without molecules being stimulated elapsed between the end of the incubations and fluorescence measurement.

Total DNA extraction, total RNA extraction, and RT-quantitative PCR. To extract total DNA, cells were scrapped in digestion buffer [100 mM sodium chloride, 10 mM tris(hydroxymethyl)aminomethane, 25 mM EDTA, 0.5% sodium dodecyl sulfate, and 0.1 mg/ml proteinase K, pH 8.0] and incubated at 50°C for 18 h. Samples were centrifuged for 10 min at 1,700 g in an equal volume of 25:24:1 phenol-chlorophorm-isoamyl alcohol saturated with 10 mM Tris, pH 8.0, and 1 mM EDTA (Sigma-Aldrich). The supernatants were incubated for 60 min in 0.5 volumes of 7.5 M ammonium acetate and two volumes of 100% ethanol at -20°C before being centrifuged at 1,700 g for 5 min. Pellets were washed in 70% ethanol, air-dried for 60 min, dissolved in diethylpyrocarbonate water, and incubated at 65°C for 60 min and then frozen at -20°C until use. Total RNA was extracted with the Total RNAagents extraction kit (Qiagen), and RNA reverse transcription was performed according to the manufacturer's protocol (Invitrogen). Sense and antisense primers for D-loop (displacement loop), PGC-1 α (peroxisome proliferator-activated receptor- α coactivator-1 α), PGC-1 β , Hadha (long-chain enoyl-CoA hydratase), Mn-SOD (manganese superoxide dismutase), PCx, and TBP (TATA box-binding protein; used as a reference gene for normalization) were designed using the Primer Express 1.5 software (Applied Biosystems; Table 1). Data calculation and normalization were performed according to de Longueville et al. (13).

Immunoblotting. An amount of 25 [MnSOD/SOD2, Hadha, mtHSP70 (mitochondrial heat shock protein 70), PCx] or 30 μ g of proteins [translocase of the outer mitochondrial membrane 40 (TOM40), translocase of the inner mitochondrial membrane 23

Table 1. Sense and antisense primers designed to amplify cDNA sequence of interest by real-time RT-quantitative PCR

	Reverse Primer	Forward Primer
mt D-loop	GCATTTGATGGCCCT	TACCATCCTCCGTGAAACCAA
PGC-1 α	CTCTCTGTTTGGCCCTTTCAGA	TGAGCAGCAAGGCTCAAGA
PGC-1 β	CCATGGCTTCGTACTTGCTTT	TCCGTGGCCCGAGATACACT
Hadha	GCATGCTATGGCAAGCTCAA	CCAAGAAGCAACACGAATATCACA
MnSOD	GTGCTCCACACGTCAATCC	CTTACAGATTGCTGCCTGCTCTAA
PCx	CGTCGGAGTTGCCAGACTTC	TGGTGGCCTGTACCAAAGG
TBP	TAGTGCTGCAGGGTGATTTCAG	CAGTTACAGGTGGCAGCATGA

mt D-loop, mitochondrial displacement loop; PGC-1 α and -1 β , peroxisome proliferator-activated receptor- γ coactivator-1 α and -1 β , respectively; Hadha, long-chain enoyl-CoA hydratase; MnSOD, manganese superoxide dismutase; PCx, pyruvate carboxylase; TBP, TATA box-binding protein. TBP was used as reference gene.

(TIM23), coenzyme Q reductase 1 (UCQR1), acetyl-CoA dehydrogenase very-long chain (ACADVL), cytochrome *c*, α -subunit of ATP synthase, β -subunit of ATP synthase, TBP] from mitochondrial fractions diluted in lysis buffer was resolved by electrophoretic migration into 4–12% NuPAGE gels run in a MOPS buffer system (Amersham). Immunoreactive proteins were revealed using ECL-PLEX technique (Amersham) according to the manufacturer's instructions. Primary antibodies were MnSOD/SOD2 (ab16956, 1/5,000; Abcam), Hadha (ab54477, 1/1,000; Abcam), mtHSP70 (804-077-R100, 1/1,000; Alexis Biochemicals), PCx (sc-67021, 1/1,000; Tebu-bio), TOM40 (sc-11414, 1/5,000; Tebu-bio), TIM23 (sc-13298, 1/5,000; Tebu-bio), UCQR1 (1/5,000; MitoSciences), ACADVL (1/5,000; Abnova), mtHSP70 (804-077-R100, 1/5,000; Alexis Biochemicals), cytochrome *c* (sc-7159, 1/2,000; Tebu-bio), α -subunit of ATP synthase (A21350, 1/10,000; Molecular Probes), β -subunit of ATP synthase (A21351, 1/10,000; Molecular Probes), and TBP (sc-204, 1/5,000; Tebu-bio).

Fatty acid β -oxidation and mitochondrial O_2 (-) measure assays. Fatty acid β -oxidation assay was assessed by the release of $^{14}CO_2$ after [^{14}C]oleate uptake (1 μ Ci/ml; PerkinElmer), as described previously (51), and normalized by protein content. A total incubation of 2 h, without TNF α or FCCP, elapsed from the beginning to the end of the experiment. Mitochondrial O_2 (-) production was measured in cells loaded with 10 μ M MitoSOX Red specific dye (Molecular Probes) for 45 min in a buffer (0.4 mM MgSO $_4$, 5.3 mM KCl, 0.44 mM KH $_2$ PO $_4$, 4.2 mM NaHCO $_3$, 138 mM NaCl, 0.34 mM NaH $_2$ PO $_4$, and 5.6 mM glucose) that does not contain TNF α or FCCP according to the manufacturer's instructions. Fluorescence signals were normalized for protein content [arbitrary fluorescence units (AFU)/ μ g proteins].

PCx assay. Differentiated adipocytes were lysed in lysis buffer (20 mM MOPS, 3 mM EDTA, 500 nM PMSF), frozen in liquid nitrogen, and thawed twice before sonication for 30 s. Cleared lysates (supernatant) were collected by centrifugation at 13,000 rpm for 5 min. A volume of 300 μ l of lysates was incubated in presence of a 1-ml reaction mixture [45 mM Tris-HCl, 50 mM KHCO $_3$, 5 mM MgCl $_2$, 2 mM ATP, 330 μ M acetyl-CoA, 2.2 U/ml citrate synthase, 10 mM NaH $^{14}CO_3$ (1 mCi), pH 7.8]. After incubation at 37°C for 4 min, a volume of 13 μ l of 10 mM pyruvate was added to start the reaction. Reaction was performed at 37°C. A volume of 500 μ l of 10% TCA was added to stop the reaction. Because acidification of the solution could lead to emission of $^{14}CO_2$ molecules, a Whatman paper imbibed of 2 N NaOH was placed onto the reaction plate, with slight agitation for 1 h at room temperature. Proteins were removed by centrifugation at 13,000 RPM for 5 min. A complete acidification of the medium was performed to assure the complete removal of unfixed CO $_2$ by addition of 360 μ l of 70% perchloric acid, followed by gentle agitation for 1 h at room temperature. Finally, a volume of 500 μ l of the final solution was added to 5 ml of scintillation liquid (Aqualuma), and the radioactivity associated with ^{14}C was determined with a scintillation counter (PerkinElmer).

Electroporation and luciferase assays. Adipocytes, differentiated in 75-cm 2 flask, were transfected by electroporation with 3 μ g of

luciferase reporter constructs and 1 μ g of a reporter construct encoding β -gal (ClonTech), using the Cell Line Nucleofector Kit L (Lonza) according to the manufacturer's instructions optimized for adipocytes. Luciferase reporter constructs were tk-4xNRF1-Luc, tk-4xNRF2-Luc, α -inhibin promoter-4xCRE-Luc, 3xERE-TATA-Luc, YY1-Luc, tk-Sp1-Luc, and tk-3xPPRE-Luc. The next day, cells were incubated with or without 10 ng/ml TNF α or 0.5 μ M FCCP in DHG-L1 + 10% FCS containing 5 μ g/ml insulin (Sigma) for 24 h. Activity of NRF1, NRF2, CREB (cAMP response element-binding protein), ERR α (estrogen-related receptor- α), YY1 (ying yang 1), Sp1 (specificity protein 1), and PPAR α (peroxisome proliferator-activated receptor- α) was determined by measuring the luciferase activity in cell lysates using a Reporter Assay System (Promega). Results were normalized for β -galactosidase activity. Overexpression of the gene encoding PCx was achieved by adipocyte electroporation with 2 μ g of an expression plasmid containing the cDNA of human pyruvate carboxylase (pEF-PC). To determine the effects of PCx overexpression on TG content in FCCP-treated adipocytes, electroporated cells were treated for 6 days with or without FCCP in DHG-L1 + 10% FCS containing 5 μ g/ml insulin before TG content was determined in cells stained with Oil Red O, as described previously (75).

Mitochondria isolation and purification. Mitochondria were isolated by subcellular fractionation (differential centrifugations) from 3T3-L1 adipocytes treated or not with 10 ng/ml TNF α or 0.5 μ M FCCP for 6 days, followed by a Nycodenz gradient to prepare highly purified mitochondrial fractions. Subcellular fractionation was performed at 4°C in HEPES-EDTA-sucrose buffer (255 mM sucrose, 1 mM EDTA, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4) according to a slightly modified, previously described protocol (28), with cell homogenate preparations from 3T3-L1 adipocytes requiring 40 changeovers through the Dounce. Mitochondrial fractions were resuspended in 1.24 Nycodenz (Gentauro) density before being added on top of a 1.05–1.24 Nycodenz density gradient and submitted to 144,203 g at 4°C for 150 min (OptimaTM LE-80K Ultracentrifuge; Beckman Coulter, rotor Sw 55Ti from Beckman). The tubes containing Nycodenz gradient were then sliced into 11 subfractions that were characterized by cytochrome *c* oxidase activity (2) and cathepsin C activity (57) assays. Protein content determination was performed by BCA assay (Pierce) on each fraction to determine the distributions of both mitochondria and lysosomes. Frequencies of both organelle populations in each fraction have been calculated according to the following expression:

$$\text{Frequency}(x) = \frac{(\text{OD}(x) - \text{OD}(\text{blank})) \times ((S(\text{mm}^2) \times H(\text{mm}) / (\text{vol}(\text{ml}) \times \text{dil}(\text{M}))) / 1000)}{1}$$

where OD (x) is optic density measured in cytochrome *c* oxidase or cathepsin C assay for the subfraction x, S is surface area of the tube, H is height of the sliced tube containing the subfraction x, vol is volume of the subfraction x, dil (M) is dilution of mitochondrial fraction used to measure activity of cytochrome *c* oxidase or cathepsin

C, and dil (x) is dilution of subfraction x used to measure activity of cytochrome c oxidase or cathepsin C.

Two-dimensional gel electrophoresis and MS analysis: mitochondrial fractions conditioning to two-dimensional gel electrophoresis. Subfractions containing the highest mitochondrial population content as well as the lowest lysosomal population content were selected to perform mitochondrial proteomic studies. Mitochondria contained in these subfractions were isolated by a centrifugation (L7-35 Ultracentrifuge from Beckman, rotor SwTi 50 from Beckman) for 10 min at 4°C at 4,236 g and then resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris; GE Healthcare) at $\pm 10 \mu\text{g}/\mu\text{l}$ and frozen at -70°C until electrophoresis. Conditions of sample preparation were chosen to mainly get “soluble mitoproteomes” since lysis buffer used is known to be poorly efficient to recover hydrophobic mitochondrial membrane proteins (58). Two-dimensional differential in gel electrophoresis (2D-DIGE) migration was performed on four independent, highly purified mitochondrial fractions per experimental condition from adipocytes treated or not (control condition) with 10 ng/ml TNF α or 0.5 μM FCCP for 6 days, as described previously (26). Samples were applied to 24-cm, nonlinear, pH 3–11, immobilized pH gradient (IPG) strips (GE Healthcare). SDS-PAGE migration was performed on 8.0–13.5%, 24-cm, 1-mm-thick polyacrylamide gradient gel.

Image analysis and statistics. CyDye gels were scanned with the Typhoon 9400 scanner (GE Healthcare) in fluorescence mode at wavelengths specific for the CyDyes. Determination of protein spot abundance as well as normalization and quantitative profiling of proteins in the 2D-DIGE gels were performed using DeCyder 6.5 software (GE Healthcare) as described in the Ettan DIGE User Manual. Protein spots that showed a statistically significant ($P < 0.001$) difference in the abundance by an ANOVA 1 test were accepted as proteins differentially abundant in mitochondria of adipocytes and adipocytes treated with TNF α or FCCP.

Mass spectrometry and protein identification. For peptide sequencing and protein identification, preparative gels, including 400- μg proteins of mixed samples, were poststained with ruthenium II Tris bathophenanthroline disulfonate overnight (7 μl ruthenium/1 liter ethanol 20%) after 6 h of fixation in ethanol-acetic acid (30–10%) and 3×30 min in 20% ethanol at 20°C (44). Spots were excised from preparative gels by using the Ettan Spot Picker (GE Healthcare), and proteins were cleaved with trypsin by in-gel digestion, as described previously (66). The gel pieces were washed twice with distilled water and then shrunk with 100% acetonitrile. The proteolytic digestion was performed by the addition of 3 μl of modified trypsin (Promega) suspended in 100 mM NH_4HCO_3 cold buffer. Proteolysis was performed overnight at 37°C . The supernatant was collected and combined with the eluate of a subsequent elution step with 5% formic acid. Then, digested peptides were desalted using C18 Geloader pipet Tips (C18 column; Proxeon Biosystems) and directly eluted on the target with a mix (1:1 vol/vol) of α -cyano-4-hydroxycinnamic acid (in 7:3 vol/vol acetonitrile-0.1% formic acid) and 2,5-dihydroxybenzoic acid (in 7:3 vol/vol acetonitrile-0.1% trifluoroacetic acid). Peptides were analyzed by using a matrix-assisted laser desorption/ionization mass spectrometer (MALDI-MX mass spectrometer; Waters) instrument piloted with MassLynx 4.0 software (Waters). Peptide mass maps were acquired in the “reflectron” mode with delayed extraction. Peaklist-generating software and release version used was ProteinLynx Global Server 2.2.5 (Waters). Parameters and thresholds used for peak picking were noise reduction. All scans combined were as follows: background threshold: 15%; background polynomial order: 5; deisotoping type: medium. The threshold for the maximum number of peaks within a mass range was 6%. External lock mass with mass-to-charge ratio (m/z) 1,618.84 from aldehyde dehydrogenase digest and internal lock mass with m/z 2,211.10 from trypsin autolysis were used. Search engine and release version was the In House Mascot Server 2.2.1 integrated to the ProteinLynx Global Server. The database searched was NCBI nr 2009 (10; *Mus Musculus*,

248974 sequences). Parameters of the database search were as follows: enzyme specificity considered, trypsin with one missed cleavage; fixed modification, carbamidomethyl; variable modification, oxidation of methionine; peptide tolerance, 100 ppm. Acceptance criteria was as follows: Mascot threshold score 66, corresponding to the identity score of the protein ($P < 0.05$); E value < 1 . If peptides were matched to multiple members of a protein family, the highest Mascot score and the most significant expected value were criteria selected to identify the protein. Protein isoforms have not been looked for. Protein identification consisted of a search accession number of protein corresponding to gi number with UniProt (www.uniprot.org).

