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Resveratrol Regulation of PI3K-AKT Signaling Pathway Genes in MDA-MB-231 Breast Cancer Cells

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Abstract. *Background: Resveratrol (RSVL), a natural compound found in grapes and other food products, has been described to exert cancer chemopreventive activities. However, the cellular and molecular basis of its anticancer activity is largely undefined. The aim of the present study was to identify RSVL target genes in the MDA-MB-231 breast cancer cell line using cDNA arrays representing genes of the PI3K/AKT signaling pathway. Materials and Methods: Total RNA from control and RSVL-treated cells was used to synthesize biotinylated cDNA probes. cDNA arrays were hybridized with the probes and signals were detected with a chemiluminescent method. Western blotting analysis was used to validate the arrays' gene expression. Results: At the cDNA level, 13 genes were altered (at least 2-fold difference) by RSVL treatment. At the protein level, both c-fos and P70S6K were also regulated. Conclusion: Using gene arrays it was shown for the first time that c-fos and p70S6 kinase were regulated by RSVL.*

Reservatol (RSVL), a natural phytoalexin antioxidant found in large quantities in grapes and berries, is attracting increasing attention for its potential as one of the most promising cancer chemopreventive agents (1-4). Numerous studies have demonstrated that RSVL exerts an anticancer activity in many cancer systems (5-7), but the precise mechanisms of its anticancer effect remain unknown. It has been shown that RSVL interferes with signal transduction pathways, modulates cell cycle-regulating proteins and is a potent inducer of apoptosis in multiple carcinoma cell lines (8-11).

The phosphatidylinositol-3-kinase (PI3K) and AKT (Protein Kinase B) signaling pathway regulates a variety of biological processes including survival, proliferation, cell growth, cell motility and glycogen metabolism (12). In cardiomyocytes, it has been shown that RSVL prevents the development of cardiac hypertrophy through an antioxidant mechanism involving inhibition of different intracellular signaling transduction pathways including the PI3K pathway (13, 14). In human cancer cells, it was suggested that RSVL could inhibit survival and proliferation of estrogen-responsive cells by interfering with the PI3K pathway through an estrogen receptor-dependent mechanism (15). Recently, we reported that RSVL-induced growth inhibition in MDA-MB-231 cells was associated with the activation of ERK signaling pathway (16). Even though several studies provided evidence for the effect of resveratrol on the expressions of individual genes in relation to different physiological states of different target cells, so far little has been done to probe the effect of resveratrol on global gene expression changes in human cancer cells (17-19). The present study was designed to further understand the chemopreventive effect of RSVL by systematical identication of the genes differentially expressed in response to RSVL treatment in the human breast cancer MDA-MB-231 cell line by analyzing a c-DNA microarray of all known genes involved in the PI3K/AKT pathway.

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Materials and Methods

Reagents and antibodies. Trans-Resveratrol, Biotin-16-dUTP (Roche, Germany) and anti-actin (clone AC-40) monoclonal antibody were obtained from Sigma (Saint Louis, USA). Phospho-p70S6 kinase (Thr421/Ser424) and phospho-AKT (Thr308), phospho-p70S6K (Thr389) and c-fos polyclonal antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). All cell culture reagents were obtained from Gibco-BRL (Paisley, UK).

Cells and culture conditions. The human MDA-MB-231 breast cancer cell line was kindly provided by Bohdan Wasylyk (IGBMC core facility, Strasbourg, France). The ER-α negative MDA-MB-231 breast cancer cell line, known to be resistant to several anticancer drugs, is p53-deficient due to a point mutation at codon 280 of the p53 gene (20). The cell line was maintained for study in RPMI-1640 medium (Gibco-BRL) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, in a 5% CO_2 incubator at 37˚C. The cells were passaged twice a week by harvesting with trypsin/EDTA and seeding into 25-cm^2 dishes.

