SUPPLEMENTAL MATERIAL & METHODS

Crucial role of fatty acid oxidation in asthmatic bronchial smooth muscle remodeling

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MATERIALS AND METHODS

Study population

A total of 21 patients with asthma and 31 non asthmatic subjects were prospectively recruited from the “Centre Hospitalier Universitaire (CHU)” of Bordeaux, France, according to the Global Initiative for Asthma [1] and the American Thoracic Society criteria [2]. Asthmatic patients were recruited from the “COBRA” cohort (COhort of BRonchial obstruction and Asthma, ethics committee number: 2008-A00294-51/1) in the Clinical Investigation Center of Bordeaux (CIC, Hôpital Haut-Lévêque, Pessac, France). Non asthmatic control subjects were recruited after surgical resection. This study received approval from the local and national ethics committees. Bronchial specimens from all subjects were obtained by either fiberoptic bronchoscopy or lobectomy in macroscopically normal areas, as previously described [3].

Cell culture

Bronchial smooth muscle (BSM) cells were obtained from patient biopsies and bronchi dissected out from lobectomy, as previously described [4]. Briefly, BSM cell culture was performed in DMEM 25 mM glucose (Gibco, Thermo Fisher Scientific, Waltham, MA), supplemented with 10% FBS (Gibco) and penicillin-streptomycin-amphotericin B 1X (100X; Gibco) and essential amino acids 1X (100X; Sigma-Aldrich, Saint-Louis, MO). The smooth muscle phenotype was confirmed by immunocytochemistry using the double staining of smooth muscle alpha-actin and calponin. Etomoxir and perhexilin were both purchased from Sigma-Aldrich and used at 10 nM each. Blocking anti-LDL-R antibody was purchased from Sigma and used at 5 µg/mL. Salmeterol, formoterol, fluticasone and budesonide were purchased from Sigma-Aldrich and used at concentrations of 10^{-7}M, 10^{-8}M or 10^{-9}M.

Bronchial epithelial cell culture was established from bronchial brushings patients' biopsies and bronchi dissected out from lobectomy, as previously described in Trian et al. [5], using PneumaCult medium (Stemcell, Vancouver, Canada).

Lentiviral infection for genetic inhibition of CPT2 expression

BSM cells were transduced with shRNA targeted against CPT2 or negative control (scrambled shRNA) at an MOI of 20. Lenti shRNA were purchased from Origene. Lentiblast (OZ Biosciences, San Diego, CA) was used as a transduction reagent following the manufacturer's recommendations. Knock-down was effective after 5 days of treatment as assessed by western blot.

Cellular oxygen consumption rate

Cellular oxygen consumption rate (OCR) was measured in intact cells at 37°C in a 2 mL thermostatically monitored chamber (1.0 x 10^6 cells/ml /run) using an Oroboros O2k instrument (Oroboros Instruments, Innsbruck, Austria). High-resolution respirometry was determined under routine conditions (in DMEM), in the presence of 6 μM oligomycin (leak respiration independent of ADP phosphorylation), or in the presence of 8 μM carbonyl-cyanide m-chlorophenylhydrazone CCCP (maximal respiration obtained in the uncoupled state). The 'reserve capacity' corresponds to the difference between the maximal respiration (CCCP-stimulated) and the basal respiration obtained in the cell culture medium. Finally, the cellular non-mitochondrial respiration was obtained after inhibition of the respiratory chain using potassium cyanide (KCN).

Cellular and mitochondrial ATP synthesis

Steady-state ATP content was measured by bioluminescence using the CellTiter Glo kit (Promega, Madison, WI), following the manufacturer's recommendations. The OXPHOS inhibitory cocktail contained 30 µM oligomycin, 5 µM rotenone and 1 mM potassium cyanide. ATP content was normalized to crystal violet staining representing cell density obtained at 570 nm.

Oxidative stress

Oxyblots were performed on total cellular lysates using an OxyBlot protein detection kit (Merck Millipore, Burlington, MA) and 2,4-dinitrophenylhydrazine (2,4-DNPH) antibody.

Cell culture substrates concentration measurement

Free fatty acid resting concentration in the cell culture medium was analysed after 48 hours following the manufacturer's recommendations from Abnova (Taipei, Taïwan). Both glucose and lactate concentrations were measured in the cell culture medium using a YSI Biochemistry Analyzer.

