Wine Phenolic Antioxidants Inhibit AP-1 Transcriptional Activity

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Some of the beneficial effects of moderate wine consumption may be related to the antioxidant properties of polyphenolic compounds containing tannins, flavonoids, and phenolic acids. Cellular actions have recently been reported and may involve the modulation of transcriptional factors such as AP-1 (activator protein-1), which controls the expression of various genes implicated in inflammation processes, cell differentiation, and proliferation. The aim of this study was to evaluate the modulation of AP-1 activity by the phenolic acids (gallic, caffeic, protocatechic, paracoumaric, sinapic, and ferulic acids) that are present in wine and to compare their modulating pathways to those of lipophilic or hydrophilic "chain-breaking" antioxidants (such as DL-α-tocopherol or trolox) vitamin C, nitric oxide, and reduced glutathione. AP-1 response was studied on a cell line (MTLN) derived from MCF-7 cells transfected with luciferase gene under TRE sequence control. After stimulation by phorbol 12-myristate 13-acetate (PMA; 100 nM, 6 h, 10^{-7} M), luciferase activity was determined by a luminescence method in the presence of luciferine/coenzyme A solution using a luminometer (LKB 1251, Finland). Antioxidants to be tested were incubated with cells in the presence or absence of PMA. Stimulation with PMA resulted in an AP-1-mediated increase in luciferase gene expression corresponding to an 8-fold increase in luciferase activity. After stimulation by PMA, a dose-dependent inhibition of AP-1 was observed with the six phenolic acids in the 20 nM−20 μM concentration range: gallic acid > caffeic > protocatechic, paracoumaric, sinapic acids > ferulic acid. Inhibition was more pronounced with phenolic acids than with DL-α-tocopherol ($IC_{50} = 5 \pm 4.5$ μM for gallic acid vs 85 ± 11 μM for vitamin E). None of the hydrophilic antioxidants inhibited PMA-induced AP-1 activation. None of the antioxidants tested in the absence of PMA stimulation induced any activation or inhibition of AP-1. Our results suggest that phenolic acids may act directly on cell signaling via inhibition of AP-1 transcriptional activity. In addition to preventing LDL oxidation in the arterial wall, our observations indicate that phenolic acids have a cell-mediated capacity to prevent some of the processes involved in atherosclerosis in a plasma concentration range compatible with nutritional intakes.

Keywords: *AP-1 transcriptional factor; red wine antioxidants; atherosclerosis*

INTRODUCTION

A regular and moderate consumption of wine may account in part for the decreased risk of coronary heart diseases observed with the "Mediterranean diet" (*1*, *2*). Growing evidence points to a major role for the phenolic compounds (PC) found in wine. PCs are products of the secondary metabolism of plants. Wine PCs have long been divided into three main classes: flavonoids (flavanols, proanthocyanidins, flavonols, and anthocyanins) with a heterocycle (2-phenylbenzopyran) structure; more simple PCs with a mono- or diphenyl structure; and tannins, which are the most complex PCs of much higher molecular weight (*3*−*5*). Belonging to the second class, phenolic acids are monophenyl components present in both red and white wine, whereas the other aforementioned PCs are essentially absent or in trace amounts in white wine (except for flavonols) (*4*). Phenolic acids are mainly found in the tartaric ester form (caftaric acid or coutaric acid) or as acylated residues of anthocyanin glycosyl substituent in the genus *Vitis*. They are released in free form during vinification (*6*). Gallic acid is the main phenolic acid (1−18 and 2−130 mg/L in white and red wine, respectively) (*7*). Caffeic acid is also present in red and white wine, but *p*-coumaric acid, ferulic acid, sinapic acid, and protocatechic acid are found in red wine only (*8*, *9*).

