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CURCUMIN ENHANCES PARAQUAT-INDUCED APOPTOSIS OF N27 MESENCEPHALIC CELLS VIA THE GENERATION OF REACTIVE OXYGEN SPECIES

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Abstract

Curcumin, the active compound of the rhizome of *Curcuma longa* has anti-inflammatory, antioxidant and antiproliferative activities. This agent has been shown to regulate numerous transcription factors, cytokines, protein kinases, adhesion molecules, redox status and enzymes that have been linked to inflammation. While curcumin has been identified as an activator of apoptosis in several cell lines, the mechanism by which it initiates apoptosis, however, remains poorly understood. We considered curcumin from the point of view of its ability to protect against oxidative stress, the latter being one factor strongly implicated in the development of Parkinson's disease. Although the etiology of Parkinson's disease remains unknown, epidemiological studies have linked exposure to pesticides such paraquat to an increased risk of developing the condition. Analysis of the neurotoxic properties of these pesticide compounds has been focused on their ability to induce oxidative stress in neural cells. Given curcumin's capacity to protect against oxidative stress, it has been considered as a potential therapeutic agent for neurodegenerative diseases such as Parkinson's disease that involve an oxidative stress component. In the present report we describe the effect of curcumin in paraquat-mediated apoptosis of N27 mesencephalic cells. We show that subtoxic concentrations of curcumin sensitize N27 mesencephalic cells to paraquat-mediated apoptosis.

Keywords

paraquat; curcumin; Parkinson's disease; oxidative stress; apoptosis

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Conflict of Interest statement

The authors declare that there are no conflicts of interest

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Introduction

Curcuma longa L. (turmeric) has been used for hundreds of years as a flavoring, coloring, and preservative. Curcumin, an important constituent of turmeric, is a nutraceutical compound reported to possess therapeutic properties against a variety of diseases ranging from cancer to cystic fibrosis (Liu et al., 1996). Curcumin has been shown to exhibit a variety of biological activities including antioxidative activity (Wei et al., 2006), scavenging of superoxide anions and nitric oxide radicals (Sreejayan and Rao, 1997), an inhibition of lipid peroxidation (Sreejayan and Rao, 1996) and lipoxygenase/cyclooxygenase activity (Huang et al., 1991). The strong antioxidant activity of curcumin makes it an interesting candidate for use in counteracting oxidative stress-induced damage. Given our interest in Parkinson's disease (PD), curcumin could be potentially useful in combating the oxidative damage triggered by neurotoxicants such as paraquat, which has been implicated in causing PD. To this extent, curcumin has been proposed as a neuroprotective agent in neurodegenerative disorders such as Alzheimer's disease (Aggarwal and Harikumar, 2008; Baum et al., 2008; Begum et al., 2008; Cole et al., 2007a) and Parkinson's disease (Cole et al., 2007; Jagatha et al., 2008; Rajeswari and Sabesan, 2008).

Recent studies have highlighted how curcumin is able to enhance cell apoptosis via a mechanism which is dependent on the generation of reactive oxygen species (ROS) (Thayyullathil et al., 2008; Su et al., 2006). However, other reports have indicated how curcumin is able to inhibit ROS, and consequently apoptosis, through its well known antioxidant properties (Chen et al., 2006; Chan et al., 2005).

Paraquat (1,1-dimethyl-4,4'-bipyridinium dichloride), a widely used herbicide, has been suggested as a potential etiologic factor for the development of PD, a neurodegenerative disorder characterized by the selective loss of dopaminergic neurons in the substantia nigra (SN) (Burke, 1998). This is accompanied by typical clinical manifestations, such as rest tremors, bradykinesia, rigidity, and postural instability. However, the causes of PD remain unknown, and over the years the mechanism of genetic inheritance versus environmental factors has been debated. Exposure to environmental agents such as paraquat have been linked repeatedly with the development of PD (Tanner et al., 1989; Tanner, 1989; Gorell et al., 1998). PQ enhances cell death through a mechanism that is dependent on ROS production and subsequent oxidative stress damage (Thrash et al., 2007; Drechsel and Patel, 2008; Chen et al., 2008; Yang and Tiffany-Castiglioni, 2005). In addition, PQ has been identified as a trigger for neural cell apoptosis (Moran et al., 2008; Gonzalez-Polo et al., 2004; Gonzalez-Polo et al., 2007a,b).

Due to its well known anti-apoptotic and antioxidant properties, and the fact that it has been proposed as a putative agent in the treatment of some neurodegenerative disorders, such as PD, we hypothesized that curcumin would be able to prevent the PQ-mediated apoptosis of rat mesencephalic N27 cells. The results that we present here differ from previous hypotheses, and show how curcumin is not able to protect against PQ-mediated neurotoxicity but enhances the PQ-mediated apoptosis of N27 cells.

Materials and methods

Cell culture

Rat mesencephalon-derived cell line (1RB₃AN₂₇; hereafter referred to as N27 cells), represents a homogenous population of tyrosine hydroxylase-positive cells with functional characteristics resembling dopaminergic neurons (Prasad et al., 1998), as an *in vitro* model for studies of dopaminergic neurodegeneration (Kitazawa et al., 2003). Diverse studies have established that N27 cells are an adequate cell culture model for studying dopaminergic neurodegeneration,

compared to PC12 and SH-SY5Y cells, as N27 cells are derived from the mesencephalon, a brain region directly affected in some neurodegenerative diseases like PD (Anantharam et al., 2002; Kaul et al., 2003; Yang et al., 2004; Kaul et al., 2005; Kanthasamy et al., 2006; Sun et al., 2006) MPP⁺ treatment in N27 cells induces acute generation of ROS in a time- and dose-dependent manner (Kaul et al., 2003; Kaul et al., 2005), and that ROS generation precedes changes in mitochondrial membrane potential or cytochrome C release. Immortalized N27 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Hyclone, Brevieres, France), 1% L-glutamine, penicillin (100 U/ml), and streptomycin (100 U/ml). Cells were seeded and maintained at a density of $1 \times 10^6/\text{cm}^2$ in 75-cm² tissue culture flasks (Corning, New York, NY) and incubated at 37°C under saturating humidity in 5% CO₂/95% air.

Reagents, treatment and induction of cell death

Confluent cells ($\approx 80\%$) in 75-cm² tissue culture flasks were trypsinized and seeded in 24-well cell culture plates to viability and for flow cytometry assay or 6 well to western blot analysis at a concentration of $2.5\text{--}3.5 \times 10^4$ cells/ml. The N27 cells were preincubated with curcumin concentrations for 24 hours prior to the addition of PQ, and then incubated for 24 hours at 37°C. When carried out, preincubation with antioxidants N-Acetyl-Cysteine (NAC) (10 mM) and Vitamin E (VitE) (200 μM), was carried out 30 mins prior to the addition of PQ or curcumin.

