*Free Radicals Research*, Vol. 35, pp. 843–856
Reprints available directly from the publisher
Photocopying permitted by license only





# α-Tocopherol Modulates Tyrosine Phosphorylation in Human Neutrophils by Inhibition of Protein Kinase C Activity and Activation of Tyrosine Phosphatases

SANDRA S. CHAN[a,*], HUGO P. MONTEIRO[b], FERNANDA SCHINDLER[b], ARNOLD STERN[c] and VIRGINIA B.C. JUNQUEIRA[d,†]

[a]*Instituto de Quımica, Universidade de São Paulo, São Paulo, Brazil;* [b]*Fundação Pro-Sangue, Hemocentro de São Paulo, São Paulo, Brazil;* [c]*New York University, Medical Center, New York, NY, USA;* [d]*Disciplina Geriatria, Departamento de Medicina, UNIFESP-EPM, Rua Pedro de Toledo, 781, 6° andar, CEP: 04038-032, São Paulo, Brazil*

Accepted by Professor H. Sies

*(Received 14 February 2001; In revised form 25 June 2001)*

α-Tocopherol augmentation in human neutrophils was investigated for effects on neutrophil activation and tyrosine phosphorylation of proteins, through its modulation of protein kinase C (PKC) and tyrosine phosphatase activities. Incubation of neutrophils with α-tocopherol succinate (TS) resulted in a dose-dependent incorporation into cell membranes, up to 2.5 nmol/$2 \times 10^6$ cells. A saturating dose of TS (40 μmol/l) inhibited oxidant production by neutrophils stimulated with phorbol myristate acetate (PMA) or opsonized zymosan (OZ) by 86 and 57%, as measured by luminol-amplified chemiluminescence (CL). With PMA, TS inhibited CL generation to a similar extent to staurosporine (10 nmol/l) or genistein (100 μmol/l), and much more than Trolox (40 μmol/l). With OZ, TS inhibited CL to a similar extent to Trolox. Neutrophil PKC activity was inhibited 50% or more by TS or staurosporine. The enzyme activity was unaffected by genistein or Trolox, indicating a specific interaction of α-tocopherol. TS or Trolox increased protein tyrosine phosphorylation in resting neutrophils, and as with staurosporine further increased tyrosine phosphorylation in PMA-stimulated neutrophils, while the tyrosine kinase (TK) inhibitor genistein diminished phosphorylation. These effects in resting or PMA-stimulated neutrophils were unrelated to protein tyrosine phosphatase (PTP) activities, which were maintained or increased by TS or Trolox. In OZ-stimulated neutrophils, on the other hand, all four compounds inhibited the increase in tyrosine-phosphorylated proteins. In this case, the effects of pre-incubation with TS or Trolox corresponded with partial inhibition of the marked (85%) decrease in PTP activity induced by OZ. These results indicate that α-tocopherol inhibits PMA-activation of human neutrophils by inhibition of PKC activity, and

*S.S. Chan is a graduate student from the Departamento de Bioquımica, IQUSP, and the recipient of a fellowship from the National Research Council (CNPq) of Brazil.

†Corresponding author. Tel./Fax: +55-11-55-746513. E-mail: junqueira-geriat@pesquisa.epm.br.

inhibits tyrosine phosphorylation and activation of OZ-stimulated neutrophils also through inhibition of phosphatase inactivation.

*Keywords*: α-Tocopherol; Neutrophil; Oxidative burst; Tyrosine phosphorylation; Protein tyrosine phosphatase; Protein kinase C

*Abbreviations*: Anti-P-Tyr, anti-phosphotyrosine antibody; AP-1, activator protein 1; CL, chemiluminescence; DTT, dithiothreitol; EGF, epidermal growth factor; GSH, glutathione reduced form; HPLC, high performance liquid chromatography; LPS, lipopolyssacharide; mAb, monoclonal antibody; MAP, mitogen-activated protein; NFκB, nuclear factor inducing immunoglobulin κ light chain in B cells; OZ, opsonized zymosan; PBS, phosphate buffered saline; PMA, phorbol myristate acetate; PKC, protein kinase C; PTP, protein tyrosine phosphatase; ROS, reactive oxygen species; TBS, tris-buffered saline; TBS-T, tris-buffered saline tween-20; TK, tyrosine kinase; TS, α-tocopherol succinate



## INTRODUCTION

The production of superoxide ($O_2^{\cdot-}$) and other reactive oxygen species (ROS) provides microbicidal and inflammatory activities of neutrophils.[1] In response to stimuli, neutrophils generate ROS by a signaling cascade that involves the assembly of cytosolic and membrane associated components to form the active NADPH oxidase complex.[2] It is well established that phosphorylation of many proteins is a primary event in the activation process.[2,3] Among these proteins are the oxidase components, $p47^{phox}$, $p67^{phox}$ and the small GTP-binding protein rac.[3–5] Protein kinase C (PKC), an ubiquitous calcium and phospholipid-dependent serine/threonine protein kinase, is known as a modulator of $O_2^{\cdot-}$ production.[2,6] PKC is activated by several agonists including, phorbol esters, diacylglycerol and oxidants.[7–10] Although PKC plays an important role in the signaling pathways involved in the activation of the neutrophil oxidase, the participation of tyrosine kinases (TKs) has also been demonstrated.[11–15] Indeed, a variety of TKs inhibitors inhibit $O_2^{\cdot-}$ production triggered by several agonists.[16] Furthermore, cell stimulation with different agonists promotes tyrosine phosphorylation in many proteins.[11–14] Activation of mitogen-activated protein kinases (MAP kinases) and Src kinases was also documented.[15] Besides phosphorylation, dephosphorylation of proteins by phosphatases has been suggested to be involved in the regulation of $O_2^{\cdot-}$ production by phagocytes.[17]

Despite the microbicidal role of ROS produced by neutrophils, in inflammatory conditions, excessive amounts of ROS may result in host cell damage. Moreover, studies using several cell types show that ROS and other oxidants may also function as signaling molecules, and amplify the inflammatory response. It has been demonstrated that oxidants increase the phosphorylation levels of cellular proteins, activating protein kinases or inhibiting protein phosphatases.[20–25] Studies using human neutrophils showed that exogenous hydrogen peroxide ($H_2O_2$) and other ROS generated during the oxidative burst activate TKs, an effect that is reversed by the thiol reductants, *N*-acetylcysteine and dithiothreitol (DTT).[18,19]

It was demonstrated that α-tocopherol, one of the most important lipid-soluble natural antioxidants, inhibits PKC activity in many cell types,[8,26,27] and suppresses the activation of the transcription factors NF-κB (nuclear factor inducing immunoglobulin κ light chain in B cells) and AP-1.[18,28–29] However, the role of antioxidant molecules such as α-tocopherol in the modulation of signaling pathways in human neutrophils remains relatively unaddressed.[30] Accordingly, we have explored the effect of α-tocopherol on the PKC- and the tyrosine phosphorylation-dependent signaling pathways in stimulated neutrophils. To explore the underlying mechanisms of the α-tocopherol-modulated production of oxidants by neutrophils, we compared its effects with the PKC inhibitor staurosporine, with the TK inhibitor genistein, and with the less hydrophobic vitamin E analog, Trolox®.

