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Polyphenol interaction with the T47D human breast cancer cell line

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Experimental and epidemiological studies indicate that antioxidant food polyphenols could have antimitotic activities, interfering with cancer initiation, progression or mortality. Circulating polyphenols are far lower than the nominal value in foods. In the rare studies dealing with polyphenol bioavailability, it was noted that their active concentrations in the blood are <1% of their food concentration. In the present study we investigated the effect of four polyphenols (resveratrol, and the flavonoids quercetin, catechin and epicatechin, major constituents of wine) in the hormone-sensitive human cancer cell line T47D, at concentrations compatible with their calculated plasma concentrations after ingestion of a moderate quantity of wine (nM or pM). Our results indicate that cell growth was decreased, with cells being arrested at the S phase of the cycle. In addition, we provide evidence of a bimodal modulation of the NO/NOS system, affecting its activity and transcription. We show that modulation of this system is sufficient to explain polyphenol action on this cell line. This result suggests a potential importance of wine ingestion and possibly the consumption of other polyphenol-rich dietary foods and drinks in the control of breast cancer cell growth.

Keywords: Resveratrol, catechin, epicatechin, quercetin, No/Nos, cell cycle.

Breast cancer initiation depends on a number of parameters, including genetic predisposition, infection, and environmental factors (Harris et al. 1997). Genetic factors relate to only 5% of new cancer cases diagnosed every year (Khoury-Collado & Bombard, 2004) indicating that transformation of the internal milieu by nutritional factors plays a predominant role in the initiation of the disease. Epidemiological evidence suggests that plant foods rich in antioxidant elements may protect or decrease the incidence of breast cancer in women (for a review see Damianaki et al. 2000).

Antioxidants are a heterogeneous class of compounds including vitamins, small (phenolic acids), or complex biomolecules (polyphenols). Polyphenols are usually found under the skin of plants, having a major antimicrobial and antioxidant function (Scalbert et al. 2002). They are purified from a number of plant extracts and brews, such as wine, olive oil, tea and spices. *In vitro*, they act as antioxidants and chelate redox-active transition metals, while they are also implicated in the inhibition of redox-sensitive transcription factors and pro-oxidant enzymes, and in the induction of phase II antioxidant enzymes. Experimental studies show that polyphenols induce cell cycle arrest and apoptosis. Epidemiological studies associate a polyphenol-rich diet with a lower risk for certain diseases, including breast, prostate, lung, colon, stomach, pancreas and liver cancer, osteoporosis and cardiovascular diseases, while studies *in vitro* confirmed these data on different cell lines (Yang et al. 1998; Damianaki et al. 2000; Hibasami et al. 2000; Kampa et al. 2000; Mouria et al. 2002; Matito et al. 2003).

Polyphenols are absorbed in the small intestine (in their generic form or after formation of glucuronides), or in the colon after partial degradation and/or transformation by the colon microflora (Scalbert et al. 2002). They enter the portal circulation and arrive at the liver. Hepatocytes either further biotransform the polyphenols to conjugated molecules (O-glucuronides, sulphate esters and O-methyl ethers) or deglucuronide them back to their native forms (Zhu et al. 1996; Donovan et al. 2001; Scalbert et al. 2002). Conjugated polyphenols are excreted with bile while unconjugated molecules enter the circulation and reach target tissues. Concentrations of circulating polyphenols are therefore far lower than their nominal values in foods. In the rare studies dealing with polyphenol bioavailability, it was noted that their active concentrations in the blood are <1% of their food concentration

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(Donovan et al. 2001). It is therefore interesting to investigate the possible biological actions of these active molecules at concentrations comparable to their calculated circulating concentration (10^{-12}–10^{-6} M) after ingestion of a moderate quantity of wine (1–2 glasses, ~240 ml; Miller & Rice-Evans, 1995). In this context, we investigated the effect of four polyphenols (the stilbene, resveratrol, and the flavonoids quercetin, catechin and epicatechin, major constituents of wine) in the hormone-sensitive human cancer cell line T47D, and provide information on their mode of action.

