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Nitric oxide activated by p38 and NF-κB facilitates apoptosis and cell cycle arrest under oxidative stress in evodiamine-treated human melanoma A375-S2 cells

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Abstract
Nitric oxide (NO) has been identified as a fundamental molecule that interplays with reactive oxygen species (ROS) in determining cell fate. As a previous study indicated that ROS was stimulated in evodiamine-induced human melanoma A375-S2 cell apoptosis, the goal of this study was to investigate the role of NO in the cells. In this study, it was found that evodiamine has a strong inductive effect on NO production synthesized by inducible NOS (iNOS) enzyme in a positive-feedback manner. The generated NO was further showed to induce apoptosis and cell cycle arrest and linked to the activation of p53 and p21. After interruption of p38 and nuclear factor-κB (NF-κB) by pre-treatment with SB203580 and PDTC, iNOS expression, NO synthesis and cell damage were all significantly blocked. It was concluded that p38 and NF-κB were critical to the NO producing system, which contributed greatly to the apoptosis and cell cycle arrest in evodiamine-incubated cells.

Keywords: *Evodiamine, nitric oxide (NO), inducible NOS (iNOS), apoptosis, cell cycle arrest, p38 MAPK, nuclear factor-κB (NF-κB)*

Introduction

Nitric oxide (NO) is a free radical that is largely synthesized by the enzyme NO synthase (NOS). Of the three NOS isoforms, two are constitutively expressed, endothelial NOS (eNOS, NOS3) and neuronal NOS (nNOS, NOS1), and one, inducible NOS (iNOS, NOS2), is regulated at the gene level by a variety of mediators [1,2]. Since iNOS is responsible for the production of sustained high levels of NO, it is often considered the primary perpetrator of autotoxicity under oxidative stress [3]. Being a reactive molecule, NO has been considered one of the appropriate bioactive molecules in the intra- and inter-cellular signal transduction for the regulation of physiological and pathological processes [4]. More and more investigations suggest that NO may have a dual action against tumour depending on its local concentration. High concentrations of NO might mediate cancer cell apoptosis and the inhibition of cancer growth, although, at relatively low concentrations of NO, tumour growth, proliferation and angiogenesis are promoted [5].

Most importantly, a large body of pharmacological and genetic evidences has demonstrated that NO

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together with reactive oxygen species (ROS) is essential for triggering cell death [6]. Oxidation products of NO, such as peroxynitrite, can induce lipid peroxidation, S-nitrosylation of thiol groups in proteins and inhibition of enzymes for mitochondrial respiration [7]. Under the simultaneous generation of NO and ROS, the cellular antioxidant capabilities were also suppressed [8].

The nuclear factor κB (NF-κB) belongs to the transcription factors family and plays a critical role in several signal transduction pathways involved in various cancers [9]. Activation of NF-κB is linked with apoptotic cell death, either promoting or inhibiting apoptosis, depending on cell type and condition [10,11]. The expressions of several target genes encoding cytokines, chemokines, growth factors, cell adhesion molecules and some enzymes including iNOS were upregulated by NF-κB. They play key roles in embryonic development, lymphoid differentiation, apoptosis, immune and inflammatory responses and development of cancer [12].

The mitogen-activated protein kinases (MAPKs) are proline-targeted serine-threonine kinases that transduce environmental stimuli to the nucleus [13]. The p38 MAPK is a member of the MAPK family that is activated by a variety of cellular stresses, including oxidative stress that is characterized by the accumulation of increased levels of ROS within the cell [14]. Of note, the activation of p38 MAPK has been recently implicated in regulating transcription factor NF-κB and iNOS expression in many studies [15,16].

Evodiamine, a quinozole alkaloid, was isolated from the dry unripe fruit of *Evodia rutaecarpa Bentham* (*Rutaceae*). It has been reported previously that evodiamine has a wide variety of pharmacological activities, such as anti-tumour growth, anti-obesity, anti-anoxic, anti-nociceptive and vasorelaxant effects [17–21]. Since it has been found that ROS was induced by evodiamine in human melanoma A375-S2 cells and contributed greatly to the apoptosis of cells [22], the objectives of this study were to investigate the possible involvement of NO under oxidative stress and the apoptotic signal transduction pathway of ROS/NO in evodiamine-treated cells.

Materials and methods

Reagents

Evodiamine was obtained from Beijing Institute of Biological Products (Beijing, China); and its purity was determined to be $\sim$ 98% by HPLC measurement. Evodiamine was dissolved in dimethyl sulphoxide (DMSO) to make a stock solution and diluted by RPMI-1640 (Gibco, Grand Island, NY) before the experiments. DMSO concentration in all cell cultures was kept below 0.001%, which had no detectable effect on cell growth or death. 3-(4,5-dimetrylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3,3-diaminobenzidine tetrahydrochloride (DAB), catalase (CAT), pyrrolidine dithiocarbamate (PDTC), N^G-nitro-L-arginine methyl ester (L-NAME), acridine orange (AO), propidium iodide (PI), 4,5-diaminofluorescein diacetate (DAF-2DA), rhodamine-123, PMSF, aprotinin and leupeptin were purchased from Sigma Chemical (St. Louis, MO). SB203580 (SB) and pifithrin-α (PFT) were obtained from Calbiochem (La Jalla, CA). Polyclonal antibodies against IκBα, phospho-IκBα, p38, phospho-p38, p53, phospho-p53, Akt, phospho-Akt, p21, iNOS, β-actin and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

A375-S2, human melanoma cells, were obtained from American Type Culture Collection (ATCC, #CRL, 1872, Manassas, VA) and were cultured in RPMI-1640 medium supplemented with 10% heat inactivated (56°C, 30 min) foetal calf serum (Beijing Yuanheng Shengma Research Institution of Biotechnology, Beijing, China), 2 mM L-glutamine (Gibco, Grand Island, NY), 100 kU/L penicillin and 100 g/L streptomycin (Gibco) at 37°C in 5% CO_2. The cells in the exponential phase of growth were used in the experiments.

