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RESEARCH ARTICLES

Protocatechuic acid induces antioxidant/detoxifying enzyme expression through JNK-mediated Nrf2 activation in murine macrophages☆

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Abstract

Protocatechuic acid (PCA) is a main metabolite of anthocyanins, whose daily intake is much higher than that of other polyphenols. PCA has biological effects, e.g., it induces the antioxidant/detoxifying enzyme gene expression. This study was aimed at defining the molecular mechanism responsible for PCA-induced over-expression of glutathione (GSH) peroxidase (GPx) and GSH reductase (GR) in J774 A.1 macrophages. New evidence is provided that PCA increases GPx and GR expression by inducing C-JUN NH_2-terminal kinase (JNK)-mediated phosphorylation of Nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2). RNA and proteins were extracted from cells treated with PCA (25 μM) for different time points. Quantitative real-time polymerase chain reaction and immunoblotting analyses showed a rapid increase in mRNA (>60%) and protein (>50%) for both the enzymes. This was preceded by the up-regulation of Nrf2, in terms of mRNA and protein, and by its significant activation as assessed by increased Nrf2 phosphorylation and nuclear translocation (+60%). By using specific kinase inhibitors and detecting the activated form, we showed that JNK was the main upstream kinase responsible for Nrf2 activation. Convincing evidence is provided of a causal link between PCA-induced Nrf2 activation and increased enzyme expression. By silencing Nrf2 and using a JNK inhibitor, enzyme enhancement was counteracted. Finally, with the ChIP assay, we demonstrated that PCA-activated Nrf2 specifically bound ARE sequences in enzyme gene promoters. Our study demonstrates for the first time that PCA improves the macrophage endogenous antioxidant potential by a mechanism in which JNK-mediated Nrf2 activation plays an essential role. This knowledge could contribute to novel diet-based approaches aimed at counteracting oxidative injury by reinforcing endogenous defences.

Keywords: Polyphenols; Protocatechuic acid; Nrf2; GSH peroxidase; Glutathione reductase; JNK

1. Introduction

In recent years, epidemiological evidence has consistently shown that polyphenols are contained in vegetables and fruit whose consumption has been associated with a reduction of cancer incidence, mortality from coronary heart disease and incidence of myocardial infarction [1-4]. The observed positive health effects associated with the consumption of polyphenols, have rendered the study of their mechanism of action a matter of great importance.

Since oxidative stress is implicated in the pathogenesis of chronic inflammatory diseases, modulating the cellular redox status by strengthening the endogenous antioxidant defences might be an effective mechanism of disease prevention. In this regard dietary polyphenols generally behave, albeit to different extents, as antioxidant compounds, e.g., they potently inhibit the in vitro and ex vivo oxidation of low-density lipoprotein (LDL) [5-7], which is considered a key event in the pathogenesis of atherosclerosis. In addition, polyphenols exert indirect antioxidant effects via induction of genes involved in the endogenous defence system [8]. Consisting of enzymatic antioxidants, such as superoxide dismutase, catalase, glutathione (GSH) reductase (GR) and GSH peroxidase (GPx), and non-enzymatic antioxidants, such as GSH, the endogenous defence system plays a relevant role in the protection of cells against oxidative damage caused by electrophiles and reactive oxidants [9]. It has recently been demonstrated that the consumption of extra virgin olive oil (EVOO) — the typical added fat of the Mediterranean diet containing a number of different polyphenols — by patients with stable coronary heart disease lowers the circulating oxidised LDL and lipid peroxide plasma levels and increases the activity of GPx, which positively correlates with olive oil polyphenol content [10]. This strongly supports the hypothesis of an association between consumption of phenol-rich olive oil and the enhancement of the endogenous antioxidant system which has been shown to be critical for cell survival and coronary heart disease prevention [11,12]. In this regard, we have previously shown that EVOO polyphenols inhibited the macrophage-mediated oxidation of LDL, and up-regulated the expression and activity of both GPx and GR in the macrophage cell

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line J774 A.1 [8]. Among the tested polyphenols, 3,4-dihydroxybenzoic acid, protocatechuic acid (PCA), showed a particularly high capability in inducing gene expression of antioxidant enzymes. PCA is found in fruit, vegetables and EVOO, but also in plant-derived beverages such as tea, white grape wine and in herbal medicine [13-16]. The PCA content varies considerably depending on the type of food, e.g., raspberry contains up to 100 mg/kg fresh weight of PCA, while its concentration in olive oil is about 0.22 mg/kg [17,18]. However, PCA is now recognized as one of the main metabolites of complex polyphenols [19], such as anthocyanins and procyanidins, that are normally found at high concentrations in vegetables and fruit, and are absorbed by animals and humans [20-23]. Since the daily intake of anthocyanins has been estimated to be much higher than that of other polyphenols such as quercetin, kampferol or myricetin (180–250 mg/d vs. 20–30 mg/d, respectively) [24,25], the nutritional value of PCA is increasingly recognized.

Although several studies have specifically addressed the biological activity of PCA, the exact molecular mechanism and the signalling pathways by which it can modulate gene expression are still unclear. In this regard, the modulation of the level of intracellular reactive oxygen species (ROS) is known to be a mechanism by which gene expression is regulated [26-29]. On the other hand, it has been well documented that polyphenols directly activate transcription factors through the modulation of specific signalling pathways [30]. In particular, the transcription factor nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2), when activated by phosphorylation due to several signal transcription pathways such as mitogen-activated protein kinases (MAPKs), protein kinase C (PKC) and PI3K [31-34], translocates into the nucleus where it binds to the antioxidant response elements (ARE) [35]. These are specific nucleotide sequences present in the promoter region of genes encoding antioxidant proteins and phase 2 detoxifying enzymes [36].

This study aimed at defining the mechanism by which PCA activates GPx and GR gene expression in the J774 A.1 cell line. Specifically, we showed for the first time that PCA induced GPx and GR up-regulation by activating Nrf2 mainly through JNK phosphorylation.

2. Materials and methods

2.1. Cell culture

J774 A.1 cells (5×10^5) were seeded in 25-cm^2 flasks (Falcon) and grown in RPMI1640 medium, containing 0.2 mmol/L glutamine, 10 U/ml antibiotics and 10% FCS at 37°C, 5% CO_2. Protocatechuic acid was purchased from Fluka. In line with our previous work [8], time-course experiments were carried out in subconfluent J774 A.1 cells incubated with 25 μM PCA. Cells cultured in the absence of PCA were used as control.