Materials and methods

Cell cultures

The human T47D cell line (DSMZ, Braunschweig, Germany) was cultured in RPMI 1640, 10% FBS (Gibco BRL, Life Technologies, Paisley, UK) at 37 °C, in an atmosphere of 5% CO_2. Catechin (+), epicatechin (−), quercetin and *trans* resveratrol (prepared from total extract of wine, by Dr Joseph Vercauteren, Laboratory of Pharmacognosy, University of Montpellier I, France) were added to cultures 1 d after seeding. Growth and viability of cells were measured by the tetrazolium salt assay (Mosmann, 1973), while cell cycle was measured by flow cytometry on a Beckton-Dickinson FACSArray apparatus (Beckton-Dickinson, Franklin Lakes NJ, USA) after fixation and PI staining, as previously described (Kampa et al. 2004).

Biochemical and enzymic assays

For the assay of nitric oxide metabolites (NOx), supernatant collected from the proliferation assays was centrifuged and frozen at −80 °C. NO was measured by assaying its stable metabolites NO_2^- and NO_3^-, as previously described (Notas et al. 2001) using the Griess reagent. Nitric oxide synthase (NOS) activity was assayed by the transformation of radioactive arginine to citrulline in cytosolic extracts (Dawes 1972; Kampa et al. 2004).

Multiplex RT-PCR

NOS transcripts after stimulation of T47D cells with polyphenols were measured by semiquantitative multiplex RT-PCR *v.* the constitutive gene of actin. Total RNA was extracted with TRIzol® (Invitrogen, Carisbad CA, USA). Specific DNA primers (500 nM, MWG Biotech, Ebersberg, Germany) and annealing temperatures were used: eNOS (forward, 5′-*AAT CCT GTA TGG CTC CGA GA*-3′, reverse, 5′-*GGG ACA CCA CGT CAT ACT CA*-3′, 58·3 °C), iNOS (forward, 5′-*ACA GGA GGG GTT AAA GCT GC*-3′, reverse, 5′-*TTG TCT CCA AGG GAC CAG G*-3′, 59·1 °C). Actin primers (100 nM, forward, 5′-*GGT GGC TTT TAG GAT GGC AAG*-3′, reverse, 5′-*ACT GGA ACG GTG AAG GTG ACA*-3′) were added to the PCR mix. PCR products were separated in 3% agarose gel and band intensities were measured using the Molecular Analyst Software (BioRad, Hercules CA, USA; Kampa, et al. 2004).

Results

Antioxidant polyphenols reduce cell growth of T47D breast cancer cells and modify the cell cycle

When T47D cells were grown in the presence of polyphenols a dose- and time-related inhibition of growth was observed (Damianaki et al. 2000). The potency of the four compounds was quercetin=catechin=resveratrol >epicatechin. IC_{50} was 0·1±0·2, 0·1±0·7, 0·1±1·2, 0·8±0·2 pM respectively, while the maximum antiproliferative activity was 69%, 73%, 56% and 75%, respectively. We investigated further whether polyphenol incubation could modify cell cycle or induce apoptosis. No significant apoptosis was found after 48-h incubation with polyphenols (10^{-7} M) while a weak reduction of cells in the G_2 phase was observed, resulting in accumulation in S phase, after flavonoids (Fig. 1A). Contrariwise, resveratrol had no effect on the cell cycle.

The system NO/NOS is involved in the antiproliferative activity of antioxidant polyphenols

Polyphenols were found to exert an antioxidant activity in T47D cells (Damianaki et al. 2000). In addition to ROS, another system active on these cells and modulated by simple phenolic structures (Kampa et al. 2004) is the NO/NOS system. NOx production was reduced after long (5-d) treatment (Fig. 1B). Catechin and resveratrol were strong inhibitors of NO production, even at 10^{-12} M. The decrease was cumulative and apparent after at least 24 h of treatment (results not shown). Epicatechin also kept NOx below basal levels, but its inhibitory effect diminished proportionally to its concentration. Contrariwise, quercetin upregulated NOx production, at 10^{-12}–10^{-10} M. At higher concentrations, it reduced NOx production by 30%.

The inhibitory effect on NO could be due to a decrease in NOS activity, or to a transcriptional regulation of NO synthases. Assays of NOS activity showed a significant decrease in total (eNOS and iNOS) activity after treatment for 24 h or longer (Fig. 1C). Surprisingly, these agents induced a transient increase in NOS activity, with resveratrol and quercetin reaching a maximum at 8 h and epicatechin and catechin at 12 h of treatment, followed by a significant fall thereafter. This bimodal NOS activity induced by polyphenols indicates a possible dual action of the agents, interfering with both NOS activity and its transcriptional regulation. We therefore performed RT-PCR experiments for NOS isoenzymes (eNOS and iNOS). Our results indicate that eNOS and iNOS transcription was induced, after 12 h of treatment (Fig. 2A), returning to the control levels, with two notable exceptions: (1) cells treated with resveratrol present a prominent increase of eNOS