Metabolites analysis by IC-MS/MS

BSM cells were incubated in DMEM without glucose (Life Invitrogen, Thermo Fisher Scientific) supplemented with 25 mM of [U-^{13}C]-glucose (Sigma-Aldrich) for 48 hours before fast filtration preparation of the samples for mass spectrometry analysis. Extraction and quenching of metabolites were performed using a specific protocol from Metatoul (Metatoul, Toulouse, France) as previously described by Heuillet M. et al. Metabolites were analysed by ionic-exchange

chromatography coupled with tandem mass spectrometry (IC-MS/MS) using a previously described method [6].

Fatty acids endocytosis

BSM cells were seeded at 10 000 cells in 8 well chamber slides. BSM cells were rapidly starved of FBS and incubated for 30 min in DMEM medium without FBS. Fluorescent BODIPY-ceramide was purchased from Life Invitrogen (Thermo Fisher Scientific) and used at 100 ug/mL following the manufacturer's recommendations. DAPI was used for nuclear staining.

Metabolic capability analysis – Biolog phenotype MicroArrays

About 20 000 asthmatic and control BSM cells were seeded into 96-well MitoPlate^{TM} S-1 and MitoPlate^{TM} I-1 BIOLOG plates. The metabolic capability of cells was assessed via redox reactions associated with cellular respiration following the manufacturer's instructions (Biolog, Hayward, CA).

Cell proliferation assays

For cell quantification, cells were trypsinized and counted at different time points. A BrdU cell proliferation colorimetric kit (Abcam, Cambridge, UK) was used following the manufacturer's instructions. BSM cells positive for Ki67 staining were analysed by flow cytometry.

Mitochondrial network immunostaining

BSM cells were seeded into Nunc Lab-Tek chamber slides at 5000 cells per chamber. TOMM20 was used for mitochondrial network staining and visualized using fluorescence microscopy using

a Zeiss objective (ZEISS, Oberkochen, Germany) and a high-resolution colour camera (CIS Corporation, Japan). Images were reconstituted using ImageJ software. The area of TOMM20 staining, representing the mitochondrial network, was quantified using ImageJ software.

Apoptosis assessment

Basal or induced apoptosis was analysed using tert-butyl-hydroxide at 100 µM for half an hour using a luminescent assay measuring caspase 3 and 7 activities (Promega) in control and asthmatic BSM cells.

Western blot

Total cell and biopsy lysis was performed using a RIPA lysis buffer (Sigma-Aldrich). Total cellular extracts were loaded onto a 4-20% SDS-PAGE gel (Bio-Rad, Hercules, CA) and transferred onto a nitrocellulose membrane. Different commercial antibodies were used directed against Porin (Abcam), Citrate Synthase (Cell Signaling, Leiden, Netherlands), TOMM20 (Santa Cruz Biotechnologies, Dallas, TX), CPT1/CPT2 (Abcam and Life Invitrogen) and LDL-R (Abcam). HRP-coupled secondary antibodies were used for revelation using a ChemiDoc imaging instrument (Bio-Rad). Protein expression was normalized using total loading protein intensity (Stain-Free system Bio-Rad).

Label-free quantitative proteomics

Cells and tissue lysates were processed using RIPA buffer. Each lysate was centrifuged and the supernatant was used for the proteomic analysis at the Mass Spectrometry facility of Bordeaux

University, as recently described [7]. Briefly, proteomic analysis was performed using an Ultimate 3000 RSLC Nano-UPHLC system (Thermo Fisher Scientific, Waltham, MA) coupled to a nanospray Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA).

These analyses were performed by the proteomics core facility at the University of Bordeaux (https://proteome.cgfb.u-bordeaux.fr/en). The steps of sample preparation and protein digestion were performed as previously described [8]. NanoLC-MS/MS analysis was performed using an Ultimate 3000 RSLC Nano-UPHLC system (Thermo Fisher Scientific) coupled to a nanospray Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific). Each peptide extract was loaded on a 300 µm ID x 5 mm PepMap C18 precolumn (Thermo Fisher Scientific) at a flow rate of 10 µL/min. After a 3 min desalting step, peptides were separated on a 50 cm EasySpray column (75 µm ID, 2 µm C18 beads, 100 Å pore size, ES803, Thermo Fischer Scientific) with a 4-40% linear gradient of solvent B (0.1% formic acid in 80% ACN) in 48 min. The separation flow rate was set at 300 nL/min. The mass spectrometer was operated in positive ion mode at a 2.0 kV needle voltage. Data were acquired using Xcalibur 4.1 software in a data-dependent mode. MS scans (m/z 375-1500) were recorded at a resolution of R = 120000 (@ m/z 200) and an AGC target of 4×105 ions collected within 50 ms, followed by a top speed duty cycle of up to 3 seconds for MS/MS acquisition. Precursor ions (2 to 7 charge states) were isolated in the quadrupole with a mass window of 1.6 Th and fragmented with HCD@30% normalized collision energy. MS/MS data were acquired in the ion trap with rapid scan mode, AGC target of 3x103 ions and a maximum injection time of 300 ms. Selected precursors were excluded for 60 seconds. For protein identification, Sequest HT and Mascot 2.4 algorithms were used through

