RESEARCH ARTICLE

Involvement of ERK, Akt and JNK signalling in H_2O_2-induced cell injury and protection by hydroxytyrosol and its metabolite homovanillic alcohol

Alessandra Incani[1,2], Monica Deiana[2], Giulia Corona[1], Katerina Vafeiadou[1], David Vauzour[1], M. Assunta Dessi[2] and Jeremy P. E. Spencer[1]

[1] Molecular Nutrition Group, School of Chemistry, Food and Pharmacy, University of Reading, Reading, UK
[2] Dipartimento di Biologia Sperimentale, Sez. Patologia Sperimentale, Università degli Studi di Cagliari, Monserrato, Italy

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The olive oil polyphenol, hydroxytyrosol (HT), is believed to be capable of exerting protection against oxidative kidney injury. In this study we have investigated the ability of HT and its *O*-methylated metabolite, homovanillic alcohol (HVA) to protect renal cells against oxidative damage induced by hydrogen peroxide. We show that both compounds were capable of inhibiting hydrogen peroxide-induced kidney cell injury *via* an ability to interact with both MAP kinase and PI3 kinase signalling pathways, albeit at different concentrations. HT strongly inhibited death and prevented peroxide-induced increases in ERK1/2 and JNK1/2/3 phosphorylation at 0.3 μM, whilst HVA was effective at 10 μM. At similar concentrations, both compounds also prevented peroxide-induced reductions in Akt phosphorylation. We suggest that one potential protective effect exerted by olive oil polyphenols against oxidative kidney cell injury may be attributed to the interactions of HT and HVA with these important intracellular signalling pathways.

Keywords:
Homovanillic alcohol / Hydrogen peroxide / Hydroxytyrosol / MAPK / Oxidative stress

1 Introduction

Epidemiological data have suggested that an association exists between diets rich in fruits and vegetables, beverages, such as wine and tea, and oils, such as olive oil and omega-3 fish oils, and a lower incidence of several pathologies [1–3]. The beneficial effect of such a diet, often referred to as the Mediterranean diet, may be due in part to the high content of phytochemicals that are present in many of these foods [4]. Extra virgin olive oil (EVOO), the principal fat component of the traditional Mediterranean diet, contains a variety of phenolic compounds, which together with other components, such as tocopherols, are thought to exert beneficial biological effects both *in vitro* and *in vivo* [5–8]. The phenolic content of olive oil is made up of simple phenols, such as hydroxytyrosol (HT) and tyrosol, flavonoids, lignans, and secoiridoids [9]. Bioavailability studies in animals and humans have demonstrated that HT is dose dependently absorbed [10] following EVOO consumption, and subject to extensive metabolic conversion to 3-*O*-methyl-HT, HT-GSH adducts [11, 12], glucuronide and sulphate conjugates [13–15]. Furthermore, secoiridoid forms have been shown to undergo hydrolysis under acidic conditions similar to those in the stomach, resulting in the production of HT [16, 17].

Oxidative stress is believed to mediate a wide range of kidney disorders, including renal impairment, acute renal failure [18, 19], obstructive nephropathy [20], hyperlipidemia [21] and glomerular damage [22]. As HT and its metabolites

Correspondence: Dr. Jeremy P. E. Spencer, School of Chemistry, Food and Pharmacy, University of Reading, P. O. Box 226, Whiteknights, Reading, RG2 6AP, UK
E-mail: j.p.e.spencer@reading.ac.uk
Fax: +44-118-931-0080

Abbreviations: ERK, extracellular-signal related kinase; **EVOO,** extra virgin olive oil; **HT,** Hydroxytyrosol; **HVA,** homovanillic alcohol; **JNK,** c-jun-N-terminal kinase; **MTT,** methylthiazolyldiphenyl-tetrazolium bromide; **TBS,** TBS Tris Buffered Saline; **TTBS,** TBS supplemented with Tween-20

have been shown to be present in the kidney in relatively high amounts [15], and express antioxidant activity *in vitro* and *in vivo* [23–28], they may be able to exert a degree of protection against oxidative kidney injury. EVOO polyphenols and HT have been shown to possess a strong antioxidant activity *in vitro* [7, 27, 29] and *in vivo* [25, 28]; they also exert an anticancer activity in different cells lines. A recent study showed an inhibition in the proliferation of colon cancer cells induced by EVOO polyphenols [30]. Moreover, HT induces apoptosis in colon cancer cells by modulating the ER stress-dependent signalling pathways [31]. HT also inhibits human promyelocytic leukaemia cells (HL-60) proliferation by inducing apoptosis and differentiation [32, 33]. The ability of HT to induce apoptosis in HL-60 is associated with an early release of cytochrome *c* from mitochondria which precedes caspase 8 activation [34].