Table I. *Differentially expressed genes in Resveratrol treated MDA-MB-231.*

No.	GenBank	Symbol / Description	Fold Change*
1	NM 005465	AKT3/ V-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)	+4.3
2	NM 004346	CASP3/ Caspase 3, apoptosis-related cysteine protease	+2.3
3	NM 002613	PDPK1/ 3-phosphoinisitide dependent protein kinase-1	+15.1
4	NM 002611	PDK2/ Pyruvate dehydrogenase kinase, isoenzyme 2	+3.9
5	NM 006244	PPP2R5B/ Protein phosphatase 2, regulatory subunit B (B56) isoform	+7.6
6	NM 002880	RAF1/ V-raf1 murine leukemia viral oncogene homolog 1	+2.7
7	NM 003161	RPS6KB1/ Ribosomal protein S6 kinase, 70 kDa, polypeptide 1	+2.2
8	NM 013257	SGKL/ Serum/glucocorticoid regulated kinase-like	+2.6
9	NM 005252	FOS/ V-fos FBJ murine osteosarcoma viral oncogene homolog	+2.3
10	NM 005938	MLLT7/ Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to 7	+3.6
11	NM 000043	TNFRSF6/ Tumor necrosis factor receptor superfamily, member 6	+2.8
12	NM 021966	TCL1A/ T-cell leukemia/lymphoma 1A	–10.2
13	NM 004322	BAD/ BCL2-antagonist of cell death	–2.5

*Fold change in expression in resveratrol-treated cells compared with control cells. +, up-regulation, –, down-regulation.

RNA extraction, probe preparation and microarray experiments. Cells ($3x10^6$) were plated into 75-cm^2 cell culture dishes in the presence of 5% FBS media and incubated in a 5% CO_2 incubator at 37˚C for 12 h (~90% confluence). The media was changed and the cells were treated with 50 μM resveratrol as treatment (2 dishes) and ethanol as control (2 dishes) in 0.5% FBS media for 12 h. Cell culture media were removed from the cell monolayer by aspiration, and cells rinsed once with ice-cold phosphate-buffered saline. Total RNA was immediately isolated using ArrayGrade Total RNA Isolation Kit following the manufacturer's procedures (SuperArray Bioscience). Biotinylated cDNA probes were synthesized from 3 μg of total RNA using a GEArray probe synthesis kit (AmpoLabeling-LPR kit) obtained from SuperArray Bioscience. The gene arrays used in this study are 3.8x4.8 cm nylon membranes containing 96 cDNA fragments from human genes associated with the PI3K-AKT signaling pathway and 10 house keeping genes for microarray signals normalization (GEArrayQ Series, SuperArray Bioscience). The membranes were pre-hybridized with 2 mL of pre-warmed GEAprehyb solution (SuperArray Bioscience) in the presence of 100 μg/ml denatured salmon sperm DNA at 60˚C for 2 h. The prehyb solution was then discarded from the hybridization tube and the membranes were incubated with 0.75 mL of GEAhyb solution (containing the entire volume of the denatured cDNA probe) overnight at 60˚C with continuous agitation. The membranes were washed and chemiluminescence's detection of signals with X-ray film was performed according to the SuperArray protocol.

Microarray data analysis. The membranes were analyzed using the GEArray expression analysis suite software provided by SuperArray Biosciences. Empty spots were used for background correction and house-keeping genes for signal normalization. The array images (representing the control and the RSVL-treated cells) were uploaded onto the software. Upon completion of the analysis, a scatter plot (log transformation plot) was created. Group 1 (control group) was plotted on the x-axis and group 2 (RSVL-treate group) was plotted on the y-axis. The boundary of the plot was set as 2-fold difference. Each symbol in the scatter plot represents one gene. Those genes that have an expression difference (between control and treatment) greater than the defined boundary appear as red for up-regulated genes and green for down-regulated genes.