Statistical analysis. Data from at least three independent experiments were analyzed by ANOVA 1 and the Holm-Sidak method. Differences between group means were considered to be statistically significant with $P < 0.05$ or less.

RESULTS

Mitochondrial ultrastructure and morphology are affected in adipocytes exposed to mitochondrial uncoupling. Electron micrographs of preadipocytes and adipocytes incubated or not with 10 ng/ml TNF α or 0.5 μM FCCP for 6 days revealed a remodeling of mitochondrial ultrastructure in adipocytes challenged with either TNF α or FCCP, which was characterized by a loss of the swelled cristae observed in adipocytes (Fig. 1). Mitochondrial morphology in adipocytes exposed to mitochondrial uncoupling or TNF α for 6 days was also assessed using MitoTracker Green, a fluorescent dye that accumulates in mitochondria irrespective of mitochondrial membrane potential (56). Mitochondrial population visualized by fluorescence confocal microscopy and phase contrast microscopy (allowing the localization of TG vesicle; Fig. 2A) revealed that interconnected and branched reticular network observed in preadipocytes is impaired in adipocytes. Mitochondrial network alterations shifting from a reticular shape to a denser pattern close to the nucleus and lipid vesicles have already been reported during 3T3-L1 adipogenesis (80). After quantification with Image J software, we also consistently noticed that mitochondria of adipocytes exposed to FCCP displayed a more fragmented network than mitochondria from untreated adipocytes (Fig. 2, B and C) because AR is decreased and EP/BP ratio is increased in cells that display a mitochondrial uncoupling (14).

Because conditions to uncouple mitochondria used in this model lead to mild uncoupling that only slightly affects mitochondrial membrane potential, as characterized previously (73), ATP content was measured in adipocytes incubated with either 0.5 μM FCCP or 10 ng/ml TNF α for 1, 3, or 6 days (Fig. 3). ATP content was found to be significantly lower in adipocytes (by 73%) than in preadipocytes. This important difference in the total abundance of cellular ATP between preadipocytes and adipocytes might be explained most likely by the high ATP consumption of differentiated cells when compared with more metabolically quiescent preadipocytes. Although ATP content was found to be higher in preadipocytes than in adipocytes, this result is in agreement with previous data obtained for porcine adipocyte differentiation (46). Unexpectedly, the ATP content in adipocytes challenged with either TNF α or FCCP was not decreased after 1 day and is even transiently higher in cells treated for 3 days when compared with untreated adipocytes. Knowing that KCl-stimulated intracellular Ca^{2+} concentration has been observed in 3T3-L1 adipocytes treated with FCCP (72), the putative effect of both molecules has been tested on

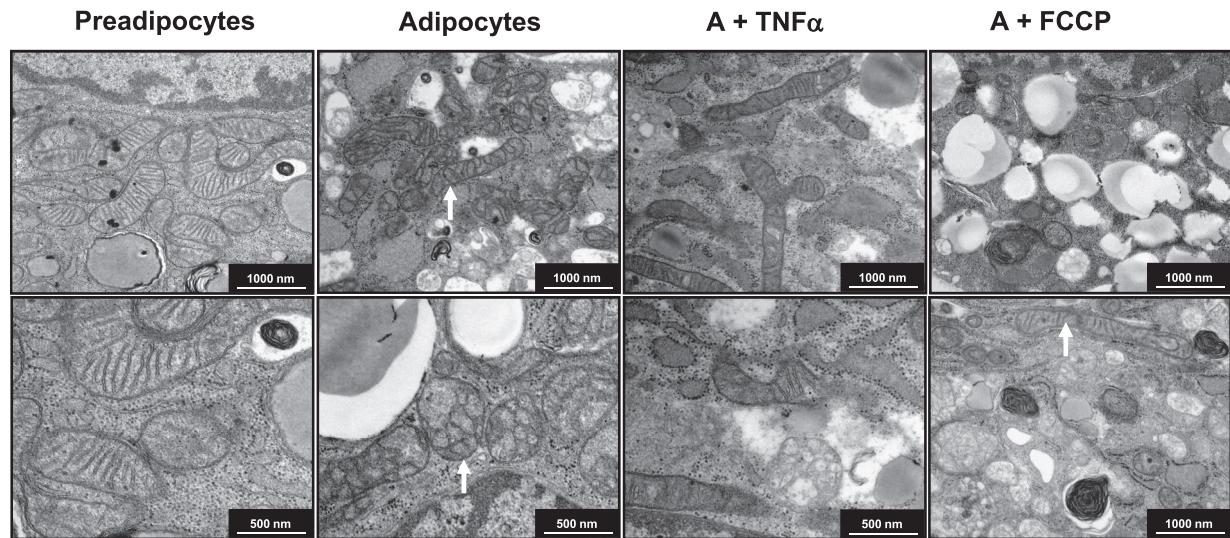


Fig. 1. Effects of $\text{TNF}\alpha$ and FCCP on mitochondrial ultrastructure. Transmission electron micrographs of 3T3-L1 preadipocytes, adipocytes, and adipocytes incubated for 6 days with 10 ng/ml $\text{TNF}\alpha$ or 0.5 μM FCCP. Scale is indicated on the corresponding image. Arrows point the modifications of cristae within mitochondria.

mitochondrial matrix Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{mt}}$), using X-rhod-5F (Fig. 4). Interestingly, after 24 h, $\text{TNF}\alpha$ triggered a strong and significant increase in $[\text{Ca}^{2+}]_{\text{mt}}$, an effect even stronger after a 72-h treatment. In these conditions, the effect of FCCP was more modest even if a significant increase was also found after 24 h of incubation. The negative control performed in preadipocytes incubated with ruthenium red, an inhibitor of the mitochondrial calcium uniporter (6), displayed a significant decrease in $[\text{Ca}^{2+}]_{\text{mt}}$ when compared with preadipocytes (Fig. 4).

Characterization of mitochondrial abundance in adipocytes exposed to mitochondrial uncoupling. Mitochondrial population abundance, assessed by flow cytometry in cells stained with MitoTracker Green (Fig. 5A), was higher in adipocytes than in preadipocytes, data that are in agreement with a previous report showing a strong increase in mitochondrial biogenesis during 3T3-L1 preadipocyte differentiation (80). Besides, mitochondrial population abundance was not modified significantly in adipocytes challenged with 0.5 μM FCCP for 6 days, a time selected to allow any putative biogenic modifications to occur in mitochondria. To support the fact that mitochondrial population abundance does not seem to be modified in adipocytes exposed to mitochondrial uncoupling, we next determined the abundance of mitochondrial DNA (mtDNA) in adipocytes challenged with 10 ng/ml $\text{TNF}\alpha$ or 0.5 μM FCCP for 6 days by assessing the abundance of the noncoding region D-loop using RT-qPCR (Fig. 5B). We found that mtDNA abundance is not modified significantly in FCCP-treated cells, whereas an increase in the abundance of mtDNA is observed in adipocytes stimulated with $\text{TNF}\alpha$. Furthermore, the Western blot analysis of the abundance of several mitochondrial proteins that cover different mitochondrial functions such as OXPHOS, mitochondrial protein import machinery, and fatty acid β -oxidation in adipocytes treated with $\text{TNF}\alpha$ or FCCP has been performed on 30 μg of proteins from cleared cell lysates of preadipocytes and adipocytes treated or not with 10 ng/ml $\text{TNF}\alpha$ or 0.5 μM FCCP for 1, 3, or 6 days (Fig. 6). Except for the ACADVL, all mitochondrial proteins were more

abundant in adipocytes than in preadipocytes, data that are in agreement with the increase in mitochondrial population abundance reported during 3T3-L1 preadipocyte differentiation (80). Among the proteins that are involved in the mitochondrial transport machinery, TOM40, which forms the protein-conducting pore of the complex TOM (77), displayed no modifications of its abundance in adipocytes challenged with FCCP for 1 or 3 days when compared with adipocytes, but this protein was found to be more abundant in adipocytes treated with FCCP for 6 days. The abundance of the protein TIM23, forming the protein-conducting pore of TIM23 complex (77), was not modified in FCCP-treated cells no matter the time of incubation (1, 3, or 6 days). In addition, in adipocytes challenged with $\text{TNF}\alpha$, the abundance of TOM40 was transiently decreased in adipocytes treated for 3 days with the cytokine, whereas TIM23 abundance was increased on *day 1* but decreased on *day 6* (when compared with adipocytes). In the protein import motor of mitochondria, the mitochondrial chaperone mtHSP70 (mitochondrial heat shock protein 70) contributes to the translocation and the folding of imported proteins (76). The abundance of mtHSP70 was not modified in adipocytes incubated with FCCP, whereas its abundance was decreased in adipocytes treated with $\text{TNF}\alpha$ for 6 days. The abundance of several proteins involved in OXPHOS, such as the subunit UCQR1 of complex III, cytochrome *c*, and α - and β -subunits of the ATP synthase, has also been analyzed. The abundance of UCQR1 was not modified in adipocytes treated with $\text{TNF}\alpha$ or FCCP for 1, 3, or 6 days, whereas the abundance of cytochrome *c* was more abundant in adipocytes exposed to either FCCP or $\text{TNF}\alpha$ for 3 days when compared with adipocytes. However, this upregulation seems to be transient since the protein was found to be less abundant in both $\text{TNF}\alpha$ - and FCCP-treated cells after 6 days of treatment. The abundance of the subunits of ATP synthase was not modified in FCCP-treated cells and was slightly decreased in cells treated with $\text{TNF}\alpha$ for 6 days. Finally, the protein ACADVL, which is involved in mitochondrial fatty acid β -oxidation, was less abundant in adipocytes challenged with the mitochondrial

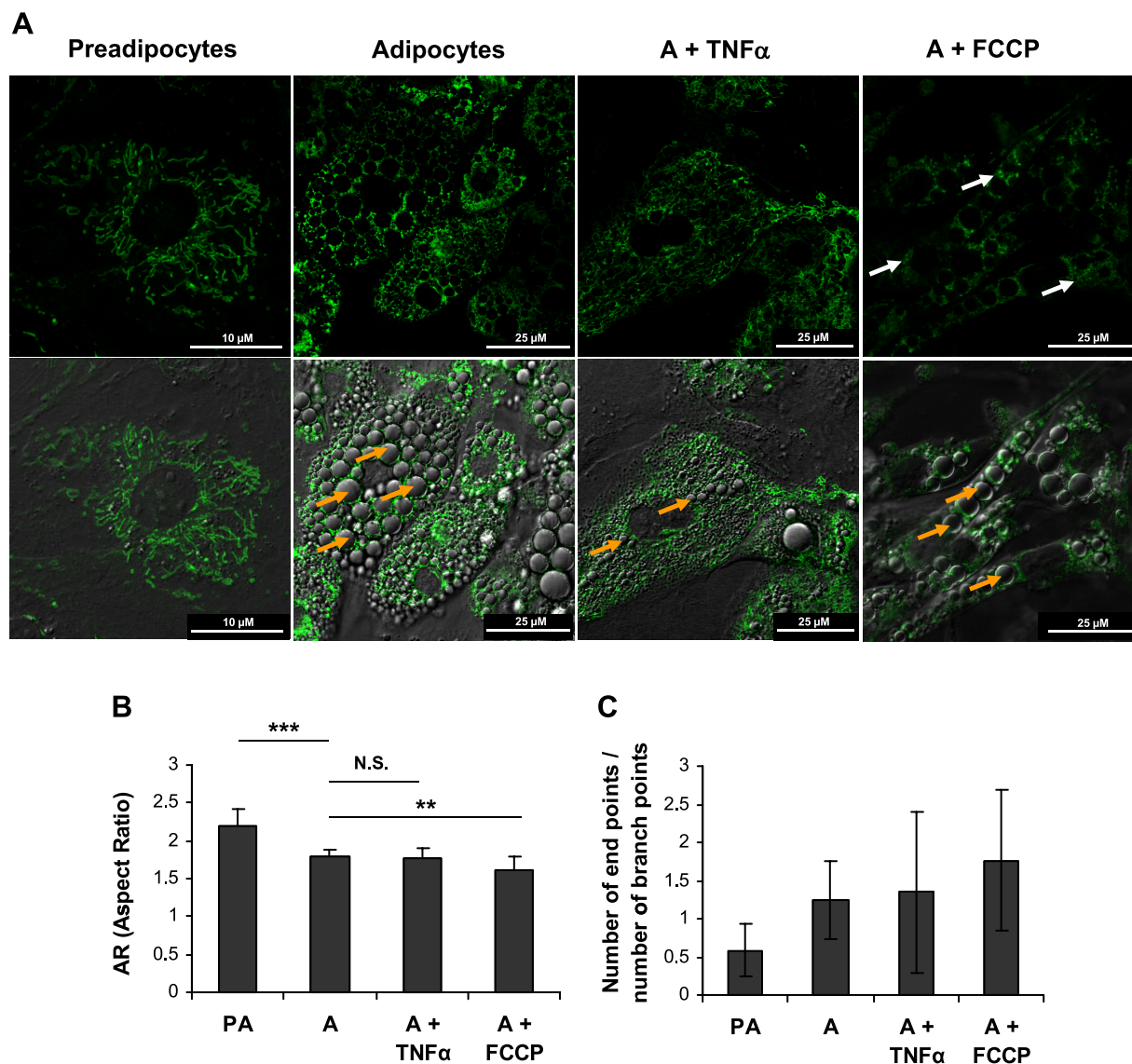


Fig. 2. Effects of TNF α and FCCP on mitochondrial morphology. **A**: mitochondrial morphology has been analyzed by confocal microscopy following staining of mitochondrial population with MitroTracker Green (70 nM, 40 min) in preadipocytes and adipocytes treated or not with 10 ng/ml TNF α or 0.5 μ M FCCP for 6 days (bar scale indicated on the corresponding image). White arrows, fragmentation of the mitochondrial network; orange arrows, localization of some lipid droplets. **B** and **C**: quantification of the mitochondrial network fragmentation has been performed in TNF α - and FCCP-treated adipocytes. The branching and reticulation status was determined on micrographs, as described previously by De Vos and Sheetz (14). **B**: aspect ratio (AR), the ratio between the bigger (major) and the smaller (minor) side for each mitochondrial fragment, has been determined using Image J software. **C**: end points/branch points (EP/BP) ratio was determined. Results are expressed in either AR (**B**) or EP/BP ratio (**C**) and represent means $\pm$ SD ($n = 12$ from 2 independent experiments). *** $P < 0.001$, statistically different from untreated differentiated adipocytes (A) as determined with an ANOVA 1 test. NS, nonstatistically different from untreated differentiated adipocytes (A), as determined with an ANOVA 1 test.

uncoupler for 6 days, whereas its abundance was not modified in adipocytes stimulated with TNF α for 1, 3, or 6 days. In conclusion, these results do not support an increased mitochondrial biogenesis in adipocytes exposed to FCCP since the abundance of many mitochondrial protein markers is unchanged in adipocytes responding to the mitochondrial uncoupler, whereas a true biogenesis of the organelle should respect stoichiometry between mitochondrial proteins.

