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Resveratrol inhibits lipogenesis of 3T3-L1 and SGBS cells by inhibition of insulin signaling and mitochondrial mass increase

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ABSTRACT

Resveratrol is attracting much interest because of its potential to decrease body weight and increase life span, influencing liver and muscle function by increasing mitochondrial mass and energy expenditure. Even though resveratrol was already shown to reduce the adipose tissue mass in animal models, its effects on mitochondrial mass and network structure in adipocytes have not yet been studied. For this purpose, we investigated the effect of resveratrol on mitochondrial mass increase and remodeling during adipogenic differentiation of two *in vitro* models of adipocyte biology, the murine 3T3-L1 cell line and the human SGBS cell strain. We confirm that resveratrol inhibits lipogenesis in differentiating adipocytes, both mouse and human. We further show that this is linked to inhibition of the normally observed mitochondrial mass increase and mitochondrial remodeling. At the molecular level, the anti-lipogenic effect of resveratrol seems to be mediated by a blunted expression increase and an inhibition of acetyl-CoA carboxylase (ACC). This is one of the consequences of an inhibited insulin-induced signaling *via* Akt, and maintained signaling *via* AMP-activated protein kinase. The anti-lipogenic effect of resveratrol is further modulated by expression levels of mitochondrial ATAD3, consistent with the emerging role of this protein as an important regulator of mitochondrial biogenesis and lipogenesis. Our data suggest that resveratrol acts on differentiating preadipocytes by inhibiting insulin signaling, mitochondrial biogenesis, and lipogenesis, and that resveratrol-induced reduction of mitochondrial biogenesis and lipid storage contribute to adipose tissue weight loss in animals and humans.

1. Introduction

Mitochondria are central actors of cell metabolism, first as the main source of cellular ATP, but importantly also as crucial players in lipid metabolism. Indeed, mitochondria provide a major crossroad for carbohydrate and lipid metabolism and storage. Mitochondria are involved in lipolytic processes through the β-oxidation of lipids, but also in anabolic processes such as lipogenesis and lipid storage in adipocytes and other tissues. In addition, they support other lipid-based pathways like steroid biosynthesis. Mitochondria are also responsible for the conversion of phosphatidylserine into phosphatidylethanolamine and further into phosphatidylcholine ([1–3], as for the conversion of cholesterol into steroids in endocrine cells [4] and for the synthesis of their own and specific lipids like cardiolipin [5–6]. Furthermore, mitochondria are very dynamic entities as they actively divide, fuse, move around and dock, or can be recycled or eliminated [7,8]. Because of all these integrated tasks, inappropriate mitochondrial structure or function may give rise to many different, otherwise unrelated pathologies such as myopathies, neuropathies, cardiopathies, obesity, diabetes, and cancers [9].

Resveratrol was discovered in 1939 by Michio Takaoka [10] and recently found to be a potent inducer of weight loss and longevity, in animals and humans [11–14]. However, the debate is still ongoing whether resveratrol may exert its functions only in the context of obesity or of a high fat diet since it had no beneficial effect on non-obese people [15–17]. Recent research efforts essentially focused on muscle and liver as target organs for resveratrol. However, adipose tissue is also affected by resveratrol exposure [12,18]. Resveratrol has been shown to inhibit PPARγ and C/EBPαβ induction in adipocytes [19,20]. Furthermore, resveratrol induced an increase of AMPK phosphorylation in C2C12 myotubes and in white adipose tissue, and an increased phosphorylation of its substrate, the acetyl-CoA decarboxylase (ACC) [21].

Abbreviations: SGBS, Simpson–Golabi–Behmel syndrome; ATAD3, ATPase family AAA domain-containing protein 3; ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase.

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Also, Sirt1 was shown to mediate the effect of resveratrol in muscle and adipocytes by the activation of PGCα [11,12,21,22], as well as of a phosphodiesterase and AMPK [21,23].

An important point to note is that resveratrol stimulates lipid uptake in muscle, which is contrary to its action in adipocytes [14]. However, it is not surprising that these two organs can work in an opposite manner because both organs have complementary tasks during metabolic adaptations and can most probably compensate each other. Especially resveratrol has been shown to promote mitochondrial biogenesis in the muscle [12], but this question was never addressed in adipocytes during adipogenesis.

Adipogenesis involves lipogenesis, a process tightly linked to mitochondrial mass, as shown *in vitro* and *in vivo* in humans by the clear correlation between mtDNA copy number and lipogenesis [24–25]. There are two major actual hypotheses concerning the contribution of mitochondria to lipid storage, not exclusive. The first is that mitochondria would supply ATP to this anabolic process [26]. The second is that the mitochondrial export of acetyl-CoA, or of citrate which is then modified to acetyl-CoA by ATP citrate lyase, allows its transformation into malonyl-CoA, by ACC, which is required to produce triglycerides by the fatty acid synthase (FAS) before being transferred into lipid droplets [26–28]. Finally, it must be pointed out that AMPK, as a central kinase involved in metabolic control, phosphorylates and thereby inactivates ACC and can contribute to mitochondrial proliferation [29–31]. In the muscle, resveratrol increases AMPK activity *in vitro* and *in vivo* [11,14,21]. In adipocytes, the impact of resveratrol on AMPK has been less studied. It may activate AMPK in differentiated 3T3-L1 adipocytes [32] or in proliferating pre-adipocytes [33,34], but nothing is known about long-term effects, especially in pre-adipocytes undergoing differentiation. Finally, resveratrol treatment is known to reduce the levels of adipogenic transcription factors, which implies that resveratrol could be also able to decrease mitochondrial biogenesis [19,20,35].

