Antioxidants induce different phenotypes by a distinct modulation of signal transduction

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Abstract **Antioxidants are known to exert a preventive activity against degenerative diseases. Here, we investigated the mechanism of action of three antioxidants: resveratrol, which causes differentiation of HL-60 cells, and hydroxytyrosol and pyrrolidine dithiocarbamate which, in the same model system, activate apoptosis. The expression profile of hydroxytyrosol-treated cells showed the up-regulation of several genes, including c-*jun* and *egr1*. Pyrrolidine dithiocarbamate activates both genes, while resveratrol increases uniquely *egr1*. A selective modulation of signalling pathway explained this finding. All antioxidants up-regulate Erk1/2, while only hydroxytyrosol and pyrrolidine dithiocarbamate activate c-Jun N-terminal kinase (JNK). Since JNK induces apoptosis by Bcl-2 phosphorylation, we investigated this event. Bcl-2 phosphorylation was increased by hydroxytyrosol and pyrrolidine dithiocarbamate and not by resveratrol. Our results indicate that the different phenotypical effects of antioxidants correlate with modulation of selective transduction pathways.**

Key words: Resveratrol; Hydroxytyrosol; Pyrrolidine dithiocarbamate; Antioxidant; Apoptosis; Signal transduction

1. Introduction

Numerous epidemiological data indicate that an increased dietary intake of antioxidants, including vitamins C and E, β-carotene and polyphenols, is associated with a diminished risk of cardiovascular diseases [1,2] and cancer [3,4]. Although the precise mechanism of action of these compounds is not understood, several lines of evidence suggest that they mainly prevent the free radical-dependent macromolecule damages, thus reducing the accumulation of structural and functional alterations in vivo [5].

Conversely, it appears clear that the biological activities of antioxidants might not be simply related to their scavenger capability. Indeed, these molecules are able to induce many phenotypical effects, ranging from cell growth arrest to activation of differentiation, induction of (or, conversely, resistance to) apoptosis and, in a few instances, stimulation of proliferation [6–12].

We previously described the effect of three antioxidants, namely resveratrol, hydroxytyrosol (dihydroxyphenylethanol, DPE) and pyrrolidine dithiocarbamate (PDTC) (Fig. 1) on the growth and survival of HL-60 cells. Resveratrol is a well-known phytoalexin occurring at high concentration in grape skin and red wine [13], while DPE is a polyphenol present in extra virgin olive oil [14]. Both compounds exert several protective effects, either in vivo or in vitro [15–18], which could explain, at least in part, the beneficial properties of the 'Mediterranean diet'. PDTC, a synthetic antioxidant, has attracted considerable interest since it is able to enhance the anticancer ability of 5-fluorouracil [19]. Particularly, the treatment with PDTC and 5-fluorouracil completely prevented the growth of tumor xenografts in athymic mice [19].

The three molecules exert different effects on HL-60 cells. We demonstrated that DPE [20] and PDTC [21] are able to activate apoptosis by causing cytochrome *c* release from mitochondria, while resveratrol induces differentiation towards a myelo-monocytic lineage by blocking the cell cycle at the checkpoint between S→G2 transition [22].

These findings moved us to investigate the molecular bases of the distinct phenotypical effects by a comparative approach employing as our model system HL-60 cells. This cell line was selected since it has been widely employed in studies devoted to the evaluation of antiproliferative, differentiation and apoptotic effects of potentially active molecules. Also, and most important, we have previously characterized in detail the phenotypical effects of all three molecules on these cells.

Initially, we studied the activity of DPE on gene transcription by means of cDNA arrays. Then, we selected the genes mostly activated and investigated their regulation by PDTC and resveratrol. This approach allowed us to identify differences of the antioxidant effects on the signalling pathways. Altogether, our results permit us to conclude that the observed different effects on the phenotype of the same cell line are, at least in part, due to specific transduction pathway modulations.

