Matrine induction of reactive oxygen species activates p38 leading to caspase-dependent cell apoptosis in non-small cell lung cancer cells

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Abstract. Non-small cell lung carcinoma (NSCLC) is one of the most refractory cancers in the clinic; it is insensitive to chemotherapy and is usually excised. However, screening natural compounds from herbs is also considered a possible method for its therapy. In the present study, we investigated whether matrine, a natural compound isolated from *Sophora flavescens* Ait. and exerting an inhibitory effect on lung cancer cells, also indicates inhibition on NSCLC cells and elucidated its molecular mechanism. Firstly, it is confirmed that matrine induces apoptosis of human NSCLC cells with anti-apoptotic factors inhibited and dependent on caspase activity. In addition, we found that matrine increases the phosphorylation of p38 but not its total protein, and inhibition of the p38 pathway with SB202190 partially prevents matrine-induced apoptosis. Furthermore, matrine generates reactive oxygen species (ROS) in a dose- and time-dependent manner, which is reversed by pretreatment with N-acetyl-L-cysteine (NAC). Additionally, inhibition of cell proliferation and increase of phosphorylation of p38 was also partially reversed by NAC. Collectively, matrine activates p38 pathway leading to a caspase-dependent apoptosis by inducing generation of ROS in NSCLC cells and may be a potential chemical for NSCLC.

Introduction

Lung cancer is the leading type of cancer worldwide with high mortality rates in men and women alike, at 29 and 26%, respectively (1). Non-small cell lung carcinoma (NSCLC) is the most frequent subtype, representing ~85% of all cases, and most patients have locally advanced or distant metastatic disease at the time of presentation (2). Although NSCLCs are relatively insensitive to chemotherapy, compared to small cell carcinoma, and are primarily treated by surgical resection with curative intent (3), chemotherapy is increasingly being used both pre-operatively (neoadjuvant chemotherapy) and post-operatively (adjuvant chemotherapy) (3,4). Most researchers believed that anticancer drugs for NSCLC may be found from natural plants (5). Several natural compounds have been found to be effective in inhibiting NSCLC in the lab and in the clinic (5). Among them, paclitaxel, the most typical compound from yew has been approved to be used clinically for cancer patients. A number of other herbal compounds such as xanthatin, lycobetaine, resveratrol, ursolic acid and lycorine are also being investigated for NSCLC (5).

Sophora species (Leguminosae), an important source of Chinese herbal drugs, has been used widely throughout China for thousands of years. As a traditional Chinese herb, the root of *Sophora flavescens* Ait. (Kushen) has long been applied for the therapy of numerous diseases, such as hepatitis (6), cardiac diseases (7) and skin diseases (8). Quinolizidine alkaloids have been found to be its chief active components in *Sophora flavescens* including matrine, oxymatrine, sophocarpine, sophoridine and other alkaloids (9). Basic and clinical studies have shown that these alkaloids possess a variety of pharmacological effects including anti-inflammation (10,11), immunity-regulation (12), antivirus (6) and antitumor action (13-15).

Matrine, with a molecular weight of 248.4 (Fig. 1), is the major quinolizidine alkaloid and has been considered a major bioactive component of the dried roots of *Sophora flavescens*. It has been found that matrine possesses a wide range of pharmacological activities, and its antitumor activity has attracted considerable attention in recent years. Cumulative data have demonstrated that matrine exerts anticancer effect on many series of human cancer cells including lung cancer (16), hepatoma (16), breast cancer (17), pancreatic cancer (18), prostate cancer (19,20) and colon cancer (21). Upregulation of protein E2F-1 and activation of caspases contribute to matrine-induced leukemia cell proliferation inhibition and apoptosis (15). For MDA-MB-231 breast cancer cells, matrine has been demonstrated to inhibit cell proliferation by reducing the ratios of Bcl-2/Bax protein and mRNA levels

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and to reduce cancer cell invasion by inhibiting the VEGF-AKT-NF-κB signaling pathway, as well as by inactivating MMPs (22).