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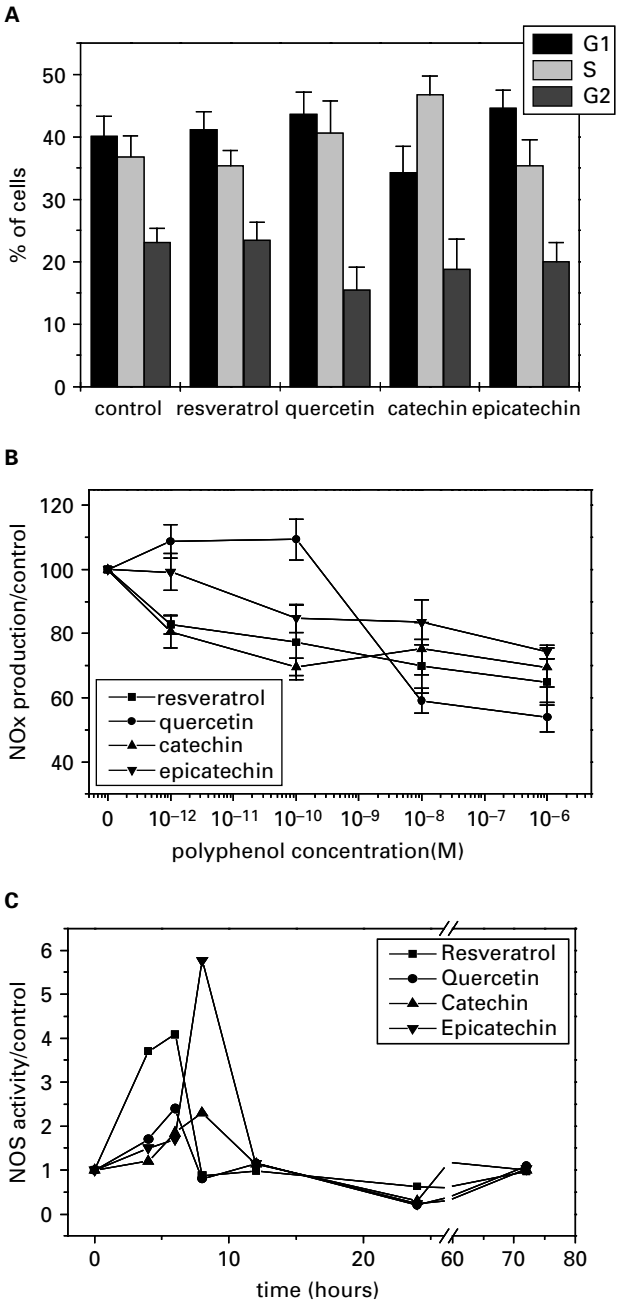

Fig. 1. Effect of wine polyphenols on cell cycle and NO/NOS activity **A**. Cell cycle analysis of T47D cells cultured for 48 h in the presence of 10^{-7} M indicated polyphenols, after fixation, PI staining and analysis by flow cytometry. Analysis of cell cycle was performed with the CELLQuest (Beckton Dickinson) and ModFit LT (Verify Software, Topsham MN, USA) software. **B**. NOx production by T47D cells, incubated for 5 d in the presence of the indicated concentrations of polyphenols. NOx was assayed with the Griess reagent in the culture medium. **C**. NOS activity of T47D cells, incubated for the indicated time-periods in the presence of 10^{-7} M-polyphenols. In B and C, results were normalized by a division of the corresponding control values. Bars indicate mean ± SEM of three different experiments in triplicate.

after 24 h (8 × control levels) and (2) cells treated with quercetin, presenting a trend to increase iNOS values even after 24 h.

The role of the NO/NOS system in cancer is not very well identified, with reports indicating a positive and a negative role of this transcellular mediator. To identify whether NO/NOS modulation by polyphenols might have a role in cell growth, we incubated cells in the presence of SNAP [(*S*)-Nitroso-*N*-acetylpenicillamine], a stable analogue of endogenous S-nitroso compounds, as a source of NO and the non-selective NOS inhibitor L-NAME [N^G-Nitro-L-arginine methyl ester hydrochloride] (both from Tocris, Bristol, UK). T47D cells treatment with polyphenols (10^{-7} M), SNAP or L-NAME (0·5 mM) resulted in reduced cell proliferation. Co-treatment experiments (Fig. 2B) showed that polyphenols reversed the SNAP effect on cell proliferation and sustained the decrease of cell number induced by NOS inhibitor L-NAME. Considering the above results, we assumed that polyphenols affect T47D proliferation *via* a long-term attenuation of the NO system.