2.2. Evaluation of PCA cellular uptake

After rinsing the cells twice with phosphate-buffered saline (PBS), the absorbed PCA was extracted by treating the monolayer with 2 ml of methanol for 30 min. The extracts were evaporated under N_2, reconstituted in 200 μl of mobile phase, and centrifuged at 18,000×*g* for 5 min. Aliquots (20 μl) were injected in a reversed-phase high-performance liquid chromatography (S200, Perkin Elmer Instruments, Waltham, MA, USA) equipped with a C18 column (250×4.6 mm), a photodiode assay detector at 280 nm, and the data station Software TotalChrom (Perkin Elmer Instruments). Gradient elution was done with solution A (3% acetic acid in water) and solution B (50% methanol–50% acetonitrile) as follows: 0–15 min: A 95% B 5% to A 70% B 30%; 15–20 min A 70% B 30% to A 60% B 40%; 20–25 min A 60% B 40%. The flow rate was 1.0 ml/min. Quantitative analyses were performed by peak area measurement compared with the external standard. To determine cellular proteins, cells were digested with 2 ml of 0.1 mol/L sodium hydroxide for 45 min and then the protein content was measured as previously described [8].

2.3. Measurement of intracellular ROS

Intracellular ROS level was determined using a fluorescence probe, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA), which is converted to highly fluorescent dichlorofluorescein in the presence of intracellular ROS. Cells were washed with PBS and incubated with freshly diluted CM-H_2DCFDA (25 μM) in PBS for 1 h and washed twice with PBS. Cell fluorescence intensity was measured on a spectrofluorometer with an excitation wavelength of 485 nm and emission wavelength of 530 nm.

2.4. RNA isolation and quantitative polymerase chain reaction

Total RNA was isolated with TRIZOL reagent (Invitrogen-Life Technologies, Carlsbad, CA, USA) as reported elsewhere [37]. Residual DNA was removed by treatment with DNase-I (Promega, Madison, WI). Quantitative real-time polymerase chain reaction (RTq-PCR) was done with gene specific TaqMan MGB probes and primers (Applied Biosystems, Carlsbad, Ca, USA) in a ABI 7700 sequence detector (Applied Biosystems). GPx, GR, Nrf2 and endogenous control TATA-box binding protein (TBP) were purchased as pre-designed assays (GPx: Mn00656767_g1; GR: Mn00439151_m1; Nrf2: Mn00477784_m1; TBP: Mm00446973_m1). All gene expressions assays have a FAM (Carboxyfluorescein) reporter dye at 5′ end of TaqMan MGB probe and a non-fluorescent quencher at 3′ end of the probe.

Expression of GPx, GR and Nrf2 genes were determined as the amount of GPx, GR and Nrf2 mRNA relative to mRNA for TBP by using the comparative C_T method described in the ABI 7700 sequence detection system, User Bulletin #2.

2.5. Protein determination by immunoblotting analysis

Whole cell extracts were prepared from cells collected and washed twice in ice-cold PBS, resuspended in 50 μl 1% TRITON X (Sigma, St Louis, MO, USA) with 2 mM Na_3VO_4 and 5 μl of a mixture of protease inhibitors (Sigma), and incubated on ice for 20 min. Cells were centrifuged at 18,000×*g* for 10 min at 4°C. Supernatants, assessed for protein concentration [38], were used for Western Blot analysis as described elsewhere [37]. Nuclear protein extracts were prepared by the Nuclear/Cytosol fractionation Kit (Medical and Biological Laboratories, Watertown, LA, USA) according to the manufacturer's instructions. For immunoprecipitation, whole-cell lysates containing 500 mg of protein were incubated for 2 h at 4°C with specific antibodies, namely anti-Nrf2, anti-c-Jun or anti-JNKs (C-JUN NH_2-terminal kinases). Then the samples were incubated with protein G-Sepharose for 30 min and the beads washed thrice with the same lysis buffer. Immunoblotting analyses were carried out using specific antibodies for Nrf2, c-Jun, GPx, GR, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), Lamin B (Santa Cruz Biotechnology, Santa Cruz, CA, USA), JNKs and phospho-JNKs (Cell Signaling, Danvers, MA, USA), phosphoserine and phosphothreonine proteins (Sigma). Blots were treated with appropriate secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology) followed by ECL detection (Amersham Bio Sciences, UK). Densitometric analysis was performed with a molecular imager FX (Bio-Rad, Hercules, CA, USA).

2.6. Gene silencing and small interfering RNA

Nrf2 and JNK expressions were inhibited with specific siRNA reagents (mouse Nrf2, mouse JNK, siGENOME SMARTpool siRNA; Dharmacon, Lafayette, CO, USA). Briefly, J774 A.1 cells were transfected with 100nM specific siRNA mixed with Lipofectamine 2000 transfection reagent (Invitrogen) in the absence of serum, according to the manufacturer's instructions. Scrambled non-targeting siRNA was used as negative control and introduced in the cells following the same protocol. At selected time points after transfection mRNA and protein were extracted to assess phospho-Nrf2, phospho-c-Jun, GR and GPx expressions.

2.7. Evaluation of kinase inhibitor effects

In a set of experiments aimed at defining the specific signalling kinase involved in Nrf2 activation, the cells were treated with specific kinase inhibitors one hour before PCA addition, at concentrations that effectively inhibited targeted pathways without any signs of cytotoxicity (see figure legends for details). Specifically, staurosporine (STS) (Sigma) against PKC; PD98059 (Biomol, Plymouth Meeting, PA, USA) against ERK1/2; SB203580 (Biomol) against p38; SP600125 against JNK1/2; and LY294002 (Biomol) against PI3K.

2.8. Chromatin immunoprecipitation assay

To demonstrate that Nrf2 bound ARE sequences of enzyme gene promoters, a chromatin immunoprecipitation (ChIP) assay was performed by using the commercial ChIP-IT kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. In brief, cellular protein and DNA were cross-linked with 1% formaldehyde in DMEM (Dulbecco's modified eagle medium) for 20 min at room temperature. DNA was sheared by sonication twenty pulses of 10 s each, followed by 10 s incubation on ice after each pulse. Sonication was done with a Vibra-cell sonicator (Sonics) at 15% power with a 3-mm tip. Isolated chromatin was run on 1.5% agarose gel to check for shearing efficiency. Subsequently, Nrf2-chromatin complexes were immunoprecipitated with anti-Nrf2 antibody. Anti-rabbit immunoglobulin G (IgG) was used as control to check specificity. Immunoprecipitates and total chromatin input were reverse cross-linked. DNA was isolated and 2 μl of DNA were used for PCR with specific primers for mouse GPx-ARE (forward CATAGATATCAATTGGCCCTTCC; reverse, CAGGATGACTTAGCAAAAACAGGT) and GR-ARE (ARE2 forward,

CCATCAAACTCGGTGA; reverse, GACTTGGGAGATAGAAGGAACG; ARE3 forward, TGA-GATTGACTGACACAATGGA; reverse, GATCACAAAAGGAAACCAACT) [39,40].