In particular, several efforts focused on how matrine inhibits lung cancer (16,23,24), suggesting that it may be a potential anticancer agent for lung cancer. Additionally, the results that matrine in combination with anticancer drugs significantly inhibited SPCA-1 (24) or A549 cells (23) indicated its potent inhibitory effect on NSCLC. However, although suppressing MAPK/ERK signal transduction was demonstrated to be involved in matrine resisting NSCLC (23), no other mechanisms were confirmed. In this study, we found that matrine caused NSCLC apoptosis due to induction of ROS generation and subsequent activation of MAPK/p38 signaling pathway.

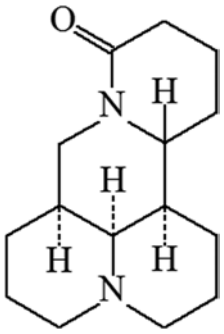

Figure 1. The structure of matrine.

Materials and methods

Materials. Matrine was from Tianyuan Biological Agent Plant (Xi'an, Shaanxi, China; purity, 98%). RPMI-1640 was purchased from Mediatech (Herndon, VA, USA). Fetal bovine serum (FBS) was supplied by HyClone (Logan, UT, USA), and 0.05% Trypsin-EDTA was from Invitrogen Life Technologies (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Sigma-Aldrich (St. Louis, MO, USA). Annexin V-FITC Apoptosis Detection kit I was from BD Biosciences (San Diego, CA, USA). Enhanced chemiluminescence solution was from PerkinElmer Life Sciences (Boston, MA, USA). Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-FMK) was purchased from ALEXIS Biochemicals Corporation (San Diego, CA, USA). The MAPK/p38 pathway inhibitor SB202190 was obtained from LC Laboratories (Woburn, MA, USA). The following antibodies were used: p38, phospho-p38, caspase-3, PARP, Bcl2, BAD (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), β-actin (Sigma).

Cell lines and culture. Human NSCLC cells A549, NCI-H358 were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). A549 and NCI-H358 cells were grown in antibiotic-free RPMI-1640 supplemented with 10% FBS and incubated in a humid incubator (37˚C, 5% CO_2).

Cell proliferation assay. Cells dispersed evenly in medium were seeded in a 6-well plate with a density of $1x10^5$ cells/well. Next day, cells were treated with various concentrations of matrine (0-100 μM) for 48 h. After incubation, MTT (5 mg/ml) was added to each well, followed by a 4 h incubation. The medium was discarded and 150 μl of dimethyl sulfoxide (DMSO) was added into each well, and incubated for 20 min. The optical density (OD) in 570 nm was measured by a BioTek multilabel counter. The cell proliferation inhibition index was calculated according to the formula: $(OD_{control} - OD_{matrine}/OD_{control}) \times 100\%$. The experiments were repeated 3 times.

Cell apoptosis analysis. A549 cells were seeded in 6-well plates at a density of $2x10^6$ cells/dish in RPMI-1640 supplemented with 10% FBS and were grown overnight at 37˚C in a humidified incubator with 5% CO_2. Cells were treated with matrine (0-50 μM) for 48 h, followed by apoptosis assay using the Annexin V-FITC Apoptosis Detection kit I. Cells without treatment were used as a control.

Caspase-3/7 activity assay. Caspase-3/7 activity was measured by the Sensolyte Homogeneous AMC caspase-3/7 assay kit (AnaSpec Systems, San Jose, CA, USA) according to the manufacturer's instructions. Briefly, cells were seeded in black 96-well plates and cultured for 24 h followed by treatment with matrine (0-50 μM) for 24 h. Then, 50 μl/well of caspase-3/7 substrate solution was added into each well. The reagents were mixed completely by shaking and the reaction was incubated at room temperature for 1 h. Finally, fluorescence intensity was measured at Ex/Em=354/442 nm using a BioTek multi-label counter.

ROS assay. A549 and NCI-H358 cells were respectively seeded at a density of $1x10^4$ cells/well in 96-well plates. Cells were incubated in the presence of various concentrations of matrine (0-50 μM) for 24 h or 30 μM matrine for the indicated time (0-24 h) with 6 replicates of each treatment followed by loading with CM-H_2DCFDA following the manufacturer's protocol. In some cases, cells were pretreated with N-acetyl-L-cysteine (NAC; 5 mM) for 1 h, and then treated with/without matrine (30 and 50 μM) for 24 h, followed by loading with CM-H_2DCFDA for 2 h. Fluorescent intensity was measured at Ex/Em=485/535 nm using a BioTek multi-label counter.