## MATERIALS AND METHODS

### Materials

dl-α-Tocopherol succinate (TS), phorbol myristate acetate (PMA), zymosan, hystopaque, protein A-sepharose CL-4B and all other routine reagents were from Sigma (St. Louis, MO). Electrophoresis reagents used were obtained from Bio-Rad (Hercules, CA) and Amersham-Pharmacia Biotech (Uppsala, Sweden). Trolox® was obtained from Aldrich (Milwaukee, WI) and [γ-$^{32}$P] ATP was from New Life Science Products (Boston, MA). A monoclonal antibody (mAb) specific for the human epidermal growth factor (EGF) receptor (Mab 108.1) was kindly provided by Dr Joseph Schlessinger (New York University Medical Center). Monoclonal anti-phosphotyrosine antibody (anti-P-Tyr) PY20 was purchased from Transduction Laboratories (Lexington, KY).

### Isolation of Human Neutrophils

Neutrophils were isolated from healthy human donors by Ficoll-Hypaque centrifugation, dextran sedimentation of red cells, and removal of the remaining red cells by hypotonic lysis.[31] Neutrophils were washed and resuspended in phosphate-buffered saline buffer (PBS) (10 mmol/l phosphate buffer, 140 mmol/l NaCl, 1 mmol/l $CaCl_2$, 0.5 mmol/l $MgCl_2$, 1 mg/ml glucose pH 7.3). Reactions were carried out in the same buffer. Cells were routinely counted and their viability was determined by Trypan blue exclusion.

### *In Vitro* Incorporation of α-tocopherol by Human Neutrophils

Neutrophils ($2\times10^6$ cells/ml) were incubated with increased concentrations of TS (0.1–40 μmol/l), under permanent shaking, for 30 min, at 37°C. After this period, cells were centrifuged at 600*g*, for 10 min at 4°C. The supernatant was removed and the pellet was washed once. Cells were resuspended in 0.5 ml of PBS and kept at −80°C until the extraction and HPLC determination of α-tocopherol incorporated into cell membranes. Saponification and extraction of α-tocopherol from neutrophil membranes were performed according to Hatam and Kayden.[32] Briefly, the suspension of cells were treated with 1 ml of 1% ascorbic acid in ethanol, and heated at 70°C for 2 min. Just after, 300 μl of saturated KOH was added and the suspension was heated to 70°C for 30 min. After cooling, 1 ml of milliQ-water and 4 ml of hexane were added to each tube. The mixture was vigorously shaken for 2 min, and the phases separated by centrifugation (1000*g*, 5 min, 25°C). The organic phase (3 ml of hexane) was removed and evaporated under an $N_2$ stream. Samples were resuspended in 200 μl of methanol/ethanol (1:1) and aliquots of 20 μl were injected into a C8 column (3.9 × 150 mm) coupled to an HPLC apparatus. The mobile phase used to separate α-tocopherol was methanol:water (97:3) containing 20 nmol/l $LiClO_4$, with a flow rate of 1.0 ml/min. Detection of α-tocopherol was done by a BIO Analytical System (BAS) electrochemical detector, using a potential of +0.8 V.[33]

### Measurement of Oxidants Production by Neutrophils Using Luminol-amplified Chemiluminescence (CL)

Neutrophils ($1\times10^5$ cells/ml) were incubated with TS (40 μmol/l), Trolox (40 μmol/l), staurosporine (10 nmol/l) or genistein (100 μmol/l) for 30 min, at 37°C, under permanent shaking. After incubation, cells were stimulated with PMA (20 ng/ml), or opsonized zymosan (OZ) (100 particles/cell). Light emission was amplified by luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (100 μmol/l). The assay was performed in a Bio-Orbit luminometer model 1251, with controlled temperature (37°C) and continuous shaking. The maximum light

emission measured was expressed as $mV/10^5$ cells.[34–36]

### Immunoblotting of Protein Tyrosine Phosphorylation

Neutrophils ($1 \times 10^6$ cells/ml) were incubated with or without TS (40 μmol/l), Trolox (40 μmol/l), staurosporine (10 nmol/l) or genistein (100 μmol/l) for 30 min, at 37°C, under continuous shaking. Cells were than stimulated with PMA (20 ng/ml) for 5 min or with OZ(100 particles/cell) for 15 min. After stimulation, neutrophils were centrifuged (600*g*, 4°C, 10 min) and cell pellets were resuspended in 300 μl of lysis buffer A (20 mmol/l Hepes, 150 mmol/l NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/l $MgCl_2$,1 mmol/l EGTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mmol/l phenylmethanesulphonyl fluoride, pH 7.5). The buffer also contained tyrosine and serine/threonine pohosphatase inhibitors (2 mmol/l sodium orthovanadata, 50 mmol/l NaF and 10 mmol/l sodium pyrophosphate). Cells were lysed in a sonic disruptor (Cole Parmer Instrument, model 4710) using three cycles of 10 s, with 15% potency.[37] The protein content of lysates was determined according to Bradford.[38] The lysate (30 μg of protein) was boiled with 3-fold-concentrated SDS/PAGE sample buffer and resolved by 10% SDS/PAGE. After electrophoresis, the gel was transferred to a 0.45 μm-pore nitrocellulose sheet, using the Pharmacia Novablot system. The transfer was carried out for 2 h at 200 mA in 48 mmol/l Tris, 39 mmol/l glycine, 0.037% SDS and 20% methanol. The nitrocellulose blots were saturated with tris-buffered saline (TBS) (10 mmol/l Tris, pH 7.5, 150 mmol/l NaCl) containing 5% BSA for 4 h, at room temperature. The blots were washed in Tris-buffered saline-Tween-20 (TBS-T) and incubated overnight with a mouse monoclonal anti-P-Tyr antibody at 4°C. After three washes with TBS-T, blots were probed with horseradish peroxidase-conjugated-anti-mouse secondary antibody. The blots were developed using the enhanced CL system ECL from Amersham.[23,37]

### Assay of PKC Activity

Neutrophils ($1 \times 10^6$ cells/ml) were incubated with TS (40 μmol/l), Trolox (40 μmol/l), staurosporine (10 nmol/l) or genistein (100 μmol/l) for 30 min, at 37°C, with shaking. Control cells, without treatment, were incubated in the same conditions. The cell lysis was performed in lysis buffer B (50 mmol/l Tris, 5 mmol/l EDTA, 10 mmol/l EGTA, 0.3% β-mercaptoethanol, 50 μg/ml PMSF, 10 mmol/l benzamidine, pH 7.5) and PKC activity was determined using the Biotrak kit from Amersham Life Sciences (Buckinghamshire).