2.9. Statistical analysis

The results are expressed as means±S.E.M. of at least four experiments performed in duplicate. Comparisons between two groups were carried out by Student's *t* test. Analysis of variance (ANOVA) followed by Student-Newman-Keuls Multiple Comparison Test were used when >2 groups were compared. Differences were considered significant when $P< .05$.

3. Results

3.1. Cellular uptake of PCA

First, we evaluated whether PCA was taken up by the cells by determining the intracellular content of the polyphenol in time. The results demonstrated that PCA entered the cells very early after the incubation was started. At Minute 5, the intracellular PCA level was 23±0.7 ng/mg of cell proteins, which did not change when incubation time was extended to 18 h (data not shown). This finding indicated that PCA would be available for modulating intracellular activities.

3.2. PCA induces the expression of GPx and GR

We have previously demonstrated that treatment of J774.A.1 cells with PCA results in enhanced mRNA expression and enzyme activity of both GPx and GR [8].

In this study we carried out experiments in order to evaluate quantitatively the increased expression of both genes, in terms of mRNA and protein levels, induced by PCA. RTq-PCR determination showed a significant increase in both enzyme mRNAs in PCA treated cells, although with a different behaviour. Specifically, GPx mRNA increased early after the beginning of PCA treatment reached a peak at Hour 1 (+90% with respect to time-matched untreated cells, post-hoc test $P< .001$) and maintained a significantly higher level than the controls at each time point. Conversely, GR mRNA increased later (+60% at Hour 2; post-hoc test, $P< .001$) and returned to the low level of controls within Hour 6 (Fig. 1, A and B). An associated increase in the GPx and GR protein levels, which reached its maximum value at Hour 6 (+53% and +63%, respectively, post-hoc test $P< .001$), was demonstrated by immunoblotting in PCA-treated cells with respect to time-matched control cells (Fig. 1, C and D).

3.3. PCA induces the expression and activation of Nrf2 in J774 A.1 cells

ARE sequences have been found in the promoter region of the two enzymes, and can be activated by binding Nrf2, a main actor in the regulation of antioxidant enzymes [39,40]. In order to determine whether PCA exerts this effects by modulating the transcription factor Nrf2 we carried out time-course experiments (0.5–6 h) in J774 A.1 cells exposed to 25 μM PCA.

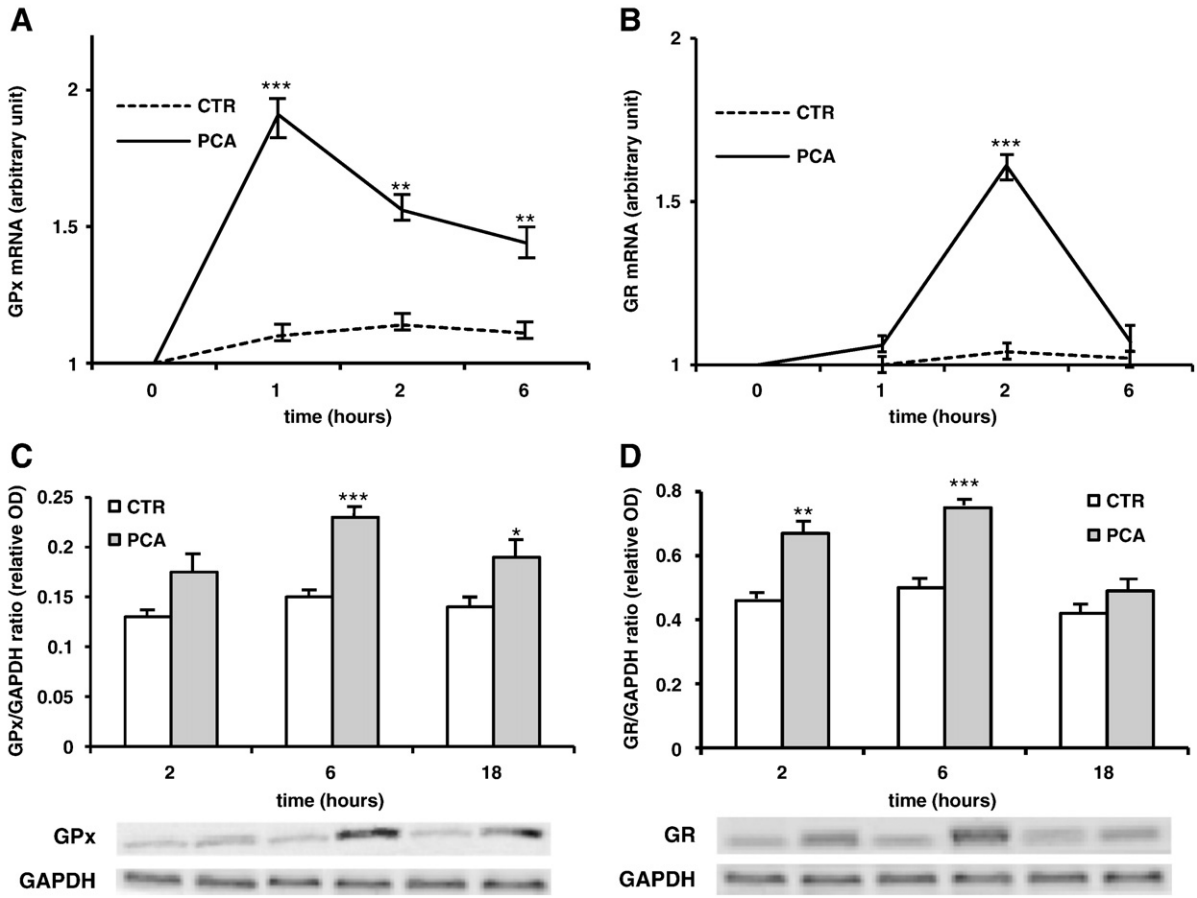
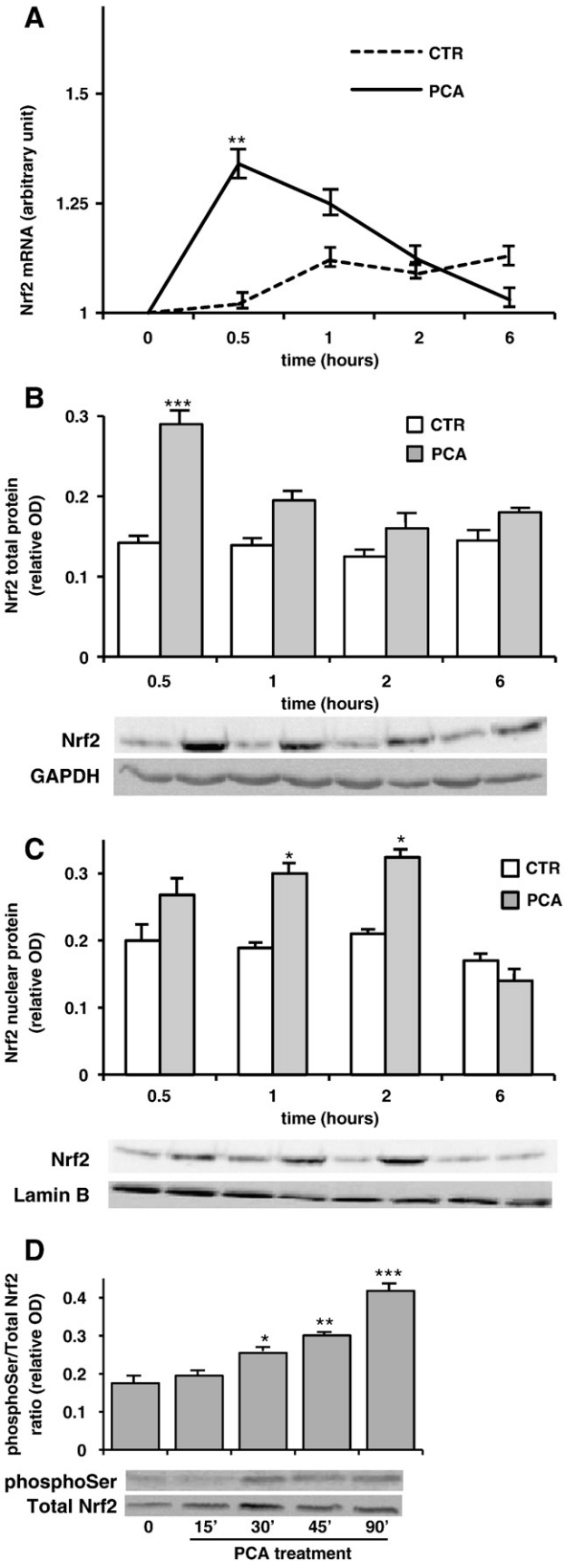