Western blot analysis. Cells ($5x10^6$) were plated into 75-cm^2 cell culture dishes in the presence of 5% FBS for 24 h. The cells were treated with 50 μM RSVL or with vehicle in 0.5% FBS-supplemented growth medium for the 0 min, 30 min, 1 h, 2 h and 24 h (P-p7056K) or 0 min, 6 h, 12 h, 24 h and 36 h (c-fos). At the end of the incubation time, cells were washed twice with ice-cold PBS buffer then the cells scraped with a rubber policeman and centrifuged to pellet the cells. The cell pellets were resuspended with 100-300 μl of ice-cold fresh RIPA lysis buffer (50 mM Tris-HCl pH 8, 0.1% SDS (w/v), 0.5% sodium deoxycholate (w/v), 1% Triton® X-100 (v/v), 150 mM NaCl, 10 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulphonylfluoride PMSF). The resuspended pellets were transferred to Eppendorf tubes and incubated in liquid nitrogen for 30 sec, then incubated in a 37˚C water bath for 1 min. Freezing and thawing was repeated 3 times and the tubes were centrifuged at 13000 xg for 5 min. The resulting supernatant was saved and the protein was determined using the Bradford method. Extracts were boiled for 3 min in 2X SDS buffer. Equal amounts of protein were loaded onto 10% SDS-PAGE according to the method of Laemmli and electrotransferred to nitrocellulose membranes. The blots were incubated with the antibodies above (1/1000 dilution) for 1 h, then incubated with the appropriate peroxidase-conjugated secondary antibodies (1/2000 dilution) for 1 h. Immunoreactive bands were visualized by incubation with luminol (according to the manufacturer's instructions; ECL Western blotting detection system, Amersham). The actin monoclonal antibody (Sigma) was used as a loading control.

Statistical analyses. Data are expressed as mean±SD. The significance of the difference between the DMSO-treated control cells and RSVL-treated cells was determined using the Student's *t*-test. A p-value of <0.05 was considered statistically significant.

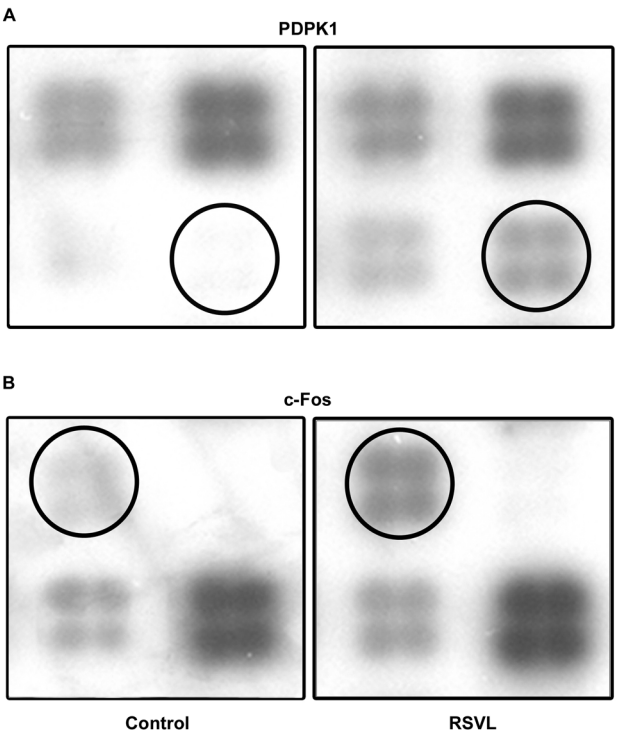


Figure 1. *Representative microarray membranes showing differential expression of PDPK1 (A) and c-fos (B) of the control and RSVL-treated MDA-MB-231 breast cancer cells. The microarray data analysis showed that PDPK1 were up-regulated (15.1-fold) and c-fos (2.3-fold).*