In vivo, the beneficial role of moderate red wine consumption may be related to the well-known antioxidant properties of polyphenols (*10*), and it has been mainly associated with an increase in plasma antioxidant capacity (*1*, *11*−*16*). Moreover, we previously reported that supplementation with red wine phenolic compounds for 14 days significantly increased antioxidant capacity of plasma and LDL vitamin E without affecting ex vivo LDL copper-induced oxidizability (*17*). By contrast, in vitro incubation of LDL with polyphenolic compounds significantly prevented LDL oxidation, in agreement with other reports (*18*−*23*).

In addition to this well-known ROS (reactive oxygen species) scavenger activity (*24*−*26*), cellular effects have been recently reported including inhibition of superoxide anion production (*27*), prevention of platelet aggregation

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(*28*, *29*), and inhibition of cell tumor proliferation (*30*−*32*). It could be postulated that the antiproliferative effect is dependent on inhibition of transcriptional factors such as nuclear factor κB (NF-κB) or activator protein-1 (AP-1). Indeed, the ubiquitous regulatory protein complex AP-1 is implicated in the inducible expression of a wide variety of genes involved in the regulation of proliferation and apoptosis as well as in cellular response to stress or in inflammation processes. The AP-1 factor is composed of protein products of members of the Fos [vFos, cFos, Fos B, Fra-1/2 (Fos-related antigen)] and Jun (vJun, cJun, Jun B, Jun D) families (*33*). The resulting heterodimeric (Fos−Jun or Jun−Fra) or homodimeric (Jun−Jun) complexes support differential transcriptional activities after binding to its palindromic DNA sequence (TRE for tetradecanoylphorbol-13-acetate responsive element) (*34*−*36*). It is generally assumed that AP-1 activation is ROS-dependent and could be modulated by antioxidants (*37*, *38*). In this context, AP-1 inhibition by polyphenols has recently been reported (principally epigallocatechin-3-gallate and flavins) isolated from green or black tea (*39*−*45*).

The aim of the present study was to evaluate the modulation of AP-1 transcriptional activity by monocyclic phenolic antioxidants originated from red wine in comparison with DL-α-tocopherol (vitamin E), trolox (6-hydroxy 2,5,7,8-tetramethyl chroman 2-carboxylic acid), and hydrophilic antioxidants such as vitamin C, glutathione (GSH), and a nitric oxide (NO) donor (*S*-nitroso-L-glutathione).

MATERIALS AND METHODS

Materials. Luciferin, 12-*O*-tetradecanoyl phorbol-13-acetate (PMA), coenzyme A, $MgCL_2$, $6H_2O$, K_2HPO_4, KH_2PO_4, ascorbic acid, Triton X-100, and NaOH were purchased from Sigma (Sigma Chemical, L'Isle d'Abeau, France). The DL-α-tocopherol was obtained from Merck (Darmstadt, Germany). DMEM (Dulbecco's modified eagle medium), phenol red, fetal calf serum, penicillin−streptomycin, and fungizone were from Life Technologies (Life Technologies, Cergy Pontoise, France). NO given as NO donor (*S*-nitroso-L-glutathione) and reduced glutathione (GSH) were from Alexis (Alexis Biochemical, Paris, France). Trolox was from Aldrich Chemical (Strasbourg, France). Cell culture flasks (150 cm^2) and plates were from Dutscher (Brumath, France). The six phenolic acids, two from the benzoic series (protocatechic acid: 3,4-dihydroxy benzoic acid; gallic acid: 3,4,5-trihydroxy benzoic acid) and four from the cinnamic series (*p*-coumaric acid: 4-hydroxycinnamic acid; caffeic acid: 3,4-dihydroxycinnamic acid; ferulic acid: 3-methoxy 4-hydroxycinnamic acid; sinapic acid: 3,5-methoxy 4-hydroxycinnamic acid) (Figure 1) were synthesized by the group of S. Labidalle (Centre d'Oenologie, UFR de Pharmacie, Toulouse, France) (*27*). The purity of the synthesized compounds was checked by IR, ^1H RMN, and ^{13}C RMN spectra as well as by thin-layer chromatography and HPLC. Element analysis was in complete agreement with theoretical values.