Western blot analysis. Treated cells were briefly washed 2 times with cold PBS. Cells were lysed in the lysis buffer [50 mM Tris, pH 7.2; 150 mM NaCl; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate (SDS); 1% Triton X-100; 10 mM NaF; 1 mM Na_3VO_4; protease inhibitor cocktail (1:1,000; Sigma)]. Lysates were sonicated for 2x15 sec and centrifuged at 13,000 x g for 2 min at 4˚C. Protein concentration was determined by bicinchoninic acid assay with bovine serum albumin as standard (Pierce Biotechnology, Inc., Rockford, IL, USA). Western blotting was carried out as previously described. The antibodies used were described above.

Statistical analysis. The results are expressed as the mean values ± standard error (means ± SE). A one-way analysis of variance (ANOVA) was used to examine differences among the matrine groups. Differences were considered statistically significant when $P<0.05$.

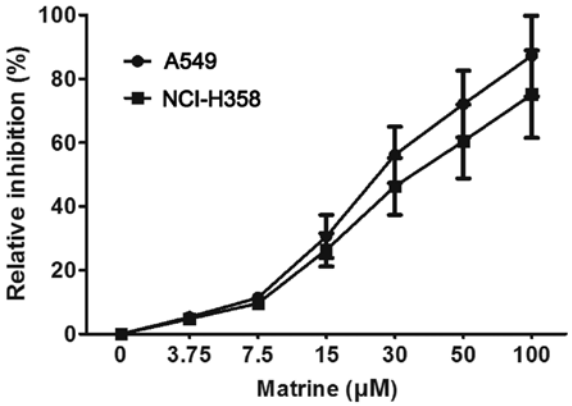

Figure 2. Matrine inhibits non-small cell lung cancer cells. A549 and NCI-H358 cells grown in 6-well plates (1x10^4 cells/well) were treated with the indicated concentrations of matrine (0-100 μM) for 48 h. Cell proliferation was evaluated using MTT assay. Absorbance at 490 nm was determined with a BioTek multilabel counter.

Results

Matrine inhibits proliferation and induces apoptosis in NSCLC cells. As shown in Fig. 2, relative inhibition curves induced by matrine in two lung cancer cells increased in a dose-dependent manner. With a logarithmic regression analysis, the concentration of matrine which results in 50% of maximal proliferation inhibition (IC_{50}) of cells was calculated. The IC_{50} value was ~25.0 μM for A549 cells, and ~34.6 μM for NCI-H358 cells.

To examine whether matrine-inhibited cell proliferation is related to induction of cell apoptosis, Annexin V-FITC and PI staining were used. As indicated in Fig. 3, the rate of apoptotic cells increased significantly (more than ~40%) from 11.8% of control to 28.4, 42.3 and 43.3%, in A549 cells treated with matrine (15, 30 and 50 μM) (Fig. 3). Matrine clearly induced cell apoptosis.

Caspase and apoptotic factors involved in matrine-induced cell apoptosis. Apoptosis is a complicated process involving several factors, and is divided into 2 different types; one is dependent on caspase, the other is not. To understand which type of apoptosis matrine induces, two key molecular proteins indicating apoptosis were detected including caspase-3 and PARP by western blot assay. The results indicated that the 17 kD cleaved-caspase-3, regarded as the marker of caspase activation in apoptosis, was expressed, as well as the 89 kD cleaved-PARP (Fig. 4A). This suggested that matrine-induced apoptosis was dependent on caspase. To further confirm this, the caspase-3/7 activity was examined using caspase-3/7 assay kit. As shown in Fig. 4B, the caspase-3/7 activity showed significant change in A549 cells with the treatment of the indicated concentration of matrine. Additionally, Z-VAD-FMK, a caspase inhibitor, pretreated for half an hour in A549 cells also reversed matrine-induced inhibition (Fig. 4C). Bcl2, exerting an anti-apoptotic function in response to a wide range of apoptotic stimuli, was also inhibited in A549 cells by matrine, while BAD, a pro-apoptotic factor of the Bcl-2 family that promotes cell death by displacing Bax from binding to Bcl-2 and Bcl-xL, expressed more by induction of matrine than the control. Taken together, matrine-induced cell apoptosis was dependent on caspase.