MTT reduction assay

The MTT assay for measuring cytotoxicity and cell growth developed by Mosman (1983) was performed following the modifications described by Hansen (1989). Viable cells with active mitochondria reduce the colourless tetrazolium salt MTT (Sigma, St. Louis, MO), producing dark blue water-insoluble formazan crystals. To perform the assay, MTT was dissolved at a concentration of 5 mg/ml in PBS. Two hours before the end of the experiment, the MTT solution was added to 24-well plates (50 μl per well) and the plates were returned to the incubator. Following the 2 h incubation period, the medium was decanted, and the formazan precipitates were solubilized with acidic isopropanol (0.04–0.1 N HCl in absolute isopropanol). Optical density (OD) was measured at 570 nm (reference wavelength 630 nm), using the extraction solution as a blank. The absorbance of the converted dye was measured at a wavelength of 570 nm with background subtraction at 630–690 nm. The absorbance of untreated cultures was set at 100%.

Flow cytometry

production, superoxide anion generation and apoptotic cell death were monitored using cytofluorometry. We used 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate; 5 μM) for the measurement of NO production, and hydroethidine (HE; 10 μM) for the determination of superoxide anion generation. Apoptotic cell death was determined by assessment of phosphatidylserine exposure, we used Annexin-V labeled with fluorescein isothiocyanate. Propidium iodide (PI; 1 $\mu\text{g}/\text{ml}$) was used to determine cell viability. All of these reagents were obtained from Molecular Probes, Eugene, OR. After exposure to different experimental conditions, cells were trypsinized and labelled with the fluorochromes at 37°C, followed by cytofluorometric analysis with a FACS scanner (Becton Dickinson, New York, NY). A total of 10000 events were analyzed for each condition.

Immunofluorescence microscopy

Cells were cultured on coverslips pretreated with poly-L-lysine. After the experimental period, the cells were first fixed with paraformaldehyde (4% w:v) and then permeabilized with Triton X-100 solution (Triton 0.2% in PBS) for 10 minutes. After blocking for 20 minutes with bovine serum albumin (BSA) solution (1 mg/ml in PBS), cells were incubated with primary antibodies

for 1.5 hours at RT with antibody directed against cleaved Caspase-3 (Cell Signaling Technology), cytochrome c (Santa Cruz Biotechnology) and 3-nitrotyrosine (3NT; Sigma-Aldrich) developed with an anti-mouse or anti-rabbit immunoglobulin Alexa[®] fluor conjugate (Molecular Probes, Eugene, OR), and finally counterstained with Hoechst 33342 (2 μM; Sigma) before mounting. Fluorescence was analysed using an Olympus IX51 microscope equipped with a DC300F camera. The quantitative measurement of the fluorescence signal was performed as described by Kirkeby and Thomsen (2005).

Western blot analysis

Following experimental treatments, the cells were rinsed twice with cold PBS and removed by scraping. Cells were lysed in buffer containing 50 mM Tris-HCl pH 6.8, 10% glycerol, and 2% SDS, heated at 95 °C for 10 minutes and then stored at -80°C until required for analysis by western blot. Protein concentration was measured based on the BCA method, using a Bicinchoninic Acid Kit (Sigma, St. Louis, MO) according to the manufacturer's instructions, with BSA used as a standard. Equal amounts of protein (40μg/condition) were resolved by 8–12% SDS-gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes according to a partially modified conventional method (Fuentes et al., 2000). Briefly, proteins were transferred (250 mA for 60 min) to PVDF membranes using a Mini Trans-Blot Cell apparatus (Bio-Rad, Hercules, CA). The procedure for immunodetection included the transfer and blocking of the membrane (60 min at room temperature) with TTBS (10 mM Tris/HCl pH 7.5, 150 mM NaCl and 0.2 % Tween-20) containing 5% BSA (Sigma). Membranes were then incubated overnight at 4°C with primary anti-IκBα antibody (diluted 1:500; Santa Cruz Biotechnology) or anti-nitrotyrosine antibody (diluted 1:1000; Sigma-Aldrich). After washing (for two 5-min periods with TTBS), membranes were incubated (60 min at RT) with peroxidase-conjugated secondary antibodies (1:5000 in TTBS with 10% non-fat dry milk). After washing (for two 5-min periods and one 10-min period), the detection of bound antibodies was visualized by chemiluminescence using the ECL-plus reagent (GE Healthcare, Bucks, UK). Actin content was analysed as a control using a rabbit polyclonal antibody (Santa Cruz Biotechnology).

RNA extraction and relative quantification of gene expression

Total RNA was extracted from N27 cells with TRI Reagent[®] (Ambion, Austin, Texas, USA) according to manufacturer's instructions. RNA was quantified using a Biowave II[®] spectrophotometer (Biochrom Ltd, Cambridge, UK), and its integrity was checked electrophoretically. Reverse transcription and amplification were performed in one step with the RNA-to-Ct[®] kit (Applied Biosystems, Madrid, Spain), according to the manufacturer's instructions. For relative quantification of gene expression, the 5' exonuclease (TaqMan) assay, which produces a directly proportional readout with the progression of PCR, was used. Amplification of cDNA derived from 50 ng total RNA was performed in a 20 μl reaction volume according to standard conditions provided by the manufacturer. Briefly, amplified cDNA of studied samples was quantified in 20 μL reactions containing 10 μl TaqMan Gene Expression Master Mix, 0.5 μl TaqMan RT Enzyme, 1 μL of the individual TaqMan Gene Expression Assay (all from Applied Biosystems), 8 μl nuclease free-H₂O and 0.5 μl total RNA. Thermal cycler conditions were 15 min at 48 °C, 10 min at 95 °C followed by 40 cycles of 30 s at 95 °C to denature the DNA and 30 s at 60 °C to anneal and extend the template. All reactions were performed in duplicate (endogenous control GAPDH was measured in triplicate) in a Model 7500 thermocycler (Applied Biosystems). The values obtained for the target gene expression were normalized to GAPDH and quantified relative to the expression in control samples. For the calculation of relative quantification, the $2^{-\Delta\Delta CT}$ formula was used, where $-\Delta\Delta CT = (C_{T,target} - C_{T,GAPDH})_{\text{experimental sample}} - (C_{T,target} - C_{T,GAPDH})_{\text{control sample}}$.

Statistical analysis

Each experiment was repeated at least three times, with satisfactory correlation between the results of individual experiments. The data shown are those of representative experiments; each result being the average of three to four culture dishes. In each experiment, differences between groups were assessed by appropriate statistical methods. Statistical significance was evaluated using the Chi-square test, and all comparisons giving a p value less than 0.05 ($p < 0.05$) were considered statistically significant. Data are expressed as the mean \pm SEM. All data were analyzed with SPSS 12.0 software (Chicago, IL) for Windows

Results

Cell viability after exposure to PQ and curcumin

The viability of N27 mesencephalic cells was evaluated after 24 hr of exposure to PQ (Fig. 1A). Cell viability was assayed by the MTT method and significant effects were found for PQ concentrations from 100 to 500 μ M. Cells were exposed for 24 hr to curcumin concentrations ranging from 0.001 nM to 50 μ M, with significant toxicity observed for concentrations in excess of 10 μ M (data not shown) (Fig. 1B).

Curcumin increases cell death in PQ-exposed N27 cells

N27 cells were co-incubated with PQ concentrations ranging from 100 μ M to 500 μ M and 10 nM curcumin for 24 hr (Fig. 2). As shown in Fig. 2A, compared to control, the presence of curcumin exacerbated the extent of cell death induced at each concentration of PQ. This effect, however, was independent of the concentration of curcumin used (Fig. 2B).