Establishment of a Stable Bioluminescent Cellular Model. The MTLN (MCF-7 transfected with luciferase and neomycin resistance gene) cell line, derived from MCF-7 cells stably transfected with p(TRE)3-tk-Luc and pAG60, which confers resistance to the neomycin analogue G418, has been previously described by Pons et al. (*46*). In this model (*47*), a reporter gene, the firefly luciferase, is under the control of three TPA-responsive elements (TRE). Briefly, the double-strand TRE sequence (5′-TCGACA*TGAGTCA*GCGCC-3′ and 5′-GATCCGCGC*TGACTCA*TG-3′) was first cloned in the *Sal*I−*Bam*HI sites of the pUC vector. In a second step, the *Bam* fragment of pVit-tk-Luc containing the herpes simplex virus promotor for thymidine kinase (tk), the firely luciferase structural gene (Luc), the SV-40 small-t-antigen intron, and an SV40 polyadenylation signal were inserted downstream of the TRE sequence. In a third step, two other TRE sequences were added to the *Sal*I site of the plasmid. Transfection experiments with p(TRE)3-tk-Luc and pAG60 were performed according to the calcium phosphate procedure. Transfected cells were first selected according to their G418 resistance; then positive clones were detected by determining photon emission in response to PMA by means of an ultrasensitive photon-counting imaging camera monitored by a computer-assisted image processor (Argus 100, Hamamatsu Photonics, Japan) (*46*, *47*).

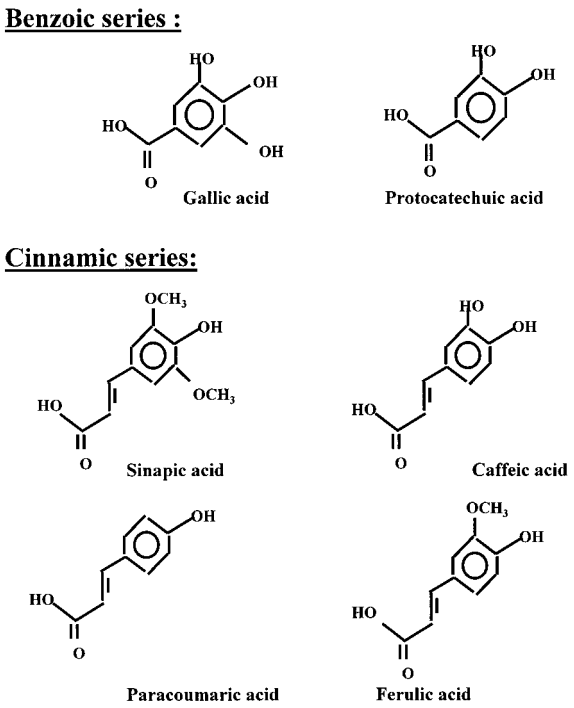

Figure 1. Structure of the differents phenolic acids tested on MTLN cells.

MTLN cells were cultured in phenol red DMEM supplemented with 5% FCS, 1% penicillin, and 0.2% fungizone in a humidified atmosphere containing 5% CO_2 at 37 °C. MTLN cells were used underneath 20 passages in the absence of G418.

Assessment of AP-1 Transcriptional Activity. MTLN cells were seeded at a density of 125 000/mL in 12-well culture cluster plates and cultured in DMEM with phenol red supplemented with 5% FCS for 48 h. AP-1 stimulation was performed after 1−8 h of PMA incubation (10^{-7} M). Antioxidant substances to be tested were dissolved in ethanol−water (9:1) solution and were then incubated in the presence of PMA at increasing concentrations. Ethanol final concentration never exceeded 1%. All separate experiments (six per phenolic acid) were carried out in duplicate and compared to control cells incubated with vehicle [ethanol−water (9:1) solution] without antioxidant or PMA. To determine the direct effect of antioxidants on AP-1 activation, the same experiments were performed in the absence of PMA.

