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Hydrogen peroxide contributes to the manganese superoxide dismutase promotion of migration and invasion in glioma cells

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Abstract

Manganese superoxide dismutase (MnSOD) is over-expressed in most brain tumours, and high MnSOD expression is associated with poor prognosis. The mechanisms still remain largely unknown. In the present study, the elevation of hydrogen peroxide (H_2O_2) level and the enhancement of glioma migration/invasion by over-expression of MnSOD were demonstrated. Subsequent studies showed that over-expression of MnSOD significantly increased the activation of mitogen-activated protein kinases (MAPKs) and phosphatidylinositol-3-kinases (PI3Ks), including AKTs, s6-ribosomal protein, ERKs and JNKs. Over-expression of MnSOD was also associated with elevations of matrix metalloproteinases-1 (MMP-1) and MMP-9 protein. The promotion of migration/invasion, activation of PI3Ks and MAPKs and up-regulation of MMPs were inhibited by the general reactive oxygen species scavenger N-acetyl-l-cysteine (NAC), over-expression of the H_2O_2-detoxifying enzyme mitochondrial catalase (mCat) and specific inhibitors of AKTs or ERKs. Collectively, our study indicated that H_2O_2 would contribute to the MnSOD-promoted migration/invasion in glioma cells through activation of AKTs and ERKs. This study provided new molecular insights into the understanding of glioma migration and invasion.

Keywords: *ROS, superoxide, MnSOD, hydrogen peroxide, glioma, invasion, migration, superoxide dismutase, signal transduction, brain*

Introduction

Gliomas are the most common primary brain tumours. Although great progress has been made in glioma treatment in the past few decades, the prognosis of patients with malignant gliomas is still poor [1,2]. The histological feature of malignant gliomas is the invasion of tumour cells in surrounding normal brain tissues [1]. However, the complete mechanisms of glioma migration and invasion are not completely defined. Understanding the mechanisms of glioma cell invasion will help modify the existing therapies and develop novel therapeutic strategies.

Reactive oxygen species (ROS), generated by the mitochondrial respiratory chain, including superoxide ($O_2^{\bullet -}$), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2), are traditionally thought to be toxic by-products of cellular metabolism. ROS can be regulated by intrinsic antioxidant enzymes [3]. Superoxide dismutase (SOD) can catalyze the superoxide into H_2O_2, which can then be catalyzed by catalase (Cat) [3]. It has been reported that manganese superoxide dismutase (MnSOD) is over-expressed in most brain tumours and high MnSOD expression is associated with poor prognosis. Kawamura et al. [4] reported the elevation of MnSOD levels in the serum of the patients with neuroblastoma. Other studies had shown that MnSOD is associated with high malignancy and poor prognosis [5].

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These authors had shown that MnSOD was highly positive in Grade IV gliomas, but negative in normal brain [5–7]. Moreover, the levels of MnSOD protein in the cerebrospinal fluids of these patients were much higher than that of the controls.

Although the association of MnSOD with the poor clinical prognosis of glioma patients has been well documented, the mechanisms are still not clearly known. In the present study, we have demonstrated that the over-expression of MnSOD can elevate the production of H_2O_2, activate the mitogen-activated protein kinases (MAPKs) and phosphatidylinositol-3 -kinases (PI3Ks), induce the expression of matrix metalloproteinases-1(MMP-1) and MMP-9 and promote the migration and invasion of U87 glioma cells. Furthermore, these processes would be restricted by efficient H_2O_2 detoxification. Our findings suggest that the elevation of H_2O_2 contributes to the MnSOD-promoted migration and invasion in glioma cells.

Materials and methods

Plasmids, antibodies and other reagents

The plasmid expressing MnSOD and the Adenovirus mitochondrial catalase (Ad-mCat) were the production of Sangon Biotech Co., Ltd (Shanghai, China). The antibodies against p-AKT, AKT, p-ERK, ERK, p-JNK, JNK, MMP-1, MMP-9, p-s6 ribosomal protein, s6 ribosomal protein, SOD and Cat were purchased from Cell Signaling Technology Inc (Boston, Massachusetts, USA). Anti-ß-actin antibodies, N-acetyl-l-cysteine (NAC), the selective PI3Ks inhibitor LY294002 and the specific ERKs inhibitor PD98059 were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Dichlorofluorescein-diacetate (DCFH-DA) and hydroethidine (HE) were purchased from Invitrogen (Carlsbad, California, USA).