However, only a few mitochondrial proteins have been analyzed in regard to the estimated 1,500 proteins that compose the organelle (78). Therefore, to further investigate whether or not mild and chronic mitochondrial uncoupling alters mitochondrial population abundance in 3T3-L1 adi-

pocytes, the effectors of mitochondrial biogenesis process have been studied.

Mild mitochondrial uncoupling does not activate transcription factors known to control mitochondrial biogenesis in 3T3-L1 adipocytes. Transcriptional mechanisms that control mitochondrial biogenesis operate mainly through the PGC coactivator family members and a subset of well-defined transcription factors such as NRF1, NRF2, ERR α , Sp1, YY1, cAMP response element-binding protein (CREB), and PPAR α (63). The selection of these genes was made for their demonstrated or suspected roles in the biogenesis of mitochondria but also to cover the different aspects of the mitochondrial biogenesis. PPAR γ and PPAR δ , two other important regulators of

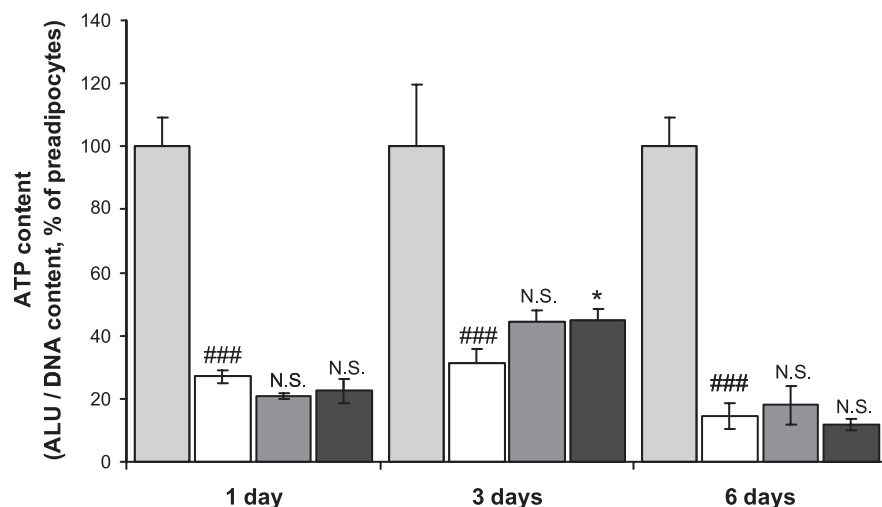


Fig. 3. Effects of $\text{TNF}\alpha$ and FCCP on cellular ATP content. ATP content was assayed in preadipocytes (light gray bars) and in adipocytes incubated with or without (open bars) 10 ng/ml $\text{TNF}\alpha$ (dark gray bars) or 0.5 μM FCCP (black bars) for 1, 3, or 6 days. Results were normalized for DNA content [arbitrary light units (ALU)/arbitrary fluorescence units (AFU), 617 nm (propidium iodide)] and expressed in %preadipocytes as means $\pm$ SD; $n = 3$. ### $P < 0.001$, significantly different from preadipocytes; * $P < 0.05$, significantly different from adipocytes. NS, not significantly different from adipocytes.

mitochondrial biogenesis, have been characterized previously in this model (73). In addition, because PGC-1 α and PGC-1 β coactivators are upregulated in 3T3-L1 adipocytes (53), we addressed the expression level of both coactivators in adipocytes and adipocytes challenged with FCCP or $\text{TNF}\alpha$ for 24 h (Fig. 7A). The relative mRNA abundance of PGC-1 α and PGC-1 β determined by RT-qPCR was significantly reduced in adipocytes treated with either 10 ng/ml $\text{TNF}\alpha$ or 0.5 μM FCCP. Besides, since PGC-1 α and PGC-1 β have the ability to orchestrate mitochondrial biogenesis through the induction and/or the activation of the transcription factors NRF1, NRF2, and ERR α (63), we next evaluated the transcriptional activity of these factors in adipocytes treated with FCCP or $\text{TNF}\alpha$. Adipocytes were transiently transfected with luciferase reporter constructs responding to NRF1, NRF2, or ERR α and then treated (or not) with 10 ng/ml $\text{TNF}\alpha$ or 0.5 μM FCCP for 24 h. In these conditions, the transcriptional activity of NRF1, NRF2, and ERR α was not modified significantly (and certainly not increased) in adipocytes incubated with $\text{TNF}\alpha$ or FCCP (Fig. 7B).

Additional nuclear factors are also involved in the maintenance and the biogenesis of mitochondria, exerting their influence on the expression of mtDNA transcriptional regulators

such as Sp1 (11), or in the expression of respiratory genes such as CREB (24), YY1 (23), and Sp1 (85). In addition, the transcription factor PPAR α has been linked to the regulation of mitochondrial oxidative metabolism, acting with ERR α on the regulation of medium-chain acyl-coA dehydrogenase gene expression (15). Because NRF1/2 factors were not differentially activated in adipocytes challenged with the uncoupler, the transcriptional activity of these regulators (CREB, YY1, Sp1, and PPAR α) has also been investigated. Using specific luciferase reporter constructs, we found that the activity of CREB and Sp1 was significantly decreased in both $\text{TNF}\alpha$ - and FCCP-treated cells, whereas the transcriptional activity of PPAR α and YY1 was not modified significantly in these conditions (Fig. 7B).

Altogether, these data clearly show that mild mitochondrial uncoupling induced by FCCP does not trigger the activation of regulators known or suspected to control mitochondrial biogenesis in 3T3-L1 adipocytes. However, we cannot exclude that these transcription factors might be differentially recruited to target gene promoters to control the expression of some nuclear genes encoding mitochondrial proteins in the various experimental conditions. In addition, the degradation of some mitochondrial proteins could also be impaired in FCCP- or

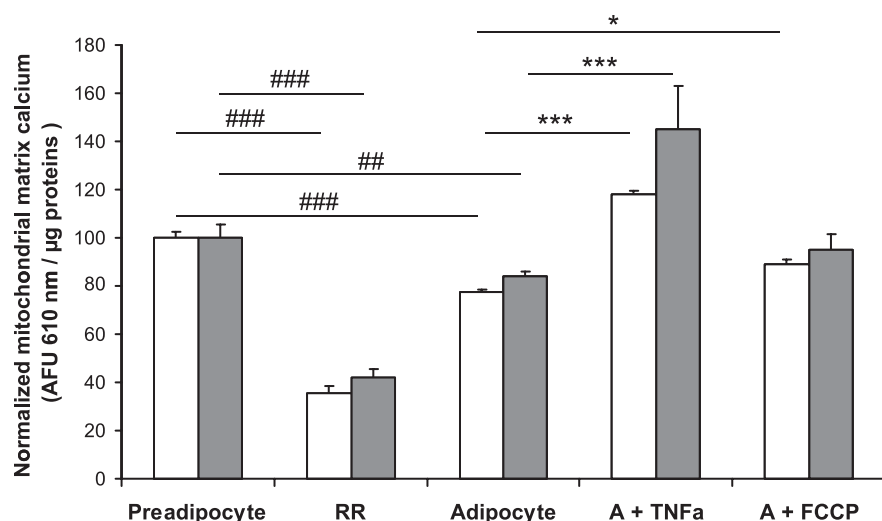
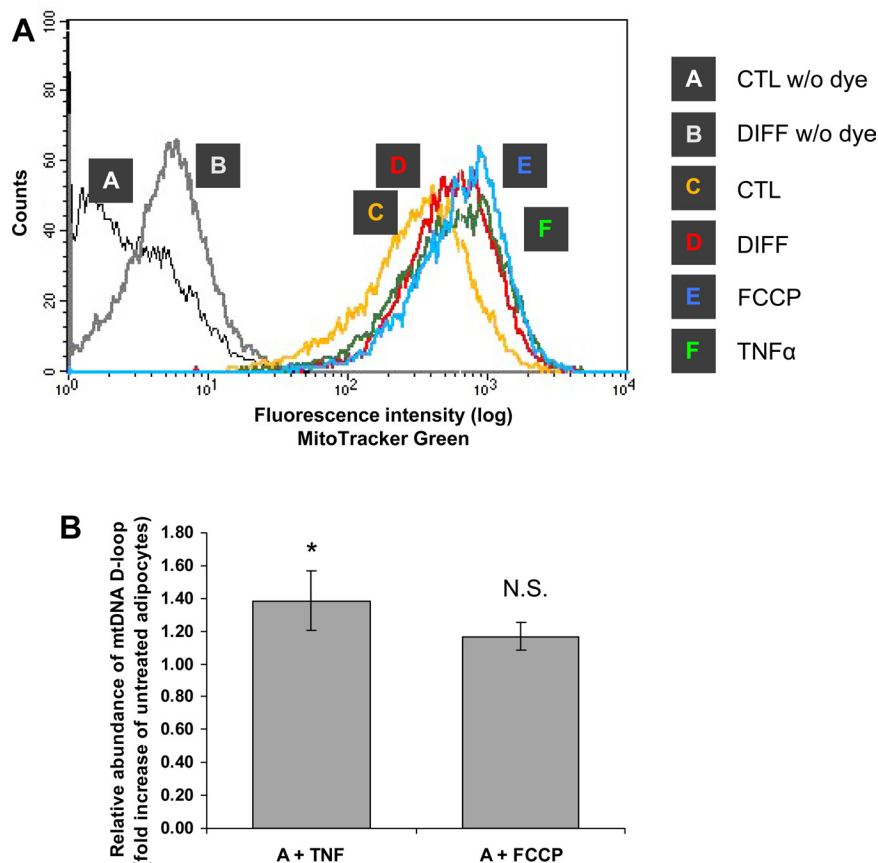


Fig. 4. Effects of $\text{TNF}\alpha$ and FCCP on mitochondrial calcium concentration. The concentration of mitochondrial matrix calcium in preadipocytes and in adipocytes incubated with or without 10 ng/ml $\text{TNF}\alpha$ or 0.5 μM FCCP for 24 (open bars) or 72 h (gray bars) using the specific mitochondrial matrix calcium probe X-rhod-5F. A negative control was performed using preadipocytes incubated for 30 min with ruthenium red (RR), a uniporter inhibitor. Results were normalized for protein content (AFU/ μg proteins) and expressed in %preadipocytes as means $\pm$ SD; $n = 3$. ## $P < 0.01$ and ### $P < 0.001$, significantly different from preadipocytes; * $P < 0.05$ and *** $P < 0.001$, significantly different from adipocytes. A + $\text{TNF}\alpha$, adipocytes incubated with $\text{TNF}\alpha$; A + FCCP, adipocytes incubated with FCCP.

Fig. 5. Effects of $\text{TNF}\alpha$ and FCCP on mitochondrial abundance. A: mitochondrial population abundance was analyzed in preadipocytes (CTL) and in adipocytes incubated or not (D) with 10 ng/ml $\text{TNF}\alpha$ (F) or 0.5 μM FCCP (E) for 6 days. Cells were then stained or not [autofluorescence signals measured from control cells without (w/o) dye] with 70 nM MitoTracker Green for 40 min, and fluorescence was analyzed by flow cytometry. B: relative abundance of displacement loop (D-loop), reflecting mitochondrial DNA abundance (mtDNA), was determined by real-time RT-qPCR in adipocytes (A) incubated for 6 days in the presence or absence of either 10 ng/ml $\text{TNF}\alpha$ (A + $\text{TNF}\alpha$) or 0.5 μM FCCP (A + FCCP). For data calculation and normalization, TATA box-binding protein (TFIID) was used as a reference gene [formula used: $2^{-(\Delta\text{C}_T \text{ D-loop} - \Delta\text{C}_T \text{ TFIID})}$]. Results were calculated and expressed as fold increase of the abundance determined in untreated adipocytes as means $\pm$ SD; $n = 4$ experiments. * $P < 0.05$, significantly different from adipocytes. NS, not significantly different from adipocytes. DIFF, differentiated cells.



$\text{TNF}\alpha$ -treated adipocytes. Indeed, we observed that some but not all mitochondrial proteins (such as TOM40 and transiently cytochrome *c*; Fig. 6) are differentially abundant in adipocytes challenged with FCCP, a finding that suggests changes in the mitochondrial protein content in adipocytes responding to a mild mitochondrial uncoupling. Therefore, comparative mitochondrial proteomes have been established between mitochondria isolated and purified from adipocytes challenged with FCCP for 6 days and mitochondria from nontreated adipocytes. Besides, to determine the specificity of changes affecting mitochondria in adipocytes incubated with FCCP, mitoproteomes have been compared with mitoproteomes generated from mitochondria purified from $\text{TNF}\alpha$ -treated adipocytes for 6 days.

Comparative mitochondrial proteomes generated from FCCP- and $\text{TNF}\alpha$ -treated adipocytes. Because the quality and purity of the organelle are crucial in a subproteomic study (22), highly purified mitochondrial fractions were prepared by differential and isopycnic centrifugations from homogenates of adipocytes incubated (or not) with $\text{TNF}\alpha$ or FCCP for 6 days. The quality of the purification of the biological sample was ascertained by enzymatic assays (Fig. 8) before 2D-DIGE on four independent replicates (per condition) of purified mitochondrial fractions from adipocytes, $\text{TNF}\alpha$ - and FCCP-treated adipocytes was performed (Fig. 9). Among the protein spots found to be differentially abundant ($P < 0.001$) between mitochondria from adipocytes incubated with $\text{TNF}\alpha$ or FCCP and mitochondria from nontreated adipocytes, 220 proteins have been significantly identified by the Mascot Software, of

which 60% corresponded to proteins from mitochondrial origin (Supplemental Table S1; Supplemental Material for this article is available online at the *AJP-Endocrinology and Metabolism* web site). The subset of identified proteins that were not identified as mitochondrial proteins might be either contaminants or proteins transiently present in the organelle, since mitochondria are dynamic organelles with a constantly changing molecular composition (83). They might also correspond to a new location of proteins.

