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Dietary Resveratrol Modulates Metabolic Functions in Skeletal Muscle Cells

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ABSTRACT

Resveratrol is one of the best known polyphenol. While its effect on endothelial blood vessel cells, cancer cells, inflammatory processes and neurodegenerative events is well documented, only little is established on the metabolic implication of this phytophenol, particularly on skeletal muscle cells. Here, we report the effect of resveratrol on mouse skeletal muscle cells (C2C12) by measurement of cell proliferation, expression of metabolic genes and their transcription factor dependency, and modulation of non-coding microRNA expression. Resveratrol slightly decreases cell proliferation, while it up regulates PGC-1α a PPAR transcription factor co-activator involved in the control of metabolic genes. It also modulates microRNAs which are expressed in skeletal muscle cells and are involved in differentiation or contraction-relaxation. Results put forward new regulatory properties of resveratrol on skeletal muscles and highlight new potential applications of this molecule in muscle performances.

Key words: Resveratrol, metabolic functions, C2C12 skeletal muscle cells, microRNA

INTRODUCTION

There are thousand of different plant polyphenols produced by plants. These non nutritional compounds may play a role as signaling molecules and exhibit defense and/or attractive properties. Interestingly, animal and human cells react to plant polyphenols exposure as they behave towards chemical drugs, i.e. by recognizing these molecules as xenobiotic/pharmacological compounds.

It is now widely admitted that dietary phenolic compounds lead to beneficial effects for health[(1,2)]. Among such positive effects of polyphenols, resveratrol (RSV) has been considered as a powerful antioxidant with a direct impact on oxidative stress and related pathologies (i.e. atherosclerotic cardiovascular diseases, cancer, neurodegenerative processes, inflammation, ageing…). For instance, the proliferative signaling pathway is modulated by polyphenols through membrane receptors (death receptors, integrin receptor…), MAP kinases, nuclear factors (NFκB, NRF2, cyclins, CDK…). Moreover, we recently demonstrated the implication of a new resveratrol-controlled regulatory pathway involving microRNAs. For example, miR-663 is directly involved in the anti-inflammatory effect of resveratrol by lowering the content of AP-1 transcription factor in human monocyte derived cells[(3)].

While these effects are well documented, and beside a study on mice physiology[(4)] and on cardiac cells[(5)], only little is so far established on the metabolic implication of this phytophenol, particularly on muscle cells. Here we report the effect of resveratrol on mouse skeletal muscle cell line (C2C12). The chosen approaches were the following: proliferation, expression of metabolic genes and their dependant-transcription factors, the modulation of non-coding microRNAs. Results demonstrate new regulatory properties of resveratrol on the metabolism of skeletal muscle cells.

MATERIALS AND METHODS

I. *Materials and Reagents*

All chemicals were of analytical grade and purchased from Sigma Chemical Reagents (La Verpillière, France). Deionized and sterile water was used throughout the experiment. A standard stock solution of resveratrol at a concentration of 80 mM was prepared in ethanol, and the sample solutions of various concentrations were prepared by appropriate dilution of the stock solution when needed.

II. *Cell lines and Culture*

C2C12 mouse skeletal muscle cell line was grown either as myoblasts (undifferentiated) or as myotubes (differentiated). Phase contrast microscopy of these cells is presented in Figure 1. The differentiation protocol was the

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following: cells were grown to confluence in DEMEM medium (4.5g/L of glucose) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.3 mM ascorbic acid, at 37°C in a humid atmosphere of 5% CO_2/95% air. When confluence was reached, differentiation of cells into myotubes was induced by replacing in the DEMEM medium, the fetal bovine serum by 5% of horse serum. Cells were allowed to differentiate during 4 days.

(I) *Cell Treatments*

Cells were seeded 24 h before treatment on 6-well plates at a density of $0{,}3 \times 10^6$ cells per well. The next day, cells were challenged with RSV or with ethanol (control). After 48 or 72 h the supernatant of each well was collected and the adherent cells collected after trypsinization with 1 mL of Trypsin/EDTA solution. These suspensions containing supernatant and trypsinized cells were homogenized and used for counting cells by trypan blue exclusion test.

(II) *Phase Contrast Microscopy*

Phase contrast pictures were taken with a Zeiss LSM 5 Pascal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). Based on morphological criteria, the pictures were used to determine the number of live cells in each group.

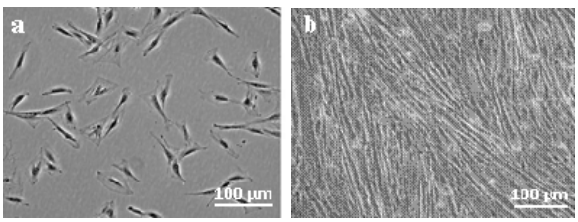

Figure 1. C2C12 cell morphologies; a: myoblasts, non-differentiated state and b: myotubes, differentiated state.