Curcumin enhances the PQ-mediated induction of apoptosis in N27 cells

We wished to characterize the type of cell death involved in the experiments outlined above. To examine whether PQ causes apoptosis of N27 cells, we performed flow cytometry experiments with Annexin V/PI on cells treated with 250 μ M PQ for 24 hours (Fig. 3A). N27 cells treated with 10 nM curcumin alone did not show any detectable evidence of apoptosis (data not shown); however, the co-incubation of N27 cells with PQ and curcumin resulted in a 34 % increase in the number of apoptotic cells detected (Fig 3B). Using fluorescence microscopy we also found that PQ induces chromatin condensation, caspase 3 cleavage, and triggers the release of cytochrome c into the cytosol (Fig 4).

Curcumin-enhanced PQ-induced apoptosis of N27 cells is a ROS-dependent mechanism

Given that an important mechanism by which many stimuli induce apoptosis is via the generation of ROS, we explored whether this was also the case in relation to PQ+curcumin-exposed cells. To assess intracellular ROS production, we used flow cytometry to measure the oxidation of HE. As anticipated, the exposure of N27 cells to 250 μ M PQ for 24 hr resulted in a significant increase in ROS production, while control cells and cells exposed to curcumin alone had relatively modest levels of ROS production compared to PQ-exposed cells (Fig 5A). Surprisingly, cells co-incubated with curcumin and PQ 250 μ M exhibited a heightened production of ROS (Fig 5B). This effect was not dependent on the concentration of curcumin used (0.1 nM to 1 μ M) (Fig. 5C). In the presence of the potent antioxidants NAC (10 mM) and VitE (200 μ M), cell viability measured using the MTT test increased significantly after PQ exposure (250 μ M) compared to cells not treated with antioxidants (Fig. 6A). However when N27 cells were co-incubated with PQ (250 μ M) plus curcumin (10 nM), in addition to the indicated antioxidants, cell viability did not increase (Fig 6B). These data suggest that cell susceptibility to PQ+curcumin stimulation might be mediated by ROS, but they also indicate that rescue of N27 cells by NAC or VitE antioxidant therapy is not a viable approach.

To expand on this result, we incubated N27 cells for 5 hr with the well known ROS generating molecule H_2O_2 (0.1 μM to 500 μM) and then measured ROS production by flow cytometry with DHE. As observed in Fig. 7A, exposure of cells to 100 μM H_2O_2 increased ROS levels in a similar manner to that obtained with 250 μM PQ +10 nM curcumin, with similar cell viability levels as measured with the MTT test (Fig. 7B). This level of oxidative stress could not be rescued by co-incubation with NAC (Fig 7B), indicating that the burst of ROS generated by the co-incubation with PQ (250 μM) and curcumin (10 nM) irreversibly initiates cell death cascades.

A NOS-dependent mechanism of curcumin-enhanced PQ-mediated cell death

Once we determined that the enhancement of cell death in PQ+curcumin-exposed cells was ROS-dependent, we considered other means by which curcumin could be able to trigger this effect. Although the molecular mechanisms underlying curcumin activity are poorly understood, one process, related to the inhibition of expression of the inducible isoform of nitrogen oxide synthase (iNOS), has been widely reported. On this basis we first assessed if indeed PQ-exposed N27 cells produce NO. As shown in Fig. 8A, flow cytometry experiments using the dye DAF-FM demonstrated that N27 cells produce NO in a manner dependent on the PQ concentration to which they are exposed. Real time PCR experiments showed that this production was accompanied by an increase in the level of iNOS mRNA expression after 24 hr exposure to 250 μM PQ (Fig. 8B), and an increase in iNOS protein expression as determined by immunofluorescence (Fig. 8C). When cells were co-incubated with 250 μM PQ and 10 nM curcumin, the nitric oxide production was partially inhibited (Fig. 9A), and iNOS mRNA expression (Fig. 9B) and protein production (Fig. 9C) after 24 hr were reduced. As the mechanism of inhibition of iNOS expression by curcumin is I κ B dependent, (Singh and Khar, 2006) we evaluated the integrity of I κ B in PQ- and PQ+curcumin-exposed cells. As shown in Fig. 10, curcumin effectively inhibited I κ B degradation, thus abrogating NF- κ B translocation and therefore iNOS expression. Overall, the data demonstrate that curcumin inhibits NO production in N27-exposed cells. Superoxide scavenging by NO to generate peroxynitrite has been proposed as a defensive mechanism in PQ-mediated cell death, thereby protecting the cell in part against oxidative stress generated by superoxide. This process does, however, also generate the toxic compound peroxynitrite. We thus assessed the production of peroxynitrite in terms of the generation of 3-NT in PQ-, curcumin- and PQ+curcumin-exposed cells. As can be seen in Fig. 11, 3-NT production is increased in PQ-exposed cells (250 μM for 24 hr) and decreased in N27 cells co-incubated with 250 μM PQ and 10 nM curcumin.

Discussion

Curcumin, a polyphenol derived from the plant *Curcuma longa*, is known to be a potent antioxidant (Menon and Sudheer, 2007; Bengmark, 2006) and could therefore have desirable preventive or putative therapeutic properties in medicine. Curcumin has thus been proposed as a potential candidate to treat neurodegenerative diseases involving oxidative stress such as PD (Jagatha et al., 2008; Zbarsky et al., 2005), Alzheimer's Disease (Ringman et al., 2005; Ono et al., 2004; Lim et al., 2001) and other neurodegenerative disorders (Calabrese et al., 2008; Cole et al., 2007; Bengmark, 2006).

PQ has been proposed as a putative etiologic factor in the development of PD (Thrash et al., 2007; Jones and Miller, 2008; Barlow et al., 2007; Dick, 2006; Dinis-Oliveira et al., 2006). The redox cycling of the pesticide PQ is characterized by the generation of ROS (Bonneh-Barkay et al., 2005) an event that has been described as a key functional element in the relation of the pesticide with PD (Miller et al., 2009; Drechsel and Patel, 2008; Chen et al., 2008; Castello et al., 2007; Yang and Tiffany-Castiglioni, 2005; Przedborski and Ischiropoulos, 2005; Thiruchelvam et al., 2005).

Antioxidant treatments have thus been proposed as potential strategies in the prevention of neuronal injury after exposure to PQ (Chen et al., 2008; Thiruchelvam et al., 2005; Reiter et al., 1997). As curcumin exhibits potent antioxidant properties, we considered that it could serve as a potential protective agent against the PQ-mediated toxicity of N27 mesencephalic cells. Surprisingly, curcumin was not able to protect N27 cells against PQ-mediated toxicity, but rather it exacerbated the cell death and dramatically decreased cell viability. This effect was clearly synergistic in nature, because curcumin itself at the concentrations used was not toxic to N27 cells. Combined use of curcumin with PQ, however, increased cell death significantly compared to levels reached with the pesticide alone.