The distribution of identified proteins according to their functional classification is represented on the pie charts compiling mitochondrial proteins that are differentially abundant in adipocytes challenged with either FCCP or $\text{TNF}\alpha$ (when compared with adipocytes; Fig. 10, A and B, respectively). Pie chart representations of identified proteins highlight the modifications of mitochondrial content in 3T3-L1 adipocytes dedifferentiated with either the cytokine or the mitochondrial uncoupler. In total, 72 mitochondrial proteins displaying differential abundance between FCCP-treated adipocytes and control adipocytes have been identified, among which 37 are found specifically in mitochondria of adipocytes treated with the mitochondrial uncoupler (Fig. 10C). Among these 37 proteins, 21 were found to be more abundant, with a fold increase ranging from +1.30 (for NADH-ubiquinone oxidoreductase 42) to +6.56 (for the carboxyltransferase domain of PCx, most likely a cleavage product), and 16 are less abundant, with the fold decrease ranging from -1.31 (for ES1 protein and hydroxysteroid dehydrogenase-like protein 2) to -2.08 (for long-chain enoyl-CoA hydratase) (Supplemental Table S1). The

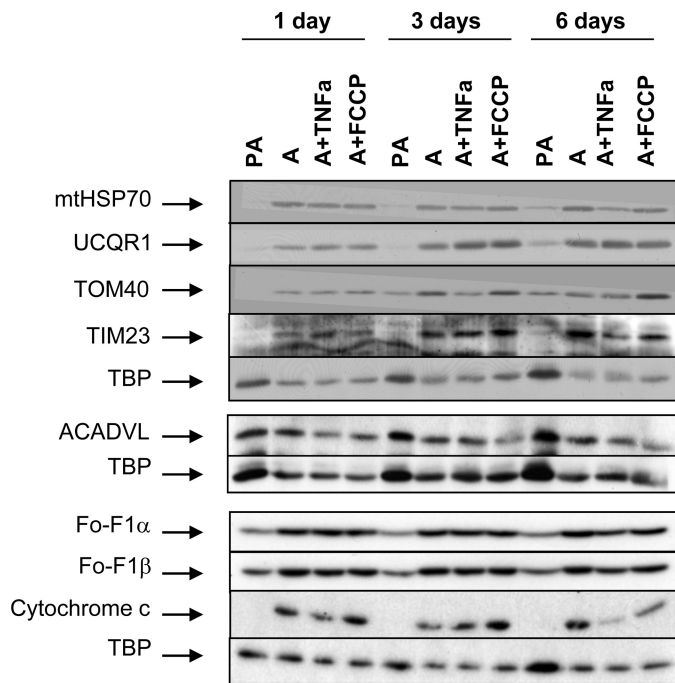


Fig. 6. Effect of TNF α and FCCP on the cellular abundance of several mitochondrial proteins. The total abundance of different mitochondrial proteins was analyzed by Western blot analysis of 30 μ g of clear cell lysates prepared from preadipocytes (PA), A, and A + TNF α or exposed to A + FCCP for 1, 3, or 6 days. Equal protein loading was controlled by the immunodetection of TATA box-binding protein (TBP). mtHSP70, mitochondrial heat shock protein 70; UCQR1, coenzyme Q reductase 1; TOM40, translocase of the outer mitochondrial membrane 40; TIM23, translocase of the inner mitochondrial membrane 23; ACADVL, acetyl-CoA dehydrogenase very-long chain; Fo-F1 α and Fo-F1 β , α - and β -subunits, respectively, of Fo -F1 ATP synthase.

mitochondrial protein content from TNF α -treated adipocytes is also modified since 13 mitochondrial proteins are specifically more abundant, ranging from +1.30 (pitrilysin metalloproteinase 1) to +3.27 (polymerase δ -interacting protein 2), whereas 28 proteins display a decrease in abundance from -1.33 (isovaleryl-CoA dehydrogenase and dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex) to -1.58 (isovaleryl-CoA dehydrogenase; Supplemental Table S1). Additionally, effects of TNF α and FCCP are only partly comparable since 35 mitochondrial proteins identified in both conditions display a similar abundance. Among these 35 proteins, 12 proteins display an increase in abundance from +1.41 for serine hydroxymethyltransferase 2 in both FCCP- and TNF α -treated cells to +2.31 for aldehyde dehydrogenase family 18 member A1 in FCCP-treated cells and +2.99 for nucleoside diphosphate kinase in TNF α -treated cells. Besides, 23 proteins display a decrease in abundance from -1.30 for citrate synthase in FCCP-treated cells and carnitine *O*-palmitoyltransferase 2 in TNF α -treated cells to -1.88 for ACADVL in FCCP-treated cells and -2.39 for PCx in TNF α -treated cells (Supplemental Table S1).

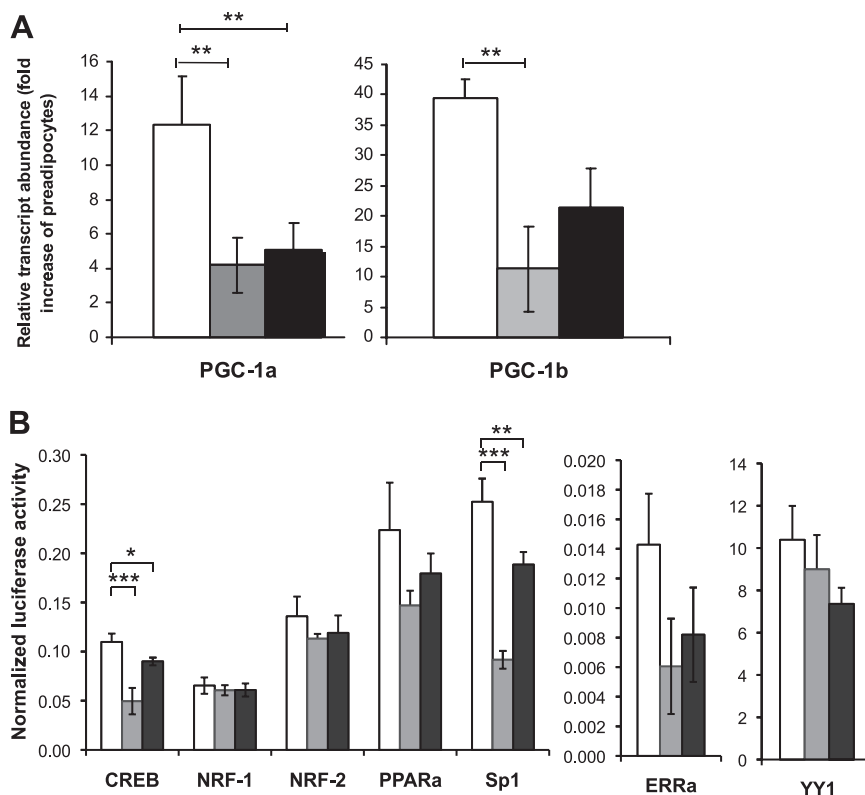
Indeed, whereas a fragment of PCx corresponding to the carboxyltransferase domain of the protein has been identified as more abundant in FCCP-treated cells, the full enzyme has been identified as less abundant in mitochondria from both TNF α - and FCCP-treated adipocytes (Supplemental Table S1). This enzyme catalyzes the ATP-dependent carboxylation of

pyruvate to form oxaloacetate, providing a substantial amount of an acetyl group and NADPH required for de novo fatty acid synthesis in adipocytes (33). The decreased abundance of mitochondrial PCx has been confirmed by Western blot (Fig. 11A) and is consistent with the downregulation of the gene encoding PCx as determined by RT-qPCR (Fig. 11B). The PCx downregulation observed for both transcript and protein abundance in adipocytes treated with FCCP has also been confirmed by measuring the activity of this enzyme. To determine the PCx activity, we modified slightly the protocol described by Atkin et al. (4) and Zhang et al. (87), as detailed in MATERIALS AND METHODS. The principle of the assay relies on the incorporation of $^{14}\text{CO}_2$ provided as NaHCO $_3$ in solution during the transformation of pyruvate into oxaloacetate catalyzed by PCx. Therefore, the rate of incorporation of this isotope is correlated to the PCx activity. We observed an increase in PCx activity in adipocytes when compared with preadipocytes, a result that is in accord with a previous study (87). Furthermore, we showed that the PCx activity seems to be decreased by 40% in adipocytes treated for 6 days with 0.5 μM FCCP when compared with untreated adipocytes, which is in accord with our results showing a decrease in the PCx protein content in these cells (Fig. 12). In addition, because modification in pyruvate dehydrogenase activity could also have an impact on de novo fatty acid synthesis and because the enzyme is strongly regulated by ATP/ADP and NAD/NADH ratios, we measured its activity in adipocytes incubated with 0.5 μM FCCP for 6 days and found that the activity of the enzyme is not significantly changed in adipocytes exposed to a mitochondrial uncoupling (data not shown).

The differential abundance of MnSOD/SOD2, mtHSP70, and Hadha has also been confirmed by Western blot analysis (Fig. 11A) and is correlated with the abundance of their transcript levels (Fig. 11B). Because the results of proteomic studies (acyl-CoA dehydrogenase very long chain/medium chain, 3-ketoacyl-CoA thiolase, Hadha, Hadha domain containing protein 2/3; Supplemental Table S1) suggest a decrease in FFA β -oxidation in adipocytes "dedifferentiated" with TNF α or FCCP, the relative rates of β -oxidation have been investigated in adipocytes challenged with TNF α or FCCP for 6 days by measuring the $^{14}\text{CO}_2$ release (Fig. 11C). Functional assays revealed a strong stimulation of fatty acid β -oxidation in TNF α -treated adipocytes for 6 days, whereas the rate of β -oxidation is not modified in adipocytes incubated with FCCP for 6 days when compared with adipocytes even if lipolysis is strongly stimulated in these conditions (73). A likely reason for this surprising result could be explained by the work of Rossmeisl et al. (61), who demonstrated that overexpression of UCP1 from the *aP2* gene promoter induces mitochondrial biogenesis in unilocular adipocytes, which is probably due to upregulation of transcription factor NRF1, a condition that could explain increased FA β -oxidation observed in these conditions.

In addition, to ascertain TNF α -induced reactive oxygen species (ROS) production suggested by the higher abundance of the antioxidant enzyme MnSOD/SOD2 in mitochondria of TNF α -treated cells, superoxide anion radicals has been evaluated in MitoSox fluorescent dye-loaded adipocytes challenged with either TNF α or FCCP for 6 days (probe loaded 45 min in the absence of FCCP or TNF α ; Fig. 11D). Indeed, mitochondrial O $_2$ (-) abundance is strongly increased after a 6-day

Fig. 7. Effects of $\text{TNF}\alpha$ and FCCP on the expression or activity of mitochondrial biogenesis regulators. **A**: relative mRNA abundance of peroxisome proliferator-activated receptor (PPAR) γ coactivator (PGC)-1 α and PGC-1 β was determined in adipocytes incubated or not (open bars) with either 10 ng/ml $\text{TNF}\alpha$ (gray bars) or 0.5 μM FCCP (black bars) for 24 h by real-time RT-qPCR. For data calculation and normalization, TBP (TFIID) was used as a reference gene. Results were calculated [formula used: $2^{-(\Delta C_T \text{ transcript} - \Delta C_T \text{ TFIID})}$] and expressed in fold change of the abundance determined in preadipocytes as means $\pm$ SD; $n = 3$ experiments. **B**: adipocytes were transiently cotransfected with luciferase reporter constructs responding to cAMP response element-binding protein (CREB), nuclear respiratory factor (NRF)1, NRF2, PPAR α , specificity protein 1 (Sp1), estrogen-related receptor- α (ERR α), or ying yang 1 (YY1) and with a β -galactosidase expression vector to account for transfection efficiency. Transiently transfected adipocytes (electroporation) were then incubated or not (open bars) with 10 ng/ml $\text{TNF}\alpha$ (gray bars) or 0.5 μM FCCP (black bars) for 24 h before luciferase and β -galactosidase activities were measured. Results were normalized for β -galactosidase activity (luciferase activity/ β -galactosidase activity) and expressed in relative light units as means $\pm$ SD; $n = 3$ experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, significantly different from adipocytes.



treatment with $\text{TNF}\alpha$, whereas a smaller increase was observed in FCCP-treated cells. However, an increase in the abundance of superoxide anion radical found in FCCP-treated cells cannot be explained by ROS production because mitochondrial uncoupling is clearly associated with ROS production decrease when evaluated on both mitochondria (37) and full cells (3).

The study of mitochondrial proteomes performed from adipocytes incubated in the presence of $\text{TNF}\alpha$ or FCCP has highlighted the reduced mitochondrial abundance of PCx, a key enzyme of lipogenesis, in adipocytes treated with those molecules. This is of particular interest and could constitute a good candidate to explain, at least in part, the TG loss in 3T3-L1 adipocytes treated with the cytokine or the uncoupler. Thus we next addressed the effect of PCx overexpression on the TG content reduction observed in adipocytes treated with either $\text{TNF}\alpha$ or FCCP.

Effect of pyruvate carboxylase on $\text{TNF}\alpha$ - or FCCP-induced TG content loss in 3T3-L1 adipocytes. 3T3-L1-differentiated adipocytes were transfected with a construct encoding pyruvate carboxylase (1 μg of pEF-PC/1,000,000 cells) and then treated (or not) with 10 ng/ml $\text{TNF}\alpha$ or 0.5 μM FCCP for 3 days before overexpression of the PCx gene was assessed by RT-qPCR (Fig. 13). Indeed, the relative transcript abundance of PCx in adipocytes transfected with pEF-PC and then incubated with either $\text{TNF}\alpha$ or FCCP is comparable with the abundance recovered for untreated adipocytes transfected with the plasmid encoding PCx or the plasmid control. Therefore, we used 1 μg of pEF-PC/1,000,000 cells to determine the putative role of PCx in the reduction of TG content in adipocytes treated with either the cytokine or the uncoupler. 3T3-L1 adipocytes were transfected with the expression plasmid encoding PCx and then treated with $\text{TNF}\alpha$ or FCCP for 6 days. At the end of the

incubations, cells were stained with Oil Red O, and quantitative analyses of TG content showed that the loss of neutral lipids induced by $\text{TNF}\alpha$ or FCCP was totally prevented in adipocytes that overexpressed PCx (Fig. 14). Although at the protein level we could only monitor an increase in PCx abundance in FCCP-treated cells after 6 days posttransfection (data not shown), these results clearly suggest that lower mitochondrial abundance of PCx might play a major role in the reduction of TG content in both $\text{TNF}\alpha$ - and FCCP-treated adipocytes.

DISCUSSION

Mechanisms affecting lipid metabolism and leading to a decrease in TG content in adipocytes exposed to mitochondrial uncoupling have been studied extensively *in vivo* and *in vitro* (38–41, 67, 68, 73). Indeed, the cell must obviously meet its own energetic needs and for that limit and reduce TG synthesis (61) as well as stimulate lipolysis and TG catabolism (47, 73). Therefore, it will use TG stores in an attempt to restore mitochondrial membrane potential and the capacity for ATP synthesis and in the mean time limit ATP consumption.

However, the impact of mitochondrial uncoupling on mitochondrial population and adaptive cell response as well as the molecular mechanism by which TG content reduction is achieved in adipocytes exposed to mitochondrial dysfunction are still largely unknown in white adipocytes.

This study was designed to assess modifications in both mitochondrial abundance and mitochondrial protein content in response to OXPHOS uncoupling induced by FCCP in 3T3-L1 adipocytes to highlight the role of mitochondrial response to TG loss in adipocytes exposed to mitochondrial uncoupling.