### Immunoprecipitation of EGF Receptor

A431 cells were solubilized in lysis buffer A, and immunoprecipitated with mAb 108.1 against the human EGF receptor, conjugated with Protein A-sepharose CL4B. After washing of immunoprecipitated receptors with kinase buffer (20 mmol/l Hepes, pH7.5, 5 mmol/l $MnCl_2$, and 2 mmol/l $MgCl_2$), 8 μCi of [$\gamma$-$^{32}$P]ATP was added to immunoprecipitates, and the mixture was incubated for 5 min at 25°C. The reaction was stopped by the addition of HNTG buffer (20 mmol/l Hepes, 150 mmol/l NaCl, 10% glycerol, 0.1% Triton X-100, pH7.5), followed by three washes with the same buffer. The labeled immunoprecipitate was resuspended in the same buffer, and kept al 4°C for later assay of protein tyrosine phosphatases (PTPs).[30]

### PTPs Assay

Neutrophils ($1 \times 10^6$ cells/ml) were incubated with TS (40 μmol/l) or with Trolox (40 μmol/l) for 30 min, at 37°C, under shaking. Control cells were incubated with PBS. Just after, cells were

stimulated with PMA (20 ng/ml) for 5 min or OZ (100 particles/cell) for 15 min. After stimulation, neutrophils were centrifuged (600*g*, 4°C, 10 min) and the pellets were resuspended with 300 µl of lysis buffer A. Cells were lysed as described previously. The protein content of lysates was determined according to Bradford,[38] and a volume of cell lysate corresponding to 100 µg of protein was mixed with 20 µl of a suspension containing [$^{32}$P] phosphorylated immunoprecipitated EGF receptors, prepared as described above. Reactions were carried out for 30 min at 37°C under shaking, and stopped by centrifugation of the immunoprecipitates (3000*g*, 2 min). After centrifugation, the supernatant was immediately aspirated and 50 µl of 2-fold-concentrated SDS/PAGE sample buffer was added to the immunoprecipitates. Samples were boiled for 5 min and resolved by SDS/PAGE (7.5% gels). After drying, gels were exposed to autoradiographic films for 2–12 h, at room temperature. The intensity of the band corresponding to the EGF receptor was quantified by densitometric analysis of linear-range autoradiograms, using a LKB Ultroscan enhanced laser densitometer.[30]

### Statistical Analysis

Values were reported as means±SD. Significant differences between groups were determined by Analysis of Variance following by Tukey's test for multiple comparison of means. The level of significance was set at $p < 0.05$ for all statistical analysis.

## RESULTS

### Incorporation of α-tocopherol into Neutrophils

Isolated human neutrophils incubated with increasing concentrations of TS (0.5–40 µmol/l), showed saturation for α-tocopherol incorporation to cell membranes. Saturating levels were obtained at a concentration of 40 µmol/l of TS (Fig. 1). Therefore, this concentration was used throughout the study.

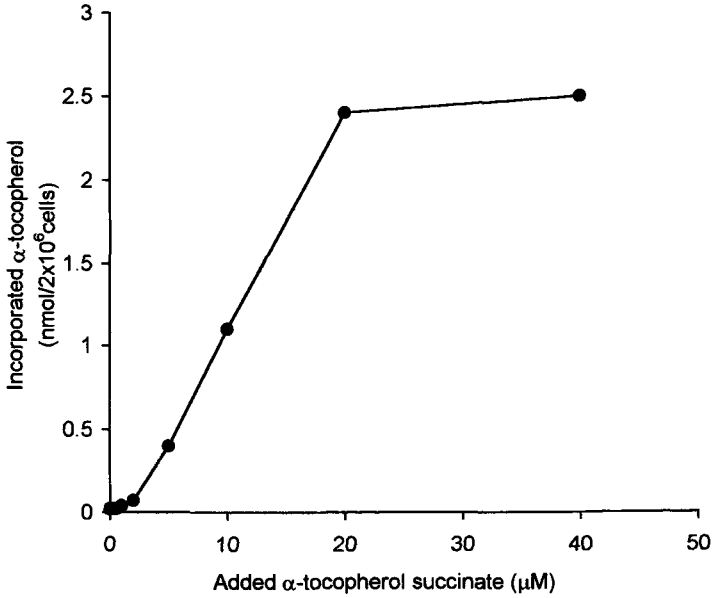


FIGURE 1 Uptake of α-tocopherol by neutrophils. Neutrophils ($2\times10^6$ cells) were incubated with increased concentrations of TS for 30 min, at 37°C, with constant agitation. Incorporated vitamin E was extracted from cell membranes and its concentration was measured by HPLC. The curve shows one representative experiment from a set of five independent experiments.

### Production of Oxidants by Stimulated Neutrophils

Both PMA (20 ng/ml), a soluble stimulus that directly activates PKC, and OZ (100 particles/cell), a phagocytic stimulus that acts in many pathways,[39] stimulated oxidant production in human neutrophils, as measured by CL. TS (40 μmol/l), or Trolox at the same concentration slightly enhanced non-stimulated CL. However, this response was not significant when compared to that from cells activated by PMA or OZ.

As shown in Fig. 2, the production of oxidants of PMA-stimulated cells was strongly (86.4 ± 0.9%) inhibited by TS (40 μmol/l), and to a lesser extent (30.5 ± 8.5%) by Trolox (40 μmol/l). When OZ was utilized as the neutrophil activator, inhibition of CL by TS and Trolox was, 57 ± 14% and 47.6 ± 2.4%, respectively (Fig. 2). In experiments, on the mechanisms involved, production of oxidants by both PMA- and OZ-stimulated neutrophils was significantly inhibited by staurosporine (10 nmol/l) and by genistein (100 μmol/l) but with differences in effectiveness (Fig. 2). Stauroporine, at low concentration, is a potent PKC inhibitor, and it completely blocked oxidant production in PMA-stimulated cells, whereas in OZ-stimulated cells, it caused a lesser inhibition (51.3 ± 5.5%). On the other hand, genistein, a TK inhibitor and antioxidant flavonoid, was a very effective inhibitor of the oxidative burst derived from cells activated by both stimuli: 83.2 ± 11.5% inhibition in PMA-stimulated cells and 95.4 ± 1.1% inhibition in OZ-stimulated cells (Fig. 2).

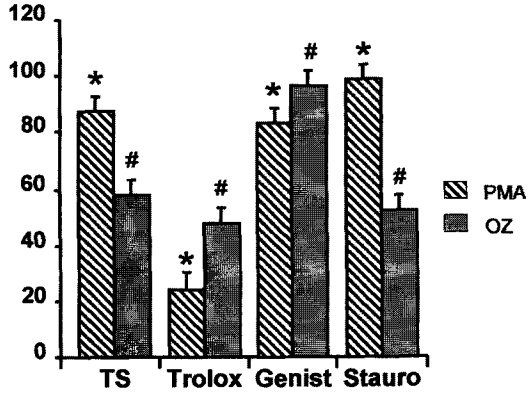


FIGURE 2 Inhibition of oxidant production in PMA- and OZ-stimulated neutrophils; by TS (40 μmol/1), Trolox (40 μmol/l), staurosporine (10 nmol/l) and genistein (100 μmol/l). Cells ($1 \times 10^5$) were incubated with the antioxidants or kinase inhibitors at the indicated concentrations for 30 min at 37°C, under continuous shaking. Treated or non-treated cells were stimulated with PMA (20 ng/ml) for 5 min or with OZ (100 particles/cell) for 15 min. Oxidant production was analyzed as described in "Materials and Methods". The % inhibition was calculated relative to oxidant production in PMA- or OZ-stimulated cells. Results are shown as means±SD of at least three independent experiments. * Values significantly different from PMA-stimulated cells ($p < 0.05$). **Values significantly different from OZ-stimulated cells ($p < 0.05$).

### PKC Activity

Production of oxidants by PMA-stimulated neutrophils was inhibited to a similar extent by the PKC inhibitor staurosporine or by TS (Fig. 2). To obtain direct evidence on the inhibitory effects of TS on PKC activity, we assayed the kinase activity in lysates from cells incubated with or without the antioxidant, and compared the effects of Trolox, staurosporine and genistein. In cells treated with TS, PKC activity decreased to 46.5 ± 5.5% of control (Fig. 3). Incubation with staurosporine even more potently decreased PKC activity to 28.3 ± 4.2% of control, whereas Trolox and genistein showed no effects on the activity of this enzyme (Fig. 3).