Measurement of intracellular NO by flow cytometry

The intracellular NO was detected using DAF-2DA as described by Habel and Jung [23] with some modifications. DAF-2DA, a nitric oxide fluorescent probe, can react with NO within viable cells to produce a fluorescent compound DAF-2T. After drug treatment, the cells were collected and resuspended in PBS and then incubated with 10 μM DAF-2DA for 45 min at 37°C. Samples were then analysed at an excitation wavelength of 485 nm and an emission wavelength of 515 nm by a FACScan flowcytometry (Becton Dickinson, Franklin Lakes, NJ).

LDH activity-based cytotoxicity assay

LDH (lactate dehydrogenase) activity was assessed using a standardized kinetic determination kit (Zhongsheng LDH kit, Beijing, China). LDH activity was measured in both floating dead cells and viable adherent cells [24]. The floating cells were collected from culture medium by centrifugation (240 × g) at 4°C for 5 min and the LDH content from the pellets was used as an index of apoptotic cell death (LDHp). The LDH released in the culture medium (extracellular LDH or LDHe) was used as an index of necrotic death and the LDH present in the adherent viable cells as intracellular LDH (LDHi). The percentage of apoptotic and necrotic cell death was calculated as follows:

$$\text{Apoptosis\%} = \text{LDHp}/(\text{LDHp} + \text{LDHi} + \text{LDHe}) \times 100$$

$$\text{Necrosis\%} = \text{LDHe}/(\text{LDHp} + \text{LDHi} + \text{LDHe}) \times 100$$

Nuclear damage observed by acridine orange (AO) staining

The changes in nuclear morphology of apoptotic cells were investigated by labelling cells with the fluorescent, selective DNA and RNA-binding dye AO and examined under fluorescence microscopy (Green fluorescence for DNA, red fluorescence for RNA) [25]. After incubation with 15 μM evodiamine for the indicated time periods with or without 10 mM L-NAME, cells were stained with 20 μg/ml AO (Sigma) for 15 min and then the morphology was observed under a fluorescence microscope (Olympus, Tokyo).

Flowcytometric analysis using PI

After treatment with drugs, 1×10^6 cells were harvested, washed with PBS, then fixed in 70% methanol and, finally, maintained at 4°C for at least 12 h. Then the cell pellets were stained with the fluorescent probe solution containing PBS, 50 μg/ml PI and 1 mg/ml DNase-free RNaseA for 30 min on ice in the dark. DNA fluorescence of PI-stained cells was evaluated by a FACScan flowcytometry. A minimum of 10,000 cells were analysed per sample and the DNA histograms were gated and analysed further using Modfit software on a Mac workstation to estimate the percentage of cells in various phases of the cell cycle [26].

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was measured by the incorporation of a cationic fluorescent dye rhodamine 123 as described [27]. After incubation with 15 μM evodiamine for the indicated time periods with or without 10 mM L-NAME, cells were collected and suspended in 1 ml PBS containing 1 μg/ml rhodamine 123 and incubated for 15 min at 37°C. The fluorescence intensity of cells was analysed within 15 min by FACScan flowcytometry.

Assessment of cell viability

A375-S2 cells were dispensed in 96-well flat bottom microtiter plates (NUNC, Roskilde, Denmark) at a density of 5×10^4 cells/ml. After 12 h incubation, the cells were treated with or without CAT, PDTC, SB, L-NAME or PFT at given concentrations 1 h prior to the administration of 15 μM evodiamine for the indicated time periods. Cell viability was measured using the MTT assay as described elsewhere [28] with a plate reader (Bio-Rad, Hercules, CA).

The percentage of cell viability was calculated as follows:

$$\text{Cell viability (\%)} = (\text{A490}_{\text{sample}} - \text{A490}_{\text{blank}})/(\text{A490}_{\text{control}} - \text{A490}_{\text{blank}})] \times 100$$

Western blot analysis

A375-S2 cells were treated with 15 μM evodiamine for 0, 6, 12 and 24 h or co-incubated with the given inhibitors for the indicated time. Both adherent and floating cells were collected and then Western blot analysis was carried out as previously described [29] with some modification. Briefly, the cell pellets were resuspended in lysis buffer, including 50 mM Hepes (pH 7.4), 1% Triton–X 100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM edetic acid, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin and lysed on ice for 60 min. After centrifugation of the cell suspension at 13,000 × g for 15 min, the protein content of supernatant was determined by Bio-Rad protein assay reagent (Bio-Rad). The protein lysates were separated by electrophoresis in 12% SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). Proteins were detected using polyclonal antibody and visualized using anti-rabbit, anti-mouse or anti-goat IgG conjugated with horseradish peroxidase (HRP) and 3,3-diaminobenzidine tetrahydrochloride (DAB) as the substrate of HRP.

Statistical analysis

The results are presented as Mean ± SD. Significant changes were assessed using Student's *t*-test for unpaired data, and p-values < 0.05 were considered statistically significant.