2. Materials and methods

2.1. Cell treatments

HL-60 cells were obtained and grown as in [22]. PDTC, tyrosol, homogentisic acid and resveratrol were obtained from Sigma Chemical Company (St. Louis, MO, USA). 2-(3,4-Dihydroxyphenyl)ethanol (DPE) was from G.F. Montedoro, University of Perugia, Italy. Re-

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Abbreviations: DPE, hydroxytyrosol; PDTC, pyrrolidine dithiocarbamate

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sveratrol and DPE were suspended in dimethylsulfoxide at a concentration of 100 mM and stored for brief periods at −80°C, while PDTC solution (100 mM in dimethylsulfoxide) was freshly prepared for each experiment. PD98059 (Erk1/2 inhibitor), SB203580 (p38 kinase inhibitor), KN62 (Ca^{2+}/calmodulin kinase inhibitor) and H8 (inhibitor of protein kinase A/C (PKA/PKC)) were from BioMol Research Laboratories (Plymouth Meeting, PA, USA). The molecules were directly added to cultures, while control cells contained the solvent alone.

2.2. cDNA expression array and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared using the Atlas pure total RNA isolation kit (Clontech, Palo Alto, CA, USA). For analyzing gene expression in HL-60 cells, the Atlas cDNA expression array (cat. # 7740-1) was employed as described in the user's manual and in [23]. Semiquantitative RT-PCR analyses were carried out as described in [23]. Primer sequences used for c-Jun, Egr1, GADD45 and RAB2 were obtained from Clontech, while the primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as in [23]. Each experiment was performed at least in triplicate.

2.3. Immunochemical techniques and c-Jun N-terminal kinase (JNK) assay

Monoclonal antibodies to phospho-Erk (Tyr204) were provided by Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Polyclonal antibodies to Bcl-2, phospho-Bcl-2 (Ser70), c-Jun, phospho-c-Jun (Ser63), JNK, Egr1 and Erk1/2 were from Santa Cruz. Immunoblotting were performed as in [24].

JNK activity was assayed as reported below. Briefly, equal amounts (0.5–1 mg) of cell extract were incubated for 4 h with anti-JNK polyclonal antibodies at room temperature. The mixtures were added with agarose A-protein, incubated for a further 2 h and centrifuged. The precipitates were washed and incubated with recombinant c-Jun protein (amino acids 1–79, Santa Cruz) for 30 min at 37°C. The reaction mixtures were then separated by sodium dodecyl sulfate electrophoresis, transferred to nitrocellulose and incubated with anti-phospho-c-Jun antibodies.

3. Results and discussion

3.1. Effect of DPE on gene expression

In order to identify genes regulated by DPE, we analyzed its activity on gene transcription by means of cDNA expression arrays. HL-60 cells were treated with 100 μM DPE for 5 h. It is to be underlined that this concentration is probably reached in vivo [25] since olive oil DPE content is up to 3 mM [26]. Moreover, we have previously showed that 100 μM DPE is able to cause the release of cytochrome *c* from mitochondria after 6 h and, thereafter, to induce apoptosis within 24 h [20]. The expression array experiments were performed in the presence of cycloheximide to identify only direct transcriptional effects and to rule out the involvement of de novo synthesized proteins. Examples of the primary data, regarding some genes of interest (see below), are reported in Fig. 2A.

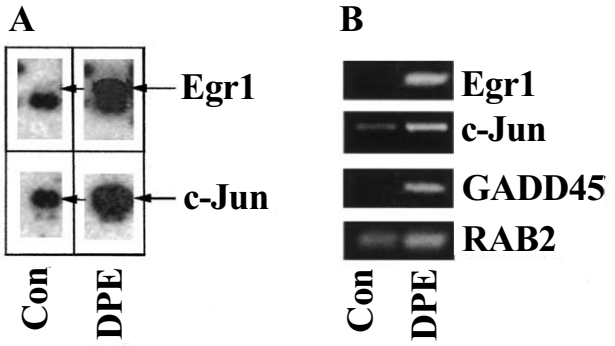

Fig. 2. Effect of DPE on gene expression. HL-60 cells were cultured with 100 μM DPE for 5 h in the presence of cycloheximide (36 μM). Untreated cells are indicated as Con. Then, total RNA was prepared and employed for investigating the expression profile by cDNA array or by RT-PCR. A: Details of cDNA arrays showing the expression level of *egr1* and c-*jun*. B: Expression of the reported gene by RT-PCR.