### Tyrosine Phosphorylation of Proteins in PMA-stimulated Neutrophils

The overall phosphorylation on tyrosine residues was notably increased after neutrophil treatment with PMA (Fig. 4). TS or Trolox alone also induced tyrosine phosphorylation, and TS further enhanced tyrosine phosphorylation of this set of proteins in cells stimulated with PMA (Fig. 4). Incubation with Trolox followed by stimulation with PMA also slightly enhanced tyrosine phosphorylation of proteins (Fig. 4).

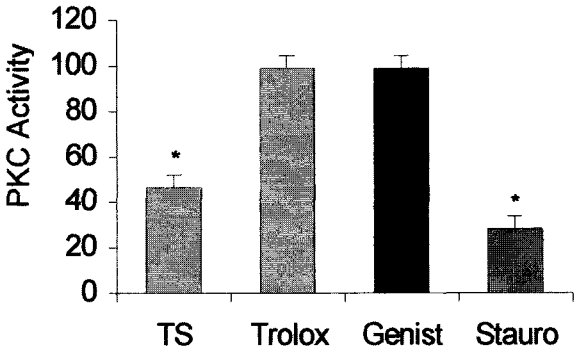


FIGURE 3 Effects of TS, Trolox, genistein and staurosporine on the PKC activity of neutrophils. Neutrophils ($1 \times 10^6$ cells) were incubated with TS (40 μmol/l), Trolox (40 μmol/l), staurosporine (10 nmol/l) or genistein (100 μmol/l) for 30 min, at 37°C, with shaking. PKC activity was assayed in aliquots of 10 μg of protein from cell lysates using the kit BIOTRAK from Amersham Life Science. Values are expressed as the percentage of activity of control cells. Results are shown as the means±SD of three independent experiments. * Values significantly different from control cells ($p < 0.05$).

Pre-incubation of neutrophils (30 min) with a low concentration of staurosporine (10 nmol/l) also led to a further increase of tyrosine phosphorylation of proteins in cells stimulated by PMA (Fig. 5). In contrast, pre-incubation with a relatively high concentration of genistein (100 μmol/l) partially inhibited tyrosine phosphorylation of the proteins induced by PMA (Fig. 5). Phosphorylation of proteins was also inhibited by pre-incubation with low concentrations of genistein (5 μmol/l) (data not shown).

### Tyrosine Phosphorylation of Proteins in OZ-stimulated Neutrophils

Strong tyrosine phosphorylation of proteins was observed in response to OZ-stimulation of neutrophils (Fig. 6). This OZ-stimulated tyrosine phosphorylation was inhibited in cells pre-incubated with TS or Trolox (Fig. 6). Even at a low concentration, staurosporine (10 nmol/l) almost completely inhibited OZ-stimulated tyrosine phosphorylation of the whole set of proteins in human neutrophils (Fig. 7). As expected, genistein (100 μmol/l) pretreatment effectively suppressed tyrosine phosphorylation induced by OZ (Fig. 7).

### Modulation of PTP Activities in Human Neutrophils

Figure 8 shows that neither TS nor Trolox significantly changed PTP activities in resting cells. PTP activity in PMA-stimulated cells decreased by 24.6 ± 3.5% as compared to the activity in resting cells. However, in cells incubated with TS, the inhibitory effect of PMA on PTP activity was not observed. Stimulation with OZ produced a strong decrease in PTP activity (to 16.0 ± 3.9% of control), an effect that was partially alleviated when cells were pre-treated with TS (to 37.7 ± 8.2% of control) or Trolox (to 31.1 ± 4.2% of control) (Fig. 8), consistent with lessened tyrosine phosphorylation (Fig. 6).

## DISCUSSION

The data reported in this paper shows that α-tocopherol inhibits PKC activity in human neutrophils. The tumor promoter PMA directly activates PKC by binding to a specific site in the regulatory domain of the enzyme. This binding displaces the inhibitory pseudosubstrate and unfolds the kinase over the membrane surface,[39] Activated PKC phosphorylates the NADPH oxides components, $p^{47phox}$ and $p^{67phox}$, resulting in production of oxidants that react with surrounding macromolecules.[40,41] Consistent with this mechanism, pre-incubation of cells with TS inhibited 80% of the oxidant production in PMA-stimulated neutrophils (Fig. 2). This action of TS was more than an antioxidant, as the antioxidant analog Trolox did not inhibit PKC (Fig. 3) and was much less effective at inhibiting oxidant generation (Fig. 2).

The PKC inhibition by α-tocopherol has been shown in other cell types, including endothelial

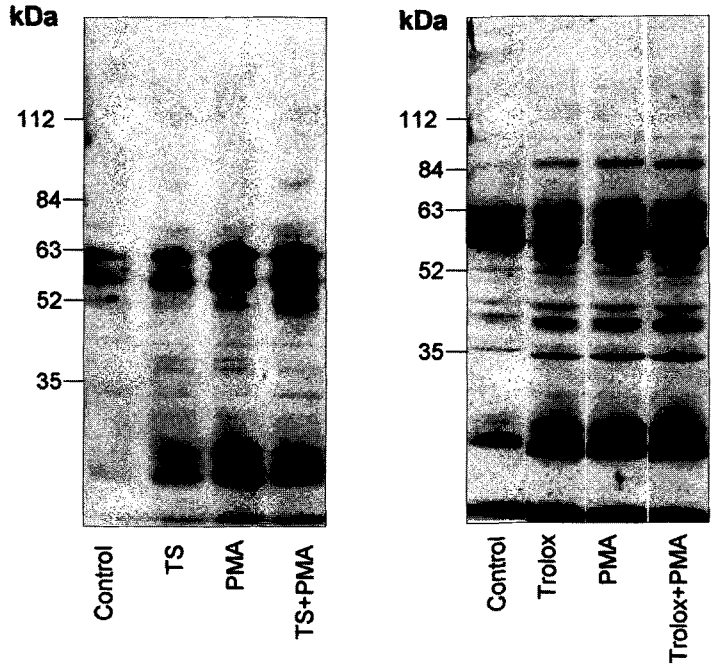


FIGURE 4 Effects of TS and Trolox on tyrosine phosphorylation in PMA-stimulated neutrophils. $1 \times 10^6$ cells were incubated with or without TS (40 μmol/l) or Trolox (40 μmol/l) for 30 min at 37°C, under shaking. After incubation, cells were stimulated with PMA (20 ng/ml) for 5 min. Cells were resuspended in lysis buffer A, and lysed by sonication. Aliquots (30 μg of protein) were subjected to SDS-PAGE. Immunoblotting was performed with a monoclonal anti-P-Tyr. A representative blot from at least three independent experiments is shown.



cells,[8] platelets, and macrophages.[27] Azzi *et al.*[26] showed that α-tocopherol, but not β-tocopherol inhibited PKC activity in smooth muscle cells, so decreasing their proliferation. They proposed that α-tocopherol acts by a non-antioxidant mechanism since β-tocopherol, a similarly potent antioxidant, did not show any effect on enzyme activity. Similar results to β-tocopherol were obtained when Trolox, an analog of α-tocopherol without the phytyl chain, was used in our experiments (Fig. 3). Cachia *et al.*[42] also demonstrated an inhibition of PKC activity of human monocytes by α-tocopherol, no effect by Trolox and poor inhibition by β-tocopherol. The authors proposed that α-tocopherol action on PKC activity is related to its property as a stabilizer of lipid bilayer membranes, hence decreasing the enzyme unfolding on the cell membrane with consequent activation. Moreover, α-tocopherol in membrane environments can react with lipid peroxides, through its antioxidant property. These peroxides were demonstrated to activate the membrane associated-PKC.[43]

When OZ is utilized as the neutrophil stimulator, multiple pathways are activated with consequent stimulation of oxidative burst. Within OZ, TS inhibited approximately 50% of the total cellular production of oxidants (Fig. 2). In this case Trolox was similarly effective, indicating an antioxidant activity as important to the mechanism of inhibition. These data, and the inhibition by the TK inhibitor genestein (Fig. 2), suggest that with OZ as stimulus TS modulates the oxidative burst in human neutrophils through tyrosine phosporylation signaling pathways.

