Diverse effects of natural antioxidants on cyclosporin cytotoxicity in rat renal tubular cells

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Abstract

Background. As is well known, the use of the immunosuppressive drug cyclosporin A (CsA) is partially restricted by its nephrotoxic effects, which include early changes in haemodynamics followed by irreversible injuries to the renal tubules. Although the mechanisms responsible for these side effects are poorly understood, an involvement of reactive oxygen species (ROS) has been suggested. In this study, we selected three natural antioxidants, resveratrol, hydroxytyrosol and vitamin E, on the basis of their scavenging capabilities, and tested their protective effects against CsA toxicity.

Methods. Immortalized rat tubular cells (RPTc) were used as the model system. Cell viability was checked with trypan blue assay, and free radical formation was measured using the fluorescent probe 2,7-dichlorofluorescein (DCF). We evaluated several oxidative stress parameters, including phospholipid peroxidation products, glutathione levels and oxygenase expression.

Results. Incubation of RPTc with 25 μM CsA induced a significant decrease in cell viability paralleled by intracellular ROS formation and alterations in lipid peroxidation. There was also an imbalance of glutathione redox state as well as upregulation of heme oxygenase-1 (HO-1). The three antioxidants, at micromolar concentration, quantitatively prevented the ROS-activated DCF fluorescent signal and membrane lipid peroxidation. Both hydroxytyrosol and resveratrol strengthened the CsA induction of HO-1 expression. Moreover, vitamin E and resveratrol counteracted CsA-induced changes in the glutathione redox state via different mechanisms, whereas hydroxytyrosol was completely ineffective. Similarly, CsA-dependent nephrotoxicity was prevented by vitamin E, while resveratrol only exerted partial protection, and hydroxytyrosol showed no protective effects.

Conclusion. Our results indicate that the diverse cytoprotective effects of the antioxidants tested in these studies were not directly related to their scavenging capabilities. These findings confirm a key role for glutathione in protecting cells from CsA-induced adverse effects and do not support a direct link between CsA-mediated ROS generation and adverse renal effects.

Keywords: cyclosporin A; hydroxytyrosol; oxidative stress; proximal tubular cells; resveratrol; ROS; vitamin E

Introduction

Cyclosporin A (CsA), a cyclic undecapeptide of fungal origin, is the most widely used immunosuppressive drug in organ transplantation and in the treatment of autoimmune disorders. However, its clinical use has been hampered by frequent reports of nephrotoxicity. In fact, moderate to severe renal dysfunction has been documented in ~30% of CsA-treated patients [1]. Although the molecular mechanisms underlying these adverse effects are poorly understood, some have suggested a possible involvement of free radicals [1,2]. In support of this, free radicals are dramatically increased in rat kidney after CsA treatment [3]. Furthermore, it has been reported that the drug induces membrane lipid peroxidation in several in vitro and in vivo experimental models [2,4], as well as in transplanted patients [1]. To explain a link between CsA treatment and production of reactive oxygen species (ROS), several hypotheses have been proposed and these include (i) upregulation of the
cytochrome P450-dependent system in the kidneys [5]; (ii) perturbation of the balance between vasodilation–vasoconstriction, which in turn is responsible for tubular hypoxia–reoxygenation [1–3]; (iii) increased formation of renal thromboxane A2; and (iv) induction of nitric oxide production [1]. In addition, a possible direct interference of the drug with the intracellular homeostasis of glutathione has been suggested [1,2,6]. Finally, it has been reported that CsA treatment induces the expression of the heat shock protein, heme oxygenase-1 (HO-1), in rat kidney [7]. These data have led to studies examining whether antioxidants [i.e. vitamin E (Vit E), N-acetyl cysteine, vitamin C and lazaroids] can neutralize the adverse effects of CsA [2].

Among natural antioxidants, polyphenols play a critical role in counteracting oxidative stress [8,9]. They are bioactive molecules present in all vegetables, and they influence plant morphology, growth and reproduction, as well as resistance to parasites and environmental stress. In addition, the antioxidant capabilities of polyphenols contribute to the general benefits of the ‘Mediterranean diet’. In recent years, great attention has been focused on two dietary polyphenols: (i) resveratrol (Resv), a phytoalexin occurring at high concentration in grape skin and red wine [10]; and (ii) hydroxytyrosol [3,4-dihydroxyphenylethanol (DOPET)], the major phenolic compound present in extra virgin olive oil [8,9].