Matrine activates MAPK/p38. Since matrine triggered a caspase-dependent apoptosis course, it is necessary to further indicate which upstream pathways regulate it. Here, we found p38 was an important pathway to mediate matrine-induced cell apoptosis. After A549 cells were exposed to matrine (0-50 μM)

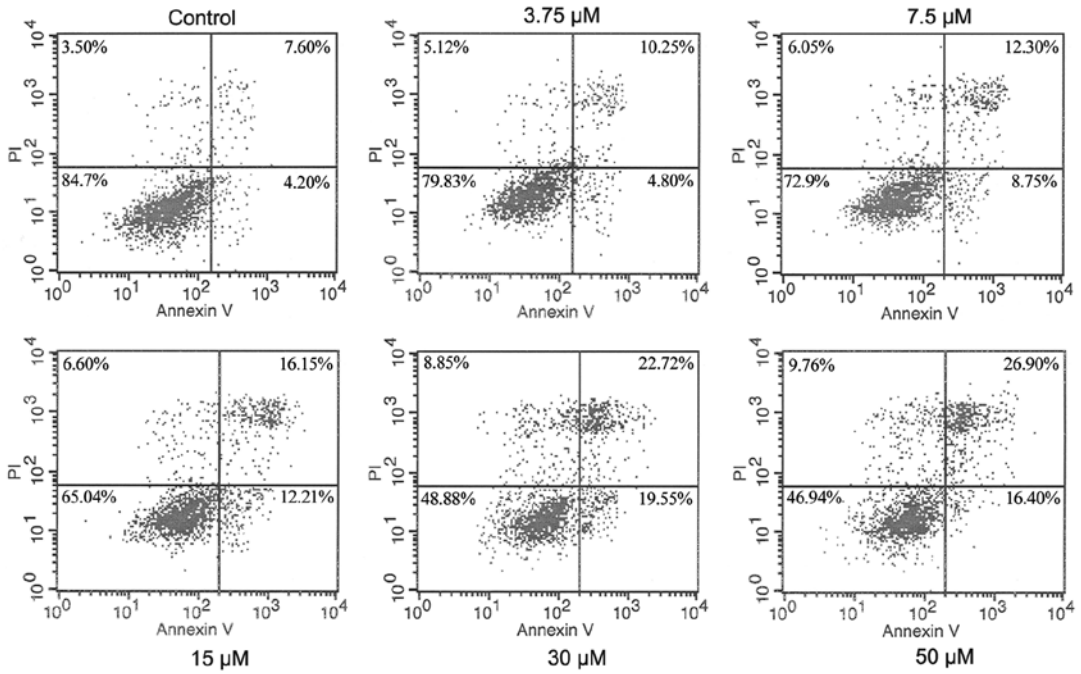

Figure 3. Matrine induces non-small cell lung cancer cell apoptosis. A549 cells grown in 6-well plates (1x10^5 cells/well) were treated with the indicated concentrations of matrine (0-50 μM) for 48 h, then stained with Annexin V-FITC and propidium iodide (PI), followed by flow cytometry analysis.