The precise mechanisms by which polyphenols exert their beneficial actions remain unclear, although these substances are powerful hydrogen-donating antioxidants and scavengers of reactive oxygen and nitrogen species *in vitro*, recent findings demonstrate a role for specific phenolic compounds in interacting selectively within signalling cascades, such as tyrosine kinase, PI 3-kinase/Akt, PKC and MAP kinase pathways, that regulate cell survival following exposure to oxidative stress [35, 36].

In this study, we investigated the protective effect of HT and one of its metabolites, homovanillic alcohol (HVA), against H_2O_2-induced kidney injury. We show that both compounds are able to inhibit peroxide-induced changes in the phosphorylation state of two pro-death signalling pathways, namely c-Jun N-terminal kinase (JNK1/2/3) and extracellular-signal relate kinase (ERK1/2) and activate the pro-survival Akt/PKB pathway. We suggest that one mode of action by which olive oil polyphenols protect kidney cells may be attributed to interactions with intracellular signalling pathways activated in response to oxidative stress [37].

2 Materials and methods

2.1 Materials

HT was obtained from Cayman Chemical (Ann Arbor, MI, USA); Methylthiazolyldiphenyl-tetrazolium bromide (MTT), HVA and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody were from Sigma (Poole, UK); Enhanced chemiluminescence (ECL) reagent and Hyperfilm-ECL were purchased from Amersham (Little Chalfont, UK). Phospho-Akt (Ser473) Antibody, Akt Antibody, phospho-SAPK/JNK (Thr183/Tyr185) Antibody and SAPK/JNK Antibody were obtained from New England Biolabs (Hertfordshire, UK); Anti-phospho-MAP Kinase1/2 (Erk1/2) and Anti-MAP Kinase 1/2 (Erk1/2) were obtained from Upstate cell signalling solution (Hampshire, UK). All the other reagents were of analytical grade and obtained from Sigma.

2.2 Cell culture and assessment of cellular injury

LLC-PK1 cells (a porcine renal epithelial cell line with proximal tubule epithelial characteristics) were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and were grown in the Medium 199 at 37°C under a humidified atmosphere of 95% air and 5% CO_2. For experimental studies cells were grown to 70% confluence in 24-well and 6-well plates. Cells were exposed to H_2O_2 (0–100 μM) in PBS. After 60 min of incubation, PBS was removed and cells were incubated for 24 h in fresh media before cell damage was assessed (MTT assay). In order to assess the protective effect of HT or HVA against H_2O_2-induced toxicity, cells were pre-treated with the tested compounds (0–10 μM, 24 h) prior to exposure to H_2O_2 (25 μM, 1 h). Cells were washed thoroughly with PBS following the removal of HT or HVA and prior to the treatment with H_2O_2. The cells were washed with PBS prior to the addition of MTT solution (0.5 mg/mL) and incubated for 1 h at 37°C. The MTT solution was removed and DMSO was added to solubilize the formazan product. The absorbance of the resulting solution was measured at 550 nm, using DMSO as a blank.

2.3 Immunoblotting

All experiments were performed on 70% confluent cells. Two sets of cell treatments were performed: first, cells were exposed to H_2O_2 (0–100 μM) in PBS for 60 min; second, cells were pre-treated with HT or HVA (0–10 μM) prior to addition of H_2O_2 (25 μM, 1 h). Following treatment, the cells were washed with ice-cold PBS prior to the addition of 120 μL lysis buffer for protein extraction (50 mM Tris base, Triton X-100 (1:100 v/v), 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 0.5 mM PMSF, 1 mM sodium ortho-vanadate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, and mammalian protease inhibitor cocktail (1:100 v/v). Cells were scraped on ice and lysates were incubated for 45 min before centrifugation at 4000 rpm at 4°C for 5 min. The BCA assay Kit was used to assess the protein content and Western immunoblotting was performed as described previously [38].

