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Visualizing inhibition of fatty acid synthase through mass spectrometric analysis of mitochondria from melanoma cells

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Fatty acid synthase (FASN) is the metabolic enzyme responsible for the endogenous synthesis of the saturated long-chain fatty acid palmitate. In contrast to most normal cells, FASN is overexpressed in a variety of human cancers including cutaneous melanoma, in which its levels of expression are associated with a poor prognosis and depth of invasion. Recently, we have demonstrated the mitochondrial involvement in FASN inhibition-induced apoptosis in melanoma cells. Herein we compare, via electrospray ionization mass spectrometry (ESI-MS), free fatty acids (FFA) composition of mitochondria isolated from control (EtOH-treated cells) and Orlistat-treated B16-F10 mouse melanoma cells. Principal component analysis (PCA) was applied to the ESI-MS data and found to separate the two groups of samples. Mitochondria from control cells showed predominance of six ions, that is, those of *m/z* 157 (Pelargonic, 9:0), 255 (Palmitic, 16:0), 281 (Oleic, 18:1), 311 (Arachidic, 20:0), 327 (Docosahexaenoic, 22:6) and 339 (Behenic, 22:0). In contrast, FASN inhibition with Orlistat changes significantly mitochondrial FFA composition by reducing synthesis of palmitic acid, and its elongation and unsaturation products, such as arachidic and behenic acids, and oleic acid, respectively. ESI-MS of mitochondria isolated from Orlistat-treated cells presented therefore three major ions of *m/z* 157 (Pelargonic, 9:0), 193 (unknown) and 199 (Lauric, 12:0). These findings demonstrate therefore that FASN inhibition by Orlistat induces significant changes in the FFA composition of mitochondria.

Fatty acid synthase (FASN, EC2.3.1.85) is the metabolic enzyme responsible for endogenous fatty acid synthesis from the small carbon precursors acetyl-CoA and malonyl-CoA.[1,2] FASN is downregulated in most normal cells, except in lipogenic tissues as liver, lactating breast, fetal lung, and adipose tissue.[3,4] The overexpression of FASN in several human malignancies, such as those of prostate, breast, ovary, melanoma, and soft tissue sarcomas,[5–18] has been therefore associated with poor prognosis. FASN inhibition reduces cell proliferation by blocking DNA replication during S-phase, promotes apoptosis,[19–22] and decreases the size of prostate, ovarian and breast cancer xenografts.[6,7,23] Previously, we have shown that the specific inhibition of FASN activity by the antiobesity drug Orlistat significantly impairs lipid synthesis, reduces proliferation, and promotes apoptosis in the mouse metastatic melanoma cell line B16-F10.[24] Moreover, the treatment of experimental melanomas produced by the inoculation of B16-F10 cells with Orlistat reduces by half their metastatic spread.[24] Recently, we have demonstrated the mitochondrial involvement in FASN inhibition-induced apoptosis in melanoma cells.[25] FASN overexpression in neoplastic tissues, in which its main role seems to provide substrate for cell membrane phospholipids synthesis,[26] suggests that this pathway is a potential target for new anticancer drugs.

Electrospray ionization mass spectrometry (ESI-MS) has been proven a powerful technique to obtain lipid profiles in biological extracts.[27,28] ESI is a soft ionization technique in which ionic species or molecules ionized in solution are gently transferred to mass spectrometers mostly in their intact forms (no or little fragmentation) for mass measurements and characterization.[29] Because of these features, the composition of electrosprayed ions provides proper snapshots of the ionic composition in solution. We have used ESI-MS with success as a fast fingerprint method for lipid mixtures and biological matrices such as vegetable oils,[27] biodiesel,[30] and media for production of bovine embryos,[31] and plant extracts.[32] Principal component analysis (PCA) is one of the oldest and most widely used multivariate

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techniques[33] for chemometric studies and has been adequately applied to metabolomic studies[34] and the grouping of samples in MS fingerprinting analysis of biological matrices.[35]

Herein, we present a comparative study of free fatty acids (FFA) composition of mitochondria isolated from control (EtOH-treated cells) and Orlistat-treated B16-F10 mouse melanoma cells. PCA was applied to the ESI-MS data to investigate sample grouping. The results indicate that treatment of B16-F10 tumor cells with Orlistat, a pharmacological FASN inhibitor, induces substantial changes in mitochondrial FFA, reflected mostly via decrease of palmitic, arachidic, behenic, and oleic acids, and the predominance of pelargonic and lauric acids. These findings indicate that FASN inhibition by Orlistat induces changes in mitochondrial FFA composition, and direct infusion ESI-MS fingerprint provides a fast and efficient method to characterize the FFA composition of mitochondria.