Cell culture and transfection

U87 cell was maintained at 37 °C in a 5% CO_2 incubator in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 25μg/ml gentamicin. Cells were trypsinized at 80% confluence in the culture dishes and then collected and seeded onto Falcon-faced coverslips in the 6-well plates. Cell transfections were performed with SignaGen® Polyjet™ *in vitro* DNA Transfection Regent (Maryland, USA) according to the manufacturer's instructions.

ROS detection by luminescence analysis

The production of ROS was monitored by fluorescent spectrophotometer SpectraMAX M2 (Molecular Devices Corp, USA) using DCFH-DA and HE. The fluorescence intensity is proportional to the amount of ROS produced by the cells. The DCFH-DA is a well-established compound to detect and quantify intracellular-produced H_2O_2 [8]. Another probe is HE, which is oxidized rapidly to the fluorescent molecule of ethidium bromide by $O_2^{\bullet-}$, is considered a good detector of intracellular $O_2^{\bullet-}$ [9]. In the present study, DCFH-DA (10 μM) and HE (10 μM) were added to the U87 cells 30 minutes before measurement. Then the cells were washed twice with Phosphate Buffered Saline (PBS) to remove the extracellular compounds, and the fluorescence intensities were measured by SpectraMAX M2 fluorescent spectrophotometer with excitation of 488nm and

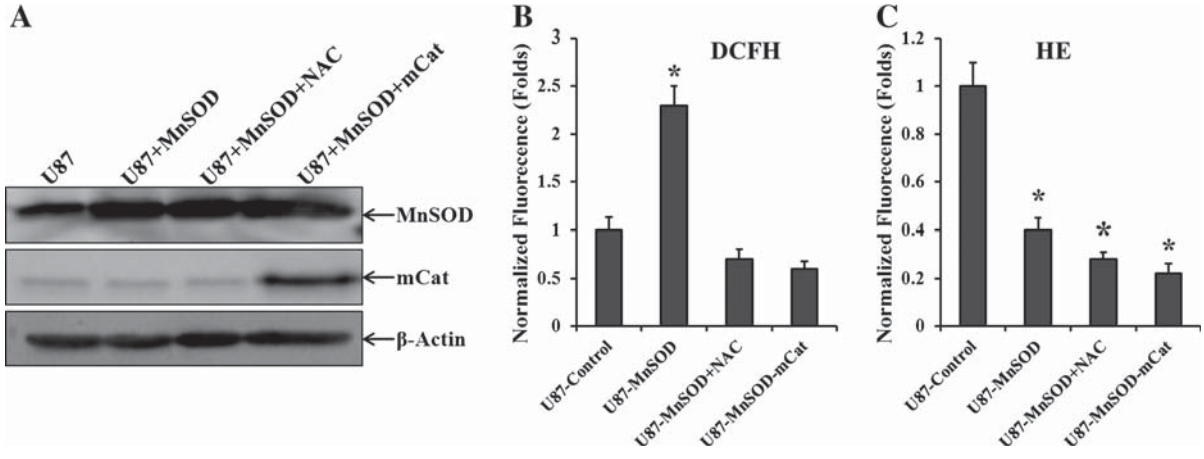

Figure 1. Intracellular H_2O_2 is elevated by over-expression of MnSOD in U87 glioma cells. (A) MnSOD was over-expressed by 3.5-folds after transfected with plasmid and the mCat elevated by infection of the Adenovirus-expressed mitochondrial catalase. (B and C) Generation of intracellular ROS was detected by the semi-quantitative technique based on the use of DCFH-DA and HE. Cell without plasmid transfection and virus infection was used as the negative control. Generation of intracellular hydrogen peroxide was detected by DCFH-DA and superoxide was detected by HE. About 10 mM NAC, in the correspondence group, was pre-loaded to the cells 30 minutes before measurement. Data from three independent experiments were analyzed, and the results are expressed as the mean ± SE. $^*p < 0.01$, statistically significant difference compared with the negative control cells by Student's *t*-test.

emission of 530 nm for DCFH-DA and excitation of 510 nm and emission of 600 nm for HE.

Cell wound healing assay

Cells suspended in 10% FBS DMEM were cultured until they reached 80% confluence. Wounds were made by sterile pipette tips. Cells were washed with serum-free PBS and then cultured in 10% FBS DMEM for the period of time frames as indicated. Selective cells were incubated with LY294002 (20 μM) and PD98059 (20 μM) in 10% FBS DMEM, if they were needed. The photos were taken at the time indicated until the wound was healed in the fastest cells. The wound area was quantified using the Cell Migration Analysis software (Muscale LLC, Scottsdale, Arizona, USA).