III. *MiRNA Micro-array Analyses*

RNAs extracted with TRIzol (Invitrogen, Carlsbad, California, USA) were subsequently subjected to DNase digestion (Turbo-DNase from Ambion, Invitrogen, Carlsbad, California, USA). MicroRNA micro-array analyses analyses were performed at the Ohio State University micro-array facility. Data were submitted to MIAME database with the accession numbers to be received after confirmation.

IV. *Gene Expression Estimation*

(I) *RNA Isolation and Reverse Transcription Step*

RNA from cultured cells was extracted and purified (from free nucleotides and contaminating genomic DNA) using RNeasy mini Kit (Qiagen) with DNAse treatment. One µg of RNA was used for reverse transcription with iScript Reverse Transcriptase (Biorad).

(II) *Real Time Quantitative PCR*

PCR reactions were performed using the qPCR MasterMix Plus for SYBR Green I with fluorescein (Eurogentec). All PCR reactions were performed with MultiGuard Barrier Tips (Sorenson BioScience, Inc.) and an Applied Biosystem Step One QPCR machine. Primers were designated to generate a PCR amplification product of 50 - 120 bp and were selected according to indication provided by the PrimerBank web site:
http://pga.mgh.harvard.edu/primerbank/) and were checked by nucleotide Blast (PubMed).

V. *Statistical Analysis*

Statistical analysis of data was performed by applying one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. Data were expressed as mean $\pm$ standard error of the mean (SEM).

RESULTS AND DISCUSSION

I. *Effect of Resveratrol on C2C12 Myoblasts Proliferation.*

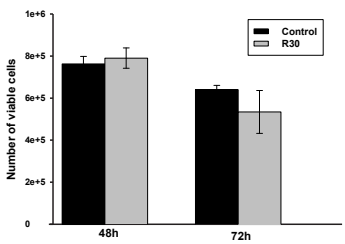

Figure 2. Effect of Resveratrol on C2C12 cell proliferation.

C2C12 myoblast cells were treated with RSV 30 µM (R30) for 48 and 72 h or with vehicle alone (0.1% ethanol). Cell proliferation was determined by counting the viable cells by trypan blue exclusion staining with a haemocytometer. Values are expressed as means ± SD of two independent experiments each performed in triplicate.

Figure 1 indicates that C2C12 cell proliferation is slightly sensitive to resveratrol only after 72 h of exposure in contrast to the inhibition obtained in tumour cell lines.[1,5]

II. *Influence of RSV on Post-transcriptional Expression of Metabolic Genes and Related Transcription Factors*

Table 1 shows that resveratrol treatment upregulated the gene expression of PGC-1α, a key co-activator of the PPAR signaling pathway and slightly PPAR γ gene (but not

statistically significant). In contrast Glut4 (a membrane glucose carrier), ACAD (a mitochondrial enzyme of the fatty acid oxidation pathway) and SREBP2 (involved in the sterol regulation metabolism) were downly expressed.

Table 1. RT-qPCR analysis of gene expression of selected genes in resveratrol-treated C2C12 cells

Gene	Relative expression level	Significance (p)	Variation
ACAD	0.843	0.041	Down
Glut4	0.471	0.001	Down
PGC1α	1.586	0.011	Up
PPARγ	1.118	NS	/
SREBP2	0.434	0.001	Down

C2C12 myoblast cells were treated for 48h with 30 μM resveratrol in 0.1% ethanol (final concentration). Relative level was compared to ethanol-treated cells. The results are the mean of 2 × 3 independent assays. They are reported on the 36B4 ribosomal protein expression gene, a choosen house keeping gene.
ACAD: acyl-CoA dehydrogenase; Glut4: glucose transporter; PGC1α: PPARγ cofactor 1-α; SOD1: superoxyde dismutase 1; PPARγ: peroxisome proliferators-activated receptor γ; SREBP2: steroid receptor element binding protein 2; NS: not statistically significant.

III. *Resveratrol and Expression of Non-coding mi RNAs on C2C12 Myoblast Cells (Table 2)*

There is more and more evidence that microRNAs are non-coding small RNAs (near 25 nt) that target specific mRNAs for cleavage and/or translational inhibition. Numerous microRNAs are expressed in a tissue specific manner and are involved in different biological processes like differentiation, immune response, cancer, neurodegenerescence. Recently, microRNAs have been implicated in various cardiovascular functions and pathologies[6]. However, until now, no information was available on skeletal muscle function regarding regulation by resveratrol. Here, we describe microRNA expression profiles in C2C12 myoblast cell lines treated or not with resveratrol.