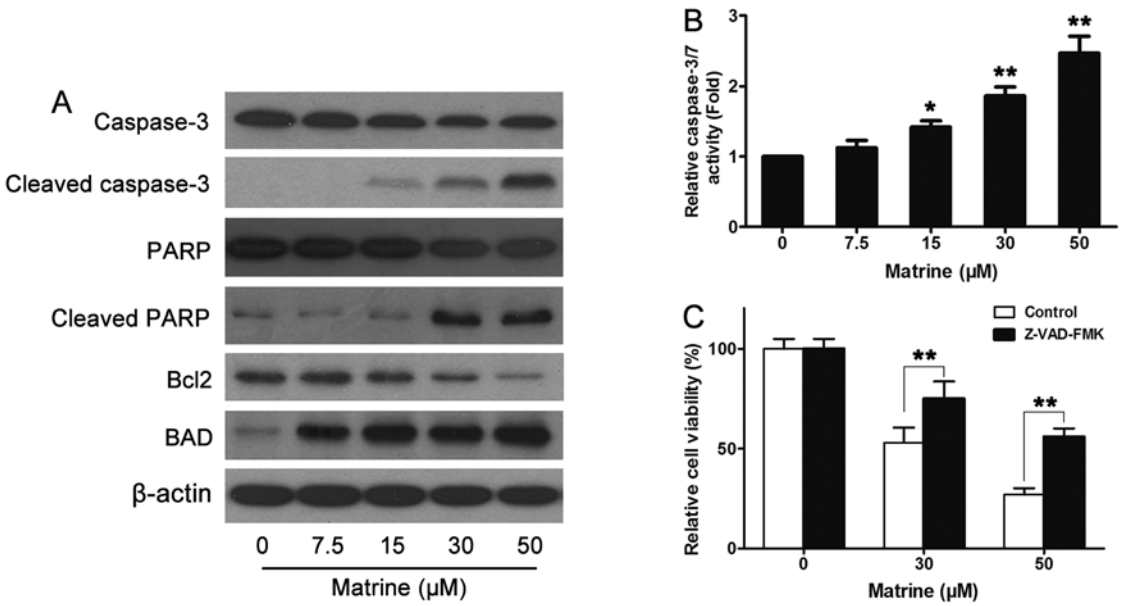

Figure 4. Matrine inhibits anti-apoptotic factors dependent on caspase. (A) A549 cells were grown in 6-well plates and treated with matrine at the indicated concentrations for 8 h, followed by western blot analysis with the indicated antibodies including caspase-3, PARP, Bcl2, BAD, β-actin. (B) A549 cells were seeded in black 96-well plates and cultured for 24 h followed by treatment with matrine (0-50 μM) for 24 h. Then, 50 μl/well of caspase-3/7 substrate solution was added into each well. The reagents were mixed completely by shaking and the reaction was incubated at room temperature for 1 h; fluorescence intensity was measured at Ex/Em=354/442 nm using a BioTek multilabel counter. (C) A549 cells were grown in 6-well plates and treated with matrine or pretreated with Z-VAD-FMK at the indicated concentrations for 24 h.

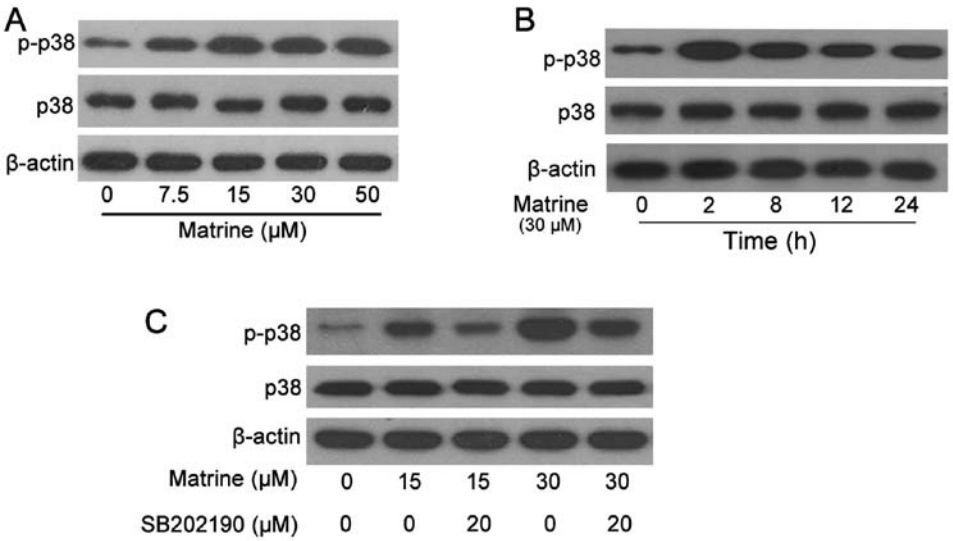

Figure 5. Matrine activates MAPK p38 pathway in non-small cell lung cancer cells. (A and B) A549 cells were grown in 6-well plates and treated with matrine at the indicated concentrations for 8 h, followed by western blot analysis with the indicated antibodies. (C) A549 cells pretreated with or without SB202190 (10 μM) for 30 min, were treated with matrine (0, 15, 30 μM) for 8 h, followed by western blot analysis with the indicated antibodies.

for 8 h, the cellular lysates were subjected to western blot analysis and the results revealed that matrine significantly increased the phosphorylation of p38 starting from 7.5 μM (Fig. 5A). With the treatment of 30 μM matrine for the indicated time (0-24 h), the phosphorylation of p38 was also upregulated and was expressed the highest at 2 h, before decreasing gradually to 24 h (Fig. 5B). Therefore, matrine activates the p38 pathway.