Our aim was to characterize the nature of cell death that occurred in response to the co-incubation of cells with curcumin and PQ. As has been previously reported, PQ exposure was able to initiate apoptosis in SH-SY5Y cells (Moran et al., 2008; Yang and Tiffany-Castiglioni, 2008; Gonzalez-Polo et al., 2007a,b; Gonzalez-Polo et al., 2004a). Although curcumin has been widely described as an inhibitor of apoptosis in several cell models (Reuter et al., 2008; Shishodia et al., 2007; Kuttan et al., 2007; Karunakaran et al., 2005), we observed here that co-incubation with PQ led to an increase of apoptosis in N27 mesencephalic cells compared to that observed in cells exposed to PQ alone. As we and other groups have previously described that the increase of apoptosis in PQ-exposed neural cells is mainly a ROS-dependent event, (Gonzalez-Polo et al., 2007a; Chen et al., 2008) we decided to evaluate ROS production after co-incubation of cells with curcumin and PQ. The results obtained indicate that the co-incubation leads to an increase in the ROS production which we characterized as a synergistic process given that curcumin itself was not able to increase ROS production in cells. This result is consistent with previously described findings that curcumin, although a potent antioxidant with ROS scavenging properties, is able to increase ROS production in different models (Javvadi et al., 2008; Cui et al., 2007; Nakamura et al., 1998). Moreover, several *in vitro* studies have shown that curcumin-induced apoptosis is associated with ROS production (Thayyullathil et al., 2008; Das et al., 2008; Jung et al., 2005; Woo et al., 2003; Bhaumik et al., 1999).

As the cell death that was observed after the PQ+curcumin exposure was found to be a ROS-dependent event, we sought to reverse this effect by using NAC or VitE, two well known antioxidants. Although both antioxidants were able to significantly protect against the cell death generated by the exposure of cells to PQ alone, we were not able to prevent cell death when the N27 cells were co-incubated with PQ+curcumin. As we have previously observed that the co-incubation generated a burst of ROS we evaluated if this effect sent the cell into a “no-way-back” cascade leading to cell death that could not be retrieved by using antioxidants. To this extent, when ROS levels exceed the cells’ defense or antioxidant capacities, injury occurs; growing evidence indicates that this burst leads to modification of proteins, lipids and DNA, altering their functions, and activating related signaling pathways, eventually leading to apoptosis of these cells. To address this question, N27 mesencephalic cells were exposed to hydrogen peroxide, a major oxidant generated when oxidative stress occurs. In experiments with the ROS-sensitive dye HE, we have observed that when ROS production in H₂O₂-exposed cells reached levels similar to those observed following the exposure of cells to PQ+curcumin, the recovery of cell viability with the antioxidants NAC or VitE could not be achieved.

To further explore the molecular mechanism that mediated the enhancement of cell death in PQ+curcumin-exposed cells, we analyzed the expression of NO and its regulation by curcumin. As superoxide has been described as the major ROS produced by the redox cycling of the pesticide PQ, (Hassan, 1984) and given that previous reports have shown that NO could act as a superoxide scavenger both *in vitro* (Day et al., 1999) and *in vivo*, (Cho et al., 2005) we analyzed the expression of NO after PQ exposure in N27 cells. We found that PQ exposure increases the production of NO in N27 cells and that this exposure leads to an upregulation of the expression of the inducible isoform of NOS in a time-dependent manner. As previously

reported (Day et al., 1999), nitric oxide interacts with superoxide to generate the strong oxidant and nitrating intermediate peroxynitrite. As curcumin is an extremely specific inhibitor of iNOS expression (Lin, 2007), we hypothesized that the heightened ROS production observed after the exposure of cells to PQ+curcumin could be due to a failure in the nitric oxide production system leading to an increase in superoxide. We observed that curcumin effectively inhibits the expression of iNOS mRNA and that this effect is due to the inhibition of I κ B degradation leading to an inhibition of the translocation of NF- κ B, the nuclear factor that initiates the expression of the inducible isoform of NOS. As this inhibition attenuates the levels of NO produced after exposure of N27 cells to PQ, this effect leads to an overall increase in ROS levels due to a failure in the scavenging of superoxide by NO. This effect was seen in the attenuation of the increase of peroxynitrite formation (as evidenced by the formation of 3-NT) after exposure of cells to PQ in the presence of curcumin.

Although a definitive mechanism for the toxicity of PQ is yet to be elucidated, a cyclic single electron reduction/oxidation of the parent molecule is a critical mechanistic event. The redox cycling of paraquat has two potentially important consequences relevant to the development of toxicity: generation of “activated oxygen” (e.g., superoxide anions, hydrogen peroxide, hydroxyl radicals) which is highly reactive to cellular macromolecules; and/or oxidation of reducing equivalents (e.g., NADPH, reduced glutathione) necessary for normal cell function. The action of superoxide is normally limited by its low lipid solubility, its limited membrane transport, and also by its removal by SOD, the rate constant of which is $2 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ (Huie and Padmaja, 1993). The production of nitric oxide after PQ exposure has been described for both in vitro (Yeh et al., 2006) and in vivo models (Berisha et al., 1994). Although the toxicity of NO is modest, it is greatly enhanced by reacting with superoxide to form peroxynitrite. The rate constant for this reaction is $6.7 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ (Beckman, 1994). This suggests that as superoxide production increases after PQ exposure, peroxynitrite formation will be favored over superoxide removal by SOD. To this extent, NO is the only known biological molecule that is produced in high enough concentrations and reacts rapidly enough to outcompete SOD for superoxide, which in fact leads to an attenuation of the superoxide levels due to a scavenging effect. The peroxynitrite formed reacts relatively slowly with most biological molecules, making itself a selective oxidant. Peroxynitrite modifies tyrosine in proteins to create nitrotyrosines, leaving a detectable footprint both in vivo and in vitro. Because in PQ mediated toxicity the generation of superoxide is the acute event due to redox cycling of the herbicide the inhibition of nitric oxide production by the iNOS expression curcumin leads to an exacerbation of the ROS due to an attenuation of the generation of peroxynitrites, which in fact enhances cell death after PQ exposure.

Curcumin has pleiotropic effects on cell death and survival. It can modulate the expression and activation of cellular regulatory systems such as NF- κ B, leading to the inhibition of iNOS expression. Although curcumin has been proposed as a putative agent to protect against the oxidative stress generated after PQ injury, (Peiro et al., 2007; Soudamini et al., 1992) our data with N27 cells do not support this hypothesis and suggest that the use of curcumin as a therapeutic agent in the case of neurodegenerative disorders must be reevaluated.

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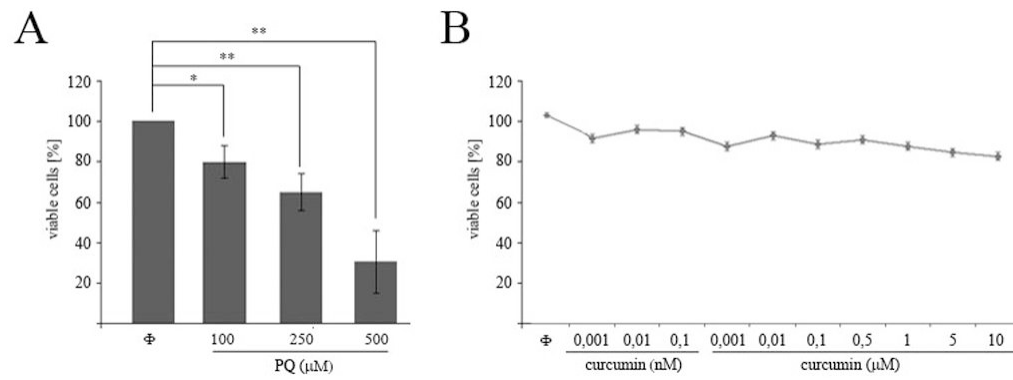


Fig. 1. Cell viability measurement after exposure to PQ or curcumin. N27 cells were exposed for 24 h to the indicated concentrations of curcumin or PQ. Cell viability measured by MTT assay after PQ (A) or curcumin (B) exposure in N27 cells. Data are expressed as the mean \pm SEM. ** $p < 0.01$.