Proteome Discoverer 1.4 Software (Thermo Fisher Scientific) for protein identification in batch mode by searching against a UniProt Homo sapiens database (71 536 entries, release March 2018). Two missed enzyme cleavages were allowed. Mass tolerances in MS and MS/MS were set to 10 ppm and 0.6 Da. Oxidation of methionine, deamidation of asparagine and glutamine, acetylation of lysine and N-terminal acetylation of the protein were searched as dynamic modifications. Carbamidomethylation on cysteine was searched as a static modification. Peptide validation was performed using the Percolator algorithm [9] and only "high confidence" peptides were retained corresponding to a 1% false discovery rate (FDR) at the peptide level. Raw LC-MS/MS data were imported into Progenesis QI (version 2.0 ; Nonlinear Dynamics, a Waters Company) for feature detection, alignment, and quantification. All sample features were aligned according to retention times by manually inserting up to fifty landmarks followed by automatic alignment to maximally overlay all the two-dimensional (m/z and retention time) feature maps. Singly charged ions and ions with charge states higher than six were excluded from the analysis. All remaining features were used to calculate a normalization factor for each sample to correct for experimental variation. Peptide identifications (with FDR < 1%) were imported into Progenesis. Univariate one-way analysis of variance (ANOVA) was performed within Progenesis LC–MS to calculate the protein p-value according to the sum of the normalized abundances across all runs. Only proteins with a p-value cut-off < 0.05 were validated. A minimum of two unique peptides matched to a protein, and a $\geq$1.2-fold change in relative abundance between the two conditions ($n = 5$ in each group) were used as the criteria for identification as a differentially expressed protein. Noticeably, only non-conflicting features and unique peptides were considered for calculation at the protein level. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [10] partner repository with the dataset identifier PXD015566. Proteins were

clustered according to their functions by using the Kyoto Encyclopedia of Genes and Genome Analysis in the search tool for retrieval of interactions between genes and proteins (STRING) database. A more global analysis of the data was performed using Ingenuity Pathway Analysis (IPA; Qiagen). We used the 'Core Analysis' package to identify relationships, mechanisms, functions, and pathways relevant to a dataset. We also used the 'regulators' package to identify predicted regulators of the proteomic changes. Comparative analyses were also performed with IPA using the 'Comparative Analysis' module.

Matrix-Assisted Laser Desorption/Ionization (MALDI) - mass spectrometry imaging analysis

Patient biopsies were frozen at -80°C and then embedded in a gel of 5% carboxymethyl cellulose. Serial cryosections (12 µm-thick) were cut from bronchial biopsies at -20°C using a NX70 Star cryostat (Thermo Fisher Scientific) and thaw-mounted onto standard glass microscope slides for MALDI - mass spectrometry imaging. These acquisitions were performed using a high performance atmospheric pressure imaging ion source named AP-SMALDI 5 AF (TransMIT GmbH) connected to an orbital trapping mass spectrometer (QExactive Orbitrap, Thermo Fisher Scientific). After MALDI - mass spectrometry imaging, we performed an α-smooth muscle actin immunostaining to localize the BSM area. We then estimated the relative lipid content within the BSM on the MALDI images. Fatty acid annotation was performed using the Human Metabolome Data Base and METASPACE software.