A number of epidemiological observations have suggested that Resv may reduce the risk of cardiovascular diseases and cancer [10]. In fact, this phytoalexin has been reported to prevent atherosclerosis by modulating the synthesis of hepatic apolipoproteins and lipids, by inhibiting platelet aggregation and the synthesis of proatherogenic eicosanoids in platelets and neutrophils [10].

The biological activities of DOPET have been explored by several groups, and were reviewed by Manna et al. [8] and by Visioli and Galli [9]. DOPET in vitro prevents low-density lipoprotein (LDL) oxidation and platelet aggregation, and inhibits 5- and 12-lipoxygenases. Experiments in our laboratory demonstrated that DOPET, which permeates cell membranes via a passive diffusion mechanism [11], exerts a protective effect on human cells. Specifically, DOPET effectively counteracted the cytotoxicity that was experimentally induced by ROS in Caco-2 intestinal cells and erythrocytes [8]. The bioavailability and metabolism of intravenously injected [14C]DOPET have been investigated in the rat [12].

The aim of the present study was to investigate the possible protective effects of Resv and DOPET (chemical structures given in Figure 1) as well as Vit E on CsA-induced nephrotoxicity. In previous work with cultured hepatocytes, Vit E totally prevented CsA cytotoxicity as well as lipid peroxidation and increased the expression and activity of several endogenous antioxidant enzymes [2,4]. Vit E also preserved renal function and structure when administered in vivo to CsA-treated rats [1,2,4]. Immortalized renal proximal tubule cells (RPTc) have frequently been selected as a model system since tubular cells represent the major target of CsA-induced nephrotoxic effects in vivo. This cell line, obtained by Hopfer and co-workers from normotensive Wistar–Kyoto (WKY) rats, retains the major phenotypic properties of proximal tubule epithelial cells [13]. In the present study, we tested: (i) whether CsA induces direct radical formation in RPTc; (ii) whether cell viability, thiobarbituric acid-reactive substance (TBARS), glutathione redox state and HO-1 induction are affected by CsA treatment; and (iii) the possible protective effects of the selected antioxidants against CsA cytotoxicity and radical formation.

Materials and methods

Reagents

CsA was obtained as Sandimmune (intravenous preparation), containing Creomphore EL and alcohol as vehicle (2:1), from Novartis (Basel, Switzerland), and as a powder from Sigma-Aldrich Chemical Co. (St Louis, MO). Dulbecco’s modified Eagle’s medium F-12 (DMEM F-12), fetal bovine serum (FBS), l-glutamine, epidermal growth factor (EGF), HEPES, dexamethasone-water soluble, insulin/transferrin–sodium selenite media supplement, penicillin–streptomycin, trypsin and phosphate-buffered saline (PBS) tablets were purchased from Gibco Life Science Technology (S. Giuliano Milanese, Milan, Italy). Resv, Vit E as well as all other chemicals were obtained from Sigma-Aldrich Chemical Co. DOPET was the generous gift of Professor Mazzoni (Department of Pharmaceutical and Toxicological Chemistry, University of Naples Federico II, Naples, Italy).

Cell culture

RPTc immortalized from normotensive WKY rats [13] were the generous gift of Professor Hopfer (Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH). Cell cultures were routinely grown at 37°C in a 5% CO2 humidified atmosphere in DMEM F-12, supplemented with 15 mM HEPES, 1.2 mg/ml
**CsA treatment of RPTc**

RPTc were seeded at a density of 10×10^3 cells/cm² and grown for 24 h to ensure culture subconfluence. Before experiments, culture medium was replaced with fresh DMEM F-12 that was serum and antibiotic free, containing different concentrations of CsA, and incubations were carried out for different periods of time. CsA was utilized either as the pharmaceutical form (Sandimmune) or as CsA powder dissolved in dimethylsulfoxide (DMSO; 0.1% final concentration). Maximum soluble CsA concentration in DMSO, without any observable precipitation, was achieved at 50 μM, and the solubility of CsA was carefully checked in all experiments.

**Evaluation of cell viability**

Cellular viability was evaluated using the trypan blue exclusion assay. At the end of incubation, cells were trypsinized, pooled with spontaneously detached cells and tested for their ability to exclude the dye. Cell viability was expressed as a percentage of non-stained cells vs total cells.

**Antioxidants**

The scavenging activity of tested antioxidants was determined by ferrie-reducing antioxidant power (FRAP) and 1,1 diphenyl-2-picrylhydrazyl (DPPH) assays as described below.