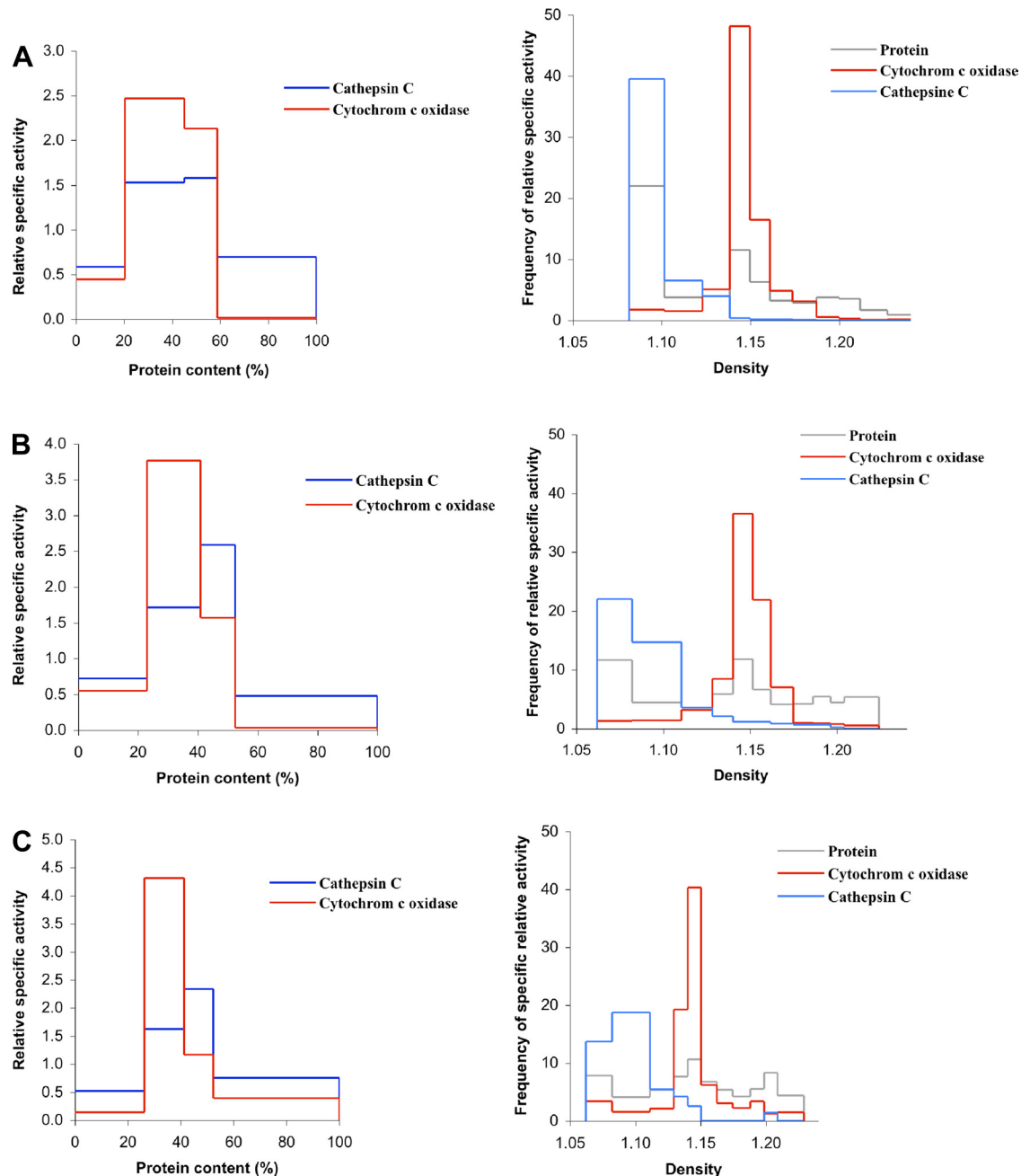


Fig. 8. Mitochondria and lysosome distributions after differential and isopycnic centrifugation. The distribution of mitochondria and lysosomes and of protein content after differential and isopycnic centrifugation (Nycodenz gradient) performed on mitochondrial fractions obtained from adipocytes (A) or adipocytes incubated with 10 ng/ml $\text{TNF}\alpha$ (B) or 0.5 μM FCCP (C) for 6 days. Cytochrome *c* oxidase and cathepsin C activities reflect the distribution of mitochondria and lysosome organelles, respectively, with lysosomes being the well-known major contaminant of mitochondrial fraction. Methods to perform cellular fractionation and isopycnic centrifugation as well as enzymatic assays are described in MATERIALS AND METHODS. It should be noted that cytochrome *c* oxidase activity has a similar distribution profile in adipocytes and in adipocytes challenged with $\text{TNF}\alpha$ or FCCP for 6 days in both differential centrifugation and Nycodenz gradient, suggesting that mitochondria from adipocytes treated or not with either $\text{TNF}\alpha$ or FCCP behave the same way during both differential centrifugation and migration into Nycodenz gradient.

The impact of mitochondrial uncoupling on mitochondria was also compared with mitochondrial effects induced by $\text{TNF}\alpha$, a proinflammatory cytokine that induces a TG content reduction in 3T3-L1 adipocytes (86).

Because both $\text{TNF}\alpha$ and mitochondrial uncoupling impair mitochondrial functions by different mechanisms (9, 10, 43, 64), we first confirmed that mitochondrial ultrastructure and morphology are affected in adipocytes challenged with either

10 ng/ml $\text{TNF}\alpha$ or 0.5 μM FCCP for 6 days, exhibiting a loss of swelled cristae observed in control adipocytes. Moreover, the increased fragmentation of mitochondrial network observed in FCCP-treated cells (Fig. 2) was expected because the maintenance of mitochondrial membrane potential, which is affected by the uncoupler (73), is essential for mitochondrial fusion events (45). These results are consistent with the impaired mitochondrial fusion and fission processes observed in

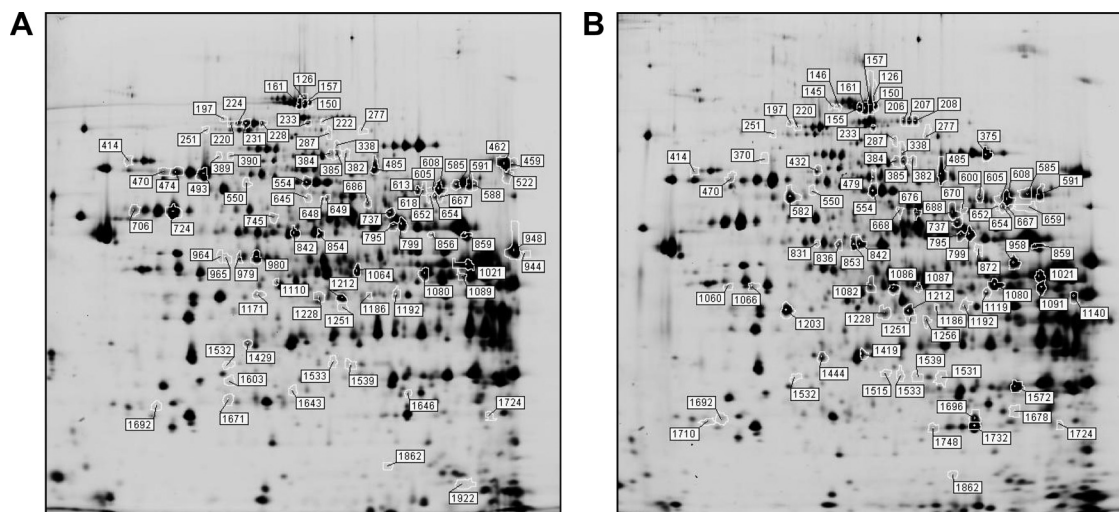


Fig. 9. Representative 2-dimensional differential in gel electrophoresis (2D-DIGE) gels showing the migration profiles of mitochondrial proteins from adipocytes treated with either 0.5 μ M FCCP or 10 ng/ml TNF α for 6 days. Proteins of purified mitochondrial fractions from adipocytes treated (or not; control cells) with either 0.5 μ M FCCP (A) or 10 ng/ml TNF α (B) for 6 days were differentially labeled with Cy3 and Cy5. An internal standard composed of equal amounts of each sample and labeled with Cy2 was performed. Labeled samples (25 μ g of each tagged sample with Cy3 or Cy5 and 25 μ g of the internal standard) were loaded on 24-cm, pH 3–11, nonlinear IPG strips and subjected to isoelectric focusing. Proteins were further separated by SDS-PAGE gradient (8.0–13.5%). Numbers allocated by the Decyder software indicate spots with significant changes in intensity ($P < 0.001$, ANOVA 1 performed on 4 independent replicates).

3T3-L1 adipocytes overexpressing UCP4 (20). In our experimental conditions, it might appear that a total of 18 days for adipocytes in culture is long, and one cannot completely rule out that some effects observed might be due to FCCP accumulation in mitochondria and the resulting toxic effect. However, a 6-day treatment of adipocytes was needed to assess the putative effect on biogenesis of mitochondria, a process known to take between 4 and 6 days when cells are exposed to nitric oxide donors (54). In addition, in FCCP-treated cells, we checked that cell viability was maintained up to 95%, as determined by both lactate dehydrogenase (which measures release of lactate dehydrogenase and thus assess the status of plasma membrane permeability) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assays (used in conditions that measure maximum succinate dehydrogenase activity) (data not shown). In addition, the FCCP effect is believed to be most likely due to mitochondrial uncoupling rather than toxicity since downregulation of PCx and mitochondrial network fragmentation could also be seen in adipocytes incubated with 0.5 μ M FCCP for shorter incubation periods (6 and 24 h; data not shown). It is important to note that 3T3-L1 adipocytes incubated with high levels of glucose and FFAs also display a decrease in mitochondrial membrane potential associated with a more fragmented mitochondrial network due to an increase in fission and decrease in fusion events (19). Further studies are then needed to investigate the mechanisms by which mitochondrial uncoupling affects mitochondrial dynamic processes in adipocytes.

Numerous studies performed in muscle cells have shown that mitochondrial dysfunction or higher energy demands induced by physical exercise lead to the stimulation of mitochondrial biogenesis (25, 79). Mitochondrial uncoupling induced by ectopic expression of UCP1 in unilocular adipocytes of *ap2-Ucp1* mice or by 150 μ M of DNP in 3T3-L1 adipocytes was also associated with an increase in mitochondrial content (59). However, 3T3-L1 adipocytes overexpressing UCP4 exhibit a decrease in the transcript levels of key regulators of mitochon-

drial biogenesis (20). In the present study, we found that the abundance of mitochondrial population (as determined by Mitotracker Green staining and quantification of mtDNA) was not significantly modified in FCCP-treated cells. Furthermore, we found that the activity of key regulators in the biogenesis of mitochondria such as NRF1, NRF2, ERR α , PPAR α , and YY1 was not increased in adipocytes treated with TNF α or FCCP. In addition, both genes encoding PGC-1 α and PGC-1 β coactivators were found to be downregulated, whereas CREB transcriptional activity, which regulates the expression of PGC-1 α (82), was also decreased. Altogether, these data support the fact that mitochondrial biogenesis is probably not quantitatively affected in adipocytes dedifferentiated with 10 ng/ml TNF α or treated with 0.5 μ M FCCP for 6 days. However, we cannot be sure about the fact that differences in the abundance for some of the mitochondrial proteins found to be more abundant in TNF α - or FCCP-treated cells are really due to transcription modifications since it could also be explained by a difference in their mitochondrial degradation (5, 36). In addition, if we do not observe the activation of some transcription factors involved in the control of expression of nuclear genes encoding mitochondrial proteins using reporter systems, it does not allow us to exclude that gene targeting by these factors could be differentially affected in FCCP- or TNF α -treated adipocytes. Indeed, specific and preferential recruitment of transcription factors to gene promoter is a complex process that might be dependent on many factors other than just the activity of the regulator itself (18). We should also add that for some actors, such as PGC-1 α , we have only transcriptional information, a readout far away and not sufficient to fully address the activity of the coactivator since posttranslational modifications and protein-protein interaction are known to control its activity (17). Finally, the selection of the transcription factors analyzed, although already relatively important in the study, does probably not cover all transcriptional regulators that could participate to the control of gene expression encoding mitochondrial proteins (63).

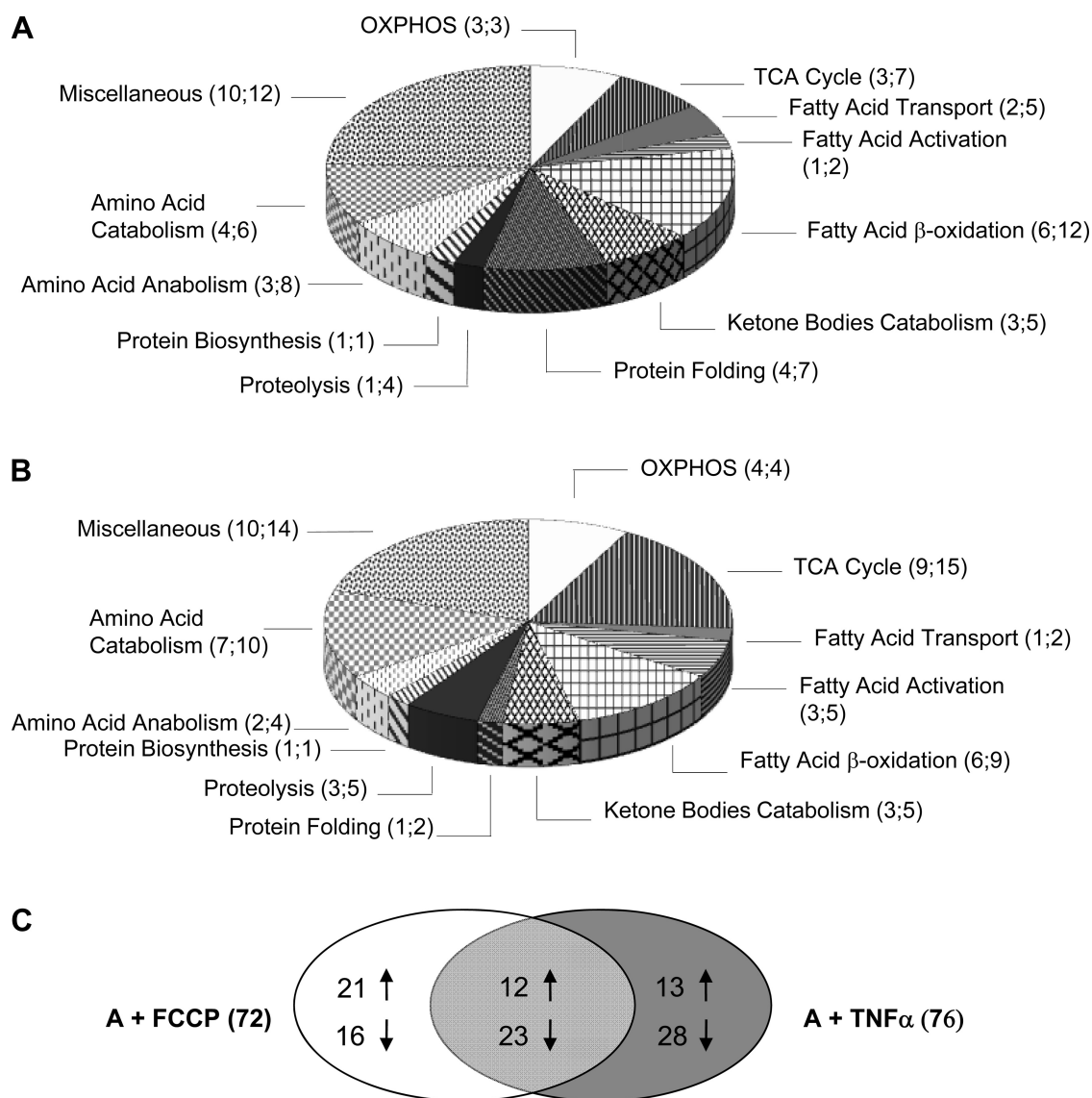


Fig. 10. Summary of mitochondrial proteins identified in both TNF α and FCCP mitoproteomes. *A* and *B*: functional classification of proteins identified as differentially abundant in mitochondrial fractions prepared from adipocytes incubated with 0.5 μ M FCCP (*A*) or 10 ng/ml TNF α (*B*) for 6 days (compared with mitochondria from nontreated adipocytes). Proteins with unknown function or proteins not involved in the selected mitochondrial metabolic processes were grouped together in miscellaneous class. The 1st number between parentheses corresponds to the number of different proteins identified in this class, whereas the 2nd number corresponds to the number of spots for which identified proteins belong to this class. *C*: distribution of mitochondrial protein spots identified exclusively in adipocytes challenged with either 0.5 μ M FCCP or 10 ng/ml TNF α or identified in both conditions. ANOVA 1 test performed on 4 independent replicates, statistically significant ($P < 0.001$). OXPHOS, oxidative phosphorylation; TCA, tricarboxylic acid.