Several stimuli of the oxidative burst of human neutrophils, including PMA, FMLP and OZ, induce tyrosine phosphorylation of proteins in these cells.[12] In the present study, tyrosine

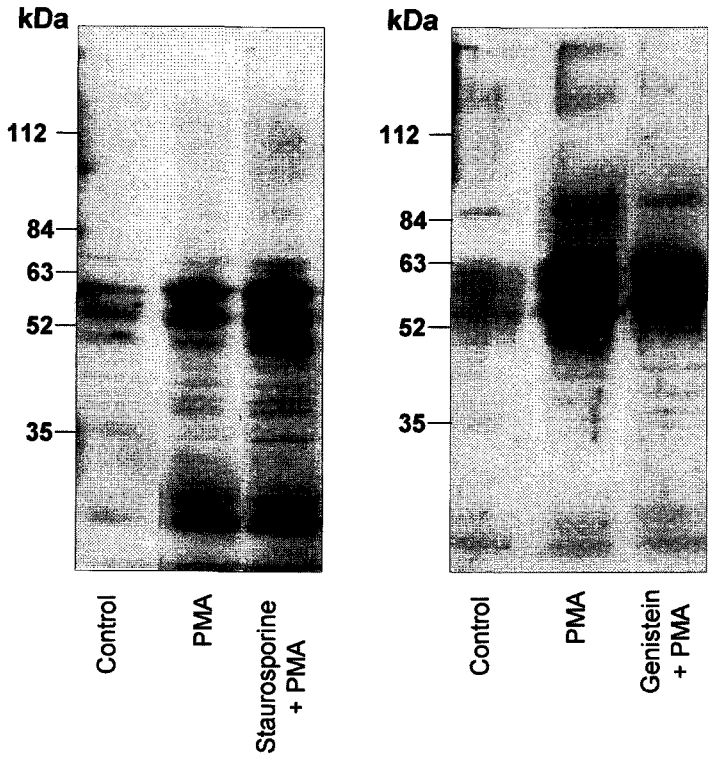


FIGURE 5 Effects of staurosporine and genistein on tyrosine phosphorylation in PMA-stimulated neutrophils. $1 \times 10^6$ cells were incubated with or without staurosporine (10 nmol/l) or genistein (100 μmol/l) for 30 min at 37°C, under shaking. After incubation, cells were stimulated with PMA (20 ng/ml) for 5 min. Cells were resuspended in lysis buffer A, and lysed by sonication. Aliquots (30 μg of protein) were subjected to SDS-PAGE. Immunoblotting was performed with a monoclonal anti-P-Tyr. A representative blot from at least three independent experiments is shown.



phosphorylation of proteins of human neutrophils was found increased with both stimuli, PMA and OZ (Figs. 4 and 6). This effect was more evident in OZ-stimulated neutrophils. The effects of α-tocopherol on tyrosine phosphorylation of proteins in OZ- or PMA-stimulated neutrophils were paradoxical. The vitamin caused a decrease of protein tyrosine phosphorylation in OZ-stimulated neutrophils, while in PMA-stimulated neutrophils it increased the tyrosine phosphorylation. Phosphorylation at serine and threonine residues of TK receptors, such as the EGF receptor, results in decreased self-phosphorylation of the receptor at tyrosine residues and its TK activity.[44] In fact, Mourad and Stern[45] have demonstrated that PKC activation by PMA decreased EGF-dependent tyrosine phosphorylation of protein in cultured HER14 cells. By this mechanism, the inhibition of PKC activity α-tocopherol, shown in the current experiments, would produce an increase in the levels of tyrosine phosphorylated proteins, as observed with PMA-stimulated neutrophils (Fig. 4). On the other hand, the inhibitory effects of α-tocopherol on tyrosine phosphorylation in OZ-stimulated neutrophils (Fig. 6) could be due to the activation of tyrosine phosphatases by the antioxidant, as shown in Fig. 8. Additionally, oxidant-dependent tyrosine phosphorylation[19] could be inhibited by the antioxidant action of α-tocopherol. The difference in the response to α-tocopherol in PMA- and OZ-stimulated neutrophils clearly shows a difference in the activating pathways by these two stimuli.

Protein kinase inhibitors are useful tools to understand the mechanisms involved in signal

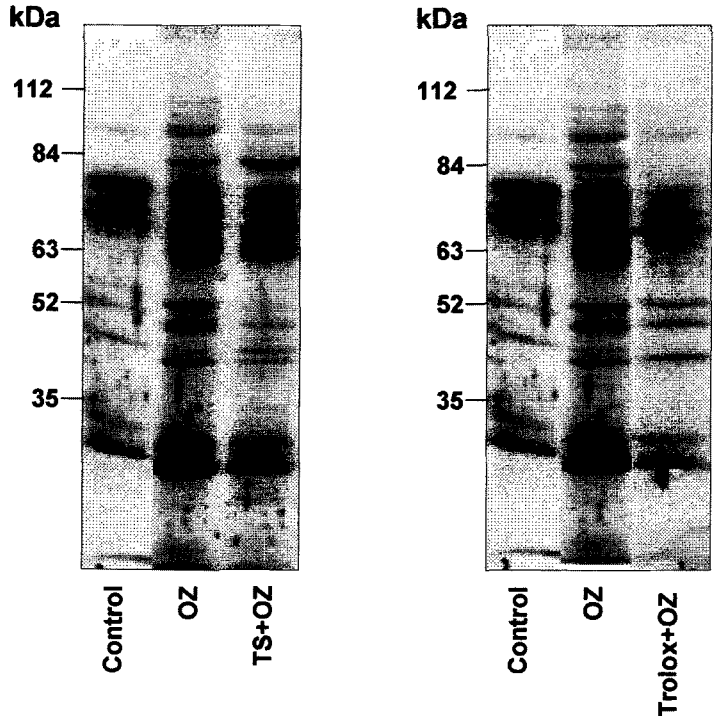


FIGURE 6 Effects of TS and Trolox on tyrosine phosphorylation in OZ-stimulated neutrophils. $1 \times 10^6$ cells were incubated with or without TS (40 μmol/l) or Trolox (40 μmol/1) for 30 min at 37°C, under shaking. After incubation, cells were stimulated with OZ (100 particles/cell) for 15 min. Cells were resuspended in lysis buffer A, and lysed by sonication. Aliquots (30 μg of protein) were subjected to SDS-PAGE. Immunoblotting was performed with a monoclonal anti-P-Tyr. A representative blot from at least three independent experiments is shown.



transduction pathways. Staurosporine, a potent PKC inhibitor decreased oxidant production by both PMA- and OZ- stimulated neutrophils, similar to TS (Fig. 2). Robinson *et al.*[46] also demonstrated that staurosporine efficiently inhibited the $O_2^{\cdot-}$ production by PMA-stimulated neutrophils, and less efficiently in fMLP-stimulated neutrophils. Genistein, a TK inhibitor, blocked almost completely the CL produced by neutrophils in the presence of both stimuli, PMA and OZ (Fig. 2). However, the observed genistein inhibition of oxidant production in PMA-stimulated cells does not agree with data of the literature. Either no effect[47] or stimulation of oxidant production[48] was observed in the presence of genistein. This conflicting result of the current experiments (at 100 μM genestein) may reflect data from Cai *et al.*[49] showing that the flavonoid genistein is a potent antioxidant, and hence scavenges the oxidants produced during oxidative burst.