MALDI Acquisition

For the MALDI-MSI (matrix-assisted laser desorption/ionization) analysis of fatty acids, a homogenous matrix layer of 1,5-diaminonaphthalene (DAN, Sigma-Aldrich) was deposited using a home-built pneumatic sprayer [11]. Briefly, 150 µL of a 5 mg/ml of DAN matrix diluted in 70% acetone (30% mQ water) was applied to the tissues under the following optimized conditions: a 0.05 mL/min matrix solution flow rate and 0.9 bar of nitrogen gas flow rate. The matrix solution was supplemented with 1-palmitoyl-d31-2-oleoyl-sn-glycero-3-phosphate (16:0-d31-18:1 PA, Avanti Polar Lipid) at 0.2 mg/ml used as internal mass calibration during MS acquisition (the selected peak with the strongest signal was 16:0-d30-18:1 PA, [M - H]- m/z 703.6697 ± 0.003) and with palmitic acid-d31 (Sigma-Aldrich) at 0.2 mg/ml used as internal standards for fatty acid normalization ([M - H]- signal (m/z 286.4275 ± 0.003)). MALDI-MSI acquisitions were performed using a high performance atmospheric pressure imaging ion source named AP-SMALDI5 AF (TransMIT GmbH) connected to an orbital trapping mass spectrometer (QExactive Orbitrap, Thermo Fisher Scientific) [12]. This latter was operated in negative ion mode at a mass resolution of 70 000 at *m/z* 400 over a mass range of *m/z* 190-2000. The ion source was equipped with a diode laser (Flare NX343, $\lambda = 343$ nm), operating at a repetition rate of 2 kHz. Imaging data were acquired in high speed continuous mode with a pixel size of 18 µm and a speed rate of 3.7 pixels/s. Fatty acid annotation was performed using the Human Metabolome Data Base (HMBD) and METASPACE software [13] based on peak exact mass and isotope profiles. MALDI images were generated using MSiReader software [14]. Normalized ion images of fatty acids were generated by dividing the given fatty acid signal by the palmitic acid-d31 signal (m/z 286.4275 ± 0.003).

Co-registration of MALDI-MS images and histology

Following the acquisition of the MALDI images, the matrix was washed from the tissue surface by immersing the tissue for 10 seconds into a bath of 50% of acetone. Tissue sections were immersed in acetone before rapid permeabilization in PBS 1X- Triton 0.01% for 5 min. After rinsing in PBS 1X-Tween 0.1%, slides were incubated with blocking solution for 20 min with PBS 1X- 0.5% BSA. Tissue sections were incubated for 45 min at room temperature with α-Smooth Muscle Actin-FITC (Thermo Fisher Scientific). Tissue sections were then washed with PBS 1X-Tween 0.1% and incubated with nuclear staining DAPI for 5 min at room temperature. Tissue sections were mounted using an aqueous mounting medium (Sigma-Aldrich). Slides were scanned by using a digital slide scanner (Nanozzomer, Hamamatsu, Shizuoka, Japan) (Supplemental figure 1). Images were read by using NDP view software (Hamamatsu). Immunohistochemistry (IHC) and MALDI images were aligned as previously described [15, 16]. Briefly, for alignment, MALDI images of ion maps of m/z 885.549 (an endogenous phospholipid) and m/z 593.8317 (a matrix-related ion) were used to reveal the contours of the tissue border (Supplemental figure 9). The MALDI image was rescaled and aligned with the tissue bright field image using the contour and tissue defaults as guides. Then, the IHC image can be cropped at the size of the MALDI image to be loaded into MSiReader software. In this way, the confocal image is aligned with the whole MSI dataset allowing us to define ROIs based on the BSM area and epithelial area. To have the same robustness for all samples despite different BSM areas, we defined a measuring box with a size of 3x3 pixels (Supplemental figure 10) and performed in each tissue 18 measurements of fatty acid abundances in the different areas presenting BSM or epithelial cells in each tissue.

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Supplemental table S1: Representation of protein expression among "mitochondrial dysfunction" annotation box

Protein	Uniprot accession number	Log2 (Fold Change)	-Log10 (p value)
CPT2	**P23786**	**1.615**	**1.789**
NDUFB11	Q9NX14	1.187	1.571
CASP8	E7EQ06	1.161	1.748
SLC27A3	H7BZH4	1.118	0.232
TXNRD2	Q9NNW7	0.891	1.054
MT-ND1	P03886	0.846	1.819
NDUFS8	E9PKH6	0.758	1.865
NDUFA10	A0A087WXC5	0.733	2.798
ACAA1	P09110	0.684	1.972
FIS1	Q9Y3D6	0.621	2.638
SDHC	Q99643	0.516	0.143
CYB5A	P00167	0.509	1.228
NDUFAF2	Q8N183	0.472	1.590
NDUFB9	Q9Y6M9	0.467	1.370
GPX4	A0A0A0MTT1	0.462	0.635
MAP2K4	P45985	0.430	0.745
NDUFA7	O95182	0.404	0.592
ACSL1	P33121	0.391	0.895
NDUFS6	O75380	0.382	0.528