Fig. 1. Effect of PCA on GPx and GR expressions in J774 A.1 cells. Time-course experiments were carried out in J774 A.1 cells treated with 25 μM PCA to determine the expression of antioxidant enzyme genes in terms of mRNA and protein. GPx (A) and GR (B) mRNAs were assessed by RTq-PCR; GPx (C) and GR (D) proteins, by Western blot. Results were normalized to GAPDH protein content. Representative blots are shown. Values are means±S.E.M. ($n=3$). ANOVA, $P< .0001$; post-hoc test, ***$P< .001$,**$P< .01$, *$P< .05$, PCA-treated (PCA) vs. untreated (CTR) cells within each time point.



3.4. Quantitative mRNA and protein determination

We determined Nrf2 mRNA level by RTq-PCR analysis, and we found that its value did not change significantly in control cells during the six hours. On the contrary, a transient up-regulation of Nrf2 gene expression detected at Hour 0.5 (post-hoc test $P< .01$ with respect to time-matched control cells) which rapidly decreased was shown in PCA-treated cells (Fig. 2A). This up-regulation was accompanied by a strong increase (post-hoc test $P< .001$ with respect to time-matched controls) in Nrf2 protein content as assessed by immunoblotting in whole-cell lysates (Fig. 2B).

3.5. Nrf2 nuclear translocation and phosphorylation

To investigate whether PCA was able to activate Nrf2, we carried out experiments aimed at determining both the nuclear level and phosphorylation status of Nrf2 in cells treated with the polyphenol. At Hours 1 and 2 of incubation with PCA, the nuclear content of Nrf2 protein significantly increased with respect to the control cells at the same time point (post-hoc test $P< .05$) (Fig. 2C).

This finding was consistent with the increase in phosphorylated Nrf2 assessed by immunoblotting analysis. Specifically, we probed anti-Nrf2 immunoprecipitated from whole-cell lysates with antibodies against phosphoserine or phosphothreonine. We found that Nrf2 was phosphorylated only at serine residues, and the amount of the phosphorylated form significantly increased from Hour 0.5 until Hour 1.5 of incubation with phenolic acid (post-hoc test $P< .05$ and $P< .001$, respectively) (Fig. 2D).

Taken together, these results suggest that PCA induced an enhanced expression and, mostly, an increased stabilization/activation of Nrf2 by phosphorylation. Worthy of note, the PCA-induced increase in phosphorylated Nrf2 (at Hour 1.5) preceded the significant up-regulation of both GPx and GR (at Hour 6).

3.6. Involvement of signalling kinases in Nrf2 activation

Since Nrf2 phosphorylation may involve several signalling kinases, we decided to investigate which upstream kinases were critical for its activation following PCA treatment. Nrf2 nuclear translocation was assessed in J774 A.1 cells treated with the phenolic acid in the presence of different kinase inhibitors. The treatment of macrophages with PD98059 (ERK1/2 inhibitor), LY294002 (PI3K inhibitor) or SB203580 (p38 inhibitor), did not counteract PCA-induced Nrf2 activation. Staurosporine (PKC inhibitor) determined a slight decrease without reaching statistical significance. Conversely, the JNK inhibitor SP600125 significantly attenuated the PCA-induced nuclear increase in Nrf2 (post-hoc test $P< .05$) (Fig. 3A), strongly suggesting the involvement of this kinase in the activation of Nrf2.