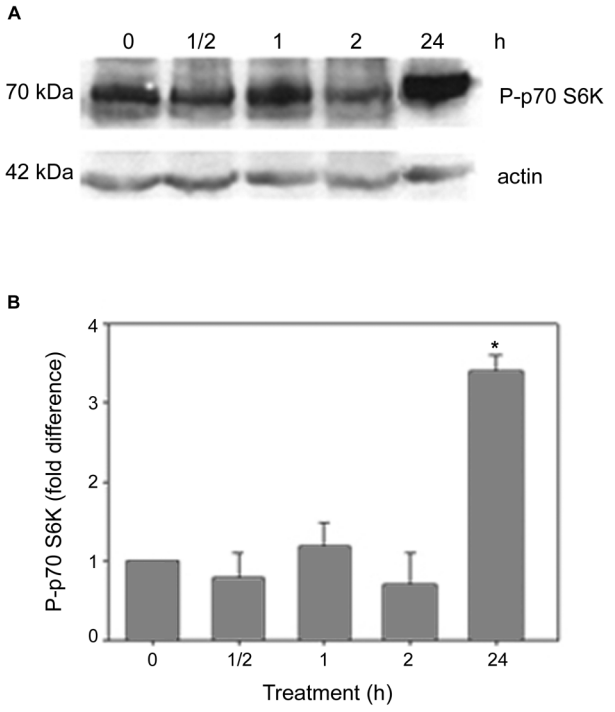


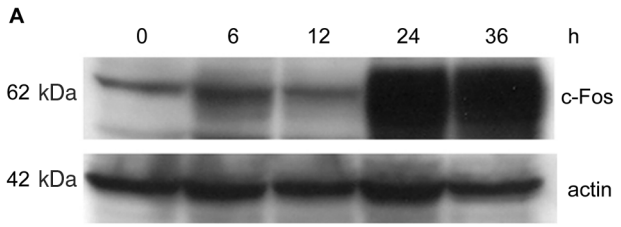
Figure 2. *RSVL phosphorylates P70S6K at (Thr421/ser424). A) Western blot analysis of extracts from MDA-MB-231 cells treated with 50 μM RSVL for the times shown, using Phospho-p70S6K (Thr421/ser424) polyclonal antibody or actin monoclonal antibody used as a loading control. B) RSVL significantly stimulated the phosphorylated p70S6K ($p<0.001$). Results are shown as mean±SD of three different experiments.*

Results

Resveratrol differentially regulated PI3K/AKT signaling pathway genes. It is well known that the PI3K/AKT pathway is involved in cell survival. Resveratrol induces significant growth inhibition of MDA-MB-231 breast cancer cells. We aimed to identify target genes which are differentially induced by RSVL in the PI3K/AKT pathway. Table I lists the 13 regulated genes that showed more than 2-fold changes in expression after treatment with 50 μM resveratrol for 12 h. The most highly expressed gene screened in this microarray experiment was *PDPK1* (phosphoinositide dependent protein kinase 1) which was up-regulated more than 15-fold. In Figure 1, representative microarray signals of RSVL-up-regulated *c-fos* (2.3-fold) and *PDPK1* (15.1-fold) genes are shown.

The effect of RSVL on p70S6 kinase. The observation that RSVL induced PDPK1 overexpression led us to investigate the impact of this up-regulation on a known target, the p70S6 kinase. Antibodies that detect p70S6K when phosphorylated on (Thr421/ser424) and (Thr389) were used. Figure 2A shows the Western blot analysis of extracts from MDA-MB-231 cells treated with 50 μM of RSVL for the indicated times using Phospho-p70S6K (Thr421/ser424) polyclonal antibody or actin monoclonal antibody. RSVL promoted phosphorylation of p70S6 kinase, with maximal promotion (around 3-fold that of the control) at 24 h. Figure 2B shows a significant increase in phosphorylation of the p70S6K protein by RSVL taking place at 24 h ($p=0.001$). As far as p70S6K (Thr389) is concerned, no effect of RSVL on this phosphorylated form was detected (Data not shown).