The effects of staurosporine on tyrosine phosphorylation levels of proteins were similar to those of TS, i.e. an increase in PMA-stimulated and a decrease in OZ-stimulated cells. These results suggest that both compounds, TS and staurosporine, increased tyrosine phosphorylation in PMA-stimulated cells through inhibition of PKC, as previously noted.[44,45] Genistein, by inhibition of TKs, decreased the tyrosine phosphorylation of proteins in both PMA-and OZ stimulated neutrophils (Figs. 5 and 7).

The levels of tyrosine phosphorylated proteins are regulated by the competitive activities of protein TKs and protein tyrosine phophatases (PTPs).[20] We have studied the effects of α-tocopherol on PTP activity in PMA- and OZ-stimulated human neutrophils (Fig. 8). Both

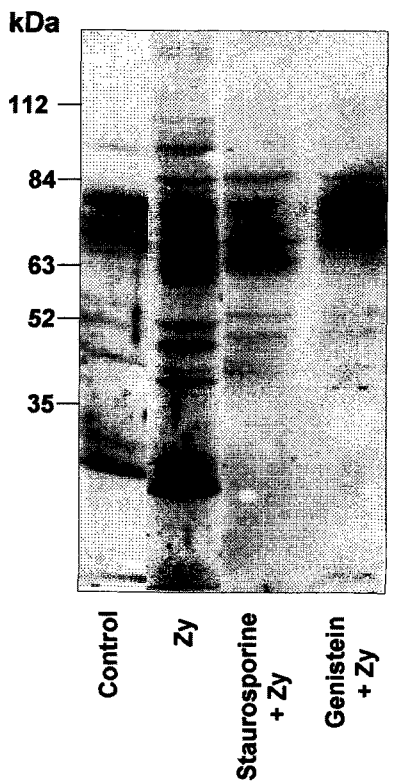


FIGURE 7 Effects of staurosporine and genistein on tyrosine phosphorylation in OZ-stimulated neutrophils. $1 \times 10^6$ cells were indicated with or without staurosporine (10 nmol/l) or genistein (100 μmol/l) for 30 min at 37°C, under shaking. After incubation, cells were stimulated with OZ (100 particles/cell) for 15 min. Cells were resuspended in lysis buffer A, and lysed by sonication. Aliquots (30 μg of protein) were subjected to SDS-PAGE. Immunoblotting was performed with a monoclonal anti phosphotyrosine antibody. A representative blot from at least three independent experiments is shown.

stimuli inhibited the PTP activity when compared to non-stimulated cells. Pre-incubation of neutrophils with TS resulted in restoration of PTP activities in stimulated cells. However, TS showed no effect on PTP activity of non-stimulated cells. The major PTPs present in hematopoietic cells, including neutrophils, are CD45 and SHP-1.[50,51] CD45 is a transmembrane PTP that translocates from specific granules to the plasma membrane upon stimulation.[52] SHP-1 (also known as PTP1C, PTP-N6, and SHPTP-1), a soluble PTP is preferentially associated with the cytoskeleton after stimulation.[53] CD45 but not SHP-1 is inactivated by oxidants, an effect that is reversed by thiol antioxidants such as DTT, *N*-acetylcysteine, or GSH.[53] This redox regulation of CD45 activity could involve oxidation of critical –SH groups present in the catalytic domain of the enzyme, resulting in decreased activity. However, PMA-inhibition of PTP activity in neutrophils[54] is unrelated to CD45, as no effects of PKC activation on CD45 could be demonstrated. Our data that is corroborated by others also shows a decrease in total PTP activity in PMA- or fMLP-stimulated neutrophils.[52,54] Taken together data from the literature suggest that the two major PTPs present in neutrophils are differently regulated. In view of the results presented, we may suggest that α-tocopherol acts through two distinct mechanisms: inhibiting PKC acitivity and/or protecting the –SH groups of CD45.

To better understand whether the effects mediated by α-tocopherol on signaling pathways in human neutrophils could be through an antioxidant, a non-antioxidant, or both mechanisms, we have compared experiments in the presence of Trolox. The lesser inhibition of oxidant production elicited by Trolox in PMA-stimulated cells as compared to that of α-tocopherol (Fig. 2), may relate to its lack of effect on PKC activity (Fig. 3), The effects of Trolox on oxidant generation and tyrosine phosphorylated proteins in OZ-stimulated neutrophils were similar to those obtained with α-tocopherol (Fig. 6), suggesting a common mechanism with this stimulus. Nevertheless, Trolox did not show any significant effect on tyrosine phosphorylation in PMA-stimulated cells (Fig. 4). Trolox and TS also increased the PTP activities in both PMA- and OZ-stimulated neutrophils (Fig. 8), suggesting an antioxidant mechanism in this response.

Despite some similar effects of Trolox and TS (decrease of tyrosine phosphorylation in OZ-stimulated cells; increased PTP activity; similar inhibition of ROS production by OZ-stimulated cells) others were identified as quite different (PKC

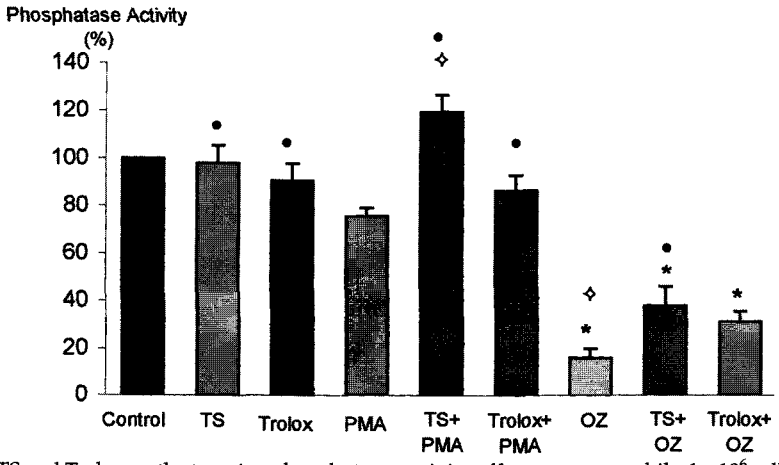


FIGURE 8 Effects of TS and Trolox on the tyrosine phosphatases activity of human neutrophils. $1 \times 10^6$ cells were incubated with TS (40 μmol/l) or with Trolox (40 μmol/l), for 30 min, at 37°C, under shaking. Cells were stimulated with PMA (20 ng/ml) for 5 min or with OZ (100 particles/cell) for 15 min. Controls of cells incubated with PBS were also performed. Tyrosine phosphatase activity was assayed by mixing neutrophil-lysate (100 μg of protein) with 20 μl of $^{32}$P-phosphorylated immunoprecipitated EGF receptors, prepared as described in Matherials and methods. Reactions were carried out for 30 min at 37°C with agitation. After centrifugation the supernatant was suspended in 50 μl of 2-fold SDS-PAGE sample buffer. Samples were resolved by SDS/PAGE (7.5% gels). After drying, gels were exposed to autoradiographic films for 3 h at room temperature. The intensity of the band corresponding to the EGF receptor was determined by enhanced laser densitometry. Values were expressed as percentage of activity from control cells. Results are shown as means±SD of at least four independent experiments. *Values significantly different from control cells ($p < 0.05$). **Values significantly different from PMA-stimulated cells ($p < 0.05$). ***Values significantly different from OZ-stimulated cells ($p < 0.05$).

activity, ROS production in PMA-stimulated cells). As mentioned above α-tocopherol, due to its lipophilic profile, could interact with PKC in the cell mebrane. On the other hand, the more hydrophilic Trolox has less influence on the membrane structure. These differences suggest that α-tocopherol acts through both antioxidant and non-antioxidant mechanisms, and Trolox by only the antioxidant mechanism. The prevalence of these mechanisms in TS-modulation of neutrophil function deserves further investigation.