Table 1 shows a short summary of the expression profiles upon addition of DPE to growing HL-60 cells; the fold induction values are a mean of three independent experiments. The genes reported are those showing the most marked up-regulation. Interestingly, only few genes are down-regulated (particularly *Cdc25A* and *NIP1*).

As reported in the table, several of the overexpressed genes (c-*jun*, *egr1*, *GADD45*) control important cellular processes, i.e. cell cycle, signal transduction, DNA repair and genome transcription, and thus they might be responsible, at least in part, for the DPE-induced phenotype. The effect of DPE on four of the up-modulated gene was also verified by RT-PCR in independent experiments (Fig. 2B). The results agree with those obtained by cDNA arrays.

3.2. Comparative effect of resveratrol, DPE and PDTC on the selected genes

Among the genes identified, we selected for further studies c-*jun* and *egr1*, since they encode transcription factors controlling key processes, like cell cycle arrest, differentiation and apoptosis [27–30].

Preliminarily, we investigated the effects of DPE on c-*jun* transcription and observed that the phenomenon was both time- and dose-dependent (data not reported). The induction of an efficacious c-Jun synthesis was then confirmed at the protein level (Fig. 3A,C), thus demonstrating a complete correspondence between the transcription and the translation of the gene. The analysis of resveratrol and PDTC effects on c-*jun* expression (Fig. 3B,C) demonstrates that the synthetic antioxidant (PDTC) markedly stimulates the up-regulation of gene transcription while resveratrol causes a very scarce induction of the gene. Finally, the phosphorylation of the newly synthesized c-Jun protein was demonstrable by using specific

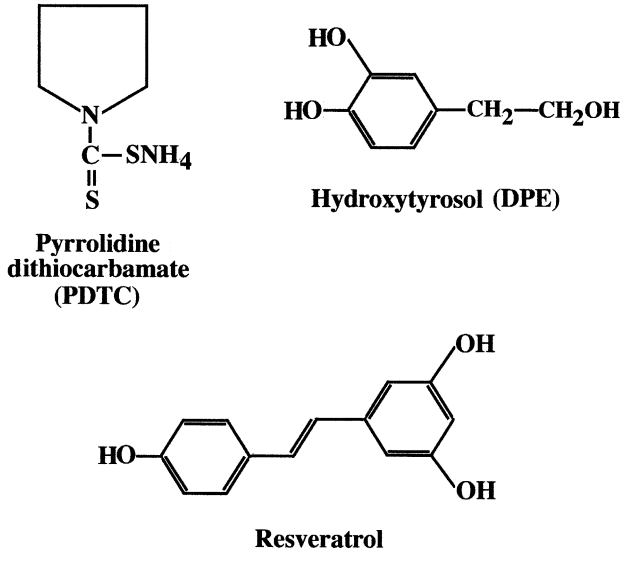

Fig. 1. Chemical formulas of PDTC, DPE and resveratrol.

Table 1
Human genes mostly induced by DPE

GenBank accession number	Description	Fold induction	Function
M62829	*egr1*	5 ± 1	Transcription factor
J04111	c-*jun*	4 ± 1	Transcription factor
M60974	*GADD45*	3.5 ± 1	DNA repair and proliferation control
M28213	*RAB2*	3 ± 1	Signal Transducer
X92812	TGFβ	3 ± 1	Growth factor
X86779	Fast kinase	3 ± 1	Apoptosis

HL-60 cells were treated for 5 h with or without 100 μM DPE in the presence of 36 μM cycloheximide. The expression profile analysis was performed as in Section 2.

antibodies (Fig. 3D), thus allowing two conclusions: (i) c-Jun was fully functional and (ii) JNKs were active.

In all these experiments the employed concentration of resveratrol and PDTC (30 μM) was similar to (or lower than) that used in several studies reported in literature [15–20,22].

The data obtained indicate a correlation between the capability of antioxidants of inducing apoptosis and the activation of signal transduction pathways leading to c-*jun* gene expression. Moreover, it is possible, although not proved by our experiments, that the accumulation of c-Jun is involved in DPE- and PDTC-dependent cell death [31].