At the end of incubation, the culture medium was removed, and cells were washed with 2 mL of the luminescence buffer (8 mM $MgCl_2$, $6H_2O$, and 15 mM potassium phosphate, pH 7.4). Cells were harvested by gentle scraping after 20 min of incubation in the same buffer with the addition of 2 mM ATP, 1% Triton X-100, and 1 N NaOH. Luciferase activity was measured on an aliquot of the cellular suspension (200 μL), and luminescence emission was assessed for 1 min after injection of 100 μL of luminescence buffer containing 0.6 mM coenzyme A and 0.6 mM luciferin. Luminescence was measured using a luminometer LKB 1251 (Wallac, Finland) (*47*). Protein concentration was determined on the supernatant fraction (*48*) so that protein-normalized luminescence signals were obtained.

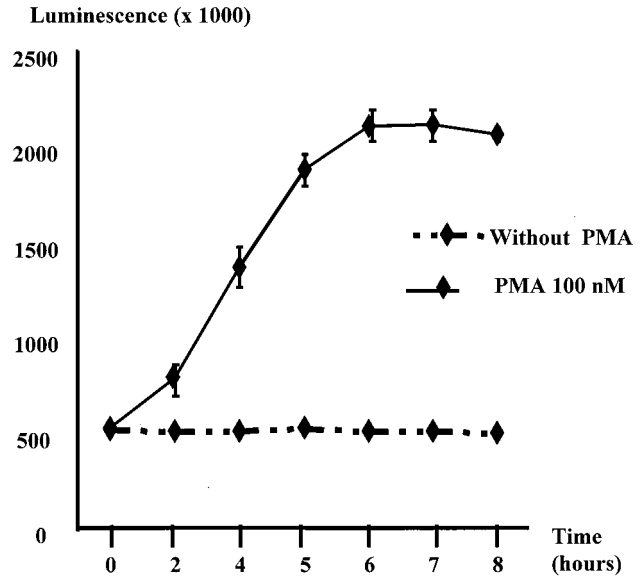

Figure 2. PMA-induced luciferase activity in the MTLN model. AP-1 stimulation was performed after incubation with or without PMA (10^{-7} M).

Statistical Analysis. All data are expressed as percentage of inhibition of PMA-induced AP-1 activity provided by the substances to be tested. Antioxidant effects are expressed as means ± SEM of six independent experiments. The statistical significances between means were assessed by a Student's test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

PMA-Induced Luciferase Expression. PMA induced an increase in luciferin-dependent luminescence that became significant after a 4-h incubation. An 8-fold increase in luciferase activity was observed after 6 h (Figure 2). In the absence of PMA, no modification of luminescence activity was shown.

Inhibition of the PMA-Induced Luciferase Expression by DL-α-Tocopherol. In the presence of PMA, low concentrations (20 and 200 nM) of DL-α-tocopherol did not influence the AP-1-induced luciferase expression. By contrast, a dose-dependent inhibition of AP-1-induced luciferase expression was observed between 2 and 100 μM with an IC50 of 85 ± 11 μM (Figure 3).

Inhibition of PMA-Induced Luciferase Expression by Phenolic Acids. All the tested phenolic acids induced a dose-dependent inhibition of AP-1-induced luciferase expression. At antioxidant concentrations higher than 200 nM, the rank order of potency was as follows: gallic > sinapic > caffeic = protocatechic = p-coumaric > ferulic acid (Figure 4). For concentrations inferior to 200 nM, sinapic acid exhibited the highest inhibitory potency ($p < 0.01$ for 20 and 200 nM). All the phenolic acids, except ferulic acid, had a more pronounced inhibitory effect than vitamin E. Indeed, the IC_{50} obtained with gallic acid was 5 ± 4.5 μM as compared with 85 ± 11 μM with DL-α-tocopherol (Figure 5) ($p < 0.01$), and inhibition by gallic acid was significant at 2 μM.

Inhibition of PMA-Induced Luciferase Expression by Hydrophilic Antioxidants. At the same concentrations (20 nM−100 μM), trolox, a water-soluble analogue of vitamin E, and the other hydrophilic antioxidants (vitamin C, GSH, and NO donor) did not inhibit AP-1 transfected cells (Figure 6).