## *Acknowledgements*

This work was supported by grants 97-02335-5 from FAPESP (VBCJ), MCT-325 from PRONEX (HPM), and 96/7927-5 from FAPESP (HPM). We also acknowledge Dr Brian Bandy for the excellent English revision of this manuscript.

## *References*

[1] Edwards, S.W. (1994) Biochemistry and physiology of the neutrophil (Cambridge University Press, New York).
[2] Babior, B.M. (1978) "Oxygen-dependent microbial killing by phagocytes", *New England Journal of Medicine* **298**, 659–668.
[3] Klebanoff, S.J. (1980) "Oxygen metabolism and the toxic properties of phagocytes", *Annals of Internal Medicine* **93**, 480–487.
[4] Thelen, M., Dewald, B. and Baggiolini, M. (1993) "Neutrophil signal transduction and activation of the respiratory burst", *Physiological Reviews* **46**, 797–821.
[5] Watson, F., Robinson, J. and Edwards, S.W. (1991) "Protein kinase C-dependent and -independent activation of the NADPH oxidase of human neutrophils", *The Journal of Biological Chemistry* **168**, 7432–7439.
[6] Nishizuka, Y. (1984) "The role of protein kinase C in cell surface signal transduction and tumor promotion", *Nature* **308**, 693–696.
[7] Stäuble, B., Bosecoboinik, D., Tasinato, A. and Azzi, A. (1994) "Modulation of activator protein-1 (AP-1) transcription factor and protein kinase C by hydrogen peroxide and D-α-tocopherol in vascular smooth muscle cells", *FEBS Letters* **226**, 393–402.
[8] Keaney, J.F., Guo, Y., Cunninghan, D., Shwaery, G.T., Xu, A. and Vita, A. (1996) "Vascular incorporation of α-tocopherol prevents endothelial dysfunction due to oxidized LDL by inhibition of protein kinase C stimulation", *Jounal of Clinical Investigation* **98**, 386–394.

[9] Kass, G.E.N., Duddy, K. and Orrenius, S. (1989) "Activation of hepatocyte protein kianse C by redox-cycling quinines", *Biochemical Journal* **260**, 499–507.
[10] Berkow, R.L., Dodson, R.W. and Kraft, A.S. (1989) "Human neutrophils contain distinct cytosolic and particulate tyrosine kinase activities: possible role in neutrophil activation", *Biochimica et Biophysica Acta* **997**, 292–301.
[11] Gomez-Cambronero, J., Huang, C.K., Bonak, V.A., Wang, E., Casneille, J.E., Shiraishi, T. and Sha'afi, R.I. (1989) "Tyrosine phosphorylation in human neutrophils", *Biochemical and Biophysical Research Communication* **162**, 1478–1485.
[12] Berkow, R.L. and Dodson, R.W. (1990) "Tyrosine-specific protein phosphorylation during activation of human neutrophils", *Blood* **75**, 2445–2452.
[13] Rollet, E., Caon, A., Roberge, C.J., Liao, N.W., Malawisla, S.E., McColl, S.R. and Naccache, P.H. (1994) "Tyrosine phosphorylation in activated human neutrophils, comparison of the effects of different classes of agonists and identification of the signating pathways involved", *Journal of Immunology* **250**, 353–363.
[14] Thompson, H.L., Shiroo, M. and Saklatvala, J. (1993) "The chemotatic factor *N*-formylmethionyl-leucyl-phenylalanine activates microtubule-associated protein (MAP) kinase and MAP kinase kinase in polymnorphonuclear leukocytes", *Biochemical Journal* **293**, 483–488.
[15] Downey, G.P., Butler, J.R., Brumell, J., Borregaard, N., Kjeldsen, L., Sue A-Quan, A.K. and Grinstein, S. (1996) "Chemotatic peptide induced activation of MEK-2, the predominant isoform in human neutrophils: inhibition by wortmannin", *The Journal of Biological Chemistry* **271**, 21005–21012.
[16] Naccache, P.H., Gilbert, C., Caon, A.C., Gaudry, M., Huang, C.K., Bonak, V.A., Umezawa, K. and McColl, S.R. (1990) "Selective inhibition of human neutrophil functional responsiveness by erbstatin, an inhibitor of tyrosine protein kinase", *Blood* **176**, 2098–2104.
[17] Cui, Y., Harvey, K., Akard, L., Jansen, J., Hughes, C., Siddiqui, R.A. and English, D. (1994) "Regulation of neutrophil responses by phosphotyrosine phosphatase", *Journal of Immunology* **152**, 5420–5428.
[18] Sundaresan, M., Yu, Z., Ferrans, V.J., Irani, K. and Finkel, T. (1995) "Requirement for generation of $H_2O_2$ for platelet-derived growth factor signal transduction", *Science* **270**, 296–298.
[19] Brumell, J.H., Burkard, A.L., Bolen, J.B. and Grinstein, S. (1996) "Endogenous reactive oxygen intermediates activate tyrosine kinases in human neutrophils", *The Journal of Biological Chemistry* **271**, 1455–1461.
[20] Monteiro, H.P. and Stern, A. (1996) "Redox modulation of tyrosine phosphorylation-dependent signal transduction pathways", *Free Radical Biology and Medicine* **21**, 323–333.
[21] Lander, H.M., Jacovina, A.T., Davis, R.J. and Tauras, J.M. (1996) "Differential activation of mitogen-activated protein kinases by nitric oxide-related species", *The Journal of Biological Chemistry* **217**, 19705–19709.
[22] Cantoni, O., Boscoboinik, D., Fiorani, M., Stäuble, B. and Azzi, A. (1996) "The phosphorylation state of MAP-kinases modulates the cytotoxic response of smooth muscle cells to hydrogen peroxide", *FEBS Letters* **389**, 285–288.
[23] Peranovich, T.M.S., Silva, A.M., Fries, D.M., Stern, A. and Monteiro, H.P. (1995) "Nitric oxide stimulates phosphorylation in murine fibroblasts in the absence and the presence of epidermal growth factor", *Biochemical Journal* **305**, 613–619.
[24] Brockhaus, F. and Brune, B. (1998) "U937 apoptotic cell death by nitric oxide. Bcl-2 downregulation and caspase activation", *Experimental Cell Research* **238**, 33–41.
[25] Fialkow, L., Chan, C.K., Rotin, S. and Grinstein, G.P. (1994) "Activation of the mitogen-actived protein kinase signaling pathway in neutrophils", *Journal of Biological Chemistry* **269**, 31234–31242.
[26] Azzi, A., Boscoboinik, D., Chatelain, E., Ozër, N.K. and Stäuble, B. (1993) "D-α-Tocopherol control of cell proliferatioin", *Molecular Aspects of Medicine* **14**, 265–271.
[27] Freedman, J.E., Farhat, J.H., Loscalzo, J. and Keaney, J.F. (1996) "Tocopherol inhibits aggregation of human platelets by a protein kinase C-dependent mechanism", *Circulation* **94**, 2434–2440.
[28] Eugui, E.M., De Lustro, B. and Rouhafza, S. (1994) "Some antioxidants inhibit, in a coordinate fashion, the production of tumor necrosis factor-α, IL-1β and IL-6 by human peripheral blood mononuclear cell", *Immunology* **6**, 409–422.
[29] Sen, C.K. and Packer, L. (1996) "Antioxidants and redox regulation of gene transcription", *FASEB Journal* **10**, 709–720.
[30] Monteiro, H.P., Ivaschenko, Y., Fischer, R. and Stern, A. (1993) "Ascorbic acid inhibits protein tyrosine phosphatases in NIH 3T3 cells expressing human epidermal growth factor receptors", *International Journal of Biochemistry* **25**, 1859–1864.
[31] Boyum, A. (1968) "Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation and of granulocytes by combining centrifugation and sedimentation at $1 \times g$", *Scandinavian Journal of Clinical and Laboratory Investigation Supplement* **97**, 77–889.
[32] Hatam, L.J. and Kayden, H.J. (1979) "A high-performance liquid chromatographic method for the determinatioin of tocopherol in plasma and cellular elements of the blood", *Journal of Lipid Research* **20**, 639–645.
[33] Lang, J.K., Gohil, K. and Packer, L. (1986) "Simultaneous determination of tocopherols, ubiquinols, and ubiquinones in blood, plasma, tissue homogenates and subcellular fraction", *Analytical Biochemistry* **157**, 106–116.
[34] Easmon, C.S., Cole, P.J., Willians, A.J. and Hastings, M. (1980) "The measurement of opsonic and phagocytic function by luminol-dependent chemiluminescence", *Immunology* **41**, 67–74.
[35] Dechalet, L.R., Long, G., Shirley, P.S., Bass, D.A., Thomas, M.J., Henderson, F.W. and Cohen, M.S. (1982) "Mechanism of luminol-dependent chemiluminescence of human neutrophils", *Journal of Immunology* **129**, 1589–1593.
[36] Halstenssen, A., Hanenberg, B., Glette, J., Sandberg, S. and Solberg, C.O. (1986) "Factors important for the measurement of chemiluminescence production by polymorphonuclear leukocytes", *Journal of Immunology Methods* **88**, 121–128.
[37] Nahas, N., Molski, T.F.P., Fernandez, G.A. and Sha'afi, R.I. (1996) "Tyrosine phosphorylation and activation of a new mitogen-activated protein (MAP)-kinase cascade in