Results

Evodiamine induced intracellular NO generation in A375-S2 cells

The intracellular NO level was examined by using DAF-2DA, a membrane-permeable derivative of the NO sensitive fluorophore DAF-2. The reaction of NO with DAF-2 yields the green fluorescent triazole derivative DAF-2T [30]. Exposure of A375-S2 cells to 15 μM evodiamine for 6 or 24 h led to a significant increase in DAF-2T signal compared with the control group in a time-dependent manner. The ratio of DAF-2T positive cells was increased from 1.61% (Figure 1A) in untreated cells to 18.09% in 6 h-treated (Figure 1B) and 50.04% in 24 h-treated cells (Figure 1C), respectively. The burst of DAF-2T

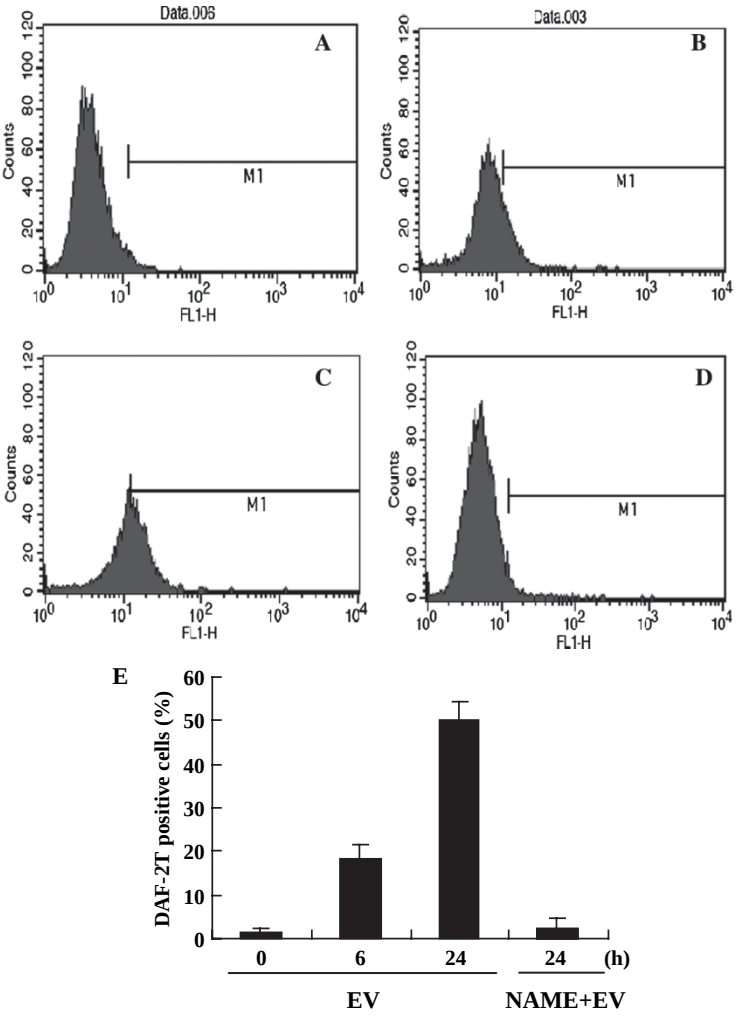

Figure 1. Production of intracellular NO was induced by evodiamine and was blocked by pre-treatment with L-NAME in A375-S2 cells. The cells were cultured in the presence of 15 μM evodiamine for 0 (A), 6 h (B), 24 h (C) or coincubated with 10 mM L-NAME for 24 h (D). DAF-2T, the fluorescent dye product of DAF-2 in reaction with NO, was measured fluorometrically at 1 h post-treatment. The corresponding linear diagram of the FACScan histograms was expressed in (E). Data from a representative experiment ($n = 3$) are shown.

fluorescence in evodiamine-treated cells at 24 h was largely reduced by 47.91% by the NOS inhibitor L-NAME (Figure 1D), a methyl ester derivate of the NOS substrate L-arginine. These results suggested that a burst of NO was stimulated in evodiamine-treated cells and was generated by the NOS enzyme.

The role of NO in evodiamine-induced cell death

Some reports have demonstrated that NO has anti-tumour activity and high concentrations of NO can inhibit cell growth and induce apoptosis [31]. To investigate the role of NO in evodiamine-induced A375-S2 cell death, ratios of apoptosis and necrosis were analysed by LDH activity-based assay. Pre-incubation with increasing concentrations of L-NAME, the number of apoptotic cells was reduced significantly from 39.51% for evodiamine alone to 29.28% or 22.40% in the presence of 5 or 10 mM L-NAME at 24 h, while the number of necrotic cells was decreased a little from 11.29% to 9.35% or 8.17% in the presence of 5 or 10 mM L-NAME, respectively (Figure 2). Therefore, NO played an essential role in evodiamine-induced cell death. Apoptosis was the predominant mechanism responsible for NO-induced cell death within 24 h.

NO induced apoptosis and cell cycle arrest in evodiamine-treated cells

To understand the pro-apoptotic effect of NO on evodiamine-treated cells, we observed the morphologic changes by AO staining. In the 24 h evodiamine-treated cells, apoptotic bodies and marked fragmented DNA in nuclei were observed (Figure 3C); whereas in the L-NAME coincubated group, cells showed rare apoptotic bodies and nuclear damage (Figure 3D). Furthermore, the cells treated with evodiamine with or without L-NAME for indicated times were stained with PI, a commonly used method to analyse DNA fragments in cells. The peak of DNA fragments in cell cycle analytic diagrams locates at the left of the normal

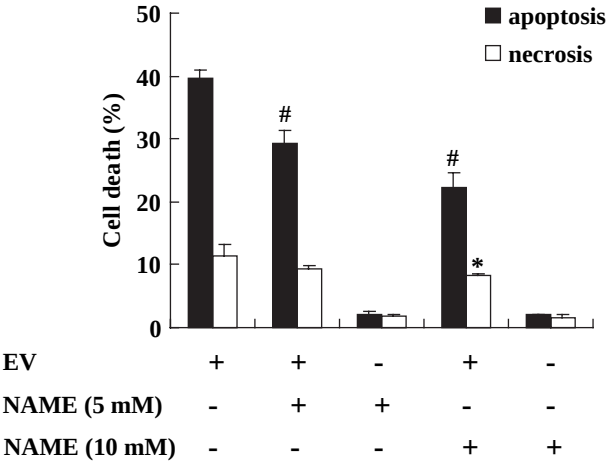

Figure 2. Effect of L-NAME on evodiamine-induced apoptosis and necrosis. The cells were pre-treated with 5 or 10 mM L-NAME (NAME) for 1 h and then incubated with 15 μM evodiamine for 24 h. The cell death rate was measured by LDH activity-based assay. Values are expressed as mean ± SD. # $p < 0.001$ vs the apoptosis percentage in groups treated with evodiamine alone. * $p < 0.05$ vs the necrosis percentage in groups treated with evodiamine alone.