To further confirm the involvement of JNK in PCA-induced up-regulation of Nrf2, we quantified its active phosphorylated form by immunoblotting using specific antibodies against phospho- and non-phospho-JNKs in time course experiments. We found that

Fig. 2. Effect of protocatechuic acid (PCA) on Nrf2 in J774 A.1 cells. Time-course experiments were carried out in J774 A.1 cells treated with PCA (25 μM) to evaluate expression, nuclear translocation and phosphorylation of Nrf2. (A) RTq-PCR determination of Nrf2 mRNA. Western blot evaluation of total (B) and nuclear (C) Nrf2 protein. Results were normalized to GAPDH and Lamin B protein content, respectively. Representative blots are shown. Values are means±S.E.M. ($n=3$). ANOVA $P< .005$; *$P< .05$, **$P< .01$, *** $P< .001$, PCA-treated (PCA) vs. untreated (CTR) cells within each time point. (D) Serine phosphorylation of Nrf2 protein detected in immunoprecipitates with anti-Nrf2 antibody as described in Materials and Methods. Values are means±S.E.M. ($n=3$) of P-Ser/Nrf2 ratio. ANOVA $P=.0001$; post-hoc test *$P< .05$, **$P< .01$, ***$P< .001$ vs. time 0. A representative blot is shown.

phosphorylated JNKs increased early (5 min) reaching a peak level at 10 min after the beginning of PCA treatment (post-hoc test $P< .001$) (Fig. 3B).

In addition, we demonstrated that the level of phosphorylated c-Jun, a specific target of JNK activity, significantly increased during the treatment with PCA (post-hoc test $P<.001$ at Hour 1)(Fig. 3C).

To provide conclusive evidence of the causal relationship between JNK phosphorylation and the increase in Nrf2 activation, we carried out the silencing of JNK gene expression in PCA-treated cells. Transfection of the cells with the anti-JNK siRNA, determined about −70% of JNK protein with respect to scrambled siRNA (data not shown), and prevented Nrf2 from being activated, as demonstrated by the significant reduction of Nrf2 translocation (post-hoc test $P< .05$), (Fig. 4A). Furthermore, the level of phosphorylated Nrf2 significantly decreased by both silencing JNK gene (post-hoc test $P< .05$), and inhibiting JNK activity (post-hoc test $P< .05$) (Fig. 4B). Finally, phosphorylated c-Jun level was reduces by both the treatment with JNK inhibitor ($P< .001$) and the silencing of JNK ($P< .01$), (Fig. 4C).

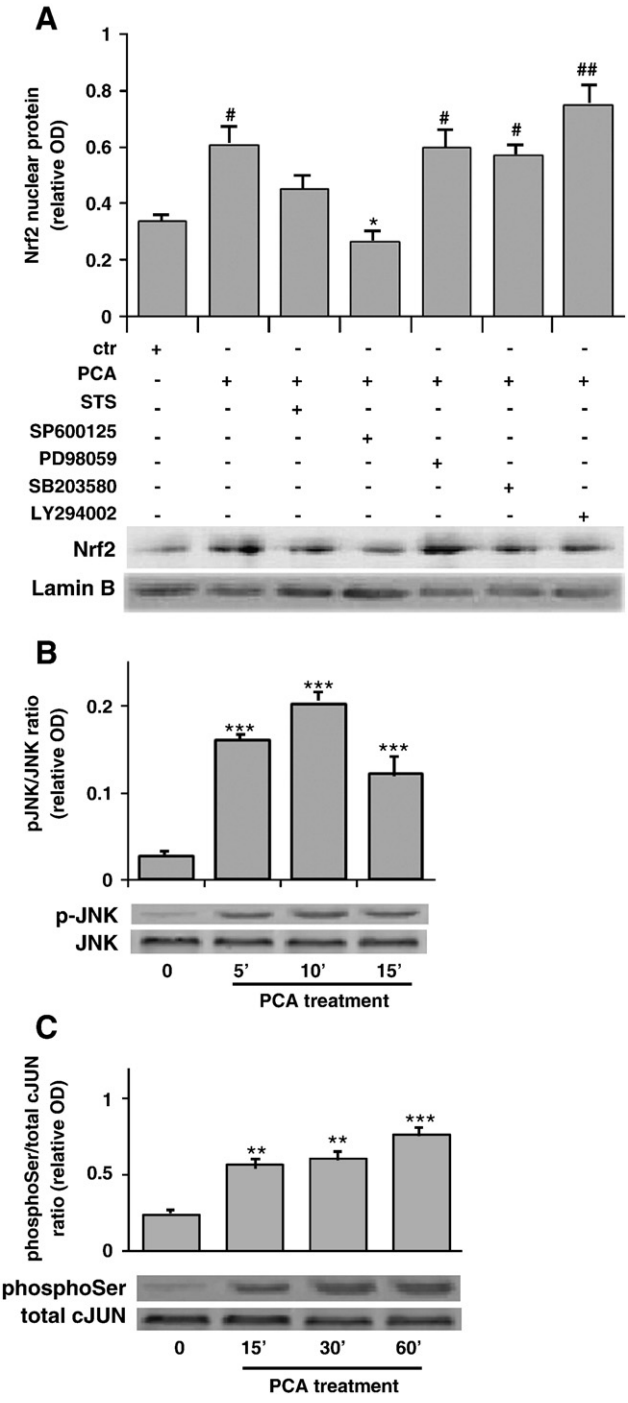


Fig. 3. Involvement of JNK in PCA-induced Nrf2 activation. (A) Experiments were carried out to determine the kinases involved in the nuclear translocation of Nrf2 in J774 A.1 cells treated with PCA (25 μM) by using specific kinase inhibitors. Cells were incubated with 0.1 μM STS against PKC, 50 μM SP600125 against JNK1/2, 10 μM SB203580 against p38, 50 μM PD98059 against ERK 1/2 or 50 μM LY294002 against PI3K, for 30 min before PCA treatment. After 2 h, cells were collected, and Nrf2 nuclear levels evaluated. Values are means±S.E.M. ($n=4$). ANOVA $P=.0003$; post-hoc test $*P< .05$ vs. PCA, PCA+SB203580, PCA+PD98059; PCA+LY294002 treated cells; $\#P< .05$, $\#\#P< .01$ vs. untreated cells (CTR). A representative blot is shown. Results were normalized to Lamin B protein content. (B) Time-course experiments were carried out to evaluate the active phosphorylated form of JNK in PCA-treated cells. Immunoprecipitates with anti-JNK antibody were analyzed using anti-nonphospho (JNK)- or anti-phospho (p-JNK)-antibodies. Values are means±S.E.M. ($n=3$) of p-JNK/JNK ratio. ANOVA $P< .0001$; post-hoc test $***P< .001$ vs. time 0. A representative blot is shown. (C) Time-course evaluation of serine phosphorylation of c-Jun protein in PCA-treated cells, detected in immunoprecipitates with anti-c-Jun antibody as described in Materials and Methods. Values (phosphoSer/c-Jun) are means±S.E.M. ($n=3$). ANOVA $P=.0056$; post-hoc test $*P< .05$, $**P< .01$, vs. Time 0. A representative blot is shown.