GPX7	Q96SL4	0.377	0.671
NDUFA9	Q16795	0.353	0.960
NDUFS5	O43920	0.333	0.849
IVD	P26440	0.318	1.131
NDUFV1	G3V0I5	0.307	0.767
ECHS1	P30084	0.300	1.144
NDUFA6	P56556	0.283	1.057
NDUFA5	Q16718	0.280	0.441
NDUFB10	O96000	0.273	2.184
NDUFS2	O75306	0.253	0.908
ECI1	P42126	0.253	0.489
HSD17B10	Q99714	0.250	1.405
HSD17B10	Q99714	0.250	1.405
ACAA2	P42765	0.250	0.893
NDUFS1	P28331	0.242	0.738
GSR	P00390	0.236	0.513
NDUFA8	P51970	0.235	1.687
NDUFA12	Q9UI09	0.234	0.380
APP	H7C0V9	0.231	0.273
ACO2	A2A274	0.220	0.917
HADH	Q16836	0.213	0.557
OGDH	Q02218	0.207	0.726
PRDX5	P30044	0.195	0.509

NDUFA11	Q86Y39	0.179	0.056
ATP5PD	O75947	0.172	0.639
NDUFS3	O75489	0.167	0.909
NDUFA13	Q9P0J0	0.161	0.458
MT-ND2	P03891	0.154	0.356
NDUFV2	P19404	0.153	0.479
CYB5R3	P00387	0.143	0.920
NDUFB6	O95139	0.138	1.078
PARK7	Q99497	0.135	0.832
PRDX3	P30048	0.124	0.635
NDUFB7	P17568	0.107	0.315
UQCRC1	P31930	0.087	0.371
CYC1	P08574	0.085	0.363
CPT1A	P50416	0.084	0.332
NDUFB4	O95168	0.083	0.117
UQCRFS1	P47985	0.081	0.242
CYCS	P99999	0.079	0.566
ECI2	A0A0C4DGA2	0.077	0.086
RHOT2	Q8IXI1	0.073	0.152
ATP5F1A	P25705	0.070	0.340
ATP5F1B	P06576	0.065	0.393
COX5B	P10606	0.061	0.063
ATP5PO	P48047	0.057	0.295

ATP5F1C	P36542	0.055	0.361
UQCRC2	P22695	0.055	0.220
NDUFB5	E7EWP0	0.045	0.198
MAOA	P21397	0.036	0.088
MAPK10	A0A286YF97	0.036	0.048
ATP5PB	Q5QNZ2	0.034	0.217
COX6B1	P14854	0.029	0.076
PDHA1	P08559	0.019	0.079
HSD17B4	P51659	0.007	0.001
HADHA	P40939	0.003	0.019
ACO1	P21399	0.001	0.012
NCSTN	Q92542	0.000	0.018
MT-CO2	P00403	-0.006	0.016
SDHB	P21912	-0.013	0.024
ATP5F1D	P30049	-0.014	0.043
HADHB	P55084	-0.014	0.009
COX4I1	P13073	-0.029	0.145
HTRA2	A0A0C4DG44	-0.045	0.078
ATP5MF	P56134	-0.058	0.285
MT-ATP6	P00846	-0.059	0.153
GPD2	P43304	-0.076	0.203
NDUFB3	O43676	-0.076	0.141
MAPK8	A6NF29	-0.092	0.243

MT-CO3	P00414	-0.094	0.027
SOD2	P04179	-0.110	0.100
ACSL3	O95573	-0.118	0.605
COX7C	P15954	-0.145	0.419
SCP2	P22307	-0.153	0.525
SDHA	P31040	-0.158	0.369
AIFM1	O95831	-0.166	0.394
ACADM	B7Z9I1	-0.179	0.581
CAT	P04040	-0.200	0.483
UQCRB	P14927	-0.213	0.600
CASP3	P42574	-0.225	1.435
VDAC3	Q9Y277	-0.240	0.524
VDAC1	P21796	-0.256	0.662
UQCRQ	O14949	-0.271	1.444
VDAC2	A0A0A0MR02	-0.315	0.713
ATP5MG	E9PN17	-0.335	1.412
COX5A	H3BRM5	-0.347	1.788
NDUFA4	O00483	-0.371	0.907
SLC27A4	Q6P1M0	-0.390	1.505
COX7A2L	E5RJZ1	-0.487	0.277
COX6C	P09669	-0.490	1.361
ATP5ME	P56385	-0.520	3.013
ACSL4	O60488	-0.882	0.427