The samples were boiled for 2 min in boiling buffer (62.5 mM Tris, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.0025% bromophenol blue). The boiled samples were run on 9% SDS-polyacrylamide gels (20 μg/lane), and the proteins were transferred to nitrocellulose membranes (Hybond-ECL, Amersham Biosciences, Buckinghamshire, UK) by semi-dry electroblotting (1.5 mA/cm^2). The nitrocellulose membrane was then incubated in Tris Buffered Saline (TBS) supplemented with 0.05% v/v Tween-20 (TTBS) containing 4% w/v skimmed milk powder for 60 min at room temperature followed by three 5 min washes in TTBS. The blots were then incubated with either anti-Akt (1:1000 dilution), anti-phospho-Akt

(Ser473) (1:1000 dilution), anti-p42/44 MAPK (1:1000 dilution), anti-phospho-p42/44 MAPK (Thr202/Tyr204) (1:1000 dilution), anti-JNK (1:1000 dilution), anti-phospho-JNK (1:1000 dilution) in TTBS containing 1% w/v skimmed milk powder (antibody buffer) overnight at 4°C on a three-dimensional rocking table. The blots were washed twice for 10 min in TTBS and then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:2000 dilution) in antibody buffer for 60 min. Finally, the blots were washed twice for 10 min in TTBS and exposed to ECL® reagent for 1–2 min as described in the manufacturer's protocol (Amersham Biosciences). The blots were exposed to Hyperfilm-ECL (Amersham Biosciences) for 2–5 min in an auto-radiographic cassette and developed. The molecular weights of the bands were calculated from comparison with pre-stained molecular weight markers that were run in parallel with the samples (molecular weights, 27 000–180 000 and 6000–45 000; Sigma). Protein bands were quantified using Quantity One software (Bio-Rad, Hertfordshire, UK).

2.4 Statistical analysis

Data are expressed as mean ± SD ($n = 18$ for each sample/condition). The statistical evaluation of the results was performed by ANOVA followed by a Bonferroni *post hoc* test using GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA, USA).

3 Results

3.1 Protection against H_2O_2-induced cytotoxicity

H_2O_2 induced a dose-dependent decrease in cell viability with an IC_{50} equal to 25 μM. This concentration was utilized in subsequent experiments addressing the putative protective effects of HT and HVA. Significant protection against H_2O_2-induced cellular injury was observed when cells were pre-treated with HT (0.3–10 μM, 24 h), with a 16.4 ± 6.5% increase in viability at 0.3 μM and 29.1 ± 7.1% at 10 μM. In contrast, HVA exerted a significant protection at 10 μM (16.6 ± 9.7%) (Fig. 1).

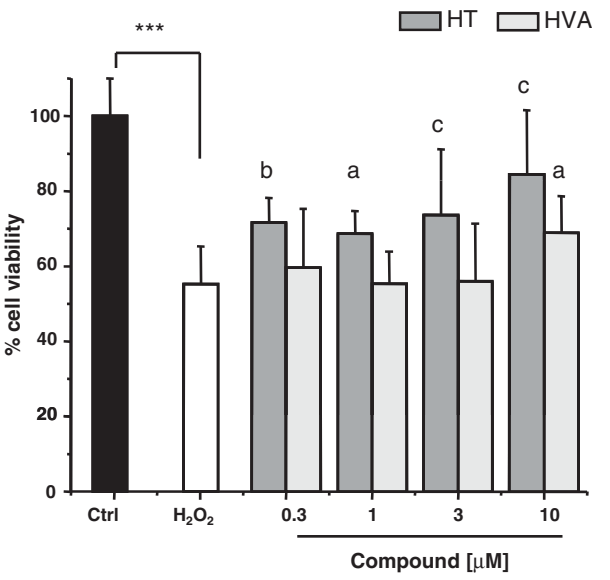

Figure 1. Protection of HT and HVA against peroxide-induced cellular injury. The level of cellular injury was assessed using the MTT assay. Cells were pre-treated with HT or HVA (0.3–10 μM) for 24 h prior to treatment with H_2O_2 (25 μM) for 1 h. ***$p < 0.001$ respect to the control; a: $p < 0.001$; b: $p < 0.01$; c: $p < 0.05$ indicate significant increases over H_2O_2 (25 μM).