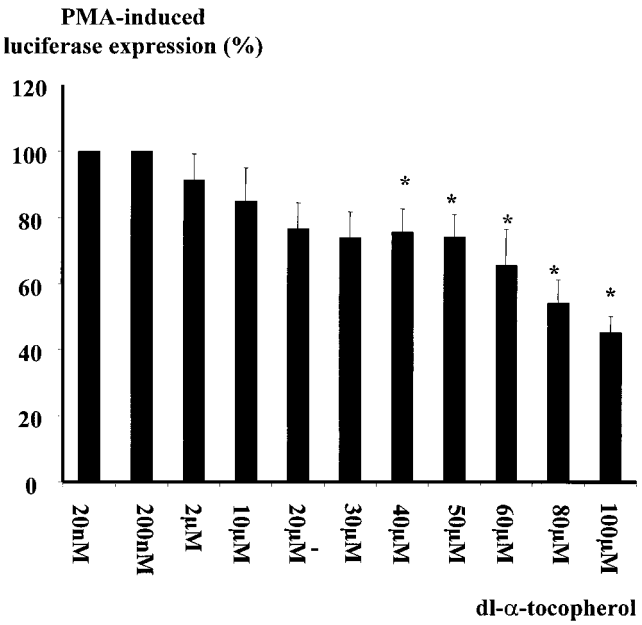

Figure 3. Inhibition of the PMA-dependent AP-1 stimulation by DL-α-tocopherol ($n = 6$).

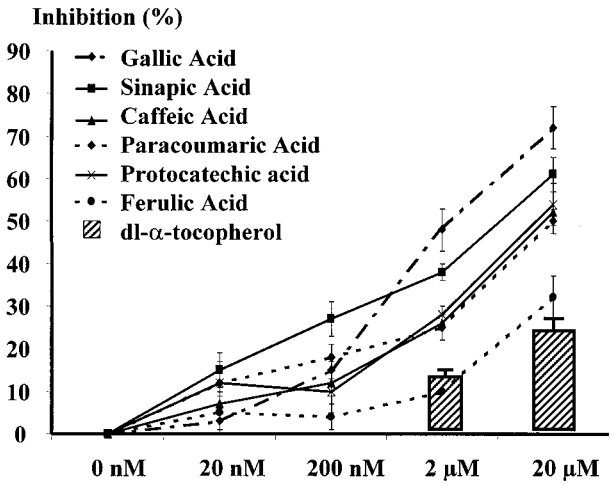

Figure 4. Inhibition of the PMA-dependent AP-1 stimulation by phenolic acids ($n = 6$). The comparative effect of DL-α-tocopherol is represented at 2 and 20 μM.

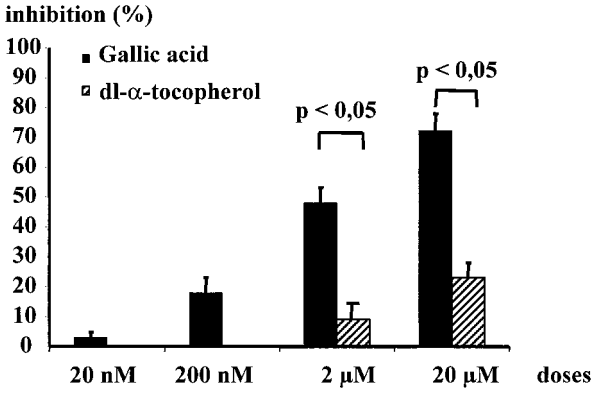

Figure 5. Comparative inhibitory effects (%) of gallic acid and DL-α-tocopherol on PMA-dependent AP-1 activation ($n = 6$).