EXPERIMENTAL

Chemical reagents and samples

For the ESI-MS experiments, all reagents used were of analytical grade. B16-F10 murine melanoma cells were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and maintained in RPMI (Vitrocell, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (FBS, Vitrocell), 100 μg/mL gentamycin (Vitrocell), 100 IU/mL penicillin (Vitrocell), and 100 mg/mL streptomycin (Vitrocell) at 37°C in a humidified atmosphere with 5% CO_2. To block FASN activity, 300 μM of Orlistat (Roche, Switzerland) was added to the culture medium supplemented with 1% FBS. Orlistat was extracted from Xenical® capsules according to Knowles *et al.*[36] Each pill was solubilized in 1 mL of ethanol (EtOH), insoluble products removed by centrifugation (12 000 *g* for 5 min) and the supernatant (250 mM of Orlistat) stored at –80°C. After 20 h of treatment with EtOH (controls) or Orlistat, 2×10^6 cells/mL were washed with PBS and mitochondria isolated as described by Abou-Khalil *et al.*,[37] with a few modifications. Briefly, cells were homogenized in a buffer containing 250 mM sucrose, 2 mM EDTA, and 0.1 mg/mL BSA, pH 7.4, on ice, using glass Dounce (# 19). Homogenates were centrifuged at 14 000 *g* for 20 min at 4°C, the pellets containing mitochondria were re-suspended in 100 μL ultrapure H_2O and lipids were extracted as described by Bligh and Dyer.[38] A total of 12 mitochondria samples were analyzed, being 6 from control, and 6 from treated cells.

General experimental procedures

ESI-MS data were collected in the negative ion mode on a API5000 mass spectrometer (Applied Biosystems/MDS Analytical Technologies, Ontario, Canada). Typical ESI-MS conditions were as follow: curtain gas, ion source gas and ion transfer voltage were 10 V, 15 and 4 kV, respectively, with a desolvation temperature of 200°C. To each sample (0.1 mL), 1 mL of a solution of methanol/toluene (7:3 v/v) and 0.1 mL of a methanol solution of ammonia (0.1% v/v) were added. This diluted solution was then directly infused into the ESI source at a flow rate of 10 μL min^{-1} via a micro-syringe pump. The mass analyzer was set to scan along the *m/z* range of 50–1000. ESI-MS/MS experiments were performed by selecting a specific ion in Q1 and then submitting it to collision-induced dissociation (CID) with argon in the collision chamber. Collision energy was optimized for each precursor ion, varying from 15 to 50 V. All ESI-MS data of the lipid extract samples were extracted using Analyst 1.4.1 software (Applied Biosystems/MDS Analytical Technologies, Ontario, Canada). Mass spectra data were accumulated over 60 s.

Statistical data treatment

The ESI-MS data were extracted using MassLynx 3.5 software (Waters, Manchester, UK). Spectra were accumulated for 30 s, centered and aligned to generate a matrix containing 15 variables (ions detected in the *m/z* 100–600 range) for the 12 samples analyzed. Although the spectra were originally scanned along the *m/z* range of 50–2000, ions of interest were detected only along the *m/z* 100–600 range. Principal component analysis (PCA) using the Unscrambler version 8.0 (CAMO Process A/S, Oslo, Norway) software was used to analyze the ESI(–)-MS fingerprint data and classify the samples.