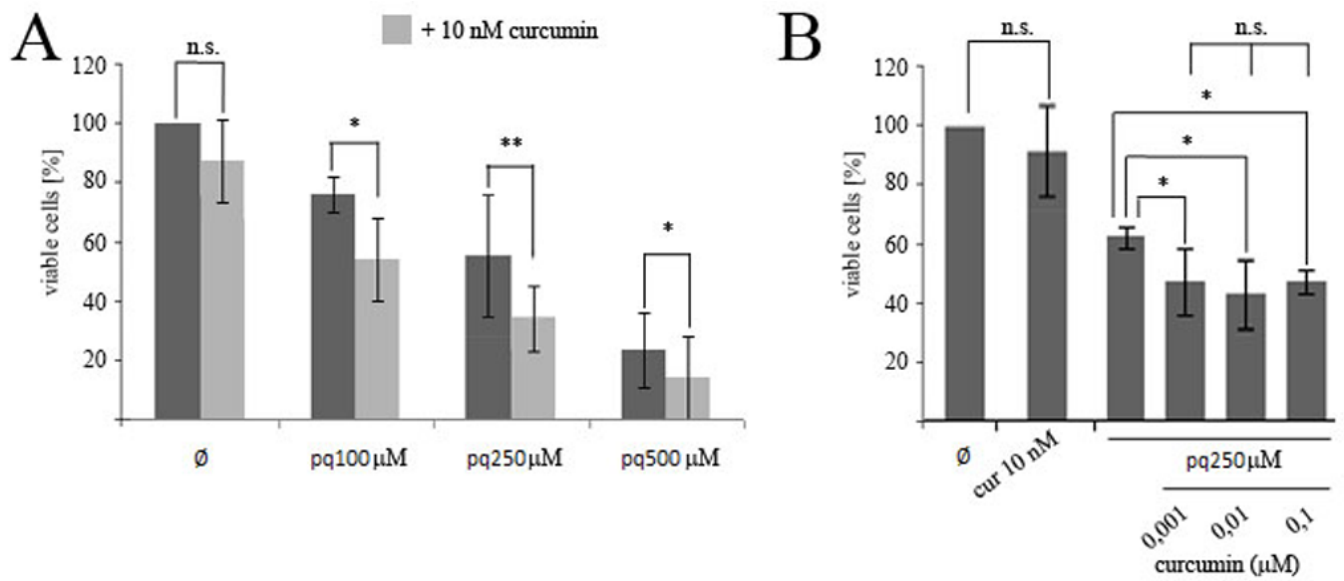


Fig. 2. Effect of curcumin exposure on the viability of PQ-exposed N27 cells. Cells were preincubated for 24h with the indicated amounts of curcumin before exposure to the indicated amounts of PQ for 24h. Cell viability was measured by the MTT assay (A,B). Data are expressed as the mean \pm SEM. n.s. not significant, * p <0.05, ** p <0.01.

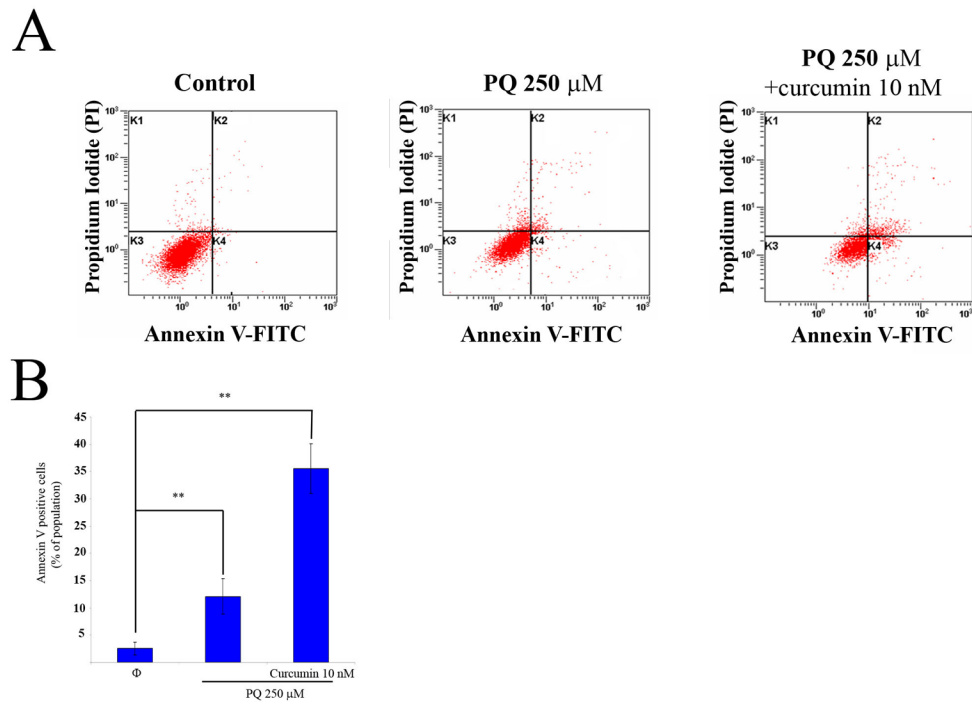


Fig. 3. Curcumin increases phosphatidylserine outsourcing induced by PQ in N27 cells. Apoptotic cells detected by flow cytometry with Annexin V/IP. (A) Representative plots of flow cytometry with Annexin V/IP of N27 cells after exposure to PQ 250 μM or PQ 250 μM plus curcumin 10 nM. (B) Measurement of phosphatidylserine outsourcing after treatment with or without exposed to PQ 250 μM for 24h. Data are expressed as the mean ± SEM. ** $p < 0.01$.