3.2 Modulation of MAP kinase and Akt/PKB signalling

Incubation of kidney cells with H_2O_2 (10–250 μM, 1 h) resulted in a change in the phosphorylation states of ERK1/2, JNK, and Akt/PKB (Fig. 2). Parallel immunoblots with a polyclonal antibody against total Akt protein levels were performed and indicated that there were no changes in total levels of these proteins. However, both the phospho and the total levels of ERK1/2 (Fig. 2A) and JNK (Fig. 2C) were found to change with peroxide treatment. The phosphorylation state of ERK1/2 was probed using a phospho-specific antibody, which recognizes the dually phosphorylated motif pTEpY within activated ERK1/2. Exposure of cells to H_2O_2 increased the ratio phospho/total of ERK1 (44 kDa) and ERK2 (42 kDa) above basal levels (Fig. 2A). The increase was significant from 25 μM. Peroxide (> 25 μM) also led to a decrease in the phospho/total ratio of Akt at Ser^{473} (Fig. 2B) and an increase in the phospho/total ratio of JNK1/2/3 (Thr^{138}-Pro-Tyr-^{185} motif (pTPpY) in the catalytic core of active JNK), as shown in Fig. 2C. In order to investigate whether HT and HVA could modulate H_2O_2-induced increases in ERK1/2 phosphorylation, cells were pre-treated with HT or HVA (0.3–10 μM, 24 h) prior to peroxide (25 μM, 1 h) treatment (Fig. 3). HT pre-treatment prior to H_2O_2 exposure resulted in a significant inhibition in the peroxide-induced increases in ERK1/2 phosphorylation at all concentrations tested (Fig. 3A), whereas HVA significantly inhibited H_2O_2-induced ERK phosphorylation at 10 μM concentration (Fig. 3B).

The influence of HT and its *O*-methylated metabolite on H_2O_2-induced Akt/PKB de-phosphorylation was determined by immunoblotting analysis with an anti-phospho-Akt polyclonal antibody, which detects Akt when phosphorylated at Ser^{473} (Fig. 4). H_2O_2 (25 μM, 1 h) treatment resulted in a marked decrease in Akt phosphorylation (Ser^{473}) with respect to the basal levels. Pre-treatment with HT (0.3–10 μM, 24 h) prior to peroxide addition prevented this inhibition of Akt phosphorylation (Fig. 4A). HVA was able