RESULTS AND DISCUSSION

Because of selective extraction, pre-concentration and ease of ionization, which led to ion suppression for other species in solution, the predominant ions in the spectra were from the FFA. ESI(–)-MS of lipid extracts (Fig. 1) display characteristic profiles of FFA anions that allow prompt differentiation

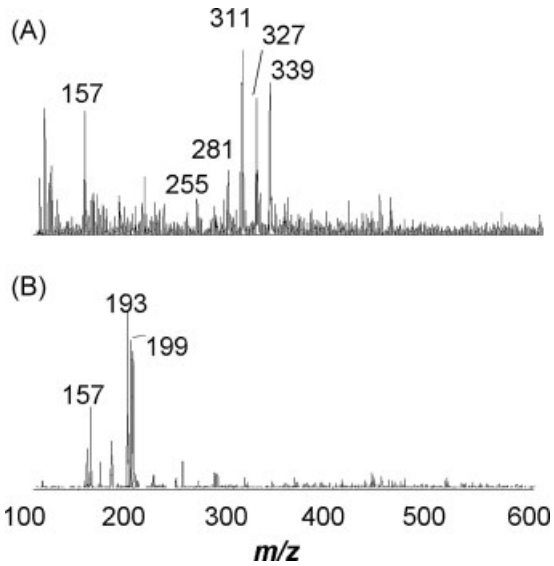

Figure 1. ESI(–)-MS fingerprints of (A) EtOH extracts of mitochondria (control) and (B) extracts of Orlistat-treated B16-F10 melanoma cells. Briefly, cells were treated with EtOH (control) or 300 μM Orlistat for 20 h, followed by mitochondria isolation. Lipids were then extracted, diluted and directly infused into the ESI source, as described in the Experimental section. ESI(–)-MS data were collected in the negative ion mode and the mass analyzer was set to scan along a *m/z* range of 50–1000. Spectra are representative of six independent scans.

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Table 1. Main FFA identified as their deprotonated molecules via ESI(–)-MS

Elemental composition	FA[a]	CN/DB[b]	$[M-H]^-$ *m/z*	Control[c]	Treated[c]
$C_9H_{18}O_2$	Pe	9:00	157	70	50
$C_{12}H_{24}O_2$	La	12:00	199	5	90
$C_{16}H_{32}O_2$	P	16:00	255	20	3
$C_{18}H_{34}O_2$	O	18:01	281	30	1
$C_{20}H_{40}O_2$	A	20:00	311	100	1
$C_{22}H_{32}O_2$	D	22:06	327	72	1
$C_{22}H_{44}O_2$	B	22:00	339	86	2

[a]Fatty acids; [b]Carbon number: number of double bonds; [c]Relative abundances.
Fatty acid abbreviations: Pe, pelargonic (9:0); La, lauric (12:0); P, palmitic (16:0); O, oleic (18:1); A, arachidic (20:0); D, docosahexaenoic (22:6); B, behenic (22:0).

between mitochondria of control (EtOH-treated cells, Fig. 1(A)) and Orlistat-treated B16-F10 cells (Fig. 1(B)). The ratios between the different FFA were calculated as in gas chromatography. The spectrum from control mitochondria (Fig. 1(A)) is characterized by six major FA ions, that is, from their deprotonated molecules of *m/z* 157 (Pelargonic, 9:0), 255 (Palmitic, 16:0), 281 (Oleic, 18:1), 311 (Arachidic, 20:0), 327 (Docosahexaenoic, 22:6) and 339 (Behenic, 22:0) (Table 1). In contrast, the spectrum for the lipid extract from mitochondria of Orlistat-treated cells (Fig. 1(B)) is rather distinctive and characterized by three major anions of *m/z* 157 (Pelargonic, 9:0), 193 (unknown) and 199 (Lauric, 12:0) (Table 1). The results presented here clearly show that treatment of B16-F10 melanoma cells with Orlistat, a pharmacological inhibitor of FASN activity, induces significant changes in the FFA profiles of mitochondria. These FFA alterations probably reflect cytosolic lipid changes, since FFA freely cross the outer mitochondrial membrane.[39] The reduced synthesis of palmitic acid promoted by FASN inhibition could consequently reduce its elongation and unsaturation products, such as arachidic and behenic acids and oleic acid, respectively.