Therefore, long-term mild mitochondrial uncoupling, although it triggers a significant loss of TG content (12), is likely accompanied by modifications in the abundance of mitochondrial proteins as well as in the mitochondrial protein content without any increase in mitochondrial population abundance as determined by Mitotracker Green staining and mtDNA abundance.

Interestingly, we observed a significantly higher abundance of mtDNA in TNF α -treated cells, whereas the content of mitochondrial genome was not affected significantly in FCCP-treated cells. To date, mechanisms regulating mtDNA copy number are not still completely identified. However, it has been demonstrated recently that ROS positively regulate mtDNA copy number in isolated yeast mitochondria (31). We observed

a strong ROS production was reflected by an increase in O₂(-) abundance in 3T3-L1 adipocytes treated with 10 ng/ml TNF α for 6 days. TNF α -induced mitochondrial ROS formation is well recognized and is attributed to TNF α -dependent accumulation of ceramide and its subsequent inhibition of electron transport chain (12, 16, 21). One can also mention that FCCP-induced mitochondrial uncoupling also slightly raises ROS abundance in adipocytes. These are surprising data, because a drop of mitochondrial membrane potential induced by uncouplers such as DNP or FCCP is known to strongly decrease ROS production in isolated mitochondria (27, 42, 70). However, although it is demonstrated that FCCP reduces ROS production (1), this is not always found in intact cells (8, 71, 74). If the reasons for these discrepancies are still unclear,

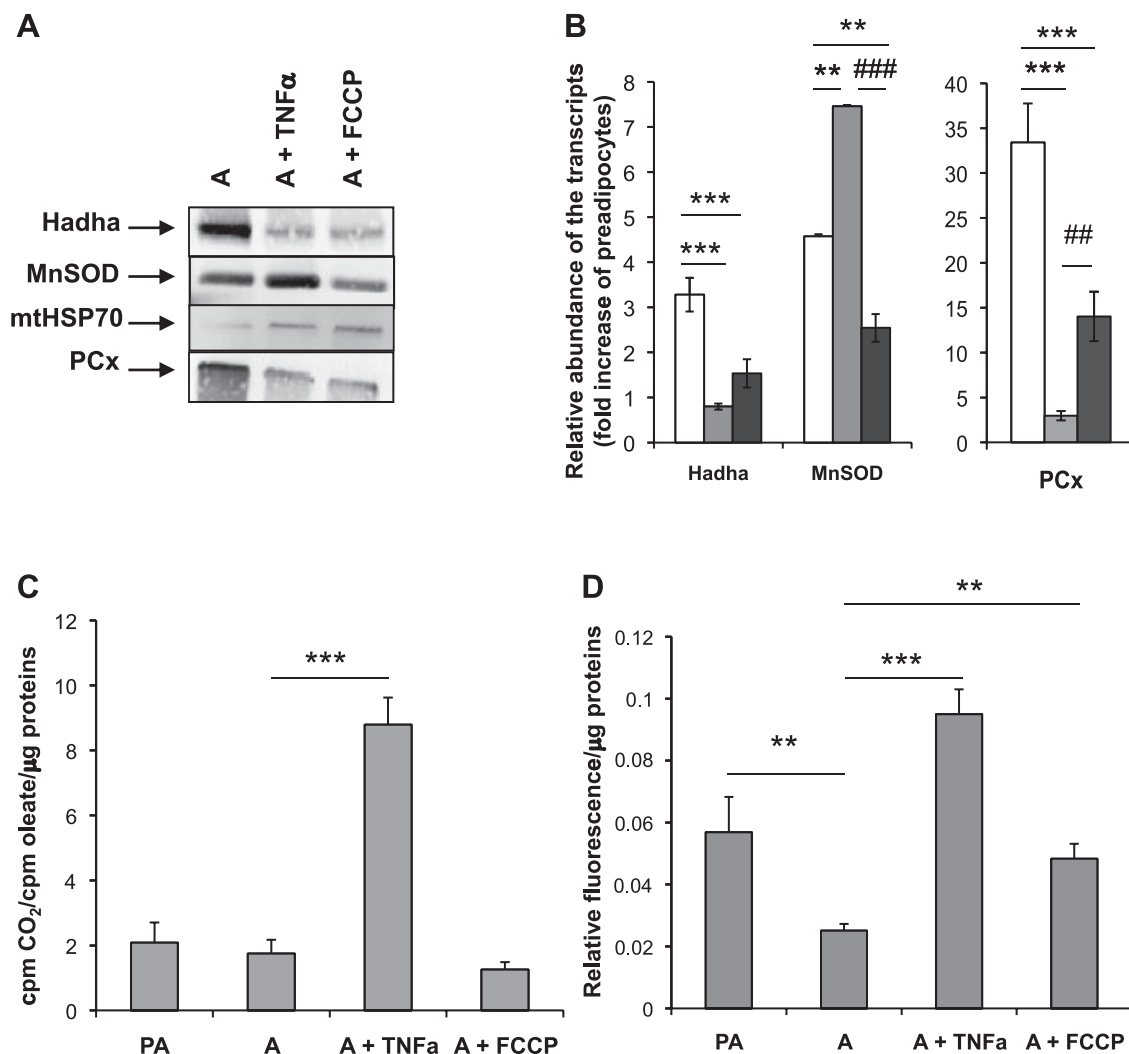


Fig. 11. Effect of TNF α and FCCP on the expression and abundance of several mitochondrial proteins as well as on fatty acid β -oxidation and O₂ $\cdot$ (-) production. **A:** validation of several mitochondrial proteins identified in the mitoproteomes by Western blot analysis for long-chain enoyl-CoA hydratase (Hadha), manganese superoxide dismutase (MnSOD), mtHSP70, and pyruvate carboxylase (PCx). Analysis was performed using the same purified mitochondrial fractions as the ones used for proteomic studies. Equal amounts of proteins have been loaded. **B:** expression of nuclear genes encoding Hadha, MnSOD, and PCx was analyzed by real-time RT-qPCR in adipocytes incubated with or without (open bars) 10 ng/ml TNF α (gray bars) or 0.5 μ M FCCP (black bars) for 6 days. For data normalization, TBP was used as reference gene [formula used: $2^{-(\Delta C_T \text{ transcript} - \Delta C_T \text{ TBP})}$]. Results are expressed in relative fold increase of the abundance determined in preadipocytes as means $\pm$ SD; $n = 3$ experiments. ** $P < 0.01$ and *** $P < 0.001$, significantly different from adipocytes; ### $P < 0.01$ and ### $P < 0.001$, significantly different between TNF α and FCCP treatment. **C:** effect of TNF α or FCCP on fatty acid β -oxidation. PA, A, and A + TNF α or A + FCCP were labeled with [¹⁴C]oleate. Results are expressed in counts/min (cpm) with [¹⁴C]oleate. Results are presented as means $\pm$ SD; $n = 3$. *** $P < 0.001$, significantly different from adipocytes. **D:** effect of TNF α and FCCP on mitochondrial O₂ $\cdot$ (-) production. Mitochondrial O₂ $\cdot$ (-) level in adipocytes treated or not (A) with 10 ng/ml TNF α (A + TNF α) or 0.5 μ M FCCP (A + FCCP) for 6 days was determined using the specific mitochondrial MitoSOX probe. Results were normalized for protein content (AFU/ μ g proteins) and expressed as means $\pm$ SD; $n = 3$. ** $P < 0.01$ and *** $P < 0.001$, significantly different from adipocytes.

differences in experimental settings (purified mitochondria vs. cell analysis), low concentrations in the uncoupler used, time of exposure, sensitivity of the assay to measure ROS, and measurement in the absence or the presence of uncoupler or secondary cell adjustments in terms of mitochondrial morphology alterations as an increase in mitochondrial fragmentation are known to trigger ROS production (84), and changes in the antioxidant expression and activity might probably explain the increased abundance of ROS found in FCCP-treated cells that does not result from an increase in ROS production per se. According to the model of Aon et al. (1), ROS accumulation would not result from an increase in ROS production in

mitochondria of FCCP-treated adipocytes but would be caused by a high increase in the oxidation state of mitochondrial ROS scavengers, impairing their ability to reduce ROS content (1). Moreover, in our study, we cannot exclude that the higher superoxide anion content found in FCCP-treated adipocytes would result from mitochondrial fragmentation observed in FCCP-treated cells (Fig. 2) and/or reduction of MnSOD/SOD2 abundance (Fig. 11A). Nevertheless, our results are consistent with the higher ROS content also found in adipocytes overexpressing UCP4 (20).

In this study, we found that several enzymes involved in FFA β -oxidation are less abundant in mitochondria of FCCP-

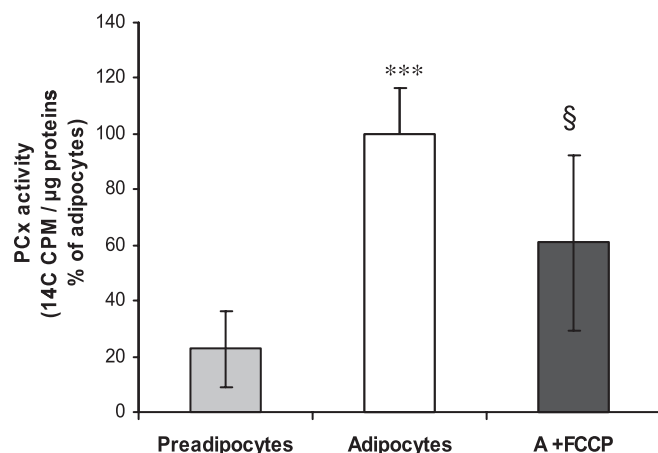


Fig. 12. Effect of FCCP on the PCx activity. Cultures of preadipocytes and differentiated 3T3-L1 adipocytes have been prepared. After differentiation, cells were incubated or not in the presence of 0.5 μ M FCCP for 6 days (A + FCCP), and PCx activity was determined. Results are measured as the radioactivity associated with 14 C incorporated into oxaloacetate normalized for protein content used in this assay (14 C counts·min $^{-1}$ · μ g proteins $^{-1}$). Results are expressed in %differentiated cells and represent the mean $\pm$ 1 SD ($n = 6$). ***Significantly different from preadipocytes as determined by an unpaired Student *t*-test with $P < 0.001$; §significantly different from adipocytes with $P < 0.05$.

treated cells than in mitochondria from control adipocytes as well as enzymes that participate in long-chain and very long-chain fatty acid transport into the mitochondrial matrix. However, FFA β -oxidation is unchanged in adipocytes exposed to mild mitochondrial uncoupling, data that are in agreement with results obtained for 3T3-L1 that ectopically expressed UCP1 (67). The discrepancy between increased lipolysis (73) and unchanged β -oxidation rate in FCCP-treated cells could be explained by the use of a low concentration of FCCP since Rossmeisl et al. (61) used DNP at 200 μ M to stimulate β -oxidation in 3T3-L1 adipocytes, a process accompanied by a

biogenesis of mitochondria. However, one cannot rule out the fact that the absence of mitochondrial β -oxidation stimulation in FCCP-treated cells might be due to the fact that the assay was performed in the absence of uncoupler and thus represents β -oxidation of FA in basal state.