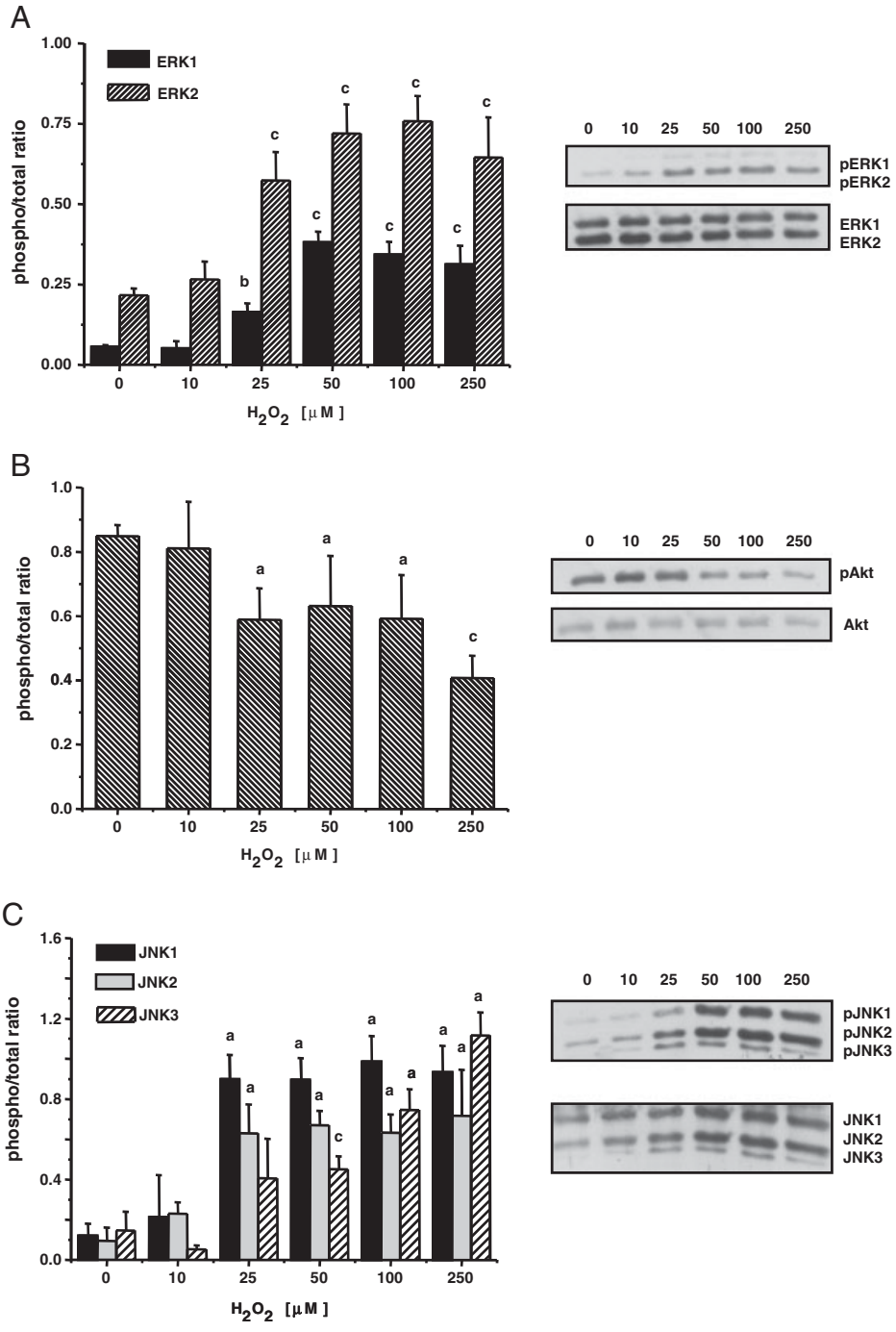

Figure 2. Modulation of cell signalling by H_2O_2 (10–250 μM) for 1 h in kidney cells. Data obtained from immunoblot experiments were analysed using Bio-Rad Quantity One 1-D Analysis software. Each column represents the mean ± SD of four independent experiments. (A) Modulation of ERK1/2 phosphorylation. (B) Modulation of Akt de-phosphorylation (C) Modulation of JNK phosphorylation. a: $p<0.001$; b: $p<0.01$; and c: $p<0.05$ indicate a significant change relative to basal levels.

to inhibit peroxide-induced Akt de-phosphorylation at the highest concentration tested (10 μM) (Fig. 4B). Parallel immunoblots with a polyclonal antibody against total Akt protein levels were performed and indicated that there was no changes in total levels of Akt. Finally pre-treatment with HT (0.3–10 μM, 24 h) significantly inhibited H_2O_2-induced JNK phosphorylation from 0.3 μM (Fig. 5A), whereas HVA inhibited JNK phosphorylation at the highest concentration (10 μM) (Fig. 5B).

4 Discussion

The kidney tubular epithelium is a major site for the processing of a wide spectrum of cytotoxic species, such as oxidized LDL, transition metals, haemoglobin, and myoglobin and potentially nephrotoxic drugs, and commonly experiences a pro-oxidant environment [39]. Indeed, the generation of oxidants such as H_2O_2 has been implicated in the pathogenesis of several forms of acute