Discussion

Experimental and epidemiological studies indicate that antioxidant food micronutrients could have antimitotic activities, interfering with cancer initiation, progression or mortality (for a review see Kampa et al. 2002). Polyphenols, antioxidant constituents of different foods and beverages, including grapes and wine, have been intensively studied in the last decade. The wine polyphenol resveratrol (a tri-hydroxy-stilbene) at micromolar concentrations was reported to possess, in addition to its antioxidant actions (Miller & Rice-Evans, 1995; Soleas et al. 1997), a variety of effects on different systems, including antimutagenic or antiproliferative properties (Jang et al. 1997). In addition to resveratrol, (red) wine contains high concentrations of the flavonoids catechin, epicatechin, and quercetin, at relative amounts 160, 70, and 9 times greater than resveratrol (Miller & Rice-Evans, 1995). In the present work, we assayed the effect of these four compounds on T47D breast cancer cell growth, at concentrations compatible with those found in biological fluids after moderate wine consumption (see above). We report that they can decrease cell growth at picomolar concentrations, in a dose- and time-dependent manner. Cell-cycle analysis revealed that flavonoid treatments result in cell-cycle arrest in the S phase, while resveratrol had no effect. Similar results have been reported for MCF-7 cells (Pozo-Guisado et al. 2002; Kim et al. 2004). Contrariwise, at micromolar concentrations, cells undergo apoptosis, and usually they arrest at G1/G0 or G2/M.

The mode of action of polyphenols is not well established. All four polyphenols interact with the oestrogen receptor (ER) (Gehm et al. 1997; Kuo et al. 1997; Damianaki et al. 2000). In addition, μM concentrations of resveratrol activate ER-independent pathways, which are

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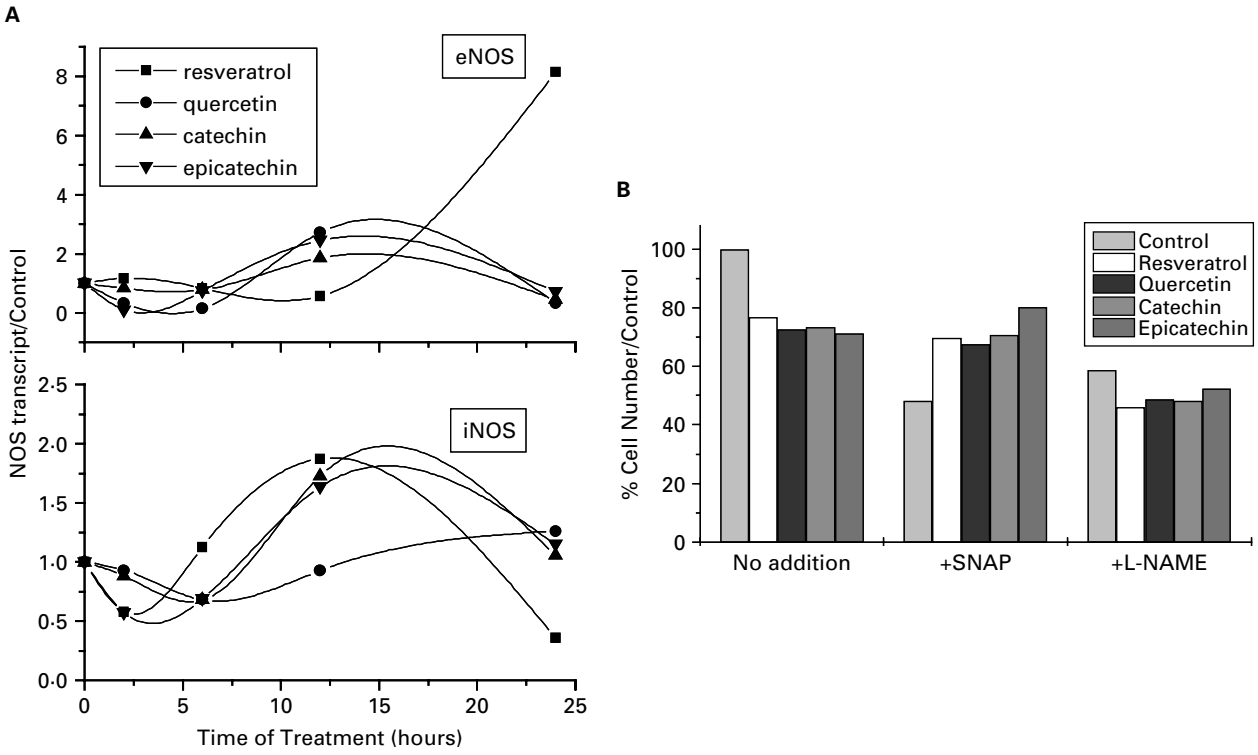