For the evaluation of biological activity, the antioxidants were dissolved in DMSO and added to the culture medium together with CsA. All antioxidants were tested at a concentration of 10 μM. At the end of incubation, several oxidative stress markers were evaluated.

**FRAP assay**

The FRAP assay is a colorimetric method based on the reduction of a ferric-tripryidyltriazine complex to its ferrous form. Different amounts of antioxidants were added to 1 ml of working solution prepared by mixing 25 ml of 300 mM acetate buffer (pH 3.6), 2.5 ml of 2,4,6-tripryidyl-s-triazine (TPTZ) solution (10 mM TPTZ in 40 mM HCl) and 2.5 ml of 20 mM FeCl₃·6H₂O. The absorbance at 593 nm was evaluated after 6 min at room temperature and the data were translated into FRAP values (μM), using a methanolic solution of Fe⁷⁺ in the range of 100–2000 μM (FeSO₄·7H₂O) for calibration.

**DPPH test**

The assay of antioxidant scavenging activity is based on the decrease of absorbance at 517 nm following antioxidant-mediated DPPH reduction. Different amounts of antioxidants were added to 1 ml of 15 μM DPPH. After 15 min at room temperature, absorbance was read and EC₅₀ values were calculated.

**Determination of reactive oxygen species**

The dichlorofluorescein (DCF) assay was performed to measure the rate of ROS production. Cells were treated with the non-polar, non-fluorescent 2,7'-dichlorodihydrofluorescein diacetate (DCFH-DA), which undergoes deacetylation by cytosolic esterases to form the polar, non-fluorescent dichlorodihydrofluorescein (DCFH). This latter product, by reacting with ROS, gives rise to the fluorescence derivative dichlorofluorescein (DCF). Stock solutions of DCFH-DA (5.0 mM) were prepared in absolute ethanol and stored at −20°C. Immediately before treatment with CsA, the cell cultures were incubated with 10 μM DCFH-DA in PBS for 30 min at 37°C. The medium was then discarded and the cells washed twice with pre-warmed PBS to remove non-incorporated DCFH-DA. After 20 h of CsA treatment, cells were gently removed by scraping into 2 ml of PBS and kept on ice. The cellular suspension was then transferred into fluorescence cuvettes and the fluorescence intensity of the probe (λ_exc 502 nm; λ_em 520 nm; band widths 5 nm) was recorded. The results were expressed as fluorescence intensity/viable cells.

**Determination of intracellular levels of glutathione**

Glutathione intracellular content was determined using the glutathione reductase/5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) recirculating assay [14]. At the end of CsA treatment, the cells were trypsinized and lysed by freezing and thawing in 200 μl of H₂O. The homogenates were precipitated by 5% (w/v) sulfosalicylic acid and centrifuged for 15 min at 2000 g (4°C). Supernatants were stored at −80°C until used. For total glutathione determination, 50 μl of sample was treated with DTNB (150 μM), NADPH (200 μM) and 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA. The reaction was initiated by addition of GSSG reductase (1.7 IU). After 10 min incubation at room temperature, the [GSH] was measured spectrophotometrically at 412 nm. We evaluated [GSSG], as previously described, following GSH derivatization by 2-vinylpyridine. For this purpose, 2-vinylpyridine (0.6% final concentration) was pre-incubated with 50 μl of cell supernatant for 1 h at room temperature in 500 μl of the same buffer.

The effects of CsA on the GSSG and GSH content and on the GSH/GSSG ratio, reported in Table 2, represent the means ± SD from nine distinct experiments, each performed in duplicate. The three values (i.e. GSSG, GSH and GSH/GSSG ratio) were also determined in extracts of cells cultured in the presence of CsA plus one of the investigated antioxidants. In this case, the results, which are reported in Figure 6, were performed in duplicate three times for each molecule. In each set of experiments, the results were expressed as the difference with respect to CsA-treated controls. The numerical values of all the analysed parameters are reported in their respective figure legends.

**TBARS assay**

The extent of lipid peroxidation was evaluated by measuring TBARS. Briefly, 250 μl of cellular suspension was combined with 500 μl of 30% trichloracetic acid, vortexed and centrifuged at 5000 g for 15 min. From this, 500 μl of the acid-soluble supernatant was added to an equal volume of 1% thiobarbituric acid in 0.05 M NaOH and the mixture was heated in a boiling water bath for 10 min. The absorbance
of the developed pink chromophore was determined at 532 nm.