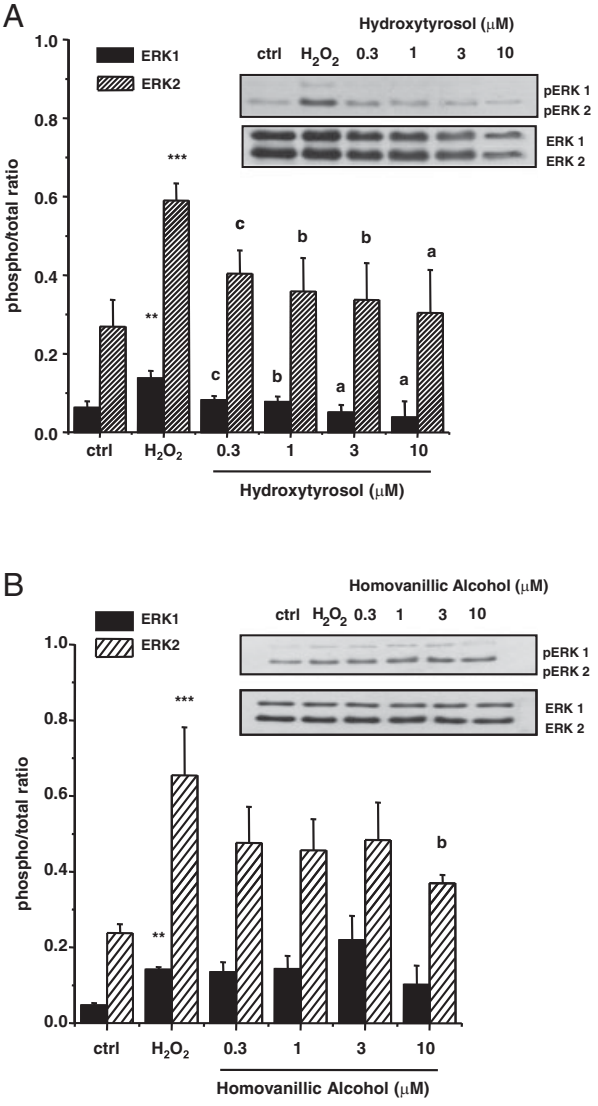

Figure 3. Modulation of H_2O_2-induced ERK phosphorylation by HT or HVA in kidney cells. Cells were treated with HT or HVA (0.3–10 μM, 24 h) prior to addition of H_2O_2 (25 μM, 1 h). Each column represents the mean ± SD of four independent experiments. Data were analysed using Bio-Rad Quantity One 1-D Analysis software. (A) Modulation by HT and (B) Modulation by HVA. ***indicates significant increase relative to basal levels ($p<0.001$); a: $p<0.001$; b: $p<0.01$; and c: $p<0.05$ indicate a significant change relative to H_2O_2-treated cells.

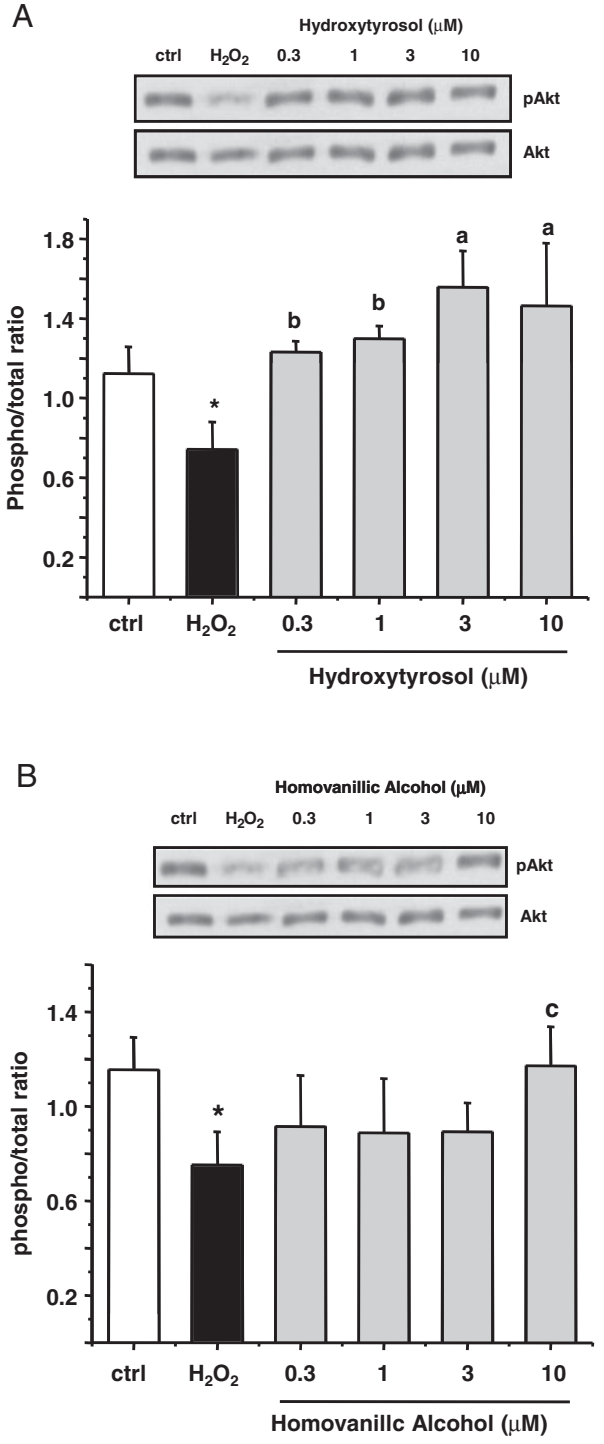

Figure 4. Modulation of H_2O_2-induced Akt de-phosphorylation by HT or HVA in kidney cells. Cells were treated with HT or HVA (0.3–10 μM, 24 h) prior to addition of H_2O_2 (25 μM, 1 h). Each column represents the mean ± SD of four independent experiments. Data were analysed using Bio-Rad Quantity One 1-D Analysis software. (A) Modulation by HT and (B) Modulation by HVA. *indicates significant decrease relative to basal levels ($p<0.05$); a: $p<0.001$; b: $p<0.01$; and c: $p<0.05$ indicate a significant change relative to H_2O_2-treated cells.