Cell invasion assay

BD BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences) was used in the Invasion Assay. Cells were seeded per insert in triplicate in 500 μl serum-free medium. Inserts were placed in wells containing 500 μl medium with 10% FBS DMEM. Selective cells were incubated with LY294002 (20 μM) and PD98059 (20 μM) in 10% FBS DMEM, if they were needed. The cells were incubated for 20 hours.

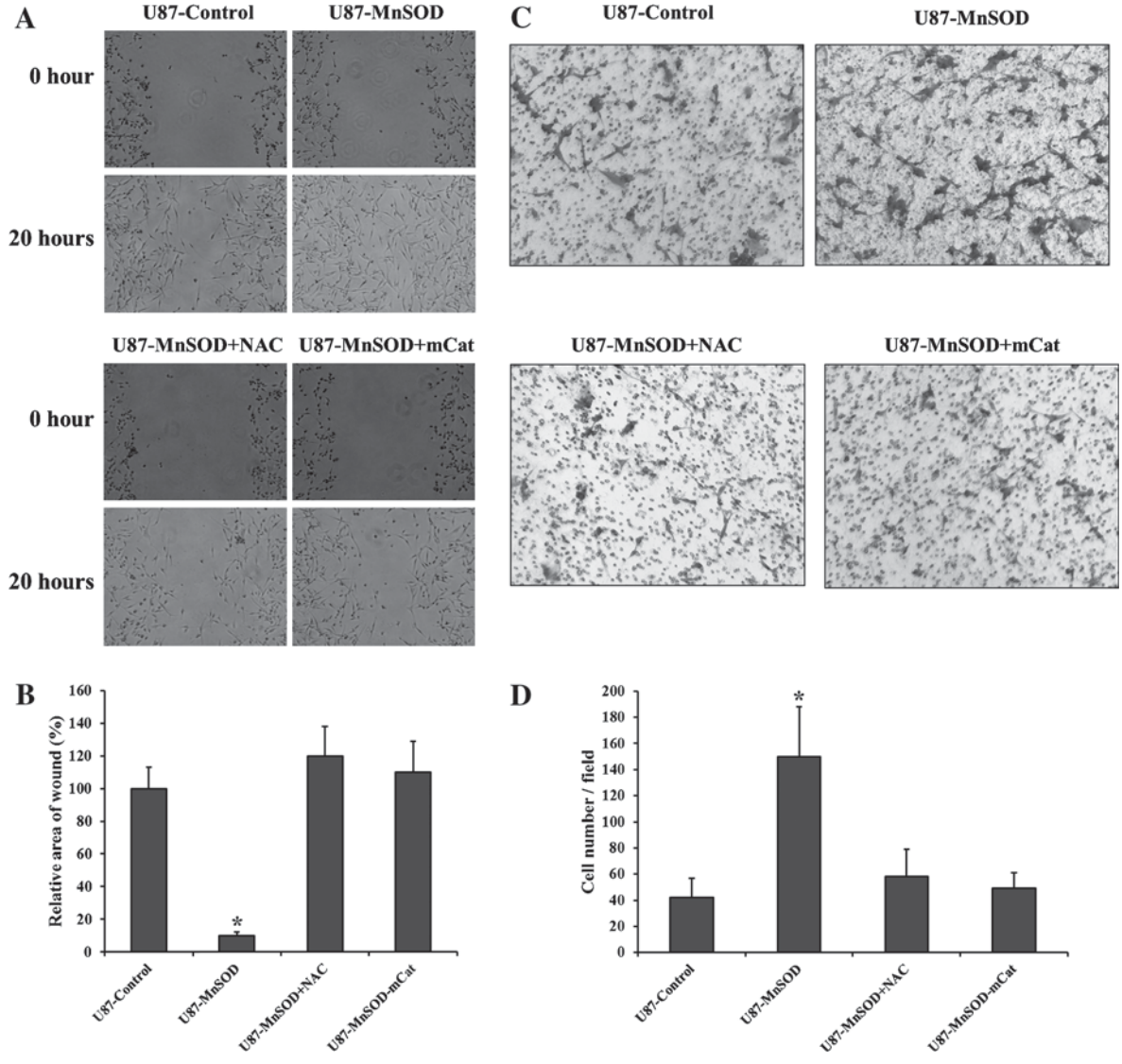

Figure 2. Over-expression of MnSOD promotes U87 glioma cell wound healing and invasion is H_2O_2-dependent. (A) Expression of MnSOD promotes migration of U87 glioma in a wound healing assay and can be reversed by pre-treating with 10mM NAC and co-expression of the H_2O_2-detoxifying enzyme mCat. (B) Quantification of remain area at 20 hours. MnSOD-transfected cells displayed significantly more migration than control cells. (C) Expression of MnSOD promotes Transwell ability of U87 glioma and can be reversed by pre-treating with 10mM NAC and co-expression of mCat. (D) The number of cells invading through the chamber was quantified. MnSOD-transfected cells displayed significantly high invasive ability than the control cells. Data from three independent experiments were analyzed, and the results are expressed as the mean ± SE. $^*p < 0.01$, statistically significant difference compared with the negative control cells by Student's *t*-test.

At the end of the culture period, cells on the upper surface of the filters were completely removed by wiping with a cotton swab. The invaded cells were calculated.