The MAPK inhibitors were generally used to further ascertain whether the tested samples functioned via this pathway. SB202190, blocking p38, pretreated the A549 cells for 1 h following the 8-h treatment of matrine, the upregulated phosphorylation of p38 was partially reversed by the inhibitors (Fig. 5C). From the cell counting data, A549 cells treated with matrine plus SB202190 showed more viability than those without inhibitors. This indicated that activation of the p38 pathway is necessary for matrine inhibition of cell apoptosis.

Matrine induces ROS generation. It is well known that ROS have a cellular signaling role in several biological systems, both in animals and plants, and in most stress-related cell progress. ROS induce programmed cell death or necrosis, induce or suppress the expression of many genes, and activate

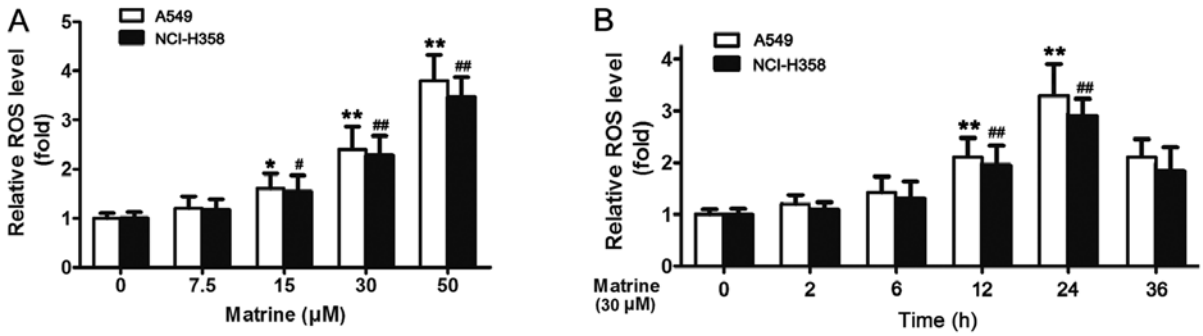

Figure 6. Matrine induces reactive oxygen species (ROS) generation in non-small cell lung cancer cells in a concentration- and time-dependent manner. A549 or NCI-H358 cells were seeded in 96-well plates, then treated with matrine at indicated concentrations for (A) 24 h or (B) with 30 μM for the indicated time. ROS in the cells were detected using CM-H_2DCFDA reagent kit. Results are presented as means ± SE, n=6. $^{*,\#}$P<0.05, $^{**,\#\#}$P<0.01, difference vs. control (0 μM matrine).