human neutrophils stimulated with various agonists", *Biochemical Journal* **318**, 247–253.
[38] Bradford, M.M. (1976) "A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding", *Analytical Biochemistry* **72**, 248–254.
[39] Faroqui, A.A., Faroqui, T., Yates, A.J. and Horrocks, L.A. (1988) "Regulation of protein kinase C activity by various lipids", *Neurochemical Research* **13**, 499–511.
[40] El Benna, J., Han, J., Park, J., Schmid, E., Ulevitch, R.J. and Babior, B.M. (1996) "Activation of p38 in stimulated human neutrophils: phosphorylation of the oxidase component p47phox by p38 and ERK but not by JNK", *Archives of Biochemistry and Biophysics* **334**, 395–400.
[41] El Benna, J.E., Dang, P.M., Gaudry, M., Fay, M., Morel, F., Hakim, J. and Gougerot-Pocidalo, M. (1997) "Phosphorylation of the respiratory burst oxidase subunit p67phox during human neutrophil activation", *Journal of Biological Chemistry* **272**, 17204–17208.
[42] Cachia, O., Benna, J.E., Pedruzzi, E., Descomps, B., Gougerot-Pocidalo, M. and Léger, C. (1998) "α-Tocopherol inhibits the respiratory burst in human monocytes", *Journal of Biological Chemistry* **273**, 32801–32805.
[43] Suzuki, Y.J., Forman, H.J. and Sevanian, A. (1997) "Oxidants as stimulators of signal transduction", *Free Radical Biology and Medicine* **22**, 269–285.
[44] Cochet, C., Gill, G.N., Meisenhelder, J., Cooper, J.A. and Hunter, T. (1984) "C-Kinase phosphorylates the epidermal growth factor receptor and reduces its epidermal growth factor-stimulated tyrosine protein kinase activity", *Journal of Biological Chemistry* **259**, 2553–2558.
[45] Mourad, E. and Stern, A. (1994) "Inhibition of epidermal growth factor-dependent protein tyrosine phosphorylation by phorbol myristate acetate is mediated by protein tyrosine phosphatase activity", *FEBS Letters* **339**, 7–10.
[46] Robinson, J.M., Heyworth, P.G. and Badway, J.A. (1990) "Utility of staurosporine in uncovering differences in the signal transduction pathways for superoxide production", *Biochimica et Biophysica Acta* **1055**, 55–62.
[47] Mocsai, A., Bánfi, B., Kapus, A., Frakas, G., Geiszt, M., Buday, L., Faragó, A. and Ligeti, E. (1997) "Differential effects of tyrosine kinase inhibitors and an inhibitor of the mitogen-activated protein kinasae cascade on degranulation and superoxide production of human neutrophil granulocytes", *Biochemical Pharmacology* **54**, 781–789.
[48] Utsumi, T., Klostergaard, J., Akimaru, K., Keisuke, E., Sato, E.F. and Utsumi, K. (1992) "Modulation of TNF-α-priming and stimultion-dependent superoxide generation in human neutrophils by protein kinase inhibitors", *Archives of Biochemistry and Biophysics* **294**, 271–278.
[49] Cai, Z., Li, X. and Katsumura, Y. (1999) "Interaction of hydrated electron with dietary flavonoids and phenolic acids: rate constants and transient spectra studied by pulse radiolysis", *Free Radicals in Biology and Medicine* **27**, 822–827.
[50] Trombridge, I.S., Ostergaard, H.L. and Johnson, P. (1991) "CD45: aleukocyte-specific member of the protein tyrosine phosphatase family", *Biochimica et Biophysica Acta* **1095**, 46–52.
[51] Okumura, M. and Thomas, M.L. (1995) "Regulation of immune function by protein tyrosine phosphatases", *Current Opinion of Immunology* **7**, 312–315.
[52] Cui, Y., Harvey, K., Akard, L., Jansen, J., Hughes, C., Siddiqui, R.A. and English, D. (1994) "Regulation of neutrophil responses by phosphotyrosine phosphatase", *Journal of Immunology* **152**, 5420–5427.
[53] Brumell, J.H., Chan, C.K., Butler, J., Borreggard, N., Siminovitch, K.A., Grinstein, S. and Downey, G.P. (1997) "Regulation of Src homology 2-containing tyrosine phosphatase 1 during activation of human neutrophils", *Journal of Biological Chemistry* **272**, 875–883.
[54] Kansha, M., Takeshige, K. and Minakami, S. (1993) "Decrease in the phosphotyrosine phosphatase activity in the plasma membrane of human neutrophils on stimulation by phorbol 12-myristate 13-acetate", *Biochima et Biophysica Acta* **1179**, 189–195.