tubular cell injury [40]. Levels of H_2O_2 in the human body may be controlled by excretion, and H_2O_2 could play a role in the regulation of renal function [41]. Human urine contains substantial concentrations of H_2O_2, usually exceeding 10 μM and sometimes 100 μM [41]. Although poorly reactive, H_2O_2 may be converted to the hydroxyl radical *via* interaction with a range of transition metal ions [42]. The aim of our study was to compare the biological effects of HT and its metabolite HVA in the same cell culture system, investigating the capacity of the two phenols

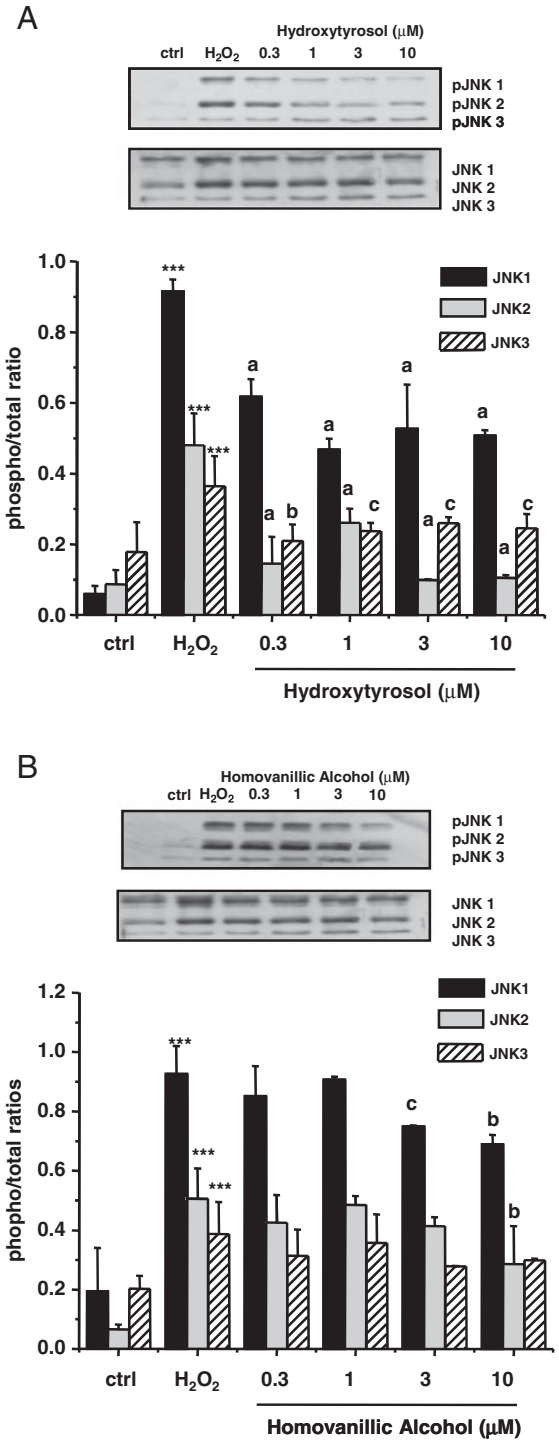

Figure 5. Modulation of H_2O_2-induced JNK phosphorylation by HT or HVA in kidney cells. Cells were treated with HT or HVA (0.3–10 μM, 24 h) prior to addition of H_2O_2 (25 μM, 1 h). Each column represents the mean ± SD of four independent experiments. Data were analysed using Bio-Rad Quantity One 1-D analysis software. (A) Modulation by HT and (B) Modulation by HVA. ***indicates significant increase relative to basal levels ($p<0.001$); a: $p<0.001$; b: $p<0.01$; and c: $p<0.05$ indicate a significant change relative to H_2O_2-treated cells.

to inhibit H_2O_2-induced oxidative damage in LLC-PK1 cells, a porcine kidney epithelial cell line that retains characteristics of the proximal tubular epithelium [43].

In this study, we show that the olive oil polyphenol, HT, evokes significant protection against hydrogen peroxide-induced cellular injury at nanomolar concentrations, whilst its *O*-methylated metabolite, HVA, expresses protection only at higher concentrations (μM). As it has been previously suggested, the level of protection afforded by nanomolar concentrations of HT in kidney cells is unlikely to result from its ability to directly scavenge peroxide and/or related radicals [7, 44]. Rather, its potential to prevent kidney cell injury appears to be due to an ability to inhibit peroxide-induced pro-apoptotic signalling [45, 46]. This mode of action is in line with previous observations with olive oil polyphenols that were shown to exert chemopreventive effects by interacting with the p38/CREB signalling pathway [30], and with the protective effects of hesperetin [47] and quercetin [48] against oxidative cellular injury.