**RNA extraction and semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR) analysis of heme oxygenase 1**

Total RNA extraction and purification, as well as RT–PCR analyses, were carried out as previously described [15]. The primers and the PCR conditions employed for each gene are shown below. All the reactions had a hot start of 10 min at 95°C and a final elongation step at 72°C for 7 min. GAPDH: 5'‐CAATGTATCCGTGTTGGATCTGACAT-3' (sense) and 5'-ATATTCATTGTCATACCAGGAATGAGC-3' (antisense); 20 cycles composed of steps at 95°C for 1 min, 58°C for 1 min, 72°C for 1 min. HO-1: 5’-TGGAAGAGGAGATAGAGCGA-3’ (sense) and 5’-TGTTGAGCAGGAAGGGCGGTC-3’ (antisense); 33 cycles composed of steps at 95°C for 1 min, 58°C for 1 min, 72°C for 1 min. Before amplifications with each specific primer pair, an aliquot of the cDNA preparation was amplified using GAPDH primers to determine the integrity of the generated cDNA. Moreover, we used five different cDNA concentrations to ensure that signals (of both GAPDH and HO-1) were proportional to input mRNA. Finally, each experiment was performed at least in triplicate, and in several cases was done in quadruplicate.

**Statistical analysis**

Due to experimental design, statistical analysis was performed using different tests. One-way analysis of variance (ANOVA) was used to compare the effect of different reagents on cell viability, TBARS and fluorescence intensity, and Dunnett’s two-tailed t-test was used for multiple comparisons vs the same CsA group. Student’s t-test was used to compare mean levels of glutathione in the CsA group vs the groups given the antioxidants (DOPET, Resv and Vit E) in the three different experiments. Comparisons of mean levels of glutathione in the CsA group vs the control group in the three experiments was made by ANOVA adjusted for each experiment. Statistical analyses were performed using SAS version 8.1 (SAS Institute, Cary, NC).

**Results**

In order to evaluate the toxic effects of CsA in our model system, RPTc were incubated overnight (20 h) in the presence of increasing drug concentrations, and cell viability was evaluated as described in Materials and methods. We compared the effects of two forms of CsA: the commercial Sandimmune (containing Cremophore EL and alcohol as vehicles) and analytical grade CsA dissolved in DMSO. As shown in Figure 2A, analytical grade CsA appeared to be significantly less cytotoxic than Sandimmune. The higher toxicity of Sandimmune was probably related to its vehicle, which has been reported to be detrimental in several cell systems [16]. The time dependency of drug cytotoxicity is reported in Figure 2B. In our model system, CsA toxicity was a relatively late event, since 95% of cells were still largely viable up to 8 h treatment in the presence of 25 μM CsA. Conversely, prolonged incubation times led to a dramatic decrease in cell viability, with 30 and 55% of cell death after 20 h incubation in the presence of analytical grade CsA and Sandimmune, respectively. On the basis of these results, unless otherwise specified, we routinely used a 20 h incubation in the presence of 25 μM CsA as either powder dissolved in DMSO or Sandimmune. Cell viability was evaluated by the trypan blue exclusion method. Data are expressed as means±SD (n=3). (B) RPTc were incubated for different times in the presence of 25 μM CsA as either powder dissolved in DMSO or Sandimmune. Cell viability was evaluated as in (A). Data are expressed as means±SD (n=3).

In order to investigate the role of ROS and the protective effects of antioxidants on CsA-induced cytotoxicity, we compared Vit E with Resv and DOPET, two phenolic compounds selected on the basis of their different scavenging capability. The antioxidant capacities of these three molecules, chemically measured as FRAP, are reported in Table 1. DOPET showed the most powerful antioxidant activity, as indicated by the highest FRAP value compared with the other compounds. These data are in agreement with their antioxidant activity measured by the DPPH quenching test. In fact, DOPET was the most effective DPPH scavenger, which is consistent with its lower EC_{50} value.

The effect of the three antioxidants on CsA-induced cytotoxicity is shown in Figure 3. Unexpectedly, only...
Vit E was able significantly to prevent the toxic effect of the drug and restored cell viability by 75%; Resv exerted only 25% protection, while DOPET is completely ineffective.