G1 phase peak, which is defined as SubG1 [32]. As shown in Figure 3E, untreated cells expressed 64.08% of G1, 12.22% of S, 17.48% of G2/M and 5.81% of SubG1. The percentage of SubG1 was obviously increased to 7.39% (Figure 3F) or 28.80% (Figure 3G) in 6 h- or 24 h-incubated cells, respectively. When coincubated with L-NAME, the percentage of SubG1 was remarkably reduced from 28.80% for evodiamine alone to 8.69% at 24 h (Figure 3H). Moreover, the percentage of the G2/M phase was observed significantly increased from 17.48% in untreated cells (Figure 3E) to 20.23% in 6 h-treated (Figure 2F) and 32.68% in 24 h-treated cells (Figure 3G), respectively. When coincubated with L-NAME, the percentage of the G2/M phase was partially reduced from 32.68% for evodiamine alone to 19.70% at 24 h (Figure 3H). Taken together, the production of NO was found to be related to the evodiamine-induced apoptosis and cell cycle arrest in A375-S2 cells.

NO induced △ψm decrease in evodiamine-treated cells

Apoptosis requires a process which concomitantly proceeds with an irreversible loss of mitochondrial membrane potential (△ψm) [33]. Our previous study showed that the drop of △ψm induced by evodiamine was greatly related to the ROS production. Since the lethal role of ROS also needed to combine with NO, the participation of NO in the regulation of △ψm was further investigated. Results showed that co-incubation with L-NAME notably reversed the loss of △ψm, as illustrated by the reduced percentage of cells in M1 zone from 43.77% for evodiamine alone to 17.96% in the presence of L-NAME at 24 h (Table I). These data indicated that the drop of △ψm was facilitated by the intracellular generated NO in evodiamine-treated cells.

Evaluate critical roles in evodiamine-induced apoptosis

CAT is a heme protein that provides a protective role which is similar to that of glutathione peroxidase because both are important means of removing hydrogen peroxide. Aerobic organisms produce protective antioxidant enzymes such as CAT, superoxide dismutase and glutathione peroxidase to help protect against the destructive effects of ROS [34]. To detoxify the effect of ROS, CAT was introduced in this study. Consistent with our previous finding, ROS act an important role in evodiamine-treated cells, as illustrated by the increased cell viability from 49.21% in evodiamine-treated cells to 75.00% in the presence of 1000 U/ml CAT at 24 h (Figure 4). Additionally, NO was showed to play a critical role in determining cell fate, as illustrated by the increased cell viability from 49.21% in evodiamine-treated cells to 68.30% in the presence of 10 mM L-NAME at 24 h (Figure 4).

Since NF-κB is reported a target for ROS and appears to be involved in the regulation of iNOS expression [35], the NF-κB inhibitor PDTC was introduced. Results showed a significant protective effect of PDTC on cell viability from 49.21% in evodiamine-treated cells to 56.33% or 64.85% in the presence of 25 or 50 μM PDTC at 24 h (Figure 4), revealing that NF-κB participated in the evodiamine-induced cell growth inhibition. Moreover, p38 MAPK has been shown to be susceptible to ROS and plays critical roles for the activation of NF-κB and iNOS [15,16]. After pre-treatment with p38 MAPK inhibitor SB203580 (SB), a remarkable protective effect on cell viability was found, from 49.21% in evodiamine-treated cells to 65.08% or 75.56% in the presence of 10 or 20 μM SB (Figure 4), respectively. These results indicated that p38 MARK was involved in evodiamine-induced cell growth inhibition. Since the tumour suppressor p53 mediates apoptosis and inhibits cell cycle progression and may also be involved in the NO-induced apoptosis, p53 inhibitor pifithrin-α (PFT) was also introduced in this MTT assay. Cell viability was shown to increase from 49.21% in evodiamine-treated cells to 58.15% or 67.41% in the presence of 10 or 20 μM PFT at 24 h (Figure 4), implicating a sufficient role of p53 in the growth inhibition induced by evodiamine.

Inhibition of ROS, NF-κB and p38 blocked NO generation in evodiamine-treated cells

As the results above revealed that ROS, NF-κB, p38 and p53 all participated in the regulation of evodiamine-induced growth inhibition, we moved to investigate the roles of them on the production of