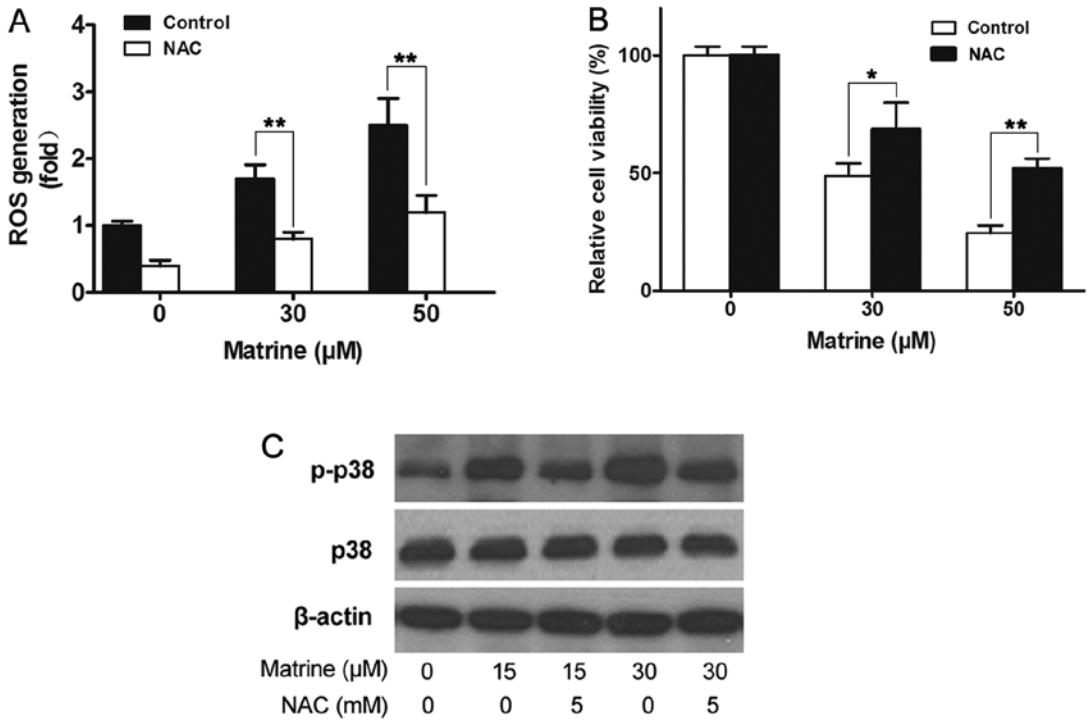

Figure 7. The apoptotic effect of matrine is attributed to induction of reactive oxygen species (ROS). (A-C) N-Acetyl-L-cysteine (NAC), an antioxidant and scavenger of ROS, reserved matrine induction of ROS and conferred resistance to matrine inhibition of cell viability. A549 cells grown in 96-well or 6-well plates, were pretreated with or without NAC (5 mM) for 30 min and then treated with matrine at the indicated concentrations for 24 h, followed by (A) ROS assay, or (B) cell counting assay (C) or western blotting. Results are presented as means ± SE, n=6. *P<0.05, **P<0.01, difference vs. control (0 μM matrine).

cell signaling cascades, such as the mitogen-activated protein kinases. Therefore, whether matrine activating MAPK is also due to inducing ROS or related to other factors, remains to be confirmed. CM-H_2DCFDA, a stable non-fluorescent molecule, specially designed to detect ROS through oxidized by oxygen radicals to form fluorescent molecule excited by specific wavelength lights was used to measure the level of ROS in A549 cells treated with matrine; the data showed that matrine induced ROS generation in a dose- and time-dependent manner (Fig. 6A and B).

NAC reverses the effect of matrine on ROS, p38 and cell viability. With the exception of ROS, several other factors including stress, EGF, radicals, IL-1, integrin can act on the MAPK pathway leading to cell proliferation, apoptosis, necrosis. Hence, it would be confirmed that matrine activating p38 pathway is mediated through inducing ROS generation if clearing ROS can reverse all or at least part of the effects mediated by matrine. NAC, a powerful antioxidant to clear the ROS, was used, then ROS generation, MAPKs pathway and cell viability were revaluated. The results of Fig. 7 suggest that ROS generation induced by matrine was completely reversed by NAC (5 mM) (Fig. 7A); second, matrine-induced increased expression of phosphorylation of p38 was also significantly reversed by NAC (Fig. 7B); third, most of the A549 cells were alive after treatment with matrine plus NAC (Fig. 7C). Based on the above results, matrine acting on the MAPK pathway is inevitably dependent on ROS.

Discussion

Non-small cell lung cancer (NSCLC) is any type of epithelial lung cancer other than small cell lung carcinoma (SCLC). It is further distinguished into three subtypes: squamous cell carcinoma, large cell carcinoma and adenocarcinoma (2,25). Since the early 2000s, a greater understanding of the molecular biology of NSCLC has led to revolutionary treatment of these carcinomas (26).