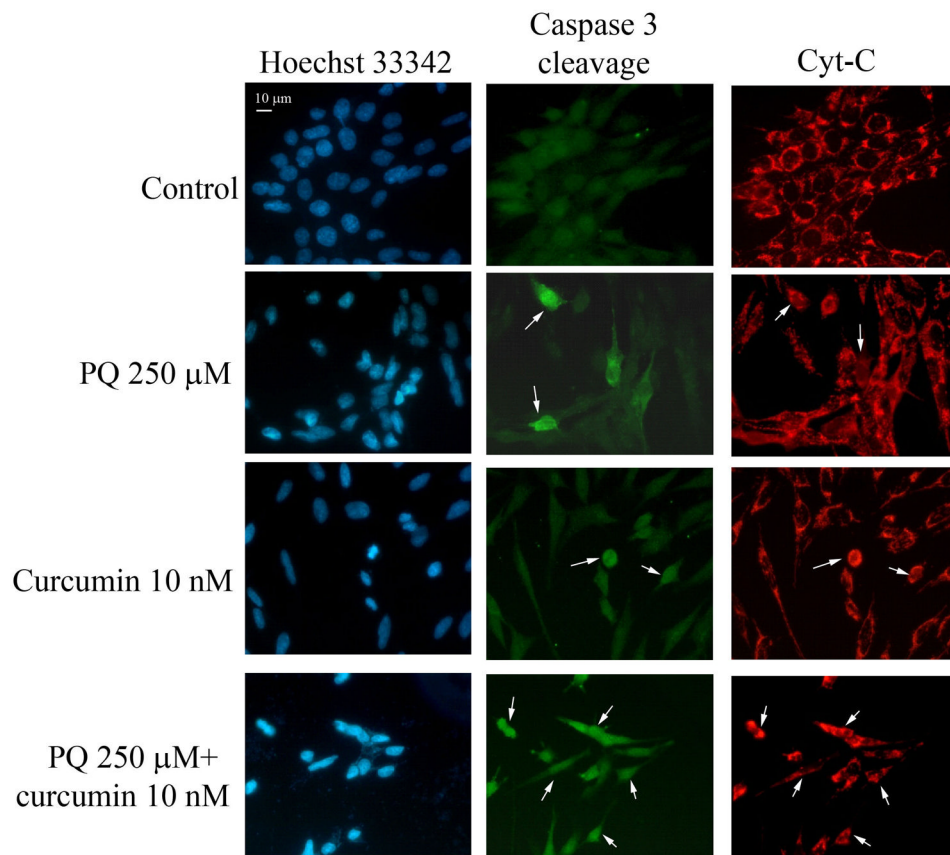


Fig. 4. Curcumin increases PQ-induced apoptotic cell death in N27 cells. Immunocytochemical analysis of active Caspase-3 (Casp-3 cleavage), released cytochrome C (Cyt-C) and chromatin condensation (Hoechst 3342) in N27 cells treated with PQ, curcumin or both.

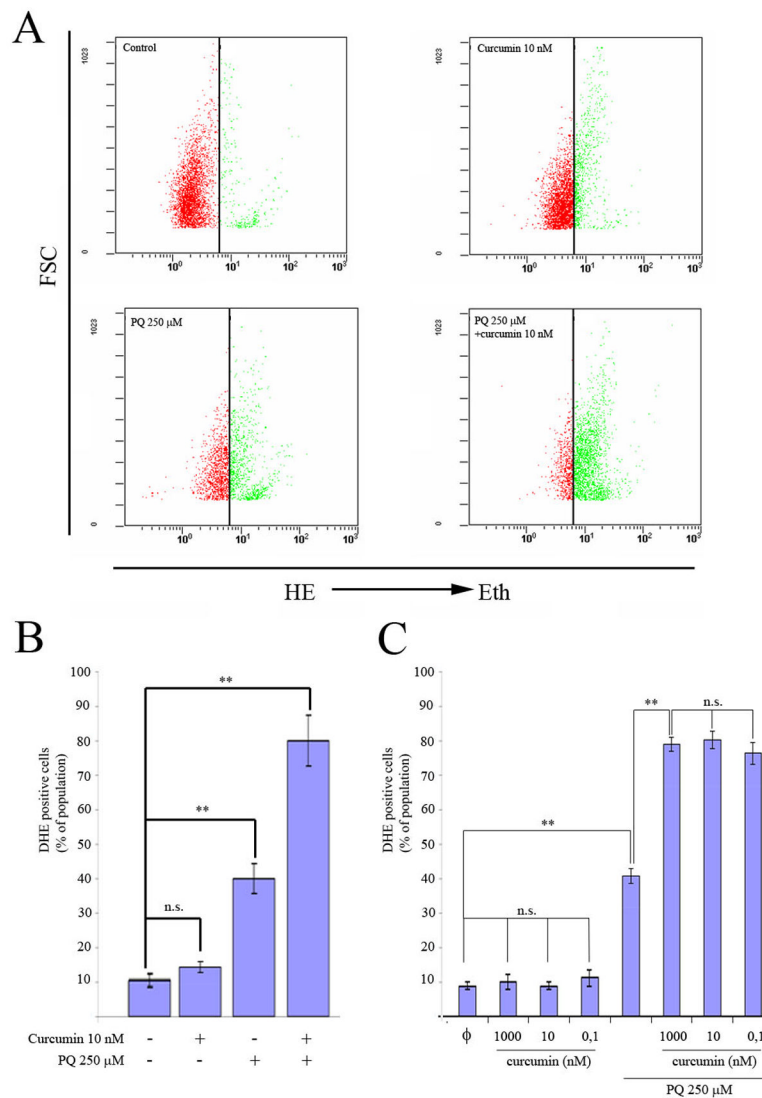


Fig. 5. Exposure to PQ plus curcumin enhances ROS generation. (A) Generation of DHE oxidation analyzed by flow cytometry in N27 cells exposed to the indicated amounts of PQ, curcumin, or both. (B) Quantification of oxidation of DHE analyzed by flow cytometry in N27 cells exposed to the indicated amounts of PQ, curcumin, or both. (C) Measurement of DHE-positive cells by flow cytometry in N27 cells treated with different curcumin-concentrations with or without exposure to PQ 250μM for 24h. Data are expressed as the mean ± SEM. n.s. not significant, * $p < 0.05$, ** $p < 0.01$.

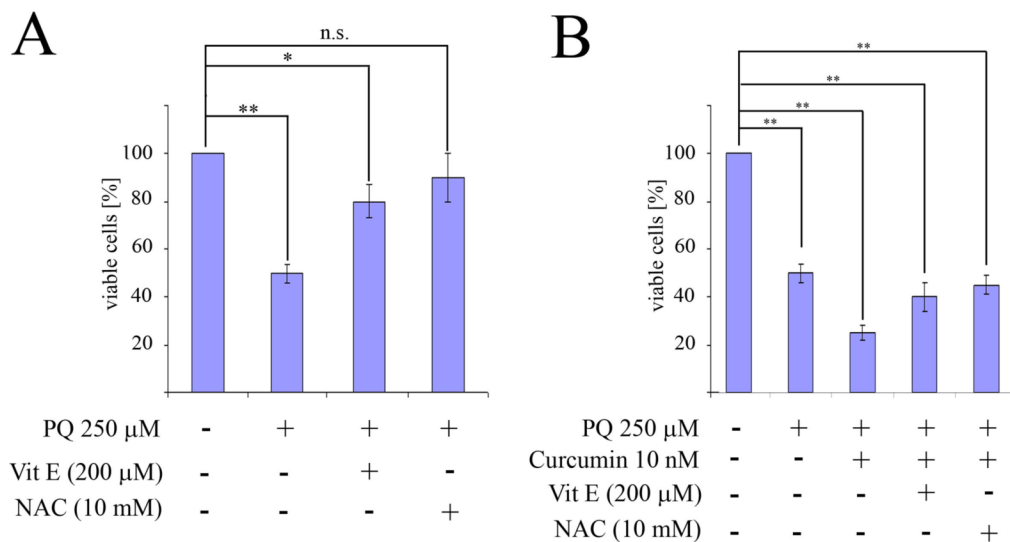


Fig. 6. Antioxidants protect N27 cells from PQ-mediated toxicity but not from toxicity induced by treatment with PQ+curcumin. Cell viability was measured by MTT assay. (A) N27 cells were preincubated for 30 min prior to PQ exposure with vitamin E (Vit E) and N-acetyl-cysteine (NAC) at indicated concentrations. (B) N27 cells were preincubated for 30 min prior to treatment with PQ or PQ plus curcumin with vitamin E (Vit E) and N-acetyl-cysteine (NAC) at indicated concentrations. Data are expressed as the mean \pm SEM. n.s. not significant, * p <0.05, ** p <0.01.