On the basis of these findings, we used the DCF assay that is routinely used in many laboratories to detect cellular radical formation in order to investigate whether CsA treatment results in ROS formation in our model system. Figure 4 shows that incubation with CsA resulted in an increase in DCF fluorescent signal, which is indicative of ROS formation. The CsA-induced fluorescence signal was completely prevented by treatment with the three antioxidants (10 μM). In spite of their different scavenging activities, the molecules equally quenched the fluorescent signal, suggesting that their concentrations largely exceeded the CsA-induced ROS formation under these experimental conditions. The antioxidant concentrations were not cytotoxic in our model system and were within the same order of magnitude as found in the ‘Mediterranean Diet’ [8,9].

Because membrane phospholipids are a major target of oxidative damage, we examined the effect of CsA on lipid peroxidation. As shown in Figure 5, cell incubation in the presence of 25 μM CsA significantly increased the TBARS concentration (30% above basal), providing confirmation that the treatment exposed cells to an oxidative microenvironment. Treatment with the three antioxidants equally counteracted the increase in TBARS formation.

A substantial body of evidence has demonstrated that increased HO-1 expression occurs as a defence mechanism during a wide range of unrelated conditions, and all are characterized by alterations in the cellular redox state [18]. Therefore, HO-1 gene transcription was evaluated in CsA-treated RPTc in the presence or absence of the tested antioxidants. As shown in Figure 6, untreated cells showed very low levels of HO-1 transcript, while CsA induced a significant increase in gene expression. The two phenolic compounds, Resv and DOPET, led to a clear upregulation of HO-1 gene transcription, while Vit E did not affect HO-1 basal expression. Moreover, when CsA was added to the cells along with either Resv or DOPET, a further increase in HO-1 expression was observed. In contrast, Vit E did not modify the effect of the immunosuppressive compound on HO-1 gene transcription. Because of the pivotal role of glutathione in cellular protection against free radical damage [2], we evaluated the intracellular GSH/GSSG ratio. It is well known that intracellular peroxides are selectively inactivated by GSH peroxidase, leading to an increase of cellular GSSG. As reported in Table 2, CsA treatment induced a significant increase in cellular GSSG and a 50% reduction in the [GSH]/[GSSG] ratio.

### Table 1. FRAP and DPPH values of natural antioxidants

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>FRAP a</th>
<th>DPPH test b</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPET</td>
<td>850 ± 51</td>
<td>2.05 × 10⁻⁷‡</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>360 ± 22</td>
<td>5.04 × 10⁻⁶‡</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>209 ± 20</td>
<td>1.75 × 10⁻⁵</td>
</tr>
</tbody>
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a FRAP values are expressed as μM Fe²⁺ equivalents.
b DPPH values are expressed as EC₅₀.
c Data from Manna et al. [8].

### Table 2. Effects of CsA on glutathione levels in RPTc a

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CsA (25 μM)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSSG nmol/mg protein</td>
<td>1.47 ± 0.48</td>
<td>2.94 ± 1.18</td>
<td>0.002</td>
</tr>
<tr>
<td>GSH nmol/mg protein</td>
<td>17.13 ± 2.72</td>
<td>16.74 ± 3.65</td>
<td>0.812</td>
</tr>
<tr>
<td>GSH/GSSG molar ratio</td>
<td>12.82 ± 4.59</td>
<td>6.55 ± 3.23</td>
<td>0.004</td>
</tr>
</tbody>
</table>

a Values are given as mean ± SD.
P-values are adjusted for experiments.
The CsA-induced alterations in GSH metabolism were differentially affected by the tested antioxidants (Figure 7): Vit E fully prevented both CsA effects, DOPET was completely ineffective and Resv only partially restored the [GSH]/[GSSG] ratio. The abilities of the three antioxidants in counteracting CsA-induced alterations in the glutathione redox state correlated well with their diverse effects against CsA-induced cytotoxicity (Figure 3). Specifically, Vit E exerted significant protection, whereas DOPET, which was unable to restore the GSH/GSSG ratio, did not provide any appreciable cytoprotection.

Discussion

Data in the literature indicate that RPTs are the major target of CsA-induced nephrotoxicity in both humans and animal models [1]. In fact, even short-term CsA treatment induces a loss of RPTc brush border and causes proximal tubule dilation [1]. In our model system, which accurately mimicked the properties of RPTc, such as basal conductance and Na-K-ATPase activity [13], CsA induced a significant formation of ROS and increased lipid peroxidation. This result confirms previous data obtained with other cellular systems [4,17]. The CsA concentration employed in this study (25 μM) was chosen to approximate the kidney levels of the drug that are reached in vivo during treatment and that lead to nephrotoxic effects [1,2].