Phenolic Antioxidants per se Did Not Modulate Luciferase Expression. In the absence of PMA, all tested antioxidants did not appear to exert any effect on the AP-1-induced luciferase expression. Incubation with phenolic acids at increasing concentration (from

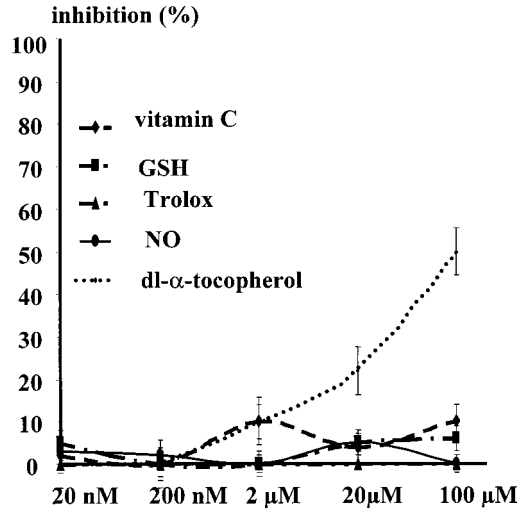

Figure 6. Lack of effect of hydrophilic antioxidants on the PMA-dependent AP-1 activation ($n = 6$). For comparaison, the DL-α-tocopherol inhibitory effect is represented.

20 nM to 20 μM) did not influence the AP-1-mediated luciferase activity. Even at 20 μM phenolic acids, inhibition was not significant: 4, 5, 0, 3, 4, and 0% for caffeic, ferulic, gallic, paracoumaric, protocatechic, and sinapic acids, respectively. A similar lack of activity was observed with the other antioxidants tested: 2, 4, 4, 1, and 2% for DL-α-tocopherol, trolox, vitamin C, GSH, and NO, respectively.

DISCUSSION

Our results show a dose-dependent inhibitory action of phenolic acids on AP-1-induced luciferase expression. The inhibition of AP-1 transcriptional activity was obtained at lower concentrations with wine phenolic compounds than with vitamin E, and it should be pointed out that inhibition was detectable with sinapic acid at concentrations as low as 20 nM. Moreover, in the same experimental conditions, hydrophilic antioxidants (trolox, NO donor, GSH, and vitamin C) did not inhibit luciferase expression.

Oxidative stress, including oxidized LDL (*49*, *50*), is widely recognized as a major activator pathway of AP-1 transcriptional activity (*51*−*56*). Moreover, some antioxidants have been reported to prevent AP-1 activation (*50*, *53*, *56*−*58*).

In our study, wine phenolic acids and vitamin E inhibited PMA-induced AP-1 activation in a dose-dependent manner and in a concentration range compatible with nutritional intakes (*15*): serum levels of vitamin E (33 ± 10 μM) (*59*) or relevant phenolic compounds (2−10 μM) (*15*). The IC_{50} was found to be 85 ± 11μM for DL-α-tocopherol and 5 ± 4.5 μM for gallic acid (Figure 4).

The inhibition of AP-1-induced luciferase expression seems to parallel the chemical antioxidant potency mainly determined by hydroxyl function number (*10*). The three substitutions with hydroxyl groups confer on gallic acid the greatest antioxidant potency among the phenolic compounds tested (3.0 mM expressed as TEAC for trolox or vitamin E equivalent antioxidant activity) (*10*, *60*). Sinapic acid, which also contains three substituents on the cyclic structure but only one hydroxy radical, exhibits a lower antioxidant capacity similar to the one or two hydroxy derivatives such as protocatechic acid, caffeic acid, ferulic acid, and *p*-coumaric acid (*10*). The antioxidant capacity has been documented in a heme/H_2O_2-dependent LDL oxidation model, showing that modification of caffeic acid by a methyl substitution of one hydroxyl group (ferulic acid) or the replacement of one OH group with an H atom (*p*-coumaric acid) results in lowered antioxidant activity (*61*). The lowest antioxidant capacity was observed with ferulic acid, and the same finding was previously reported by our group in copper-induced LDL oxidation (*17*) and in modulation of NADPH oxidase (*62*). Recently, we showed that gallic acid had the highest specific antioxidant activity at submicromolar concentrations and that gallic and caffeic acids were the most potent antioxidants in Cu^{2+} or AAPH oxidation generation systems (*63*).