Various members of the MAPK family (ERK, JNK, p38) and Akt/PKB signalling pathways have been implicated in cell survival signalling in response to different toxic influences [49]. The ERK pathway is mainly induced in response to mitogens and growth factors [50] and plays a major role in regulating cell growth, survival and differentiation in different cell lines [51], whilst JNK and p38 pathways are activated in response to chemical and environmental stress and their activations are most frequently associated with the induction of apoptosis [51]. The PI3 kinase/Akt pathway also plays a critical role in the regulation of cell survival and its activity has recently been reported to be involved in preventing renal proximal tubular cell apoptosis [52]. We observed that exposure to hydrogen peroxide resulted in the increased phosphorylation of ERK1/2 and JNK1/2/3 and the inhibition of Akt phosphorylation in renal epithelial cells. These effects may be linked as ERK1/2 has been suggested to contribute to cell death through the suppression of Akt phosphorylation/activation [50]. Although ERK1/2 has been reported to be de-phosphorylated on exposure to peroxide, for example in cardiomyoblasts [48] and fibroblasts [47], in renal cells peroxide has been shown to result in increased ERK1/2 phosphorylation [50]. In addition, increases in ERK1/2 phosphorylation has been linked to tissue damage and diminished renal function in a mouse model of cisplatin-induced nephrotoxicity [53].

Previous studies have shown that specific polyphenols are capable of exerting protective effects against oxidative kidney injury [39]. For example, HT and HVA inhibit H_2O_2-induced oxidative damage in kidney cells with HT exerting a greater effect against lipid peroxidation than HVA [54]. The greater protection afforded by HT may reside in the fact that hydrophilic catechols may form catechol quinone electrophiles, thereby possessing properties similar to those of lipophilic arylating tocopherols quinones, including an ability to undergo Michael additions with cellular thiols such as cysteinyl containing proteins [55]. Hydrophilic catechols,

such as HT, are much more readily oxidized *in vivo* than lipophilic tocopherols, and their hydrophilicity allows the catechol quinone electrophile to react with available thiol nucleophilic groups in enzymes and signalling molecules within the hydrophilic environment of the cell [55]. Consequently, some of the biological effects of HT may be attributed to the rapid generation of the arylating catechol quinone as an oxidation product [55].

Both the protection afforded by HT and HVA are linked to their interactions within the MAP kinase and PI3 kinase signalling cascades. Both compounds inhibited the activation of ERK in response to H_2O_2 treatment, although in line with cell injury observations, HT was effective at nanomolar concentrations, whilst HVA was protective at micromolar levels. These observations agree with previous studies, which have shown that the ability of hesperetin to protect fibroblasts against oxidative injury [47] and quercetin to protect cardiomyoblasts against peroxide damage [48], are linked to their actions of ERK1/2.

We also observed that similar concentrations of HT prevented H_2O_2-induced inhibition of Akt phosphorylation. This agrees with studies in cardiomyoblasts where quercetin, but not its two *O*-methylated derivatives, was able to modulate the phosphorylation state of Akt [48]. Finally, nanomolar concentrations of HT were also capable of strongly inhibiting peroxide-induced increases in the phosphorylation of JNK1/2/3, whereas HVA was active at micromolar levels. Many oxidants capable of inducing apoptosis are also known to be potent activators of JNK [56], and the eventual release of cytochrome *c* and the inhibition of the anti-apoptotic protein Bcl-2 [45]. Previous studies have highlighted that polyphenols inhibit apoptosis is through the suppression of oxidant-induced JNK phosphorylation [57, 58].

In summary, our results demonstrate that both HT and HVA can exert protection against peroxide-induced renal epithelial injury, and that this protection is linked to their potential to modulate the activation of ERK, Akt and JNK. These protective effects appear to be physiologically relevant in that both HT and HVA have been found to be present in the circulation at low micromolar concentrations following oral intake [59]. Therefore, we suggest that one potential protective mode of action of olive oil polyphenols against oxidative kidney cell injury may be attributed to the actions of HT and its *O*-methylated metabolite, on signalling pathways relevant to apoptosis, notably the MAP kinase and PI3 kinase signalling.

The authors have declared no conflict of interest.

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