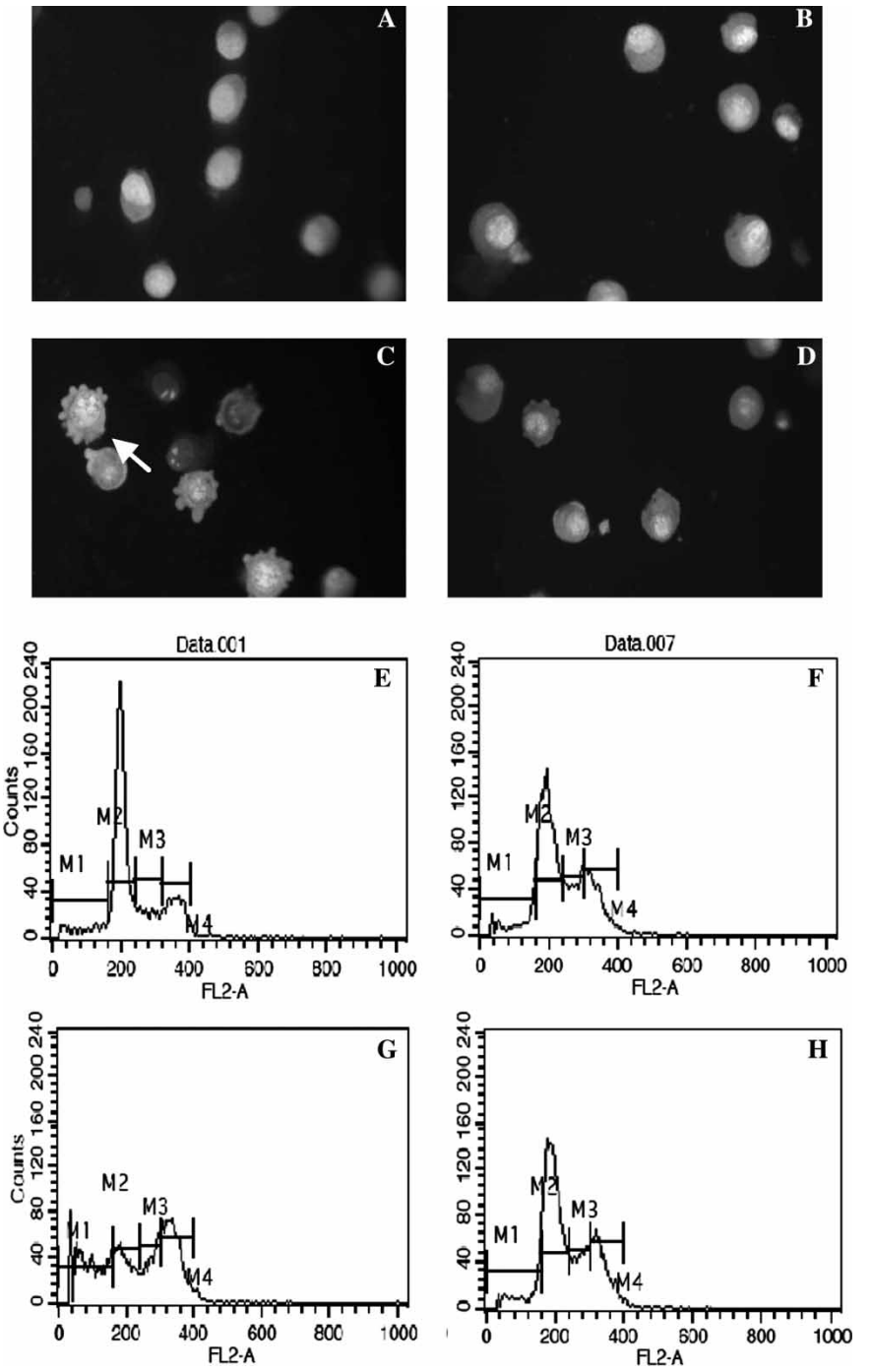

Figure 3. Apoptosis and cell cycle arrest were induced by evodiamine and were inhibited by L-NAME addition in A375-S2 cells. The cells were treated with 15 μM evodiamine for 0, 6, 24 h or coincubated with 15 μM evodiamine and 10 mM L-NAME for 24 h. Then the cellular morphologic changes were observed under a fluorescence microscope by AO staining (A: 0 h for EV; B: 6 h for EV; C: 24 h for EV; D: 24 h for L-NAME+EV; arrow indicates the apoptotic body; ×200 magnification). The DNA content was analysed by flow cytometry after PI staining (E: 0 h for EV; F: 6 h for EV; G: 24 h for EV; H: 24 h for L-NAME+EV). Data from a representative experiment ($n=3$) are shown.

intracellular NO. The DAF-2T fluorescence in evodiamine-treated cells was reduced from 50.04% to 27.98% by 1000 U/ml CAT (Figure 5C), 31.33% by 50 μM PDTC (Figure 5E), 31.57% by 20 μM SB (Figure 5F) and 45.16% by 20 μM PFT (Figure 5D), respectively. These data showed that the intracellular NO generation was probably regulated by ROS, NF-κB and p38; whereas p53 had little effect on it.

p38 and NF-κB activation were required for iNOS expression under oxidative stress in evodiamine-treated cells

As the results from Figure 4 indicated that p38 and NF-κB were both activated in evodiamine-treated cells, the expressions of p38, phospho-p38, IκBα and phospho-IκBα were further examined by Western

Table I. FACScan analysis of the inductive effect of NO on △ψm dissipation. The cells were incubated with 15 μM evodiamine for 0, 6 and 24 h or coincubated with 15 μM evodiamine and 10 mM L-NAME for 24 h. After loading with rhodamine-123 1 μg/ml, cells were measured for △ψm by FACScan flowcytometery. The cells in M1 zone represent the cells which already undergo △ψm dissipation. Data from a representative experiment ($n=3$) are shown. Values are expressed as mean±SD. $^{\#}$ $p<0.001$ vs evodiamine-treated group at 24 h.

Group	Time (h)	M1 (%)
Evodiamine	0	0.95±2.67
	6	8.18±4.83
	24	43.77±5.96
Evodiamine+L-NAME	24	17.96±1.42$^{\#}$

blot analysis. Results showed that the expression of phospho-p38 was increased time-dependently; whereas the expression of p38 did not change after evodiamine treatment (Figure 6A). Pre-incubation with CAT obviously suppressed the elevation of phospho-p38 (Figure 6A), suggesting an essential role of ROS in the activation of p38. As shown in Figure 6B, a time-dependent phosphorylation of IκBα was observed after evodiamine treatment and was reversed by the addition of CAT and SB, indicating that ROS and p38 are indispensable for IκBα degradation. To further verify the plausible role of ROS, NF-κB and p38 in the induction of NO production, CAT, PDTC and SB were applied and the expression of iNOS was detected by Western blot analysis. Results showed that CAT, PDTC and SB could all markedly block the induction of iNOS (Figure 6C), confirming the inductive effects of ROS, NF-κB and p38 on the expression of iNOS. Importantly, L-NAME was found to present its