Western blot

Cell extracts were prepared with the cell lysis buffer (10 mM Tris-HCl, pH 7.4, 1% SDS and 1 mM Na_3VO_4). Protein concentrations were determined by the NanoDrop 2000 (Thermo Fisher Scientific Inc, USA). Fifty micrograms of proteins was subjected to Western blotting system.

Statistical methods

The Student's *t*-test was utilized for determining the significance of differences of cell migration, invasion and ROS generation among various treatments. The differences were considered to be significant at a $p < 0.05$.

Results

Intracellular H_2O_2 is elevated by over-expression of MnSOD in U87 glioma cell

Collective evidence shows that H_2O_2 contributes to the migration of cells [10]. In our previous study, MnSOD was over-expressed by 3.5-folds in the U87 glioma cells (Figure 1A). The ROS was detected by redox-sensitive dye DCFH-DA and the HE. As shown in Figure 1B, the intracellular H_2O_2 detected by DCFH-DA was significantly elevated by the over-expression of MnSOD and inhibited by 10mM NAC pre-treatment and mCat over-expression (Figure 1A). However, the superoxide detected by HE was significantly inhibited by the over-expression of MnSOD and then further decreased by 10mM NAC pre-treatment and mCat over-expression (Figure 1C). These results suggested that in spite of decreasing the intracellular superoxide, the over-expression of MnSOD could increase the intracellular level of H_2O_2.