Red wine phenolic compounds may modulate AP-1 transcriptional activity at any of three well-known regulation steps: PKC activation, MAPK (mitogen-activated protein kinase) pathways, and/or binding of AP-1 to DNA. Astruc et al. (*47*) previously showed that PKC activation is a specific key event in the activation of luciferase gene expression in the MTLN cell line. Indeed, neither the TPA 4α−isomer (known to be inefficient in PKC activation) nor the PKA pathway modulators induce luciferase expression. In addition, PKC inhibition by Staurosporine (1−30 nM) or GF109203X (25−1000 nM) prevents AP-1 transcriptional activity (*47*). On the other hand, PKC activation could be prevented by antioxidants, including vitamin E (*64*−*66*) and flavonoids, which include quercetin (*67*, *68*). Although, there is no report on a direct phenolic acid-dependent PKC inhibition, this modulation remains a suitable hypothesis to explain AP-1 inhibition by wine phenolic acid in our model.

The MAPK pathway includes JNK (c-Jun NH_2-terminal kinase), ERK (extracellular signal regulated kinase), and the p38 kinase (*69*−*71*). Oxidative stress is able to induce MAPK activation (*72*−*77*). This ROS-dependent activation of MAPK may be widely prevented by lipophilic antioxidants, including vitamin E (*78*). Recently, it has been reported that flavonoids from green and black tea inhibit p38 (*40*) and ERK (*44*) activation. By contrast, JNK could be directly stimulated by phenolic antioxidants (*55*, *79*, *80*). Since phenolic compounds alone did not induce any luciferase activity in MTLN, a direct stimulatory effect on AP-1 could be ruled out. In contrast, our result suggests that phenolic acid, as with flavonoids and vitamin E, could decrease the MAPK pathway.

Finally, the binding to TRE is known to be dependent on the reduced state of two cystein residues on Jun and Fos. This could result in an up-regulation of AP-1 activity in the presence of antioxidants such as *N*-acetyl cystein (NAC) or thioredoxine (*81*). Similar increases in AP-1 binding have been described with phenolic antioxidants such as butyl hydroxytoluene (BHT) and butyl hydroxyanisole (BHA) (*82*), DL-α-tocopherol (*83*−*86*), and *RRR*-α-tocopheryl succinate (*87*). This mechanism played only a minor role in the present study since only an inhibitory effect occurred. Transcriptional activity is dependent on the equilibrium of both the Jun−Fos and the Jun−Fra heterodimers. In addition, this increase in DNA binding does not necessarily result in an enhancement of gene transcription (*88*). Further experiments including PKC translocation and phosphorylation assay (*89*), and AP-1 gel shift assay would more clearly specify the molecular mechanism of AP-1 transcriptional inhibition by phenolic compounds.

In summary, AP-1 transcriptional activity appears to be the result of opposite modulations targeted at one or several of the three major steps of regulation that are dependent and regulated by cell redox status (*90*).

The potential inhibition of AP-1 transcriptional activity could be relevant in the prevention of atherosclerosis. On one hand, vitamin E (*91*−*93*) and phenolic acids (*17*, *94*, *95*) prevent LDL oxidation, which is considered as a key event in atherosclerosis (*96*, *97*). Moreover, DL-α-tocopherol (*89*, *98*, *99*) and phenolic acids (*62*) may inhibit ROS production, and this cell-mediated effect could be in part independent of the chemical antioxidant capacity. Finally, our results show that vitamin E and wine phenolic compounds could negatively regulate AP-1 transcriptional activity. This down-regulation is compatible with the beneficial effects against atherogenesis since, in association with NF-κB, AP-1 regulates the expression of genes implicated in the atherogenic process such as proinflammatory cytokines, chemokines, adhesion molecules, cytokine receptors, NO synthases, lipoxygenases, cyclooxygenases, growth factors, and antioxidant enzymes (*100*).

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