In order to evaluate our hypothesis, we studied the effect of two DPE structural analogs, which are unable to activate apoptosis [20], on c-*jun* expression. Particularly, we compared the effect of DPE with that of tyrosol and homogentisic acid. Both the analogs neither induced programmed cell death [23] nor up-regulated c-*jun* transcription (Fig. 3E), thus confirming the hypothesis of a possible correlation between these two events.

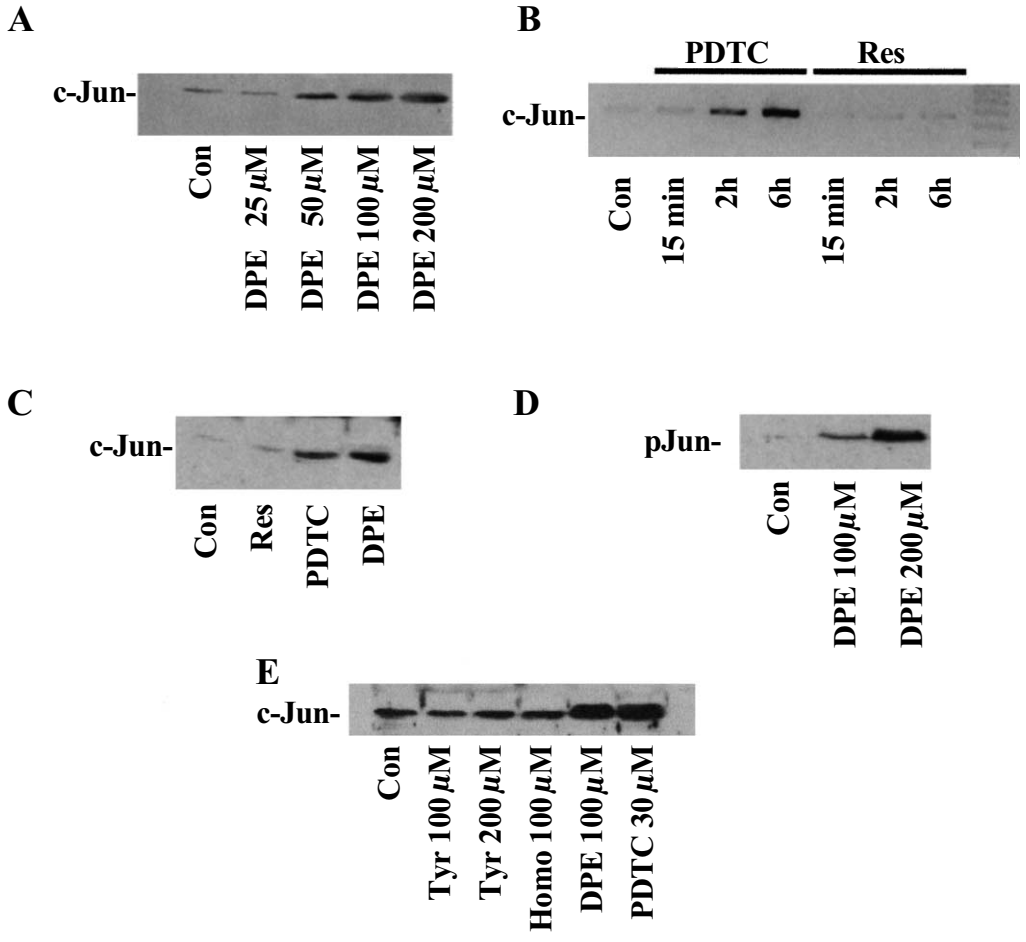

Fig. 3. Effect of DPE, PDTC and resveratrol on c-*jun* expression. A: HL-60 cells were cultured in the presence of the indicated amounts of DPE for 8 h. Then, the c-Jun level was determined by immunoblotting. Con, untreated cells. B: HL-60 cells were cultured with 30 μM PDTC and 30 μM resveratrol (Res) in the presence of cycloheximide (36 μM) for different time periods. Then, c-*jun* expression was determined by RT-PCR. C: HL-60 cells were cultured without (Con) or with 100 μM DPE, 30 μM PDTC or 30 μM Res for 8 h. The c-Jun level was determined by immunoblotting. D: HL-60 cells were cultured with DPE for 8 h. The phospho-c-Jun level was determined by immunoblotting. E: HL-60 cells were cultured with the indicated amounts of tyrosol (Tyr), homogentisic acid (Homo), DPE and PDTC for 8 h. Then, the level of c-Jun was determined by immunoblotting.