Most SCLCs acquire multi-drug resistance, while NSCLCs tend to be intrinsically resistant to chemotherapy (27). Less than 5% of SCLC patients currently survive five years past initial diagnosis, but 15% of patients with NSCLC survive 5 years (27). Historically, response rates rarely exceeded 20% prior to the development of cisplatin, with an increase to 20-40% during the cisplatin-combination era. In the post-cisplatin era, new antitumor drugs such as gemcitabine, vinorelbine, paclitaxel and docetaxel have been improved with the intention of increasing response rates to as high as 50-60% (28,29). Except for the above-mentioned chemicals, several compounds from natural herbs including lycobetaine, resveratrol, indirubin, ursolic acid have been considered potential anticancer drugs for NSCLC (5). Matrine, an alkaloid from *Sophora flavescens* Ait., was also found to be an anticancer agent, and possibly showed higher inhibition on lung cancer cells than any other cancer cells. Additionally, matrine inhibition of lung carcinoma angiogenesis via suppression of MAPK/ERK signal transduction suggested that the MAPK signaling pathway plays a critical role in it (23).

It is well known that MAPKs contain three main family members, respectively known as the c-Jun NH2-terminal kinase (JNK), the p38 and the extracellular signal-regulated kinase (ERK). Each of the MAPK cascade pathways works by the same three-tier manner. The MAPK kinase kinase (MAPKKK), activated by environmental or extracellular stimulus, activates MAPK kinase (MAPKK) which sequentially activates MAPK through gradient phosphorylation. Phosphorylation of the MAPK leads to activation of the corresponding substrates, which regulate the transcription factor and control cell proliferation, differentiation, motility and apoptosis (30,31).

Previously, MAPK/ERK was demonstrated to be involved in matrine suppressing HUVEC cell migration induced by A549 cancer cells. Matrine inhibition of phosphorylation of ERK induces antiangiogenic effects leading to the elimination of lung carcinoma (23). Thus, in the present study, we focused on elucidating whether MAPK/p38 also involves matrine induction of apoptosis in NSCLC and how it acts.

p38, known as stress-activated protein kinases (SAPKs), is widely expressed in most tissues and participates in several different stress signaling pathways that control a spectrum of cellular processes (31,32). Most of the data demonstrated that activation of the p38-MAPK signaling induced cell apoptosis and death in cancer cells (33,34). Increase of caspase-3/7 activity and overexpression of cleaved caspase-3 and cleaved PARP make it clear that matrine induces a caspase-dependent apoptosis in NSCLC cells, as well as Z-VAD-FMK, a cell-permeant caspase inhibitor that irreversibly binds to the catalytic site of caspase, can reverse inhibition of proliferation in part. Then, matrine significantly increasing phosphorylation of p38 disclosed p38 also mediates matrine induction of apoptosis.

Free radicals and reactive molecules containing oxygen are collectively known as ROS and induce oxidative stress in cells (35). ROS, such as hydrogen peroxide (H_2O_2) and superoxide (O^{2-}), are found higher in most tumors and cancer cells than in normal tissues and cells (36). Thus, ROS are conventionally regarded as cytotoxic and mutagenic, and induce cell death, apoptosis and senescence in high levels (35). On the contrary, ROS also function as signaling molecules to mediate cell growth, migration, differentiation and gene expression in low levels (35). In the present study, matrine was demonstrated to induce cell apoptosis in NSCLC cells, then found to significantly induce ROS generation. These two combined with the finding that cell death and ROS generation were reversed by NAC, suggested that induction of ROS was one of the critical reasons causing cancer cell apoptosis by matrine.

Several downstream signaling pathways mediated by ROS were tracked, including MAP kinases (37), JAK/STAT (38), NF-κB (39), ion channels (40) and angiogenesis (41). Therefore, we also tried to explain whether matrine activates p38 by inducing ROS. As expected, using NAC to clear ROS could partially inhibit the level of phosphorylation of p38, indicating that MAPK/p38 was involved in matrine-induced cell apoptosis.

Collectively, the mechanism of matrine-induced NSCLC cell apoptosis was presented in this study. Matrine could stimulate ROS generation in NSCLC cells and subsequently activated p38, resulting in a caspase-dependent cell apoptosis by the indication of inhibition of Bcl2 and by activating caspase-3 and PARP. Matrine may be a promising agent for chemoprevention and treatment in NSCLC patients.

Acknowledgements

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