The effect of RSVL on c-fos expression. Cells were treated with 50 μM RSVL for the time shown in the presence of 0.5% FBS. Figure 3A shows Western blot analysis with c-fos antibodies. RSVL significantly promoted the expression of c-fos as early as 6 h of treatment, reaching a maximum level (around 5.5-folds that of control) after 24 h ($p=0.004$). This overexpression persisted even after 36 h of treatment. Actin levels served as controls for protein loading. Figure 3B shows changes in the mean band optical density as analyzed by Chemi Genius Syngene (Bioimaging System). Data are expressed as mean±SD. This shows that a



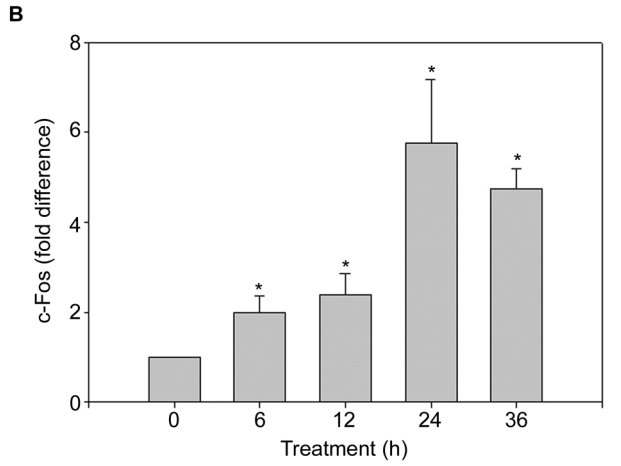


Figure 3. *A) Western blot analysis of extracts from MDA-MB-231 cells treated with 50 μM RSVL for the times shown, using c-fos polyclonal antibody or actin monoclonal antibody. B) RSVL significantly promoted the expression of c-fos as early as 6 h of treatment, reaching a maximum level (around 5.5-fold that of control) after 24 h (p=0.004). Results are shown as mean±SD of three different experiments.*

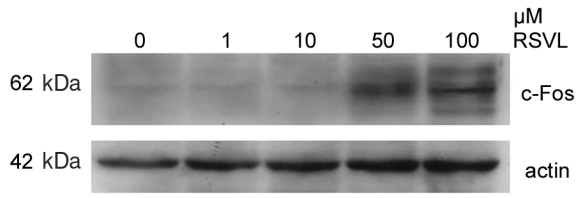


Figure 4. *Western blot analysis of extracts from MDA-MB-231 cells treated with different doses of RSVL (0, 1, 10, 50, 100 μM), using c-fos polyclonal antibody or actin monoclonal antibody used as loading control.*

significant expression of c-fos was achieved in 6 (p=0.009) and 12 (p=0.008) hours following RSVL treatment, with a significant promotion of its phosphorylation peaking at 24 h (p=0.004) and 36 h (p=0.001).

Discussion

Gene expression analysis *via* the DNA array presented here is part of our ongoing research on resveratrol-regulated gene expression in human breast cancer (16, 21). In this study we investigated the regulation by RSVL of the relative expression of genes associated with PI3K-Akt signaling in MDA-MB-231 human breast cancer cells. The PI3K-Akt pathway is involved in various neoplastic diseases and represents an attractive target for drug development for the treatment and/or the prevention of cancer (22). Several studies demonstrated the involvement of this signaling pathway in resveratrol-induced growth inhibition of different cell types (23, 24). We used a PI3K-Akt pathway-focused microarray containing 96 genes known to be involved in PI3K-Akt signaling pathways and 10 house keeping genes used for signal normalization during microarray data analysis. We show here that the most highly expressed gene screened in this microarray experiment was *PDPK1* which was up-regulated more than 15-fold. This kinase functions as a master kinase, phosphorylating and activating PKB/Akt, p70S6K and RSK and regulates cell size independently of cell number or proliferation (25). To see if this high increase in PDPK1 expression detected in the array was real, we analyzed some of its known target genes, namely the AKT and p70S6K proteins. AKT was not phosphorylated in MDA-MB-231 cells in response to resveratrol under our cell culture conditions (data not shown). However, we were able to detect three-fold increases in the phosphorylation of p70S6 Kinase (Thr421/ser424). As far as p70S6K (Thr389) is concerned, we did not detect any effect of RSVL on this protein (Data not shown). Another gene which was up-regulated by RSVL was the CASP3 (Caspase 3, apoptosis-related cystein protease). The Casp3 protein is one of the key executioners of apoptosis, as it is either partially or wholly responsible for the proteolytic cleavage of many key proteins such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP) (26). This result is very interesting and in line with our previous data (16) in which we showed that RSVL induces PARP cleavage in MDA-MB-231 under similar conditions. On the other hand, the caspase-3 overexpression seen in MDA-MB-231 is in agreement with the recent finding of Shimizu *et al.* (27) who demonstrated that resveratrol induced apoptosis of human malignant B-cells by activation of caspase-3. However, Pozo-Guisado *et al.* (28) demonstrated that resveratrol-induced apoptosis in the estrogen receptor positive human breast cancer MCF-7 cells involves a caspase-independent mechanism with down-regulation of Bcl-2 and NF-kappaB.