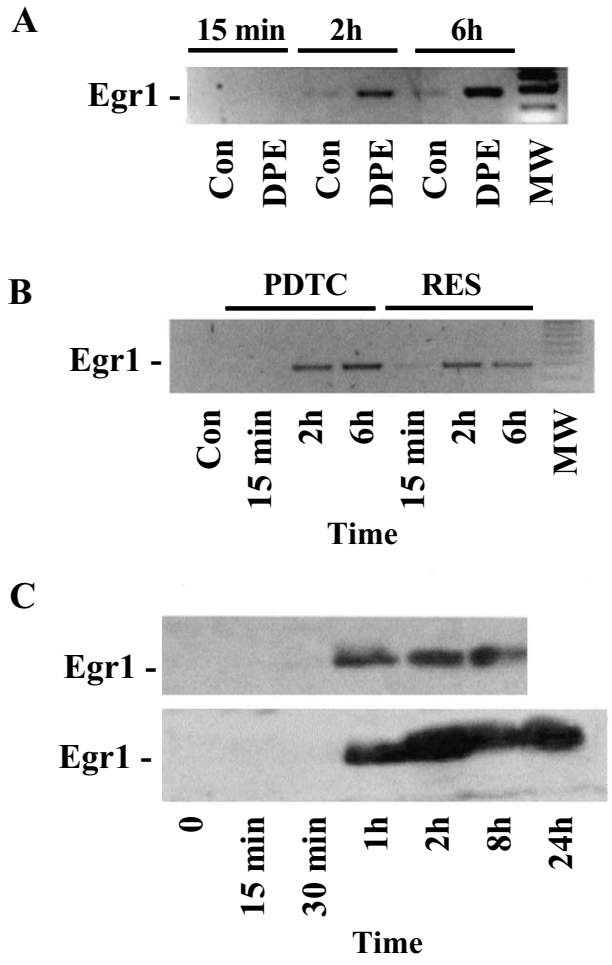

Fig. 4. Effects of DPE, PDTC and resveratrol on *egr1* expression. A: HL-60 cells were cultured with 100 μM DPE in the presence of cycloheximide (36 μM) for the reported time periods. *egr1* expression was evaluated by RT-PCR. MW, molecular weight. B: HL-60 cells were cultured with 30 μM PDTC and 30 μM resveratrol (Res) in the presence of cycloheximide for different time periods. *egr1* expression was evaluated by RT-PCR. C: HL-60 cells were cultured with 100 μM DPE and 30 μM Res for the reported time periods. Egr1 level was determined by immunoblotting. Importantly, the point at 24 h for DPE was not reported due to the death of cells by apoptosis.

Then, we analyzed the effects of DPE, resveratrol and PDTC on *egr1* up-regulation. In this case, all the compounds were able to activate the expression of the gene (Fig. 4A,B). Comparing the level of the induced protein, the effect of resveratrol, on the basis of the immunoblotting signal, was stronger than that of DPE. Moreover, in the case of resveratrol the Egr1 up-regulation persists, at least, up to 24 h (Fig. 4C). This result is quite surprising since, generally, the activation of the *egr1* gene is rapid but transient [32,33]. The data on DPE also indicate a marked increase in Egr1 protein (Fig. 4C), although the 24 h point could not be taken into account due to the apoptosis of cells.