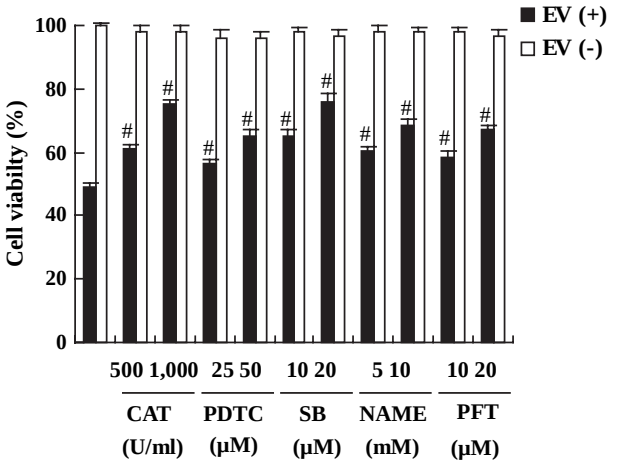

Figure 4. Protective effects of CAT, PDTC, SB203580, L-NAME and pifithrin-α on evodiamine-induced cell death. The cells were cultured in the presence or absence of CAT, PDTC, SB203580 (SB), L-NAME (NAME) or pifithrin-α (PFT) at indicated concentration for 1 h prior to the addition of 15 μM evodiamine and then incubated for 24 h. The viability of cells was determined by MTT assay ($n=3$). Values are expressed as mean± SD. $^{\#}p<0.001$ vs the viability in the group treated with evodiamine alone.

suppressive effect on the expressions of phospho-p38, phospho-IκBα and iNOS, suggesting the positive role of NO in the stimulation of p38, NF-κB and iNOS.

p53 and p21 were involved in the NO-mediated signal transduction in evodiamine-treated cells

To further demonstrate that p53 participated in the regulation of NO-induced cell damage, the expressions of p53 and phospho-p53 were both detected by Western blot analysis. Results showed that the level of phospho-p53 was significantly elevated at 6 h coincident with the increased expression of p53, and both were gradually reduced after 6 h in evodiamine-treated cells, suggesting that p53 might be upregulated by evodiamine in both transcriptional and post-transcriptional step and had a short half-life in cells. Coincubation with CAT, SB or L-NAME at 6 h could apparently downregulate the expressions of p53 and phospho-p53 at 6 h (Figure 7A), indicating the inductive roles of p38 and NF-κB in the activation of p53 under oxidative stress.

The cell cycle inhibitor p21 was reported to play a central role in the control of cell cycle and to be activated by p53 [36]. As shown in Figure 3 that G2/M phase arrest was induced in evodiamine-treated cells, we postulated the involvement of p21 in cells. Western blot analysis revealed that the expression of p21 was significantly increased at 12 h and was markedly suppressed by the addition of CAT, SB or PFT (Figure 7B), showing that p21 was activated dependently on p38 and p53 in the cells.

Since Akt/PKB plays an important role in cell survival and has an inhibitory effect on p53 [37], we next examined whether the proapoptotic effects of ROS and NO were related to the degradation of Akt/PKB. The level of phospho-Akt was significantly lower in cells treated with evodiamine and was remarkably reversed by CAT and L-NAME (Figure 7C). However, the total Akt level did not change in cells whether treated with the inhibitors or not (Figure 7C). These results demonstrated that ROS and NO might target the post-transcriptional modulation of Akt and result in its inactivation.

Discussion

Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. Disturbances in this normal redox state can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids and DNA [38]. Our previous study showed that oxidative stress was induced in evodiamine-treated A375-S2 cells as illustrated by