The second important gene which was induced at the array level and was validated by Western blotting analysis is c-*fos*. The data shows that the c-fos protein level is remarkably induced by this dietary phytoestrogen. This

induction was observed in a dose- and time-dependent manner. To our knowledge, this is the first report indicating that RSVL up-regulates the expression of c-*fos* in human breast cancer cells. However, Wolter *et al.* (29) using the colon cancer Caco2 cell line demonstrated that elevated protein levels of c-Fos were observed together with an increase in DNA-binding activity after RSVL treatment. The proto-oncogene c-*fos* plays a relevant role in the regulation of normal cell growth, differentiation, and cellular transformation processes and its expression is rapidly induced by different extracellular stimuli including mitogens and hormones. The nuclear protein encoded by c-*fos* interacts with c-*jun* family members to form the heterodimeric activating protein-1 transcription factor complex. The *fos-jun* heterodimers binding to activating protein-1 sites located within mammalian gene promoters regulates gene expression in a specific manner depending on cellular and promoter context as well as interacting proteins (30). Moreover, c-*fos* binds to sites identified in the regulatory region of target genes modulating the late response expression of critical factors for cell cycle re-entry. The exact mechanism of c-fos up-regulation by RSVL in MDA-MB-231 cells is not well understood. It is well documented that the transcription of c-*fos* is controlled by multiple *cis*-elements present in the gene promoter: the cAMP-response element that binds to cAMP-response element-binding protein, the Sis-inducible enhancer that is recognized by the signal transducers and activators of transcription (STAT) group of transcription factors, the serum-response element that mediates c-fos induction by growth factors, and other extracellular stimuli leading to activation of MAPK pathways (31). It was shown that RSVL-induced growth inhibition in MDA-MB-231 cells was associated with activation of the MAPK pathway (16). Therefore, resveratrol-induced c-*fos* expression may be linked to the activation of the MAPK pathway and has interesting implications for the treatment of ER-negative breast cancer.

More interestingly, the results of the microarray show that the most highly depressed gene was *TCL1A* (T-cell leukemia/lymphoma 1 A) which was down-regulated by more than 90%. TCL1 is a protooncogene responsible for the development of prolymphocytic T-cell leukemia and is also overexpressed in human B-cell malignancies. The inhibition of TCL1 protooncogene by RSVL may explain its antiproliferative effect in breast cancer cells. TCL1 expression was also inhibited by RSVL in an osteosarcoma cell line (data not shown). It is well known that TCL1 protein causes cell survival by interacting with Akt protein, functioning as an Akt kinase co-activator (32). Information obtained from this data will enable us to gain further insights into the effect of RSVL on this highly metastatic and ER-negative breast cancer cell line.

Conclusion

The use of the focused DNA microarrays (pathway-specific microarrays) correlated well with Western blotting-based analysis and enabled us to identify two new RSVL target genes. Because of their focused design, data handling is easier and more straightforward, as compared with classical gene arrays which contain too many genes. Therefore, this type of gene array is reliable and sensitive and may be an ideal tool for studying gene expression associated with a biological pathway or disease state.

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