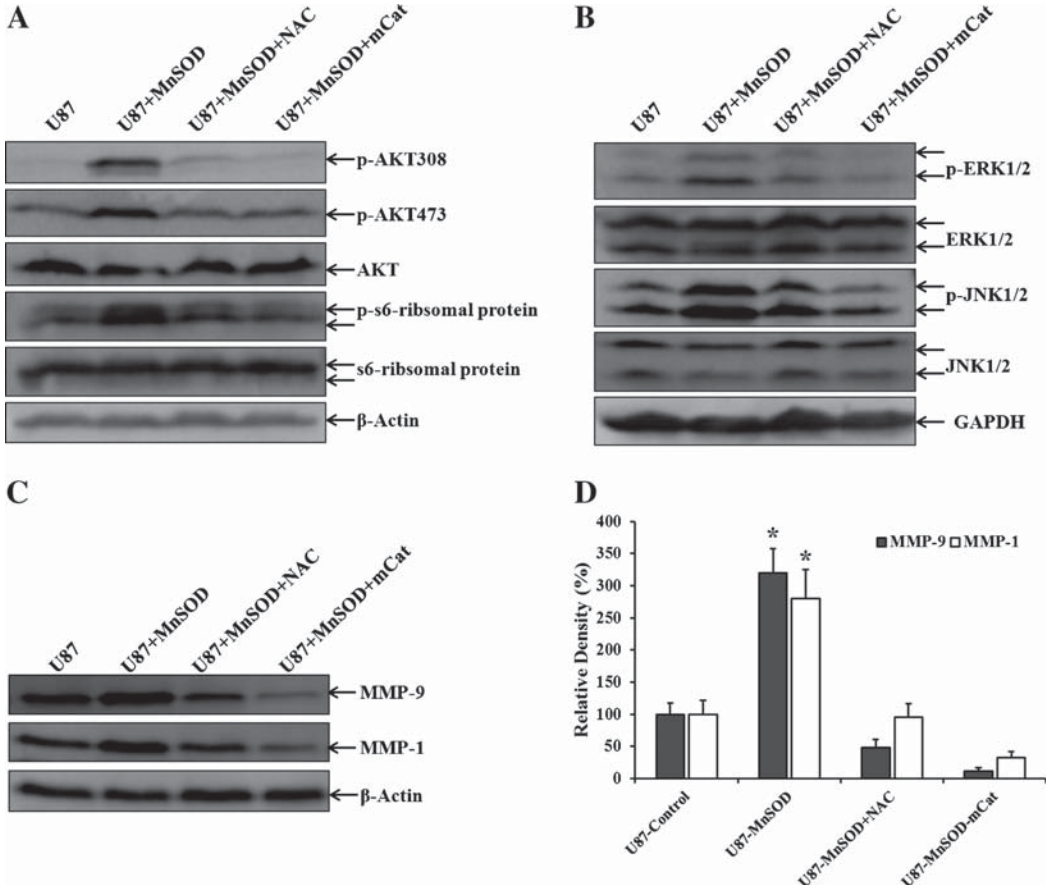

Figure 3. MnSOD expression activates PI3Ks and MAPKs signalling pathways and is H_2O_2-dependent. (A) PI3Ks including AKT and s6-ribosomal protein were activated by MnSOD expression and inhibited by pre-treating cells with NAC or over-expression of the mCat. (B) MAPKs including ERK1/2 and JNK were activated by MnSOD expression and inhibited by NAC or mCat. (C) Western blot shows that over-expression of MnSOD is associated with elevations of MMP-1 and MMP-9. The expression ofMMP-1 and MMP-9 was significantly decreased by NAC pre-treatment and over-expression of the mCat. Data presented are a typical representation of three experiments. (D) Quantification of MMP-1 and MMP-9 protein. Data from three independent experiments were analyzed, and the results are expressed as the mean ± SE. $^*p < 0.01$, statistically significant difference compared with the negative control cells by Student's *t*-test.

Over-expression of MnSOD promotes U87 glioma cell migration and invasion and is H_2O_2-dependent

Migration and invasion are important aspects that reflect the ability of cancer cells. We investigated the role of H_2O_2 in migration using the classic wound healing assay in U87 glioma cells. It was apparent that MnSOD over-expression caused a significant increase of migration in U87 glioma cells (Figures 2A and B). Because over-expression of MnSOD results in an increase of intracellular H_2O_2, this increase was reversed by pre-treating with 10mM NAC and co-over-expression of the H_2O_2-detoxifying enzyme mCat (Figures 2A and B). These results suggested that the increase in the intracellular level of H_2O_2 would be responsible for promoting the migration of U87 glioma cells.

The histological feature of malignant gliomas is the invasion of tumour cells in surrounding normal brain tissues. The role of H_2O_2 in glioma invasion was measured by using the classic Transwell assay. As Figures 2C and D show, invasion of U87 glioma cells was enhanced when MnSOD was over-expressed. Pre-treating cells with 10mM NAC and over-expression of the mCat, which scavenged the intracellular H_2O_2, also significantly decreased the Transwell cell number (Figures 2C and D). These data indicated that the increase in the intracellular level of H_2O_2 would promote U87 glioma cell invasion *in vitro*.

Over-expression of MnSOD can activate the PI3Ks and MAPKs and elevate MMPs and is H_2O_2-dependent in U87 glioma cell

Previous studies have shown that the activation of MAPKs and PI3Ks is essential for cell migration [11–14] and that H_2O_2 can stimulate MAPKs in several cell types. To examine if cell signalling pathways have been activated by H_2O_2 in U87 glioma cells, we examined the activation status of AKT, s6-ribosomal protein and the MAPK ERK1/2 and JNK. The over-expression of MnSOD significantly increased the overall activation of AKT and s6-ribosomal protein (Figure 3A), ERK1/2 and JNK (Figure 3B). These activations of PI3Ks and MAPKs were inhibited by pre-treating cells with