Fig. 2. Effect of wine polyphenols on NOS isoenzyme transcription (A) and the effect of NO system on T47D cell growth **A.** Effect of 10^{-7} M-polyphenols on the transcription of endothelial (eNOS, upper panel) and inducible (iNOS, lower panel) nitric oxide synthase. Cells were incubated for the indicated time periods, mRNA was isolated and reversed transcribed. Results of multiplex (NOS isoenzyme+actin) PCR, normalized for the enzyme modulation in control (non-treated) cells. **B.** Effect of SNAP [(*S*)-Nitroso-*N*-acetylpenicillamine], a NO-donor and L-NAME [N^G-Nitro-L-arginine methyl ester hydrochloride], a non-selective NOS inhibitor, on T47D cell growth. Control (non-polyphenol-treated) and polyphenol-treated (10^{-7} M, 6 d) cells are presented. Results were normalized as per non treated cells.

also involved in cell growth inhibition (Levenson et al. 2003). In mammary cell lines S30, T47D and LY2, resveratrol exerts mixed oestrogen agonist/antagonist activities in the absence of oestradiol but, in its presence, an anti-oestrogenic action is detected (Bhat et al. 2001). In MCF-7 cells, resveratrol reverses E2 signalling by interfering with post-translational modifications of p53, leading to apoptosis (Zhang et al. 2004) and modifies the expression of a gene panel, interacting with BRCA1 signalling, BRCA1, BRCA2, ERalpha, ERbeta, p53, p21(waf1/cip1), CBP/P300, RAD51, pS2 and Ki67 (Le Corre et al. 2004). Moreover, E2 and quercetin can regulate the expression of growth-related genes, such as c-fos, *via* the G protein-coupled receptor homologue GPR30 (Maggiolini et al. 2004). Finally, polyphenols can interact with NO synthesis (Kawada et al. 1998; Visioli et al. 1998; Kampa et al. 2000; and the present report) which modulates subsequently a number of transcription factors, coordinating the spatial and temporal patterns of gene expression.

The role of NO in cancer is ubiquitous (see Wink et al. 1998, for a discussion). NO is reported to inhibit cell proliferation, to induce differentiation, and to decrease the metastatic spread of different tumour cell lines (Bani et al. 1995; Reveneau et al. 1999) although this effect seems to be related to the type and the origin of cells (Adami et al. 1998). An increased expression of NOS is reported in different human cancers (Ambs et al. 1998) suggesting that NO synthesis may facilitate tumour metastasis and angiogenesis (Thomsen et al. 1995; Duenas-Gonzalez et al. 1997). A more detailed analysis of NOS expression pattern revealed a discrete role for the two different isoforms of NO synthases (eNOS and iNOS). Expression of inducible nitric oxide synthase (iNOS) by tumour cells has been suggested to abrogate metastasis in several tumour models, whereas constitutive NOS expression correlated positively with tumour grade in human breast carcinoma (Tschugguel et al. 1999).

Polyphenols can interact with the production (Visioli et al. 1998; Kampa et al. 2000) or with some biological effects of NO (Ahmad et al. 1997; Ho et al. 1997). Our results indicate that all four polyphenols inhibit cell growth by modifying NOS expression and NOS activity. Experimental data support a cell-specific mechanism of NO/NOS regulation by wine antioxidants. Quercetin, galangin, apigenin, and naringenin exerted anti-inflammatory properties in macrophages by markedly decreasing iNOS expression and PGE2 release (Raso et al. 2001). Furthermore, treatment of RAW 264.7 macrophage cells with

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quercetin inhibited LPS-induced NO production and reduced iNOS expression (Chen et al. 2001). Resveratrol, through the inhibition of ER-independent NF-kappaB mobilization, produced the same effect in leukaemic B-cells (Cho et al. 2002; Quiney et al. 2004). In hepatocellular carcinoma cells a strong induction of both eNOS and iNOS mRNA transcripts after a 2-h treatment with polyphenols was found with a subsequent increase in NOx levels (G. Notas, unpublished observations). Similar results are reported for pulmonary epithelial cells (Hsieh et al. 1999a, b).