Part of the mitochondrial response to TNF α or FCCP in 3T3-L1 adipocytes is the decreased abundance of PCx, an enzyme that serves an anaplerotic role for the Krebs cycle in mammalian cells when intermediates are removed for different biosynthetic purposes (35). The expression of PCx gene, under the control of PPAR γ , is markedly increased in 3T3-L1 preadipocyte differentiation as well as its protein level and its activity, whereas its expression is repressed by TNF α (32). Therefore, the lower PCx gene expression in both TNF α - and FCCP-treated adipocytes is consistent with the decrease in PPAR γ gene expression in adipocytes exposed to the cytokine or the mitochondrial uncoupler (73). In adipocytes, PCx is known to participate in de novo fatty acid synthesis and glyceroneogenesis (33). According to its prolipogenic role in adipocytes, this enzyme was suggested to contribute to the metabolic switch controlling fuel partitioning toward lipogenesis in fat cells (34). The lower mitochondrial abundance of PCx in TNF α - and FCCP-treated adipocytes displaying reduced lipogenesis (73) thus suggests a putative role of PCx in TG reduction. In fact, we showed that the overexpression of PCx in adipocytes prevents TG loss induced by TNF α or FCCP. These results support a role for the decrease in PCx activity, at least in part, in TG content decrease induced by either the cytokine or the mitochondrial uncoupler. However, because TG content in adipocytes at some point is a balance between TG synthesis and TG catabolism, we cannot completely exclude the possibility that TG loss in FCCP-treated adipocytes could also reflect an increase in lipid catabolism, as evidenced by stronger lipolysis in these conditions (73), leading to endogenous lipid as substrates that would be needed by the cells to maintain a higher rate of electron entry into the

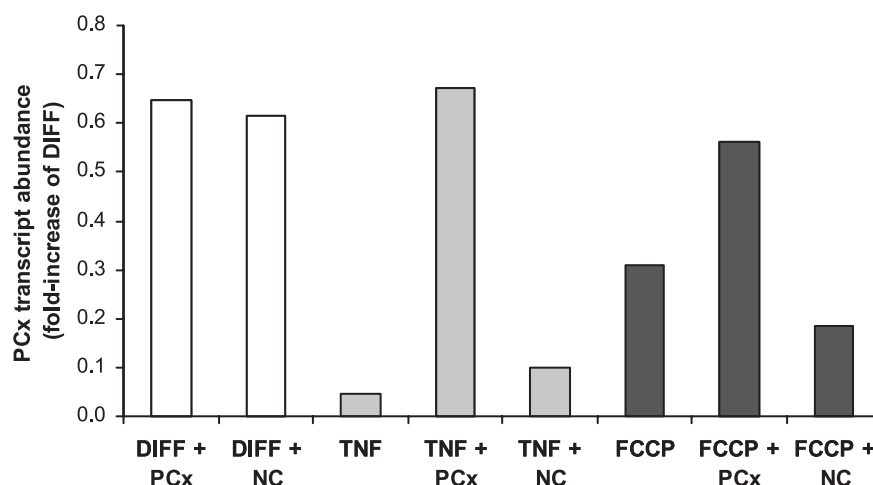


Fig. 13. Effect of the transfection of plasmids expressing PCx on the relative abundance of PCx transcripts in adipocytes treated with TNF α or FCCP. Adipocytes were transiently transfected (electroporation; Nucleofector AMAXA) or not with 1 μ g/1,000,000 cells of an expressing plasmid (pEF-PC) encoding PCx or a plasmid control (pEF) and then treated or not with 10 ng/ml TNF α or 0.5 μ M FCCP for 72 h. Then, total RNA was extracted from cells, reverse transcribed, and amplified in the presence of PCx primers and SYBR green. TBP was used as a reference gene for data normalization [formula used: $2^{-(\Delta C_T \text{ PCx} - \Delta C_T \text{ TBP})}$]. Results are expressed in relative fold increase of the abundance determined in adipocytes electroporated only with transfection solution. TNF α and FCCP represent TNF α - and FCCP-treated adipocytes electroporated only with transfection solution. DIFF/TNF α /FCCP + NC, adipocytes and adipocytes treated with either TNF α or FCCP and transfected with plasmid control (negative control). DIFF/TNF α /FCCP + PCx, adipocytes and adipocytes treated with either TNF α or FCCP that overexpress the transgene encoding PCx.

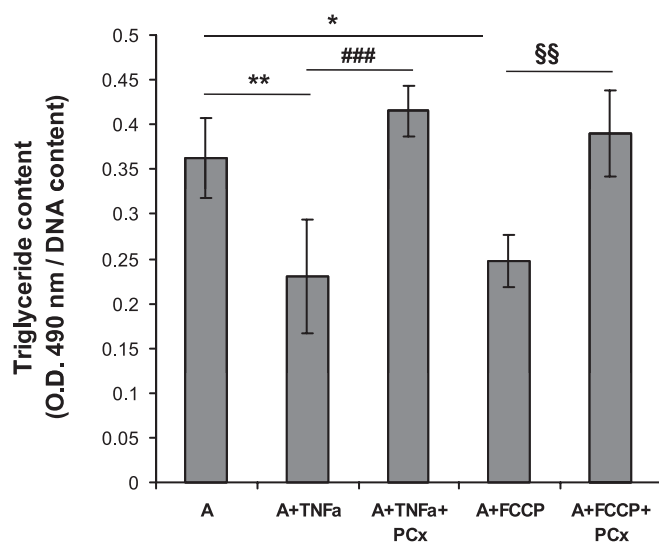


Fig. 14. Role of PCx in triglyceride content decrease in TNF α - and FCCP-treated adipocytes. Adipocytes were transiently transfected or not with 1 μ g/1,000,000 cells of pEF-PC-expressing PCx and then treated or not with 10 ng/ml TNF α or 0.5 μ M FCCP for 6 days. On day 6, triglyceride vesicles were stained with Oil Red O, and the relative triglyceride content was determined by measuring the absorbance of cell monolayers at 490 nm with a spectrophotometer. DNA was stained with propidium iodide, and the total DNA content was determined by measuring the fluorescence at 617 nm. Results were normalized for DNA content [optical density (OD) 490 nm/AFU 617 nm]. Values are means $\pm$ SD; $n = 3$. * $P < 0.05$ and ** $P < 0.01$, significantly different from adipocytes; ### $P < 0.001$ significantly different from A + TNF α ; §§ $P < 0.01$ significantly different from A + FCCP. A, A + TNF α , and A + FCCP: control adipocytes. TNF α - and FCCP-treated adipocytes electroporated only with transfection solution. A + TNF α + PCx, A + FCCP + PCx: TNF α - and FCCP-treated adipocytes overexpressing gene encoding PCx.

electron transport chain to drive mitochondrial membrane potential in response to mitochondrial uncoupling. Moreover, we also have to mention that AMP-activated protein kinase, an energetic sensor activated in adipocytes treated with FCCP, might also control the TG content in these conditions since compound C and STO-069 clearly increase the TG content of cells exposed to mitochondrial uncoupling (data not shown), an effect that could be mediated by the relief of the inhibitory effect of the kinase on the acetyl-CoA carboxylase, as shown for the action of dominant negative mutant of the AMP-activated protein kinase in the INS-1D β -cell line (55). These new findings fit perfectly well with the fact that the lower membrane potential will increase demand on lipid catabolic pathways by stimulating lipolysis (and reducing FA synthesis to save ATP), thereby reducing TG stores (73) in an attempt to supply reducing equivalents at a rate appropriate for restoring membrane potential. Repression of pyruvate carboxylase could thus be interpreted as a survival tactic of repressing anabolic pathways to support a redirection of limited carbon units to catabolism rather than storage (68).

A crucial question to address is to determine whether the effects observed for FCCP represent a true impact of mitochondrial uncoupling or a toxic cell response due to FCCP accumulation over long incubation periods. To address this question, we analyzed both PCx abundance and mitochondrial fragmentation in adipocytes incubated with 0.5 μ M FCCP for shorter incubations (6 and 24 h). Although we cannot completely rule out that some effects observed in the study might

be due to FCCP toxic effects rather than consequences of mitochondrial uncoupling, the fact that cell viability was maintained (>95%) in FCCP-treated adipocytes for 6 days combined with the fact that we had already found a downregulation of PCx after a 6-h treatment and an increase in mitochondrial fragmentation within a 24-h incubation time (data not shown) is compatible with a cell response to mitochondrial uncoupling.

The constant ATP level in FCCP-treated cells for 1 day followed by a slight increase in ATP content in cells treated for 3 days might appear inconsistent. However, this apparent discrepancy might be explained by the fact that a decrease in fatty acid synthesis in FCCP-treated cells reflects a partitioning of media glucose toward oxidation in response to a lower membrane potential. This observation supports the fact that adipocytes appear to minimize ATP utilization in response to mild and chronic mitochondrial uncoupling. Indeed, we and others have shown recently that fatty acid synthesis is decreased in FCCP-treated cells (61, 73). However, several enzymes that participate in amino acid biosynthesis, a major ATP-consuming anabolic pathway, are more abundant in mitochondria of TNF α - and FCCP-treated adipocytes than in mitochondria from control adipocytes. Although this appears to be paradoxical with an attempt to minimize energy-consuming processes, the increase in mitochondrial abundance of ornithine aminotransferase and aldehyde dehydrogenase family 18 member A1, both of which are involved in L-proline synthesis, is consistent with the increased proline production observed in adipocytes overexpressing UCP1 or treated with FCCP proposed by the stoichiometric network model of adipocyte intermediary metabolism of Si et al. (68).

In conclusion, our results clearly indicate that 3T3-L1 adipocytes exposed to a mild mitochondrial uncoupling do not respond to a stimulation of mitochondrial biogenesis. However, prolonged treatment with 0.5 μ M FCCP triggers modifications in mitochondrial protein content, affects mitochondrial ultrastructure, and leads to a reduction in TG content in adipocytes. Indeed, changes in the abundance of many mitochondrial proteins have been identified in the mitochondria of adipocytes incubated either with TNF α or exposed to FCCP. Although we clearly observed that some modifications are common to both treatments, 69% of the modifications of about 70–80 proteins identified as mitochondrial proteins differentially abundant in one of these conditions can be considered as a specific molecular signature at the mitochondrial level for adipocytes exposed to mitochondrial uncoupling or the proinflammatory cytokine. Part of the mitochondrial modifications is a decrease in PCx abundance, which appears to be closely related to TG content reduction in adipocytes incubated with TNF α or exposed to a long-term and mild mitochondrial uncoupling. These results highlight a new mechanism by which the effects of mitochondrial uncoupling might limit TG accumulation in adipocytes.

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Present address of A. De Pauw: Institute of Experimental and Clinical Research, Pole of Pharmacology and Therapeutics (FATH 5349), Univ. of Louvain Medical School, Ave. E. Mounier 52, 1200 Brussels, Belgium.

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DISCLOSURES

The authors express no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

A.D.P., P.R., and T.A. did the conception and design of the research; A.D.P., S.D., S.T., M.D., E.D., and A.K. performed the experiments; A.D.P., S.D., M.D., E.D., A.K., and T.A. analyzed the data; A.D.P., S.D., S.T., M.R., and T.A. interpreted the results of the experiments; A.D.P. and S.D. prepared the figures; A.D.P., S.D., S.T., P.R., M.R., and T.A. drafted the manuscript; S.D. and T.A. edited and revised the manuscript; T.A. approved the final version of the manuscript.

REFERENCES

1. Aon MA, Cortassa S, O'Rourke B. Redox-optimized ROS balance: a unifying hypothesis. *Biochim Biophys Acta* 1797: 865–877, 2010.
2. Appelmans F, Wattiaux R, De Duve C. Tissue fractionation studies. 5. The association of acid phosphatase with a special class of cytoplasmic granules in rat liver. *Biochem J* 59: 438–445, 1955.
3. Aronis A, Melendez JA, Golan O, Shilo S, Dieter N, Tirosh O. Potentiation of Fas-mediated apoptosis by attenuated production of mitochondria-derived reactive oxygen species. *Cell Death Differ* 10: 335–344, 2003.
4. Atkin BM, Buist NR, Utter MF, Leiter AB, Banker BQ. Pyruvate carboxylase deficiency and lactic acidosis in a retarded child without Leigh's disease. *Pediatr Res* 13: 109–116, 1979.
5. Azzu V, Mookerjee SA, Brand MD. Rapid turnover of mitochondrial uncoupling protein 3. *Biochem J* 426: 13–17, 2010.
6. Bae JH, Park JW, Kwon TK. Ruthenium red, inhibitor of mitochondrial Ca²⁺ uniporter, inhibits curcumin-induced apoptosis via the prevention of intracellular Ca²⁺ depletion and cytochrome c release. *Biochem Biophys Res Commun* 303: 1073–1079, 2003.
7. Bray GA, Tartaglia LA. Medicinal strategies in the treatment of obesity. *Nature* 404: 672–677, 2000.
8. Brennan JP, Southworth R, Medina RA, Davidson SM, Duchon MR, Shattock MJ. Mitochondrial uncoupling, with low concentration FCCP, induces ROS-dependent cardioprotection independent of KATP channel activation. *Cardiovasc Res* 72: 313–321, 2006.
9. Chen GD, Kermany MH, D'Elia A, Ralli M, Tanaka C, Bielefeld EC, Ding D, Henderson D, Salvi R. Too much of a good thing: long-term treatment with salicylate strengthens outer hair cell function but impairs auditory neural activity. *Hear Res* 265: 63–69, 2010.
10. Cho SY, Park PJ, Shin ES, Lee JH, Chang HK, Lee TR. Proteomic analysis of mitochondrial proteins of basal and lipolytically (isoproterenol and TNF- α)-stimulated adipocytes. *J Cell Biochem* 106: 257–266, 2009.
11. Choi YS, Lee HK, Pak YK. Characterization of the 5'-flanking region of the rat gene for mitochondrial transcription factor A (Tfam). *Biochim Biophys Acta* 1574: 200–204, 2002.
12. Corda S, Laplace C, Vicaut E, Duranteau J. Rapid reactive oxygen species production by mitochondria in endothelial cells exposed to tumor necrosis factor- α is mediated by ceramide. *Am J Respir Cell Mol Biol* 24: 762–768, 2001.
13. de Longueville F, Atienzar FA, Marcq L, Dufrane S, Evrard S, Wouters L, Leroux F, Bertholet V, Gerin B, Whomsley R, Arnould T, Remacle J, Canning M. Use of a low-density microarray for studying gene expression patterns induced by hepatotoxins on primary cultures of rat hepatocytes. *Toxicol Sci* 75: 378–392, 2003.
14. De Vos KJ, Sheetz MP. Visualization and quantification of mitochondrial dynamics in living animal cells. *Methods Cell Biol* 80: 627–682, 2007.
15. Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr Rev* 20: 649–688, 1999.
16. Di Paola M, Cocco T, Lorusso M. Ceramide interaction with the respiratory chain of heart mitochondria. *Biochemistry* 39: 6660–6668, 2000.
17. Fan M, Rhee J, St-Pierre J, Handschin C, Puigserver P, Lin J, Jaeger S, Erdjument-Bromage H, Tempst P, Spiegelman BM. Suppression of mitochondrial respiration through recruitment of p160 myb binding protein to PGC-1 α : modulation by p38 MAPK. *Genes Dev* 18: 278–289, 2004.
18. Ferreira ME, Hermann S, Prochasson P, Workman JL, Berndt KD, Wright AP. Mechanism of transcription factor recruitment by acidic activators. *J Biol Chem* 280: 21779–21784, 2005.
19. Gao CL, Zhu C, Zhao YP, Chen XH, Ji CB, Zhang CM, Zhu JG, Xia ZK, Tong ML, Guo XR. Mitochondrial dysfunction is induced by high levels of glucose and free fatty acids in 3T3-L1 adipocytes. *Mol Cell Endocrinol* 320: 25–33, 2010.
20. Gao CL, Zhu JG, Zhao YP, Chen XH, Ji CB, Zhang CM, Zhu C, Xia ZK, Peng YZ, Guo XR. Mitochondrial dysfunction is induced by the overexpression of UCP4 in 3T3-L1 adipocytes. *Int J Mol Med* 25: 71–80, 2010.
21. García-Ruiz C, Colell A, Mari M, Morales A, Fernández-Checa JC. Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species. Role of mitochondrial glutathione. *J Biol Chem* 272: 11369–11377, 1997.
22. Gauthier DJ, Lazure C. Complementary methods to assist subcellular fractionation in organellar proteomics. *Expert Rev Proteomics* 5: 603–617, 2008.
23. Goffart S, Wiesner RJ. Regulation and co-ordination of nuclear gene expression during mitochondrial biogenesis. *Exp Physiol* 88: 33–40, 2003.
24. Gopalakrishnan L, Scarpulla RC. Differential regulation of respiratory chain subunits by a CREB-dependent signal transduction pathway. Role of cyclic AMP in cytochrome c and COXIV gene expression. *J Biol Chem* 269: 105–113, 1994.
25. Graham BH, Waymire KG, Cottrell B, Trounce IA, MacGregor GR, Wallace DC. A mouse model for mitochondrial myopathy and cardiomyopathy resulting from a deficiency in the heart/muscle isoform of the adenine nucleotide translocator. *Nat Genet* 16: 226–234, 1997.
26. Gustin C, Delaive E, Dieu M, Calay D, Raes M. Upregulation of pentraxin-3 in human endothelial cells after lysophosphatidic acid exposure. *Arterioscler Thromb Vasc Biol* 28: 491–497, 2008.
27. Gyulhandanyan AV, Pennefather PS. Shift in the localization of sites of hydrogen peroxide production in brain mitochondria by mitochondrial stress. *J Neurochem* 90: 405–421, 2004.
28. Hamer I, Jadot M. Endolysosomal transport of newly-synthesized cathepsin D in a sucrose model of lysosomal storage. *Exp Cell Res* 309: 284–295, 2005.
29. Harper JA, Dickinson K, Brand MD. Mitochondrial uncoupling as a target for drug development for the treatment of obesity. *Obes Rev* 2: 255–265, 2001.
30. Hood DA. Mechanisms of exercise-induced mitochondrial biogenesis in skeletal muscle. *Appl Physiol Nutr Metab* 34: 465–472, 2009.
31. Hori A, Yoshida M, Shibata T, Ling F. Reactive oxygen species regulate DNA copy number in isolated yeast mitochondria by triggering recombination-mediated replication. *Nucleic Acids Res* 37: 749–761, 2009.
32. Jitrapakdee S, Slawik M, Medina-Gomez G, Campbell M, Wallace JC, Sethi JK, O'rahilly S, Vidal-Puig AJ. The peroxisome proliferator-activated receptor- γ regulates murine pyruvate carboxylase gene expression in vivo and in vitro. *J Biol Chem* 280: 27466–27476, 2005.
33. Jitrapakdee S, St Maurice M, Rayment I, Cleland WW, Wallace JC, Attwood PV. Structure, mechanism and regulation of pyruvate carboxylase. *Biochem J* 413: 369–387, 2008.