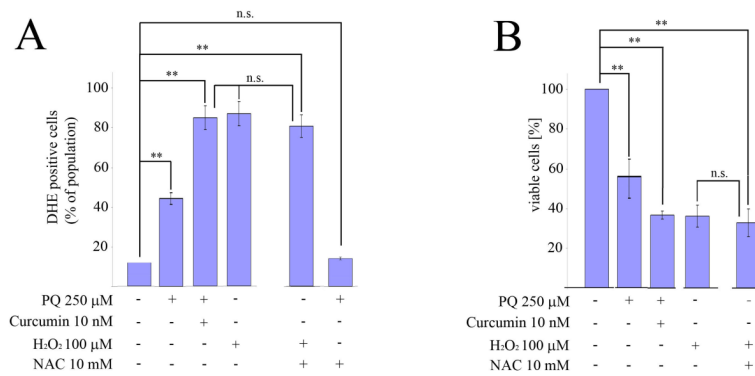


Fig. 7. Effect of oxidative stress generated after treatment with PQ plus curcumin cannot be rescued by co-incubation with antioxidants. (A) Flow cytometry analysis of ROS generation by N27 cells co-incubated with 10 mM NAC for 30 min prior to treatment with H₂O₂. (B) The MTT assay was used to measure the viability of N27 cells co-incubated with 10 mM NAC for 30 min prior to treatment with H₂O₂ at the indicated concentrations. Data are expressed as the mean \pm SEM. n.s. not significant, ** p <0.01.

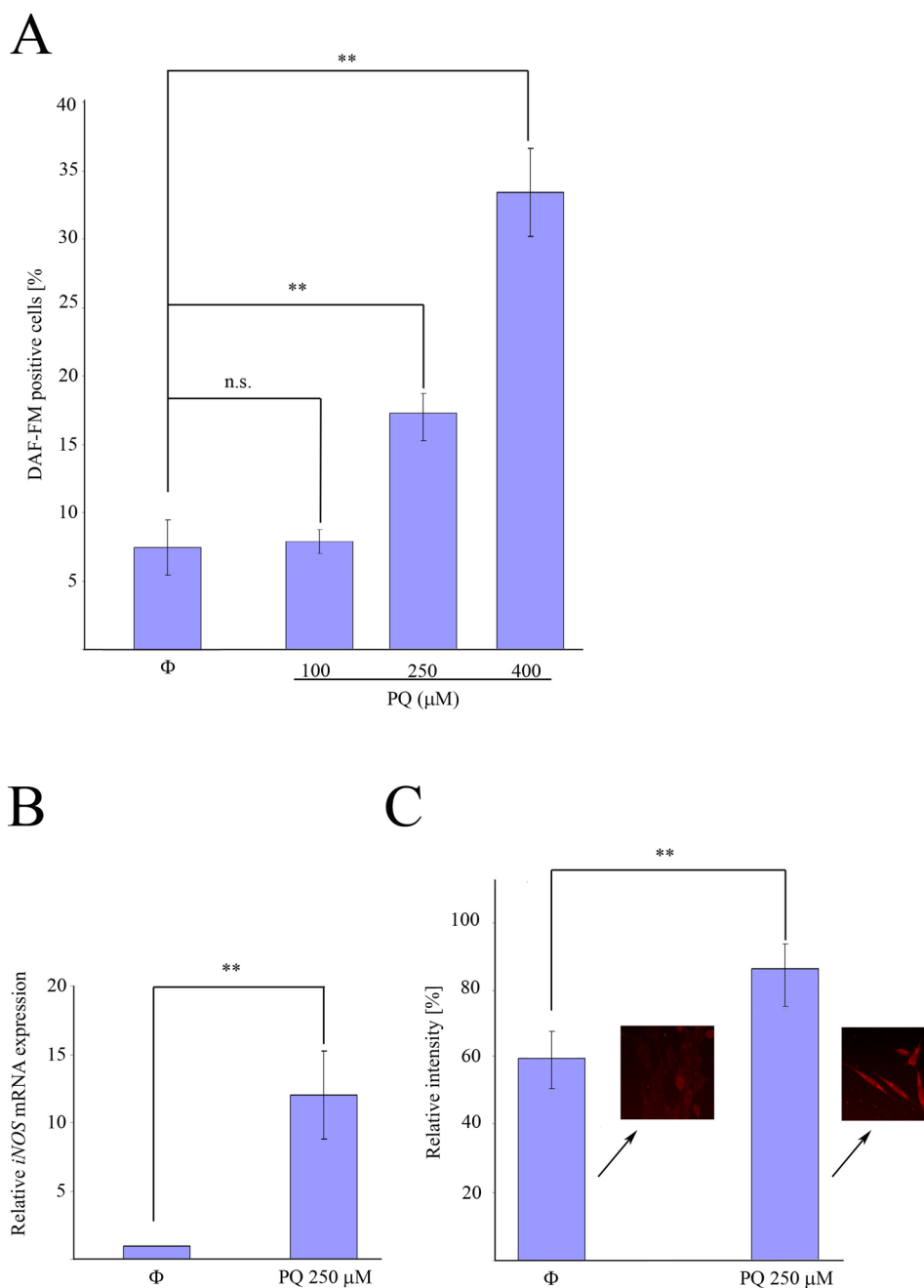


Fig. 8. PQ exposure induces an increase of iNOS mRNA and protein expression in N27 cells. (A) Analysis of DAF-FM-positive cells by flow cytometry after PQ treatment at indicated concentrations. (B) Quantification of iNOS mRNA expression by real-time RT-PCR after incubation with 250µM PQ for 24h.(C) Relative quantification of iNOS fluorescence signal by immunocytochemistry in N27 cells exposed to 250 µM PQ. Data are expressed as the mean \pm SEM. n.s. not significant, ** p <0.01.