Since FFA provided from both intracellular production and extracellular uptake can be quickly driven to membrane synthesis, such reduction in FFA content in Orlistat-treated B16-F10 cells could, at least in part, explain their decreased rates of proliferation.[24,25] The presence of medium-chain FFA, such as lauric and unknown FFA detected by the ion of *m/z* 193 (Fig. 1(B)), also indicates that these FFA are poorly metabolized in these cells. Besides, lauric acid-coated nanoparticles have also been shown to induce apoptosis in human melanoma cells[40] and inhibit proliferation in LNCaP prostate carcinoma cells.[41] Accumulation of lauric acid could therefore contribute to the apoptotic cell death observed in B16-F10 following FASN inhibition.[24,25]

PCA reveals the relationships between samples without peak-choice bias and without neglecting a significant amount of the information in the spectra. Herein, PCA was used

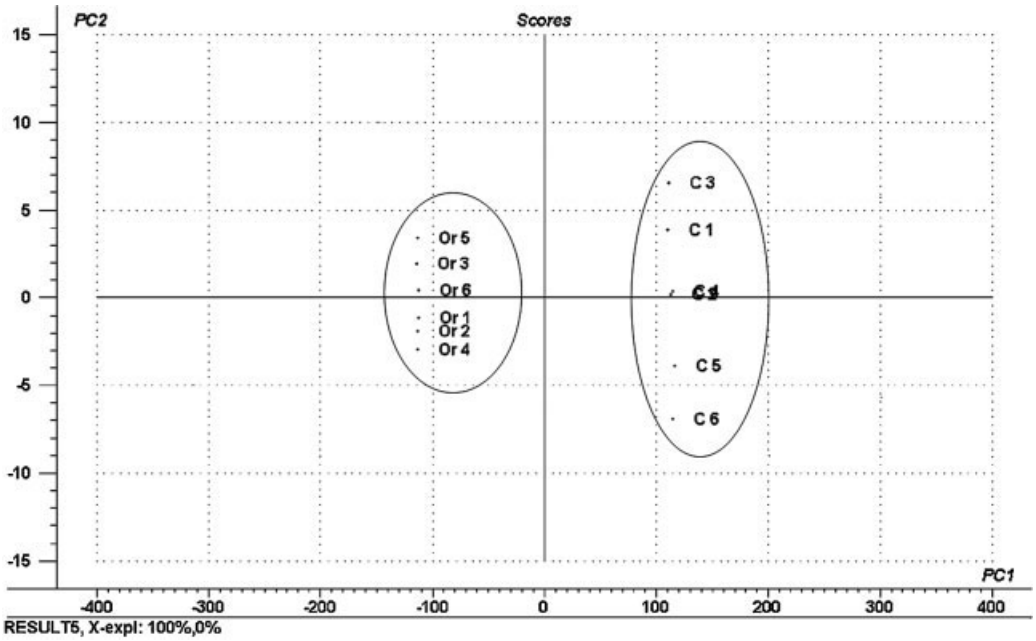

Figure 2. PCA scores plot for the ESI(–)-MS data of (A) EtOH extracts of mitochondria (control C) and (B) extracts of Orlistat-treated B16-F10 melanoma cells (Or). Each spot on the plot represents six independent scans.

to treat the MS data so as to statistically evaluate the performance of ESI(–)-MS in differentiating mitochondria isolated from control or from Orlistat-treated B16-F10 tumor cells. Figure 2 shows the PC1 versus PC2 scores plot. Note that indeed the two classes of samples are quite clearly grouped: control (C) or treated cells (Or). PCA describes the underlying orthogonal variables in the set of multivariate data as a set of principal components (PC), being a linear additive model in which each PC accounts for a portion of the total variance of the data, re-synthesizing the data as a function of two or three PCs. Plotting our data in the space provided by these PCs (scores plot) permits the rapid discrimination of these samples and clear visualization of their similarities and differences.

ESI(–)-MS provides therefore a simple and powerful screening method to specifically detect FFA in extracts of mitochondria. Direct infusion ESI(–)-MS fingerprinting was shown here to be capable of revealing FFA changes in mitochondria isolated from low quantities of cultured tumor cells (2×10^6 cells/mL). These findings have demonstrated that FASN inhibition by Orlistat induces significant changes in the FFA composition of mitochondria.

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