3.7. PCA induces GPx and GR expression through Nrf2 activation

To acquire a convincing prove of a causal relationship between the enhanced expression of the antioxidant enzymes and the activation of Nrf2 in cells treated with PCA, we carried out experiments following two approaches, namely, the Nrf2 gene silencing and JNK inhibition

3.7.1. Nrf2 silencing

As expected, PCA-treated cells, transfected with the anti-Nrf2 siRNA, (but not with siRNA negative control, scrambled siRNA), showed about 95% reduction of Nrf2 mRNA expression, as detected by RTq-PCR. A remarkable reduction of Nrf2 nuclear protein was also found in anti-Nrf2-siRNA-transfected cells, with respect to scrambled-siRNA-transfected cells(data not shown).

As a consequence of Nrf2 knockdown, the expression of GPx and GR significantly decreased in terms of both mRNA (−48% and −72%, respectively; post-hoc test $P< .001$)(Fig. 5, A and B), and protein levels (−42% and −60%, respectively) (post-hoc test $P< .05$ and $P< .001$, respectively) (Fig. 5, C and D).

3.7.2. JNK inhibition

The kinase inhibitor SP600125 strongly counteracted the increased expression of GPx and GR observed in PCA-treated cells in terms of both mRNA (−57% and −55%, respectively) (post-hoc test $P< .001$) and protein levels (−37% and −56%, respectively) (post-hoc test $P< .01$ and $P< .001$, respectively). This finding was confirmed by silencing JNK with the anti-JNK siRNA in PCA-treated cells. In fact, in these cells, GPx and GR mRNA decreased by 41% and 47%, respectively, with respect to untransfected cells (post-hoc test $P< .001$). As for protein levels the two enzymes behaved in the same manner (−37% GPx and −49% GR, post-hoc test $P< .01$ and $P< .001$, respectively) (Fig. 5,A–D).

3.8. Nrf2 binds specific ARE sequences in GPx and GR promoters

ChIP assay was used to demonstrate the PCA-induced binding of Nrf2 to the ARE sequences contained in the promoter regions of GPx and GR.

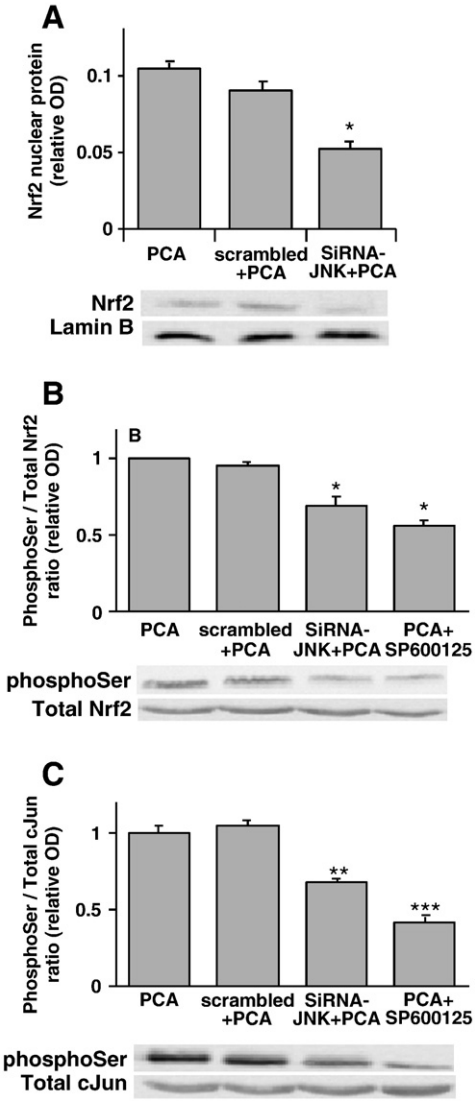


Fig. 4. Anti-JNK siRNA transfection in PCA-treated J774.A1 cells. To evaluate the effect of JNK silencing/inhibition on Nrf2 and c-Jun activation in PCA-treated cells, pre-confluent cells were transfected with anti-JNK-siRNA (100 nM) for 18 h, or treated with the specific inhibitor (50 μM) for 30 min, then PCA (25 μM) was added. (A) Western Blot evaluation of Nrf2 nuclear protein 2 hours after PCA treatment. Results were normalized to Lamin B protein content. ANOVA P=.037. (B) Serine phosphorylation of Nrf2 protein detected in immunoprecipitates with anti-Nrf2 antibody as described in Materials and Methods. ANOVA P< .0139. (C) Serine phosphorylation of c-Jun protein detected in immunoprecipitates (see Materials and Methods for details). ANOVA P=.0006. Values are means±S.E.M. (n=3). Post-hoc test *P< .05, **P< .01, ***P< .001 vs. untransfected or scrambled transfected PCA-treated cells. Representative blots are shown.

Activation of Nrf2 by PCA enhanced the recruitment of Nrf2 to the GPx and GR promoters as demonstrated by the increased amplification of the specific promoter regions (Fig. 6). Activated Nrf2 was present at very low or negligible levels in control cells, and no amplification was observed by using GPx and GR specific ARE primers. Immunoprecipitation with rabbit IgG failed to select the promoters.

4. Discussion

Using the macrophage cell line J774 A.1, we have herein provided new evidence that the phenolic acid PCA increases the expression of GPx and GR, mainly by inducing the JNK-mediated phosphorylation of the transcription factor Nrf2, a major regulator of antioxidant/detoxifying enzymes. This finding further confirms and expands the concept that PCA improves antioxidant cellular defences in critical target cells such as macrophages whose dysfunction has been implicated in many pathophysiological processes that entail inflammation and atherogenesis [41,42].