3.3. *Modulation of signal transduction pathways by resveratrol, DPE and PDTC*

The above reported findings prompted us to analyze the activity of the antioxidants on signalling pathways which might induce c-*jun* and *egr1*. By using a series of kinase inhibitors, we demonstrated that DPE-dependent *egr1* up-regulation is due exclusively to Erk1/2 pathway activation (Fig. 5A). Identical findings were obtained by studying *egr1* modulation by resveratrol and PDTC (data not reported). The result allows the hypothesis that, at least in HL-60 cells, all the antioxidants activate the Erk1/2 pathway. This view was confirmed by the direct analysis of the phosphorylated forms of the kinases by immunoblotting, after DPE stimulation (Fig. 5B). While the inhibition of this pathway abrogates the up-regulation of *egr1*, it was inefficacious towards c-*jun* up-regulation, which was not affected by any of the inhibitors employed (Fig. 5C). This permits us to exclude a role of Erk1/2, p38 kinase, PKA, PKC and Ca^{2+}/calmodulin kinase in the antioxidant-dependent c-Jun accumulation.

Since there are no specific JNK inhibitors available we could not know whether the stimulation of c-*jun* transcription was due to the activation of the JNK pathway. Moreover, since resveratrol did not induce c-*jun* expression under the employed experimental conditions, it is conceivable that the phytoalexin did not activate JNK. In order to address this view, we analyzed JNK activity by an enzymatic assay. We observed a significant JNK activation by DPE (Fig. 5D) and PDTC (not reported) but not by resveratrol (Fig. 5D). This result suggests a possible correlation between the apoptotic effect exerted by the two antioxidants (DPE and PDTC) and JNK activation. In accordance with this mechanism, resveratrol, which does not activate JNK, does not induce programmed cell death.

While the pro-apoptotic effect of JNK activation has been widely reported in the literature [34], it is not definitely established whether c-Jun accumulation might correlate with this activity. c-Jun up-regulation causes a complex array of phenotypical effects, including proliferation, cell death and differentiation [27,28,31]. Thus, we were looking for other putative molecular mechanism(s) linking antioxidant → JNK activation → apoptosis.

In this scenario, it is important to underline that one of the effectors of JNK-related cell death is the phosphorylation (at Ser70) and inactivation of Bcl-2 [35,36]. This protein exerts its anti-apoptotic effect by maintaining the structure of the external membrane of mitochondria and, in turn, by hampering the release of cytochrome *c* [34]. Therefore, we evaluated the effect of DPE, resveratrol and PDTC on Bcl-2 phosphorylation. As shown in Fig. 5E, while PDTC and DPE markedly increased Bcl-2 phosphorylation, resveratrol did not modulate this event.

Our study reports a significant number of novel observations. First, we demonstrated, for the first time, that resveratrol, DPE and PDTC enhance directly the expression of a pivotal transcriptional factor, i.e. *egr1*, while only DPE and PDTC up-regulate the transcription of the c-*jun* gene. Second, the three antioxidants modulate, in a different fashion, kinases involved in two transduction pathways, namely Erk1/2 and JNK. Third, DPE and PDTC, but not resveratrol, activate the phosphorylation of Bcl-2.

When we compare these molecular events with the antioxidant-dependent phenotypical effects, it appears probable that a different regulation of signal transduction pathways might be the principal cause of different cellular effects. This also strongly suggests that specific molecular interactions, only partially related to the general scavenger capability, are at the basis of the phenotype observed. Moreover, the molecular