As mentioned above, the processes of adipogenesis and lipogenesis require mitochondrial biogenesis, which itself requires, among many others, the correct expression of the mitochondrial inner membrane protein ATAD3 (ATPase family AAA domain-containing protein 3). ATAD3 is a vital ATPase involved in mitochondrial biosynthesis [36–38,26], but whose exact functions are still unknown. However, its knock-down is lethal during the early development of *Caenorhabditis elegans*, *Drosophila melanogaster* and mouse, at times where mitochondrial neo-biogenesis occurs, indicating a major role in cell growth and differentiation. In cancer cells, ATAD3 was often found to be overexpressed, but also to be inhibitory for cell cycle and apoptosis, as well as conferring chemoresistance (for review see Ref. [38]).

Our aim in the present study was to elucidate if and how resveratrol regulates lipogenesis and mitochondrial mass in adipocytes *in vitro*. To this end we used the murine 3T3-L1 cell line and the human SGBS cell strain, two well-established model systems for studying adipocyte biology [39–41]. Both cell types are characterized by a high capacity for adipogenic differentiation and show accumulation of lipid droplets derived from *de novo* lipogenesis. Mitochondrial mass was shown to increase during adipogenesis and lipogenesis in these models and seems to precede lipid storage ([42–44,26]. In addition to lipogenesis, mitochondrial mass and mitochondrial proteins during the onset of adipocyte differentiation, we investigated selected components of lipid metabolism and insulin signaling, including ACC, AMPK and Akt. Finally, we asked whether resveratrol effects depend on the expression level of ATAD3. Our data suggest that resveratrol triggers closely related effects on insulin signaling, mitochondrial biogenesis and lipogenesis.

2. Materials and methods

2.1. Cells culture and treatments

Cells used in this studied were murine 3T3-L1 cells, purchased from ATCC, and human SGBS (Simpson–Golabi–Behmel syndrome, [40]) kindly provided by Pamela Fischer-Posovszky. 3T3-L1 and SGBS preadipocytes were cultured in a Dulbecco's Modified Eagle's Medium (DMEM, Gibco™, high glucose—4.5 g/l) containing 10% FBS (Biowest) at 37 °C and 5% CO_2 until confluence and maintained for 48 h (day 0). 3T3-L1 cells were induced to differentiate using MDI induction medium (DMEM containing 10% FBS and 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1 μM insulin) for 2 days, followed by insulin medium (DMEM containing 10% FBS and 1 μM insulin) for 2 days. The medium was subsequently replaced with fresh culture medium (DMEM with 10% FBS) containing insulin (1 μM) every 2 days until the terminal differentiation.

Human SGBS pre-adipocytes [40] were maintained in DMEM/Ham's F12 (1:1) medium (Invitrogen, Paisley, UK) containing 10% fetal calf serum (FCS; Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 33 μM biotin and 17 μM pantothenate. To differentiate SGBS cells into adipocytes, near confluent cells were washed three times with PBS and cultured in FCS-free differentiation medium: DMEM/Ham's F12 (1:1) medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 33 μM biotin, 17 μM pantothenate, 10 μg/ml human transferrin, 10 nM insulin, 100 nM hydrocortisone, 0.2 nM triiodothyronine, 25 nM dexamethasone, 500 μM 3-isobutyl-1-methylxanthine (IBMX) and 2 μM rosiglitazone (Cayman Chemical, Ann Arbor, MI, USA). After 4 days, this medium was replaced with differentiation medium excluding dexamethasone, IBMX and rosiglitazone, which was changed every 3–4 days.

Resveratrol (Sigma) was prepared at 100 mM in DMSO and oleic acid (Sigma) was prepared at 0.5 mM complexed to BSA at a 3:1 M ratio [45].

To generate ATAD3 over-expressing cells, 3T3-L1 cells were transfected with untagged ATAD3 expression vector (pCDNA3™) [26] and selected for G418 resistance at a concentration of 1 mg/ml of media. After 15 days of selection, resistant cells were grown as a pool in medium containing 200 μg/ml of G418.

2.2. Confocal microscopy analysis

The cells on glass coverslips were incubated with 200 nM MitoTracker Green FM at 37 °C in a 5% CO_2 incubator for 30 min then 1 μg/ml Hoechst 33342 in the dark at 37 °C for 10 min. After staining, cells were washed twice with pre-warmed phosphate-buffered saline (PBS), and fresh DMEM medium was added, and cells were placed on the microscope stage. Images were collected with a Leica TCS SP2 AOBS (Acoustico Optical Beam Splitter) inverted laser scanning confocal microscope equipped with a water immersion objective ×63 (HCX PL APO 63.0 × 1.20 water corrected). Laser excitation was 351–364 nm for Hoechst and 488 nm for MitoTracker Green FM. Fluorescence emission adjusted with AOBS was 390–470 nm for Hoechst, 500–550 nm for MitoTracker Green. Confocal pinhole (Airy units) was 1 for all channels. Each experiment was performed on a randomly chosen field. Raw images merging and zooming were obtained by Image J software (imagej.nih.gov).

2.3. Oil-Red-O staining

Cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. After fixation, cells were washed two times with distilled water and one time with 60% isopropanol. The plates were dried for 30 min at room temperature and then cells were stained with filtered Oil-Red-O working solution (stock solution: 3 mg/ml in isopropanol; working solution: 60% Oil-Red-O stock solution and 40% distilled water) for 10 min at room temperature, washed 4 times with distilled water, and plates were dried and scanned for images. Oil-Red-O dye was extracted using 100% isopropyl alcohol and measured by spectrophotometry at 500 nm.