The two enzymes, together with other phase II enzymes such as GSH synthetase and GSH-S-transferase, have the main role of catalyzing reactions aimed at scavenging/detoxifying electrophile/nucleophile compounds, as well as maintaining the optimal cellular level of the reduced form of GSH. GSH is an essential intracellular detoxifying/reducing agent and plays a pivotal role in antioxidant defences by preserving the thiol groups in intracellular proteins, and by reducing the burden of biomolecular adducts from the cellular environment [8].

We have previously demonstrated [6,8] the inhibitory activity of PCA towards cell-mediated oxidation of LDL due to both the direct antioxidant activity and the specific induction of mRNA expression of GPx and GR in macrophages which improves the endogenous antioxidant system. In this study, we demonstrated that the increased expression of the two enzymes was accompanied by an increase in the enzymatic proteins, which was consistent with the increased enzymatic activity we had previously shown [8].

It is well known that the transcriptional activation of antioxidant/detoxifying enzymes is regulated by common upstream regulatory elements called ARE, or electrophyle response elements, present in the promoter region of those genes [36]. There is a fair amount of evidence that ARE sequences regulate the cellular defence system, being in turn strictly regulated by transcriptional factors, such as the E2-related factors Nrf1 and mainly Nrf2 [43]. The latter is a member of the cap'n'collar family of basic leucine zipper transcription factors ubiquitously expressed [29]. Under homeostatic conditions, Kelch-like ECH-associated protein 1 (Keap1) sequesters Nrf2 in the cytoplasm by forming heterodimers [44]. When the interaction between Nrf2 and Keap1 is disrupted, Nrf2 is allowed in the nucleus where it binds to the ARE sequences, inducing the expression of genes encoding for the majority of phase II detoxifying and antioxidant enzymes [45]. These enzymes were, in fact, found to be abrogated in $Nrf2^{-/-}$ mice [46].

It has been reported that different compounds, e.g., tea polyphenols [47,48], increase phase II enzyme expression via the activation of Nrf2 and its binding to ARE sequences.

Consistently, in this study, we have demonstrated that in macrophages PCA induced not only a significant, but temporary, increase in mRNA expression of Nrf2 early after treatment but mostly a strong increase in Nrf2 protein likely due to the stabilization of Nrf2 molecules, which allows the transcription factor to escape degradation. Worthy of note, we proved that the up-regulation of Nrf2 expression was accompanied by a strong activation of the transcription factor resulting in a massive translocation to the nucleus, the place where it can exert its effect, and, to the best of our knowledge, this represents the first evidence of PCA-induced activation of Nrf2 in macrophages.

Various mechanisms have been speculated for Nrf2 activation and various inducers have been shown to activate Nrf2 differentially [49,50]. The activation of Nrf2 has been demonstrated to be mediated by mechanisms that lead to either its stabilization or the up-regulation of the Nrf2 gene thus increasing the level of cellular Nrf2 [51]. On the basis of our results, we can hypothesize that the activation of Nrf2 by PCA depended partially on the up-regulation of Nrf2 gene transcription and mainly on the stabilization of the transcription factor we observed in our experimental system. Specifically, the post-translational modification of Nrf2 protein, in particular phosphorylation by different signalling kinases, has been demonstrated to be the main regulator of Nrf2 activity, and phosphorylated Nrf2 (P-Nrf2) is, actually, the common form found