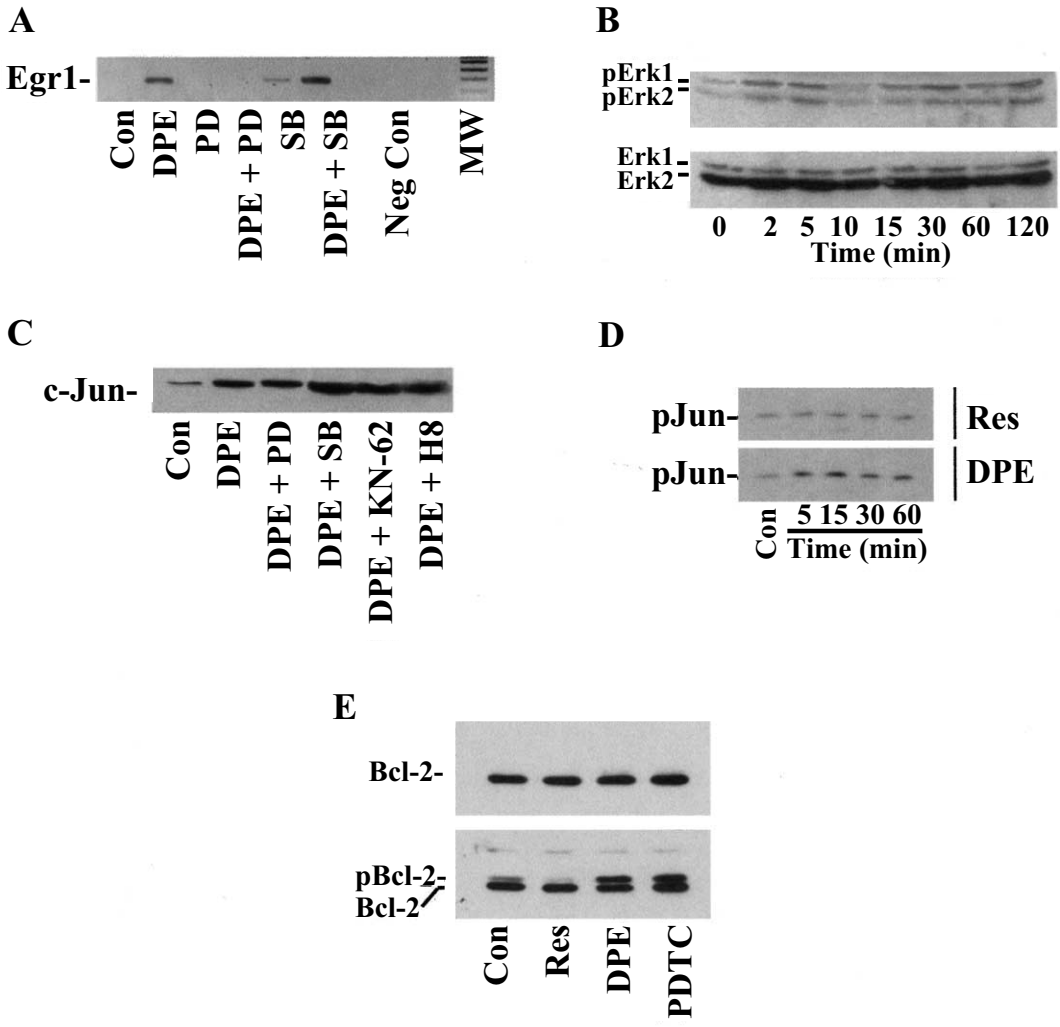

Fig. 5. Effects of DPE, PDTC and resveratrol on signal transduction pathways and Bcl-2 phosphorylation. A: HL-60 cells were grown for 1 h with or without the reported kinase inhibitors and then 100 μM DPE was added. The concentration of the inhibitors was the following: PD98059 (PD), 25 μM; SB203580 (SB), 20 μM. *egr1* expression was evaluated by RT-PCR. Neg Cont means a PCR reaction carried out without cDNA. B: HL-60 cells were grown with 100 μM DPE for different time periods. Phospho-Erk1/Erk2 (top) and total Erk1/Erk2 (bottom) were determined by immunoblotting. C: HL-60 cells were grown for 1 h with or without the reported kinase inhibitors and then 100 μM DPE was added. The concentration of the inhibitors was the following: PD98059 (PD), 25 μM; SB203580 (SB), 20 μM; KN62, 10 μM; H8, 25 μM. c-*jun* expression was evaluated by RT-PCR experiments. D: HL-60 cells were grown without or with 30 μM resveratrol (Res) or 100 μM DPE for different time intervals. Then, JNK activity was determined as reported in Section 2. E: HL-60 cells were grown without (Con) or with 100 μM DPE, 30 μM PDTC or 30 μM Res for 5 h. Phospho-Bcl-2 (top) and Bcl-2 (bottom) levels were established by immunoblotting.

basis of the observed differences will also be beneficial for a rational therapeutic use of antioxidants.

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