In Table 2, we have selected four microRNAs whose levels changed significantly ($p < 0.05$) following resveratrol treatment of C2C12 cells. These selected microRNAs have numerous putative target genes and are the following: miR-20b, miR-21, miR-27 and miR-149. MiR-20b shows 813 putative genes. Resveratrol decreased its expression by -33%. MiR-20b which modulates VEGF is strongly down regulated by resveratrol (6-fold variation) in rat cardiomyocytes. The expression of miR-21 which may target 142 genes, increased 2-fold following resveratrol treatment. In rat heart resveratrol up regulated this microRNA by near 4-fold[6]. MiR-21 has been shown to regulate the ERK/MAP kinase signaling pathway. MiR-27b appears to target 737 putative genes and is up regulated (2-fold) by resveratrol. In contrast, resveratrol down regulated miR-27b in rat heart by 1.4-fold[6]. One target of miR-27b may be FoxO1 transcription factor. MiR-149 shows 185 putative genes and resveratrol strongly decreased its expression. Among miR-149 putative targets, we found several important genes in skeletal muscles such as Map3k13 of the JNK pathway ; Prdx6 a specific skeletal muscle gene ; Atp2a2 in the contraction/relaxation cycle ; and Stard3 gene in involved in the binding and transport of cholesterol.

Table 2. Expression level in mouse skeletal C_2C_{12} myoblasts of selected microRNAs resulting from microRNA chips screening and putative gene targets. Effect of resveratrol treatment

microRNA	miR-20b	miR-21	miR-27b	miR-149
Fold changes after resveratrol treatment	0.67	1.93	2.97	0.026
Variation	down	up	up	down
Parametric p-value	0.0087745	0.0141262	0.303593	0.0135363
Current putative target genes	813	142	737	185
Selected possible key target genes	Map3k5[1]	Bcl2[4]	Pparγ[6]	Map3k13[9]
	Myold[2]	Ppara[5]	Ppara[5]	Prdx6[10]
	Myf[3]		FoxO1[7]	Atp2A2[11]
			Igf1[8]	Stard3[12]

[1]Map3k5: Mitogen-activated protein kinase kinase5; [2]Myold: Myosin ID; [3]Myf: Myogenic factor 5; [4]Bcl2: anti-apoptotic activity; [5]Ppara: Peroxisome proliferator-activated receptor alpha; [6]Pparγ: Peroxisome proliferator-activated receptor gamma; [7]FoxO1: Forkhead box 01 involved in the transformation of Arf-1-myoblasts; [8]Igf1: Insulin-like growth factor 1; [9]Map3k13: JNK signaling pathway; [10]Prdx6: Expressed in skeletal muscle; [11]Atp2A2: Involved in the contration/relaxation cycle regulation; [12]Stard3: binds and transports cholesterol. The data result from microRNA chips screening. They are the mean of 3 samples. The changes obtained by screening were not compared with qRT-PCR approach.

IV. *Conclusions and Perspectives*

In skeletal muscle cell lines, resveratrol only slightly decreased C2C12 cell proliferation, but up regulated PPAR transcription factor complex encoding gene (PGC-1α). Moreover, we identified new signaling pathways modulated by resveratrol which implicate the regulation of microRNAs. Related to this the lack of up regulation of PPARα gene by resveratrol (data not shown) is coherent with the up regulation of miR-27b, which is a potentially target PPARα (cf. Table 2). Interestingly, from microRNA screening, miR-133 appeared down regulated by resveratrol in C2C12 treated cells (results not shown). MiR-133 is known to stimulate myoblast differentiation[8]. Thus, results are in agreement with the cell proliferation inhibition by resveratrol after a long period of culture (cf. Figure 2). The effect of resveratrol on differentiated C2C12 (myotubes) is in progress.

The determination of the part taken by microRNAs in resveratrol sensitivity can have some pharmacological interests to improve resveratrol efficiency in various major diseases. In addition, Bastin *et al*., have reported that exposure to human fibroblasts resveratrol triggers pharmacological correction of fatty acid utilization in human fatty acid oxidation-deficient cells[7].

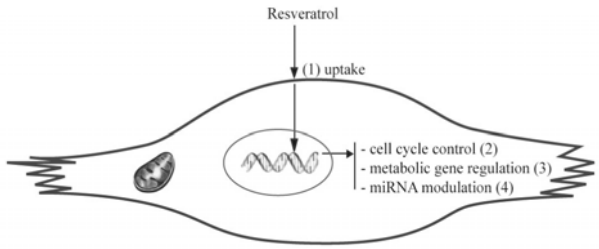

Figure 3. Functions and metabolic pathways modulated by resveratrol in C2C12 mouse skeletal muscle cell line.(1)Resveratrol penetrates in C2C12 cells (unpublished data).(2)Moderated inhibition of cell proliferation in myoblasts (this work) but no apoptosis (unpublished data).(3)PPAR/PGC-1a dependent gene expression encoding for mitochondrial metabolism.(4)Transcription factors dependent differentiation, contraction-relexation.

Our present data summarized in Figure 3 support the objective to develop nutraceutical and functional foods based on dietary resveratrol with the aim to prevent or reverse muscular dysfunctions associated with various diseases.

ACKNOWLEDGMENTS

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