In our system, ~50% cell death was observed after 20 h exposure to 25 μM CsA. In order to investigate a possible relationship between ROS formation and CsA-induced cytotoxicity, we tested three natural antioxidants. The antioxidants used in this study, endowed with high scavenging power and low toxicity in vivo, effectively counteracted CsA-induced free radical production and membrane lipoperoxidation. However, the effective protection of these antioxidants was not paralleled by expected equivalent decreases in CsA cytotoxicity. Indeed, the decrease in ROS and TBARS formation was associated with a comparable cytoprotective effect only in the presence of Vit E. In addition, Resv exerted only a partial protection, and DOPET, the antioxidant endowed with the highest radical scavenging activity (see Table 1), failed to exert any protection at all. In agreement with these findings, recently unpublished in vivo experiments showed that DOPET failed to prevent CSA nephrotoxicity, measured as decreased glomerular filtration rate, even though it exerted powerful antioxidant effects in this model.

In the present system, the CsA-induced imbalance of the glutathione redox state was also differentially affected by the three antioxidants. Vit E effectively counteracted the alterations in glutathione metabolism, probably by modulating both the expression and activity of glutathione peroxidase [17], while Resv and DOPET did not exert significant effects. These findings correlated well with the different cytoprotective effects exerted by the tested antioxidants, and confirm the critical role of glutathione in CsA cytotoxicity observed in different cellular models [17]. In fact, our results are in agreement with previous findings that suggest a possible direct interference of the drug with the intracellular homeostasis of glutathione [1,2,6].

HO-1 induction is a relevant step in the cellular adaptation to stresses inflicted by pathological events, including UVA irradiation, heavy metals, inflammation and pro-oxidant states [18]. It has been reported that an increased expression of this enzyme occurs in tubules of CsA-treated rats [7]. Accordingly, in our model system, CsA upregulated HO-1 expression. Moreover, both DOPET and Resv were able to induce the expression of...
this gene, confirming data obtained with other polyphenolic antioxidants. The mechanism through which the two polyphenols upregulate HO-1 transcription is still poorly understood but it may rely on the complex effects of the molecules on specific pathways which modulate HO-1 promoter activity. The combination of CsA plus DOPET or Resv caused a higher expression of HO-1, suggesting either a simple cumulative effect or a possible amplificatory mechanism. In contrast to the two polyphenols, Vit E did not upregulate HO-1 expression and did not interfere with the effects of CsA on HO-1 expression. Altogether, these findings indicate that the two polyphenols and Vit E show clearly different phenotypical effects, and that the actions of Resv and DOPET on HO-1 expression are probably unrelated to their antioxidant properties.

Taken together, our results strengthen the view that ROS-independent pathways may be the primary cause of CsA-mediated toxicity. Also of importance are the distinct effects of the presently used antioxidants on various signalling processes. Recent data from our laboratory demonstrated that DOPET modifies the expression profile of promyelocytic cells, resulting in a significant upregulation of several genes. This effect is linked to the selective modulation of signalling pathways, such as Erk1/2 and c-Jun N-terminal kinase (JNK) [15]. We have also reported that Resv upregulates p21 by an Erk1/2-dependent mechanism in K562 cells [19]. On the other hand, Vit E in cultured hepatocytes induces an increased expression and activity of several enzymes such as MnSOD, CuZnSOD and catalase [17]. In this context, it is interesting that several alternative roles recently have been attributed to Vit E, that are independent of its radical chain-breaking function [20]. For instance, it has been demonstrated that Vit E in some instances affects cellular signalling and gene expression. Moreover, several enzymes, such as protein kinase C, 5-lipoxygenase and phospholipase A2, are inhibited at the post-translational level whereas phosphatase 2A and diacylglycerol kinase are activated. Finally, it has been reported that Vit E activates human pregnane X receptor, resulting in the activation of genes coding for cytochromes P450, which are involved in CsA catabolism [20]. Therefore, it is entirely possible that Vit E protection is due to non-antioxidant effects of this molecule.

In conclusion, ROS generation induced by CsA was not necessarily related to its nephrotoxicity. Moreover, the generalization that antioxidants exert protective effects against the adverse effects of CsA, which has been proposed by several authors and shared by nephrologists, is called into question by the present findings.

Conflict of interest statement. None declared.

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