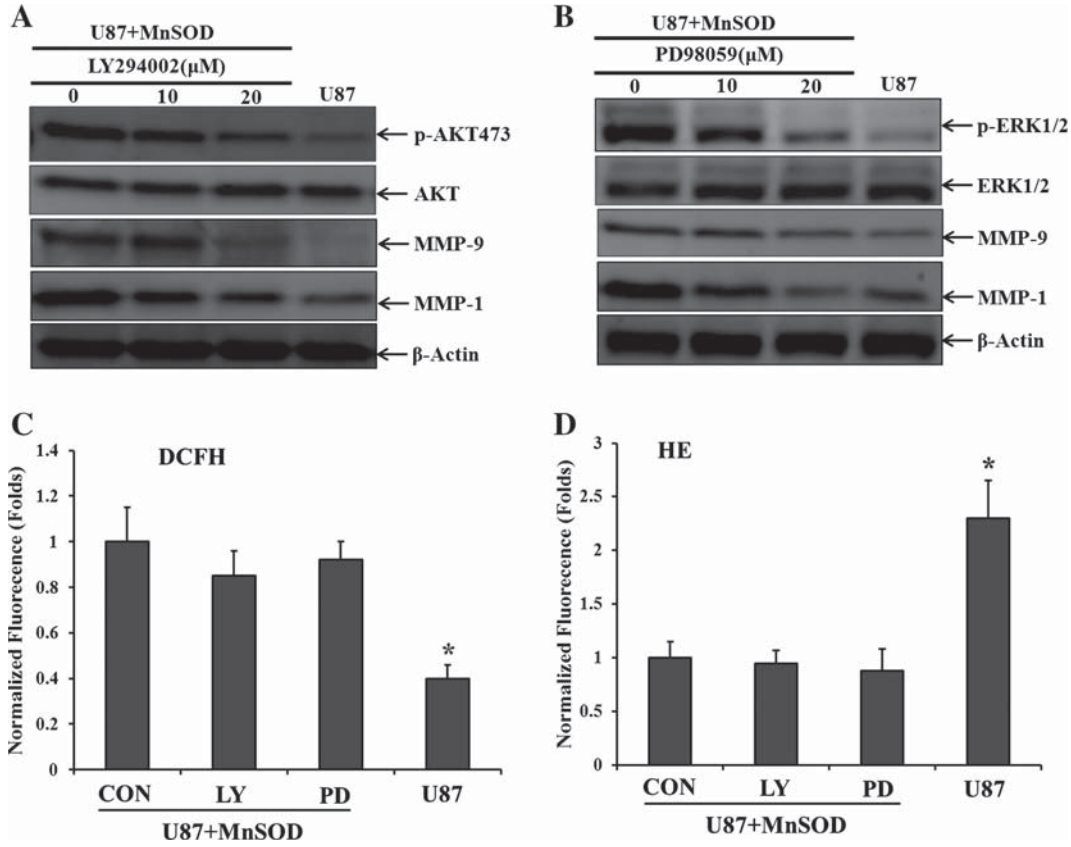

Figure 4. Inhibition of the activation of AKTs and ERKs decreases expression of MMPs without influencing the production of ROS in MnSOD over-expression cells. (A) Treating MnSOD over-expression cells with LY294002 (10 and 20 μM) for 24 hours can inhibit the activation of AKT and decrease the protein level of MMP-1 and MMP-9. (A) Treating MnSOD over-expression cells with PD98059 (10 and 20 μM) for 24 hours can inhibit the activation of ERK1/2 and decrease the protein level of MMP-1 and MMP-9. (C and D) LY294002 (20 μM) and PD98059 (20 μM) have no significant influence in the generation of H_2O_2 detected by DCFH and superoxide detected by HE. Data from three independent experiments were analyzed, and the results are expressed as the mean ± SE. $^*p < 0.01$, statistically significant difference compared with MnSOD over-expression U87 cells by Student's *t*-test.

10mM NAC and over-expression of the mCat (Figures 3A and B). These results suggested that H_2O_2 possesses the activation of PI3Ks and MAPKs pathways, which would contribute to the promotion of invasion and migration of U87 glioma cells.

The ability to remodel and degrade the extracellular matrix reflects the ability of tumour cells to invade the extracellular matrix. Over-expression of MnSOD was observed to be associated with elevations in MMP-1 and MMP-9 protein production, in our present study (Figures 3C and D). Furthermore, the increase in MMP-1 and MMP-9 following over-expression of MnSOD was shown to be H_2O_2-dependent. Expression of MMP-1 and MMP-9 was significantly decreased by NAC pre-treatment and over-expression of the mCat (Figures 3C and D). These data indicated that the effects of MnSOD on MMP-1 and MMP-9 expression were due to the elevation of intracellular H_2O_2 level.