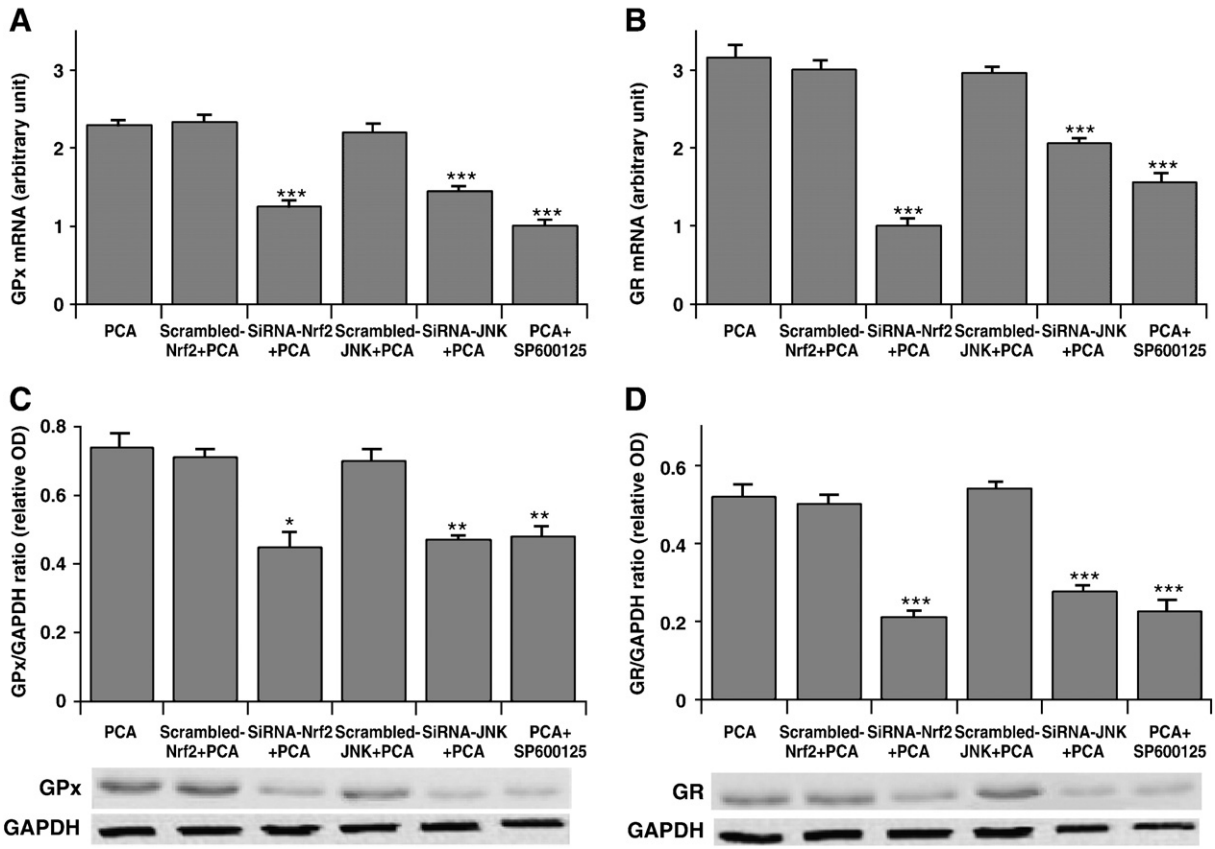


Fig. 5. Effects of Nrf2 and JNK silencing on GPx and GR expressions in PCA-treated J774.A1 cells. To evaluate the effect of Nrf2 or JNK silencing on PCA-induced enzyme over-expressions, pre-confluent cells were transfected with anti-Nrf2-siRNA or anti-JNK-siRNA (100 nM) for 18 h, then PCA (25 μM) was added. Cells transfected with scrambled siRNAs were used as negative control. To evaluate the effect of JNK inhibition on PCA-induced enzyme overexpressions, cells were incubated with 50 μM SP600125 for 30 min before PCA treatment. GPx (A) and GR (B) mRNAs were assessed by quantitative RT-PCR at Hour 2 of PCA treatment. ANOVA $P<.0001$. GPx (C) and GR (D) proteins were evaluated by Western blot at Hour 6 of PCA treatment. Results were normalized to GAPDH protein content. ANOVA $P=.0072$ and $P=.0006$ for GPx and GR, respectively. Values are means±S.E.M. ($n=3$). post-hoc test * $P<.05$, ** $P<.01$, *** $P<.001$ *vs* PCA-treated cells. Representative blots are shown.

in the nucleus [31]. P-Nrf2 shows, in fact, an increased stability against cytoplasmic degradation and at the same time a low affinity for Keap1, which brings about the rupture of the heterodimer and allows for Nrf<.2 nuclear translocation. In the light of this, and to definitely confirm PCA-induced Nrf2 activation/stabilization, we demonstrated that the serine phosphorylation of Nrf2 really occurred in PCA treated cells. Since several signalling kinase pathways may be involved in Nrf2 phosphorylation, we also demonstrated which upstream kinases were mainly responsible for the activation of the transcription factor. The use of specific kinase inhibitors allowed us to identify JNK as the kinase mostly involved in this process, in fact the JNK inhibitor was able to significantly counteract the translocation and the phosphorylation of Nrf2 in PCA-treated cells. Furthermore, we found the level of active phosphorylated JNK to be significantly higher in PCA-treated cells than in the controls. The PCA-mediated activation of JNK was also confirmed by showing the phosphorylation of c-Jun, which is a specific target of JNK activity and was thus used as positive control. Finally, to provide the conclusive evidence of JNK involvement in Nrf2 activation, we specifically silenced JNK gene expression demonstrating that in anti-JNK siRNA-transfected cells the activation/translocation of Nrf2, as well as the phosphorylation of c-Jun, following PCA treatment did not occur. Taken together, these results indicate that PCA was able to activate the JNK MAP kinase which in turn had a pivotal role in activating Nrf2 in the macrophage cell line. This finding was in agreement with literature data demonstrating that PCA can activate different signalling kinases in different cell types [52-54] and added further convincing evidence

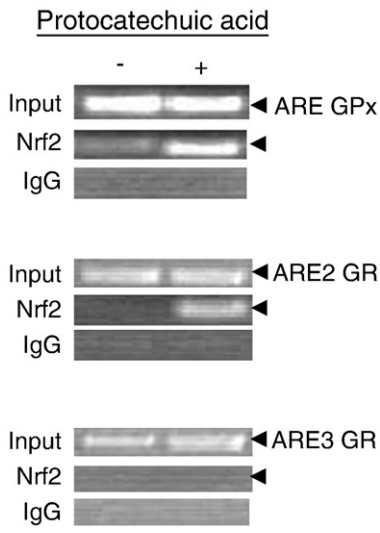


Fig. 6. PCA enhances the binding of Nrf2 to GPx-ARE and GR-ARE. ChIP assays were performed to examine the interaction of Nrf2 with ARE sequences in the promoter regions of the antioxidant enzymes in J774.A1 cells untreated or treated with PCA for 1 h. The cross-linked chromatin was immunoprecipitated with rabbit IgG or anti-Nrf2 antibodies. Immunoprecipitated DNA was analyzed by PCR with primers specific for GPx-ARE, GR-ARE2 and GR-ARE3. Representative images of three independent experiments are shown.

that many effects of polyphenols such as anti-tumour, anti-inflammatory, and anti-atherosclerotic activities, can be explained not only on the basis of their antioxidant properties but also on the basis of their efficacy in modulating signalling pathways.

Polyphenols can have a pro-oxidant effect under certain condition; on the other hand, ROS level can act as intracellular stress sensor modulating several cell activities among which Nrf2 [55,56]. In order to evaluate the possible involvement of ROS in the modulation of Nrf2 activity, we carried out experiments to determine intracellular ROS in PCA-exposed J774 A.1 with time, demonstrating that no production of ROS occurred during the treatment with phenolic acid. The level of ROS remained, in fact, unchanged in PCA treated cells with respect to the control at each time point (data not shown).

Consequently, we excluded that the activation of Nrf2 could be related to changes of the intracellular redox environment.

By silencing JNK or Nrf2 genes, we showed that a causal link existed between JNK-mediated Nrf2 activation and the increase in GPx and GR expression observed in PCA-treated cells. The inhibition of JNK, in fact, by both the JNK inhibitor SP600125 and the short-interfering anti-JNK siRNA, counteracted the PCA-induced increase in antioxidant enzymes. In the same fashion, the down-regulation of Nrf2 expression by short-interfering siRNA, inhibited the enhancement of GPx and GR expression induced by PCA.

Finally, by using the ChIP technique, we provided clear evidence that Nrf2, activated by PCA treatment, specifically bound specific ARE sequences, which have been recently demonstrated to be contained in the promoter regions of the two enzymes [39,40].

In conclusion, our study demonstrates for the first time that PCA improved the macrophage endogenous antioxidant potential by a mechanism in which Nrf2 activation by JNK MAP kinase plays an essential role. The new knowledge achieved on the molecular mechanisms that allow PCA to exert potentially protective effects against oxidative injury could have important implications for the design of novel diet-based preventive approaches to counteract oxidative stress-induced pathologies.

It must be underlined that the physiological in vivo context in which dietary polyphenols exert their influence is undoubtedly much more complex than that available from an in vitro system. However, this is a suitable tool for the identification of the molecular mechanisms of action of polyphenols and may be considered a useful step before translating the study to appropriate in vivo models.

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