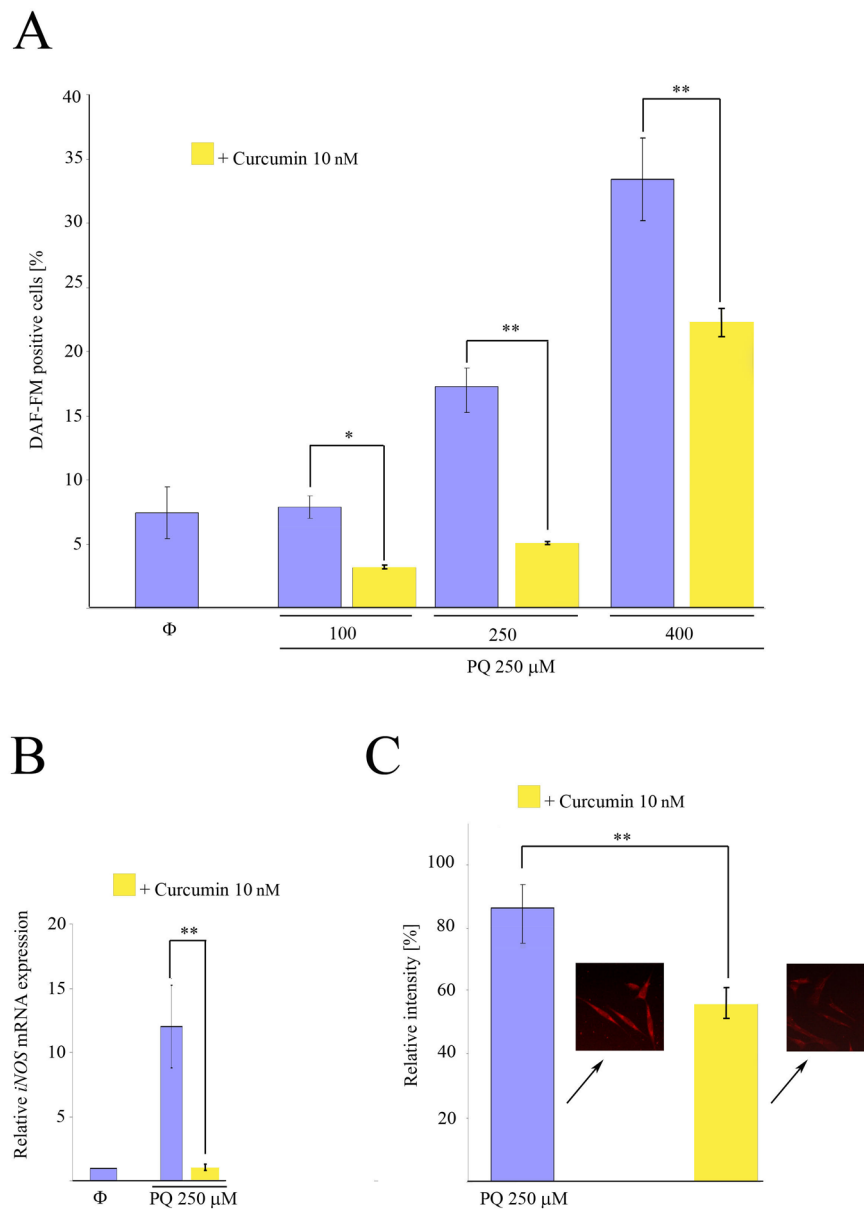


Fig. 9. Inhibition by curcumin of NO production, and expression of iNOS mRNA and protein in N27 cells after treatment with PQ. (A) Analysis of DAF-FM positive N27 cells by flow cytometry after exposure to PQ or PQ plus curcumin at the indicated concentrations. (B) Quantification of iNOS mRNA expression by real-time RT-PCR after incubation with 250 μ M PQ or 250 μ M PQ plus 10 nM curcumin for 24h. (C) Relative quantification of iNOS fluorescence signal by immunocytochemistry in N27 cells exposed to 250 μ M PQ or 250 μ M PQ plus curcumin 10 nM for 24h. Data are expressed as the mean \pm SEM. n.s. not significant, ** p <0.01.

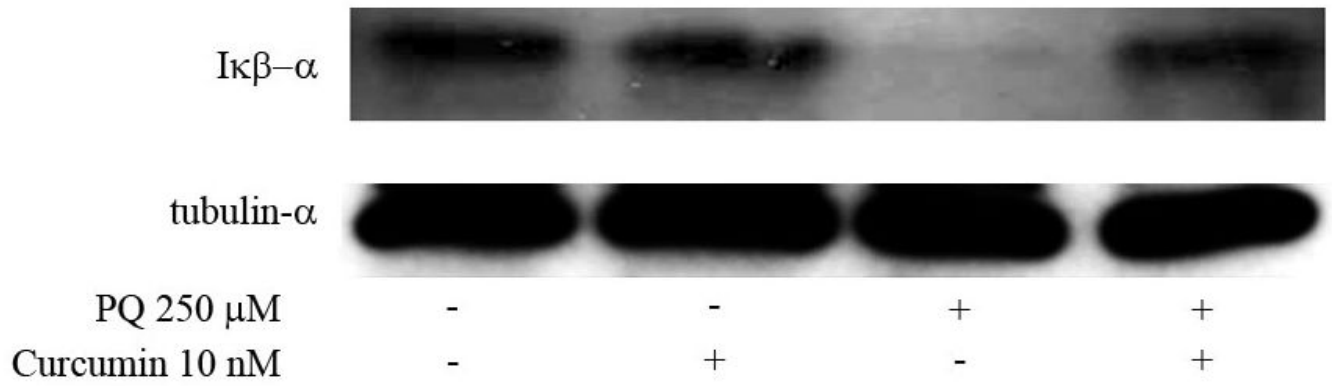


Fig. 10. Curcumin inhibits the PQ-induced degradation of IκBα. Western blot analysis of IκBα expression in N27 cells treated with PQ 250μM, 10nM curcumin or both. α-tubulin was used for equal loading.

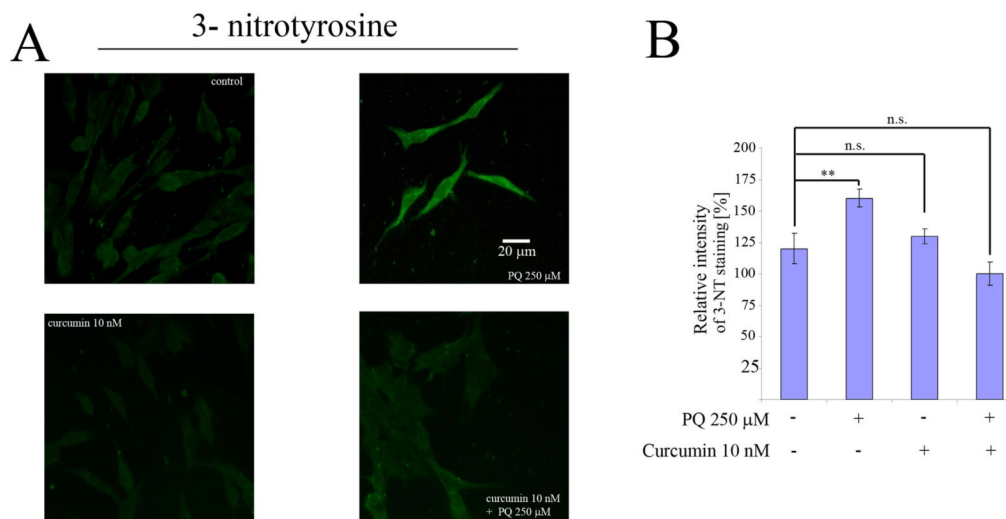


Fig. 11. Inhibition by curcumin of PQ-induced 3-Nitrotyrosine (3-NT) formation. (A) Immunocytochemical analysis of 3-NT formation in rat N27 cells after exposure to 250 μM PQ, 10 nM curcumin, or both. (B) Relative quantification by immunocytochemistry of 3-NT fluorescence signal after exposure of N27 cells to 250 μM PQ, 10 nM curcumin, or both. Data are expressed as the mean ± SEM. n.s. not significant, ** $p < 0.01$.