Activation of AKT and ERK by MnSOD is responsible for the elevation of MMPs and promotion of migration and invasion in U87 glioma cell

The effects of MAPKs and PI3Ks activation in MnSOD-induced migration and invasion of U87 cells were investigated. As illustrated in Figures 4A and B, specific inhibitors, including LY294002 for AKTs and PD98059 for ERKs, were applied to study the role of MnSOD over-expression in U87 cells. Inhibition of AKTs by LY294002 or ERKs by PD98059 could reduce the MMP-1 and MMP-9 protein production. No significant change in the H_2O_2 detected by DCFH and superoxide detected by HE was found

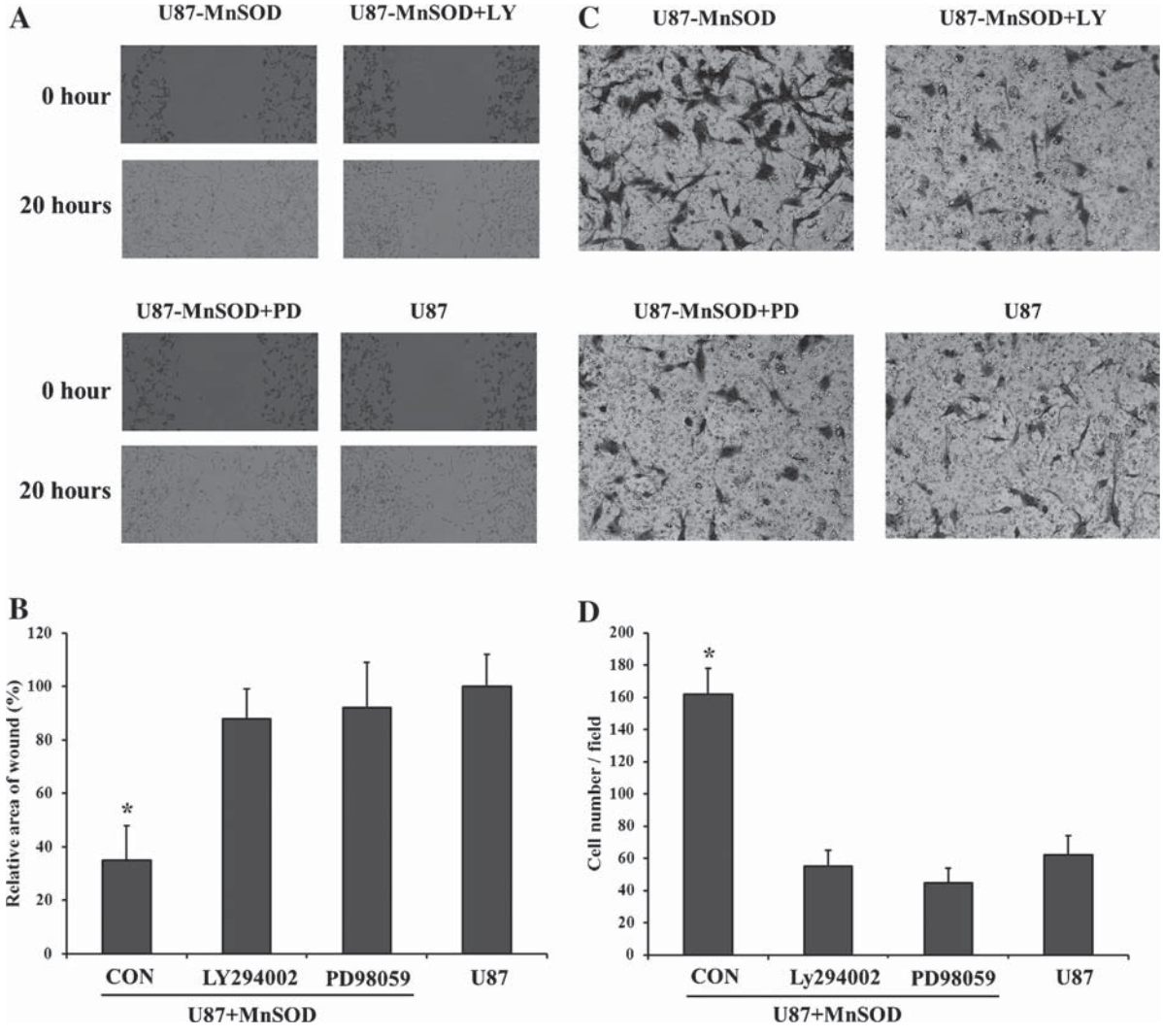

Figure 5. Inhibition of the activation of AKTs and ERKs decreases the migration and invasion of MnSOD over-expression U87 cells. (A and B) Expression of MnSOD promotes migration of U87 glioma in a wound healing assay and can be decreased by incubating the cell with LY294002 (20 μM) and PD98059 (20 μM). (C and D) Expression of MnSOD promotes Transwell ability of U87 glioma and can be decreased by incubating the cell with LY294002 (20 μM) and PD98059 (20 μM). Data from three independent experiments were analyzed, and the results are expressed as the mean ± SE. $^*p < 0.01$, statistically significant difference compared with U87 cells by Student's *t*-test.