34. Jitrapakdee S, Vidal-Puig A, Wallace JC. Anaplerotic roles of pyruvate carboxylase in mammalian tissues. *Cell Mol Life Sci* 63: 843–854, 2006.
35. Jitrapakdee S, Wallace JC. Structure, function and regulation of pyruvate carboxylase. *Biochem J* 340: 1–16, 1999.
36. Kaser M, Langer T. Protein degradation in mitochondria. *Semin Cell Dev Biol* 11: 181–190, 2000.
37. Kim GJ, Fiskum GM, Morgan WF. A role for mitochondrial dysfunction in perpetuating radiation-induced genomic instability. *Cancer Res* 66: 10377–10383, 2006.
38. Kopecky J, Clarke G, Enerback S, Spiegelman B, Kozak LP. Expression of the mitochondrial uncoupling protein gene from the aP2 gene promoter prevents genetic obesity. *J Clin Invest* 96: 2914–2923, 1995.
39. Kopecky J, Hodny Z, Rossmeisl M, Syrový I, Kozak LP. Reduction of dietary obesity in aP2-Ucp transgenic mice: physiology and adipose tissue distribution. *Am J Physiol Endocrinol Metab* 270: E768–E775, 1996.
40. Kopecky J, Rossmeisl M, Flachs P, Bardova K, Brauner P. Mitochondrial uncoupling and lipid metabolism in adipocytes. *Biochem Soc Trans* 29: 791–797, 2001.
41. Kopecky J, Rossmeisl M, Hodny Z, Syrový I, Horakova M, Kolarova P. Reduction of dietary obesity in aP2-Ucp transgenic mice: mechanism and adipose tissue morphology. *Am J Physiol Endocrinol Metab* 270: E776–E786, 1996.
42. Korshunov SS, Skulachev VP, Starkov AA. High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett* 416: 15–18, 1997.
43. Krauss S, Zhang CY, Lowell BB. The mitochondrial uncoupling-protein homologues. *Nat Rev Mol Cell Biol* 6: 248–261, 2005.
44. Lamanda A, Zahn A, Röder D, Langen H. Improved Ruthenium II tris (bathophenanthroline disulfonate) staining and destaining protocol for a better signal-to-background ratio and improved baseline resolution. *Proteomics* 4: 599–608, 2004.
45. Legros F, Lombès A, Frachon P, Rojo M. Mitochondrial fusion in human cells is efficient, requires the inner membrane potential, and is mediated by mitofusins. *Mol Biol Cell* 13: 4343–4354, 2002.
46. Luo GF, Yu TY, Wen XH, Li Y, Yang GS. Alteration of mitochondrial oxidative capacity during porcine preadipocyte differentiation and in response to leptin. *Mol Cell Biochem* 307: 83–91, 2008.
47. MacLellan JD, Gerrits MF, Gowing A, Smith PJ, Wheeler MB, Harper ME. Physiological increases in uncoupling protein 3 augment fatty acid oxidation and decrease reactive oxygen species production without uncoupling respiration in muscle cells. *Diabetes* 54: 2343–2350, 2005.
48. Matsumoto T, Kano K, Kondo D, Fukuda N, Iribe Y, Tanaka N, Matsubara Y, Sakuma T, Satomi A, Otaki M, Ryu J, Mugishima H. Mature adipocyte-derived dedifferentiated fat cells exhibit multilineage potential. *J Cell Physiol* 215: 210–222, 2008.
49. Mercy L, Pauw A, Payen L, Tejerina S, Houbion A, Demazy C, Raes M, Renard P, Arnould T. Mitochondrial biogenesis in mtDNA-depleted cells involves a Ca²⁺-dependent pathway and a reduced mitochondrial protein import. *FEBS J* 272: 5031–5055, 2005.
50. Muñoz-Málaga A, Bautista J, Salazar JA, Aguilera I, Garcia R, Chinchon I, Segura MD, Campos Y, Arenas J. Lipomatosis, proximal myopathy, and the mitochondrial 8344 mutation. A lipid storage myopathy? *Muscle Nerve* 23: 538–542, 2000.
51. Muoio DM, Seefeld K, Witters LA, Coleman RA. AMP-activated kinase reciprocally regulates triacylglycerol synthesis and fatty acid oxidation in liver and muscle: evidence that sn-glycerol-3-phosphate acyltransferase is a novel target. *Biochem J* 338: 783–791, 1999.
52. Nawrocki AR, Scherer PE. Keynote review: the adipocyte as a drug discovery target. *Drug Discov Today* 10: 1219–1230, 2005.
53. Nie Y, Wong C. Suppressing the activity of ERR α in 3T3-L1 adipocytes reduces mitochondrial biogenesis but enhances glycolysis and basal glucose uptake. *J Cell Mol Med* 13: 3051–3060, 2008.
54. Nisoli E, Clementi E, Paolucci C, Cozzi V, Tonello C, Sciorati C, Bracale R, Valerio A, Francolini M, Moncada S, Carruba MO. Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. *Science* 299: 896–899, 2003.
55. Okazaki Y, Eto K, Yamashita T, Okamoto M, Ohsugi M, Noda M, Terauchi Y, Ueki K, Kadowaki T. Decreased insulin secretion and accumulation of triglyceride in beta cells overexpressing a dominant-negative form of AMP-activated protein kinase. *Endocr J* 57: 141–152, 2010.
56. Pendergrass W, Wolf N, Poot M. Efficacy of MitoTracker Green and CMXrosamine to measure changes in mitochondrial membrane potentials in living cells and tissues. *Cytometry A* 61: 162–169, 2004.
57. Peters TJ, Müller M, De Duve C. Lysosomes of the arterial wall. I. Isolation and subcellular fractionation of cells from normal rabbit aorta. *J Exp Med* 136: 1117–1139, 1972.
58. Reinders J, Zahedi RP, Pfanner N, Meisinger C, Sickmann A. Toward the complete yeast mitochondrial proteome: multidimensional separation techniques for mitochondrial proteomics. *J Proteome Res* 5: 1543–1554, 2006.
59. Rossmeisl M, Barbatelli G, Flachs P, Brauner P, Zingaretti MC, Marelli M, Janovska P, Horakova M, Syrový I, Cinti S, Kopecky J. Expression of the uncoupling protein 1 from the aP2 gene promoter stimulates mitochondrial biogenesis in unilocular adipocytes in vivo. *Eur J Biochem* 269: 19–28, 2002.
60. Rossmeisl M, Kovar J, Syrový I, Flachs P, Bobkova D, Kolar F, Poledne R, Kopecky J. Triglyceride-lowering effect of respiratory uncoupling in white adipose tissue. *Obes Res* 13: 835–844, 2005.
61. Rossmeisl M, Syrový I, Baumruk F, Flachs P, Janovská P, Kopecký J. Decreased fatty acid synthesis due to mitochondrial uncoupling in adipose tissue. *FASEB J* 14: 1793–1800, 2000.
62. Ryden M, Arvidsson E, Blomqvist L, Perbeck L, Dicker A, Arner P. Targets for TNF-alpha-induced lipolysis in human adipocytes. *Biochem Biophys Res Commun* 318: 168–175, 2004.
63. Scarpulla RC. Transcriptional paradigms in mammalian mitochondrial biogenesis and function. *Physiol Rev* 88: 611–638, 2008.
64. Schulze-Osthoff K, Bakker AC, Vanhaesebroeck B, Beyaert R, Jacob WA, Fiers W. Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. *J Biol Chem* 267: 5317–5323, 1992.
65. Senocak FS, Si Y, Moya C, Russell WK, Russell DH, Lee K, Jayaraman A. Effect of uncoupling protein-1 expression on 3T3-L1 adipocyte gene expression. *FEBS Lett* 581: 5865–5871, 2007.
66. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* 68: 850–858, 1996.
67. Si Y, Palani S, Jayaraman A, Lee K. Effects of forced uncoupling protein 1 expression in 3T3-L1 cells on mitochondrial function and lipid metabolism. *J Lipid Res* 48: 826–836, 2007.
68. Si Y, Shi H, Lee K. Metabolic flux analysis of mitochondrial uncoupling in 3T3-L1 adipocytes. *PLoS One* 4: e7000, 2009.
69. Spiegelman BM, Flier JS. Obesity and the regulation of energy balance. *Cell* 104: 531–543, 2001.
70. Starkov AA, Fiskum G. Regulation of brain mitochondrial H₂O₂ production by membrane potential and NAD(P)H redox state. *J Neurochem* 86: 1101–1107, 2003.
71. Stöckl P, Zankl C, Hütter E, Unterluggauer H, Laun P, Heeren G, Bogengruber E, Herndler-Brandstetter D, Breitenbach M, Jansen-Dürr P. Partial uncoupling of oxidative phosphorylation induces premature senescence in human fibroblasts and yeast mother cells. *Free Radic Biol Med* 43: 947–958, 2007.
72. Sun X, Zemel MB. Effects of mitochondrial uncoupling on adipocyte intracellular Ca²⁺ and lipid metabolism. *J Nutr Biochem* 14: 219–226, 2003.
73. Tejerina S, De Pauw A, Vankoningsloo S, Houbion A, Renard P, De Longueville F, Raes M, Arnould T. Mild mitochondrial uncoupling induces 3T3-L1 adipocyte de-differentiation by a PPARgamma-independent mechanism, whereas TNFalpha-induced de-differentiation is PPARgamma dependent. *J Cell Sci* 122: 145–155, 2009.
74. Tretter L, Adam-Vizi V. Moderate dependence of ROS formation on DeltaPsi_m in isolated brain mitochondria supported by NADH-linked substrates. *Neurochem Res* 32: 569–575, 2007.
75. Vankoningsloo S, Piens M, Lecocq C, Gilson A, De Pauw A, Renard P, Demazy C, Houbion A, Raes M, Arnould T. Mitochondrial dysfunction induces triglyceride accumulation in 3T3-L1 cells: role of fatty acid beta-oxidation and glucose. *J Lipid Res* 46: 1133–1149, 2005.
76. Voisine C, Craig EA, Zufall N, von Ahsen O, Pfanner N, Voos W. The protein import motor of mitochondria: unfolding and trapping of preproteins are distinct and separable functions of matrix Hsp70. *Cell* 97: 565–574, 1999.
77. Wagner K, Mick DU, Rehling P. Protein transport machineries for precursor translocation across the inner mitochondrial membrane. *Biochim Biophys Acta* 1793: 52–59, 2009.
78. Wallace DC. Mitochondrial diseases in man and mouse. *Science* 283: 1482–1488, 1999.

79. **Wei YH, Lee HC.** Mitochondrial DNA mutations and oxidative stress in mitochondrial diseases. *Adv Clin Chem* 37: 83–128, 2003.
80. **Wilson-Fritch L, Burkart A, Bell G, Mendelson K, Leszyk J, Nicoloro S, Czech M, Corvera S.** Mitochondrial biogenesis and remodeling during adipogenesis and in response to the insulin sensitizer rosiglitazone. *Mol Cell Biol* 23: 1085–1094, 2003.
81. **Wlodek D, Gonzales M.** Decreased energy levels can cause and sustain obesity. *J Theor Biol* 225: 33–44, 2003.
82. **Wu H, Kanatous SB, Thurmond FA, Gallardo T, Isotani E, Bassel-Duby R, Williams RS.** Regulation of mitochondrial biogenesis in skeletal muscle by CaMK. *Science* 296: 349–352, 2002.
83. **Yates JR 3rd, Gilchrist A, Howell KE, Bergeron JJ.** Proteomics of organelles and large cellular structures. *Nat Rev Mol Cell Biol* 6: 702–714, 2005.
84. **Yu T, Robotham JL, Yoon Y.** Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. *Proc Natl Acad Sci USA* 103: 2653–2658, 2006.
85. **Zaid A, Li R, Luciakova K, Barath P, Nery S, Nelson BD.** On the role of the general transcription factor Sp1 in the activation and repression of diverse mammalian oxidative phosphorylation genes. *J Bioenerg Biomembr* 31: 129–135, 1999.
86. **Zhang HH, Halbleib M, Ahmad F, Manganiello VC, Greenberg AS.** Tumor necrosis factor- α stimulates lipolysis in differentiated human adipocytes through activation of extracellular signal-related kinase and elevation of intracellular cAMP. *Diabetes* 51: 2929–2935, 2002.
87. **Zhang J, Xia WL, Ahmad F.** Regulation of pyruvate carboxylase in 3T3-L1 cells. *Biochem J* 306: 205–210, 1995.