The role of NO in the mediation of the polyphenol antiproliferative effect is ambiguous. In spontaneous mammary tumours in C3H/HeJ mice, the sequential activation of NOS, GC and MAPK pathways mediated C3L5 cell migration, an essential step in invasion and metastasis, while L-NAME prevented this effect (Jadeski et al. 2003). Interestingly, DETA-NONOate, an NO donor, induced cytostasis in the human breast cancer cells MDA-MB-231, in G(1) phase of the cell cycle, associated with the down-regulation of cyclin D1 and hypophosphorylation of the Rb protein (Pervin et al. 2001). Furthermore, in human breast cancer cell lines (BT-20 or MCF-7), NO induced apoptosis and triggered a time-dependent activation of caspases 1, 3, and 6 (Umansky et al. 2000). Here we report similar results, as both NO donor (SNAP) and NOS inhibitor (L-NAME) suppressed cell proliferation. However, the cytostatic effect of polyphenols was reversed only by elevation of NO levels (Fig. 2C).

The mechanism underlying the regulation of NO/NOS system by polyphenols is not clear. Polyphenols could interact with NOS enzyme molecules. Nevertheless, the transcriptional activation of NOS isoforms implies a more complicated pattern of interaction and a more complicated action on NO system through multiple cross-talk points. In addition to an antioxidant effect, there is an emerging view that polyphenols and their metabolites do not act as conventional hydrogen-donating antioxidants but may exert modulatory actions in cells through actions at protein- and lipid-kinase signalling cascades (Williams et al. 2004). Indeed, resveratrol decreased both Akt (essential for the mobilization of the Raf-MEK-ERK signalling pathway, leading from proliferation to cell cycle arrest; Zimmermann & Moelling, 1999) and FAK phosphorylation in T47D cells (Brownson et al. 2002), stimulated PI-3 kinase activity in MCF-7 cells (Pozo-Guisado et al. 2004) while both resveratrol and quercetin up-regulated p21(CIP1)/WAF1 protein levels in an ER-independent manner, resulting in inhibition of cell growth (Choi et al. 2001; Levenson et al. 2003). Recently, breast cancer cell proliferation was found to depend on the NO/Rb pathway, cell migration and angiogenesis were blocked *via* VEGF/PI3K/AKT/NO/ICAM-1 pathway, while breast cancer cell apoptosis was induced through the NO/ROCK/FOXO3a signalling cascade (Radisavljevic, 2004a, b). Even if the full mechanism of uptake, transport, metabolism and intracellular signalling of polyphenols remains unidentified, as these agents influence cell viability and differentiation, our results provide evidence that NO could mediate the biological effects of flavonoids.

Polyphenols signalling include also the activation of transcription factors. As described above, polyphenols can act as agonists or antagonists of ER, or the AhR receptor systems (Kampa et al. 2004). These effects could be mediated also *via* NO system, as NO suppression correlates with p53 induction and p53 stabilization, restoring tumour suppression function of p53 (Calmels et al. 1997). There is also evidence that polyphenols down-regulate hypoxia-inducible factor-a (HIFa) expression, through AKT and MAPK cascades (Cao et al. 2004). HIF-a stability depends also on minor changes in NO concentration and cell confluency (Thomas et al. 2004). Hence, flavonoids could target multiple intracellular systems, in order to access the transcriptional machinery and determine the fate of cancer cells.

Taking into account the concentrations of these polyphenols in red wine (5·4 μM and 302 μM of resveratrol and quercetin, respectively; Miller & Rice-Evans, 1995) and the volume of the interstitial fluid (~40 l), after the ingestion of 0·5 l of wine, their concentrations could reach 70 nM and 3800 nM, assuming no metabolism or excretion of the substances. Therefore a possible effect of these substances, after ingestion of a moderate quantity of wine, must be detected at the low nanomolar or even the picomolar range, as reported here. The present results suggest a potential importance of wine ingestion and possibly the consumption of other polyphenol-rich dietary foods and drinks in the control of breast cancer cell growth, although further investigations are necessary to confirm their effect and to elucidate their mode of action.

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