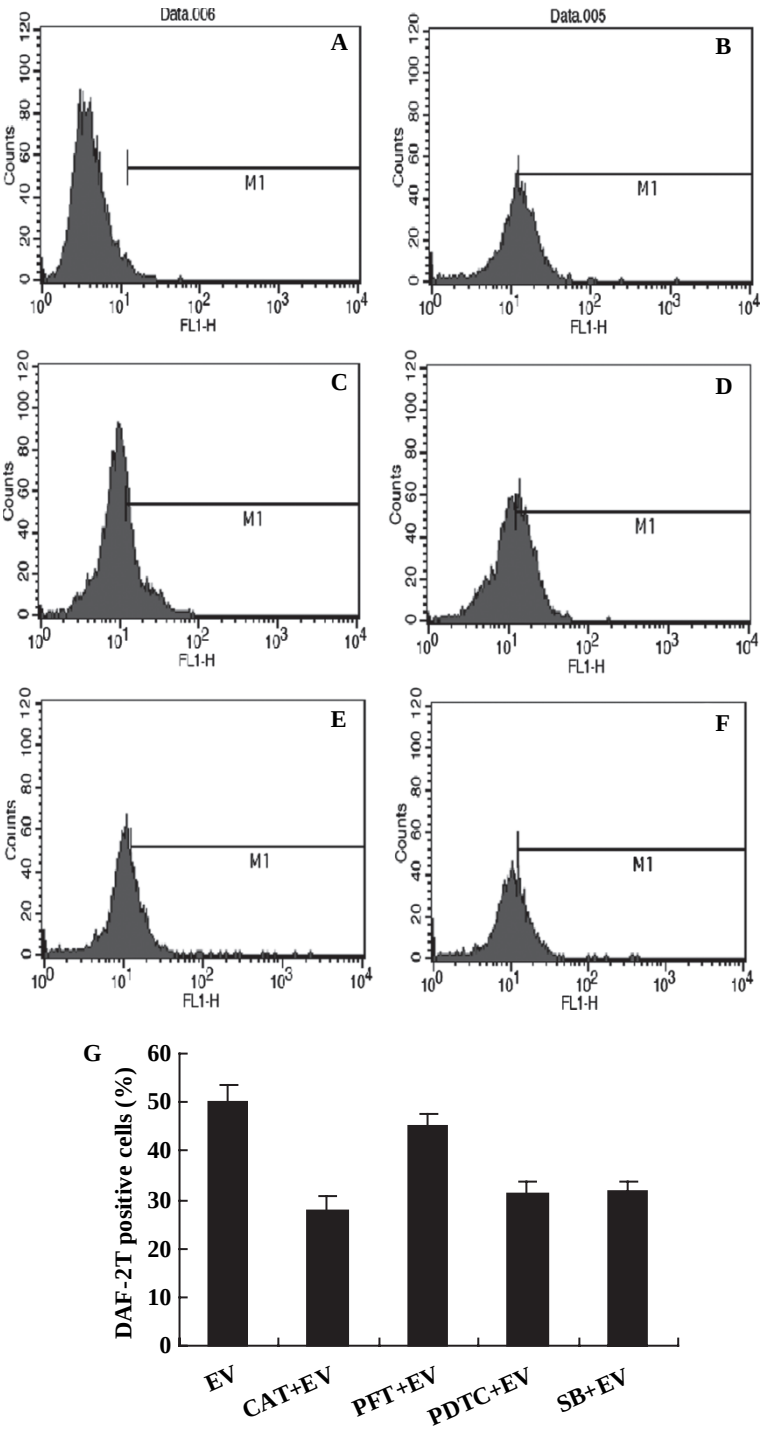

Figure 5. Effects of CAT, PDTC, SB203580 and pifithrin-α on evodiamine-induced NO production. The cells were cultured in the presence or absence of 1000 U/ml CAT, 20 μM pifithrin-α (PFT), 50 μM PDTC or 20 μM SB203580 (SB) for 1 h prior to the addition of 15 μM evodiamine and then incubated for 24 h. The cells were loaded with DAF-2DA and examined by flow cytometry. The FACS histograms of the treated cells were presented (A: 0 h for EV; B: 24 h for EV; C: 24 h for EV+CAT; D: 24 h for EV+PFT; E: 24 h for EV+PDTC; F: 24h for EV+SB). The corresponding linear diagram of the FACScan histograms was expressed in (G). The values shown are mean±standard errors ($n=3$ of individual experiments).

the elevated intracellular ROS level and the decreased cellular antioxidant capacity [22]. In this study, we further investigated the possible role of NO, another free radical, in evodiamine-treated A375-S2 cell apoptosis.

NO is produced by NOS enzymes, which convert the amino acid L-arginine to nitric oxide and L-citrulline. NO-mediated apoptosis or programmed cell death has been demonstrated in a variety of tumour cell types [39]. The present study showed

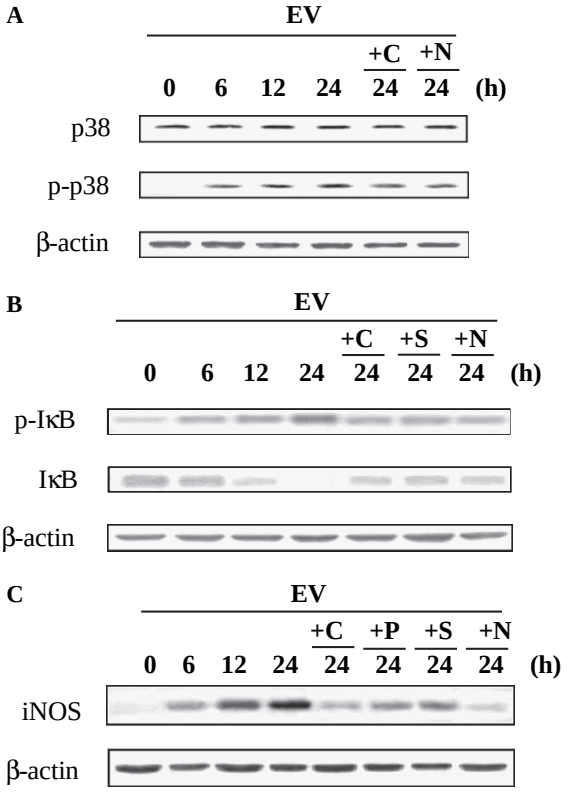

Figure 6. NF-κB and p38 participated in the ROS-induced NO production in evodiamine-treated A375-S2 cells. The cells were treated with 15 μM evodiamine for the indicated time periods in the presence or absence of 1000 U/ml CAT (C), 50 μm PDTC (P), 20 μM SB203580 (S) or 10 mM L-NAME (N), followed by Western blot analysis for detection of p-IκBα, IκBα (A); p38, p-p38 (B); and iNOS (C) expressions.

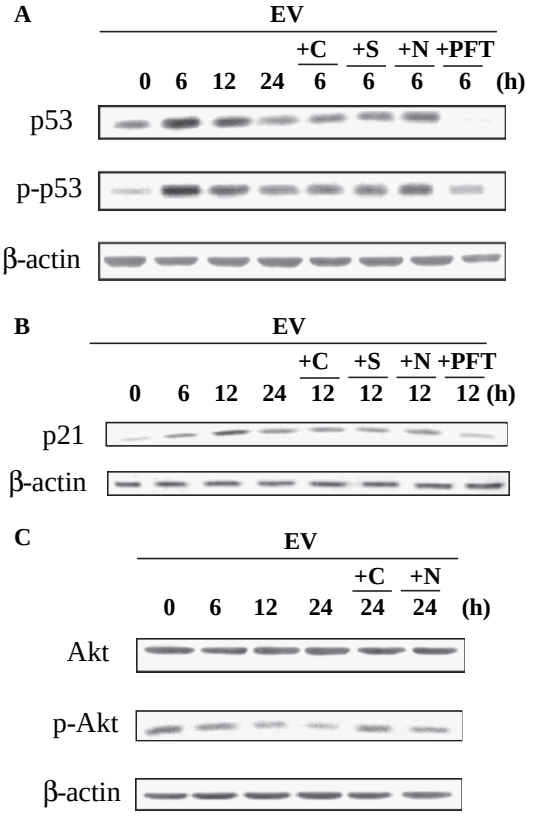

Figure 7. Involvement of p53, p21 and Akt in evodiamine-treated A375-S2 cells. The cells were treated with 15 μM evodiamine for the indicated time periods in the presence or absence of 1000 U/ml CAT (C), 20 μM SB203580 (S), 10 mM L-NAME (N) or 20 μM pifithrin-α (PFT), followed by Western blot analysis for detection of p53, p-p53 (A); p21 (B); Akt and p-Akt (C) expressions.