(Figures 4C and D). However, LY294002 and PD98059 could decrease the migration (Figures 5A and B) and invasion (Figures 5C and D) of U87 cells, which are promoted by over-expression of MnSOD. These results showed that inhibition of the activation of AKTs and ERKs could decrease the migration and invasion of U87 cells by MnSOD over-expression, without influencing the production of H_2O_2.The presented data suggested that the activation of AKTs and ERKs would be the downstream signals of H_2O_2-enhanced migration and invasion in U87 glioma cells.

Discussion

MnSOD is essential to the vitality of mammalian cells. Most studies have concluded that MnSOD has anti-proliferative effects and tumour suppressor characteristics [15,16]. The over-expression of MnSOD has been shown to enhance the sensitivity of oral cancer in response to radiation while protecting the normal tissues [17]. However, a large number of studies have declared that MnSOD is elevated in most of the malignant tumours [7,15,18,19]. In addition, increased MnSOD levels are also associated with poor prognosis and resistance to therapy of several tumours in the central nervous system, gastrointestinal tract, head and neck [15]. Connor et al. [20] found that MnSOD can enhance the invasive and migratory activity of HT-1080 fibrosarcoma cells. Previous studies have shown that the U87 gliomas with over-expression of MnSOD by 3.5-folds significantly enhanced their migration and invasion.

Elevated rates of ROS have been detected in almost all cancers, particularly H_2O_2 can act as second messenger in cellular signalling. There is growing evidence showing the correlation between H_2O_2 and cell migration. H_2O_2 was identified as key regulator of neutrophil chemotactic migration [19], hepatic pro-fibrogenic cells [10] and breast cancer cells [21]. Connor et al. [20] figured out that the pro-migratory/invasive phenotype of MnSOD-expressing cells is H_2O_2-dependent. Our studies have shown that although the superoxide detected by HE was significantly inhibited by the over-expression of MnSOD, the intracellular H_2O_2 detected by DCFH-DA was significantly elevated. The promotion of migration and invasion of U87 glioma cells by MnSOD can be reversed by pre-treating with NAC or co-over-expression of the H_2O_2-detoxifying enzyme mCat. These results suggested that the increase in the intracellular level of H_2O_2 would be responsible for the promotion of migration and invasion in U87 glioma cells.

Activation of MAPKs and PI3Ks is essential for cell migration [11–14]. Researchers have shown that ROS may activate MAPKs and PI3Ks cascades in the malignant progression of cancer cells [21–23]. MMPs are vital to the process of cellular invasion, studies have shown that the ROS leads to increased expression of MMPs and correlates with enhanced metastasis [24–26]. Our observation shows that over-expression of MnSOD significantly increased the overall activation of AKT and s6-ribosomal protein, ERK 1/2 and JNK. Over-expression of MnSOD is associated with elevations in MMP-1 and MMP-9 protein production. Although the activation of MMPs needs to be investigated, the promotion of migration/invasion, activation of PI3Ks and MAPKs and up-regulation of MMPs were inhibited by pre-treating cells with NAC, over-expression of the mCat and the specific inhibitor of AKTs or ERKs. These data suggest that H_2O_2 plays key role in the activation of intracellular signalling pathways, which contribute to the invasion and migration of U87 glioma cells.

In summary, the results obtained from current studies suggest that the H_2O_2 would contribute to the MnSOD-promoted migration and invasion in glioma cells. In the present study, the elevation of H_2O_2 level and the enhancement of glioma migration and invasion by over-expression of MnSOD are clearly demonstrated. This study also provides evidence that the activation of PI3Ks and MAPKs and up-regulation of MMPs after MnSOD over-expression might be due to the elevation of the intracellular H_2O_2. Thus, our studies provide new molecular insights into the understanding of glioma migration and invasion.

Declaration of interest

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