that NO was apparently induced from 6 h incubated with evodiamine and was persistently increased until 24 h in cells. Both the evident detection of iNOS expression and the potent prevention of NO production by the non-specific iNOS inhibitor L-NAME at 24 h implicated that evodiamine induced NO generation mainly through the activation of iNOS enzyme. Interestingly, L-NAME was also found to suppress evodiamine-induced iNOS expression strongly at 24 h, suggesting that the iNOS expression might be regulated by a NO-dependent mechanism, as reported [40]. These findings indicated that a positive-feedback mechanism played an important role in iNOS-induced NO production in evodiamine-treated cells. Since NO is a small molecule and can freely diffuse through aqueous solutions or membranes, reacting rapidly with metal centres in cellular proteins and with reactive groups in other cellular molecules, the large amount of NO can be greatly harmful to cells [41]. As shown by LDH assay and AO or PI staining, the generated NO was found to contribute greatly to the apoptosis and cell cycle arrest. Mitochondria are the cells' energy converters and particularly vulnerable to free radical molecules as the sources of free radicals and their exceedingly intricate structures [42]. In this study, the mitochondria were destructed by the induced NO as assessed by the dissipation in $\triangle\psi m$. Therefore, the induced NO might directly target mitochondria to initiate apoptosis in evodiamine-treated A375-S2 cells.

NF-κB belonging to the transcription factors family exists in most cells as homodimeric or heterodimeric complexes of p50 and p65 sub-units. Once activated, NF-κB stimulates the expressions of enzymes including iNOS which generates NO [43]. The p38 MAPK pathway is activated in response to a variety of environmental stresses and can sense oxidative stress in cells. It has been recently implicated in regulating NF-κB and iNOS expressions. Our study indicated that the inductions of cell death and NO generation were both dependent on the activations of NF-κB and p38, as assessed by MTT assay and measurement of NO level. It has been generally accepted that, under unstimulated conditions, the nuclear localization of NF-κB is blocked by the binding of the NF-κB inhibitory proteins (IκB). The activation of NF-κB results in phosphorylation, ubiquitination and proteasome-mediated degradation of IκB proteins, followed by nuclear translocation and DNA binding of the NF-κB [44]. The activation of p38 MAPK is induced by a

signalling cascade that leads to phosphorylation of both tyrosine and threonine residues, which in turn induce a conformational change that exposes the active site for substrate binding [45]. Thus, the apparently increased expressions of phospho-IκBα and phospho-p38 under the control of ROS confirmed the activations of NF-κB and p38. Furthermore, the significant inhibitory effect of SB on the phosphorylation of IκBα suggested that p38, located at the upstream of NF-κB, contributed to the activation of NF-κB through the regulation of IκBα degradation. The activations of both p38 and NF-κB were shown critical to the NO synthesis as demonstrated by the suppressive effect of PDTC and SB on iNOS expression at 24 h. Interestingly, L-NAME was found to be effective at preventing the phosphorylations of p38 and IκBα. Therefore, the sustained phosphorylation of p38 and degradation of IκBα were encouraged by the initial synthesized NO and might result in the constantly activated NF-κB, which facilitated the magnification of NO signalling by the induction of iNOS transcription.

p53 is the 'guardian of genome integrity', since it elicits block in the cell cycle, DNA repair and eventually apoptotic response after stress insults compromising genomic integrity, oncogene activation and hypoxia [46]. p53 levels are normally very low in the cells, since it is constantly ubiquitinated by murine double minute 2 (Mdm2) and then degraded by the proteasome [47]. Mdm2 contains two potential Akt phosphorylation sites, thus may be counter-regulated by Akt [48]. In situations of cellular stress, p53 degradation is stopped and its levels rapidly rise. p21 is induced by p53 when DNA damage has occurred, thereby permitting a cell cycle arrest until DNA repair occurs [49]. In this study, apoptosis and cell cycle arrest were indicated to be induced in evodiamine-treated cells as shown by the PI staining; while p53 and p21 under the regulations of both p38 and NF-κB were found to be involved in the process as assessed by Western blot analysis. Moreover, the increased expressions of p53 and the phospho-p53, which might stabilize p53, and the reduced phosphorylation of Akt, which might lead to the loose regulation of Mdm2, all played key roles in the activation of p53. Our findings are consistent with the reports that endogenously generated NO caused oxidative DNA damage by p53 accumulation [50,51].

In summary, this study suggested that evodiamine triggered intracellular NO production. Together with our previous finding that ROS was induced in evodiamine-treated cells, evodiamine was found to exert its cytotoxic effect on A375-S2 cell growth through the synergy effects of ROS and NO. The mechanism under the induction of NO investigated that the transcription factor NF-κB, located at the downstream of p38 MAPK, stimulated the expression of iNOS and contributed to the generation of NO. Through a positive feedback manner, the intracellular NO was markedly augmented and rapidly activated p53 and p21 in concert with the degradation of Akt, thus leading to the apoptosis and cell cycle arrest in A375-S2 cells.

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