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Silver nanoparticles down-regulate Nrf2-mediated 8-oxoguanine DNA glycosylase 1 through inactivation of extracellular regulated kinase and protein kinase B in human Chang liver cells

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ABSTRACT

Recently, we reported that silver nanoparticles (AgNPs) induced reactive oxygen species (ROS) generation and the resultant oxidative stress contributes to the cell damage associated with AgNPs. 8-Oxoguanine (8-oxoG) is sensitive marker of ROS-induced DNA damage. 8-Oxoguanine DNA glycosylase 1 (OGG1) is an important DNA repair enzyme that recognizes and excises 8-oxoG. The aim of the present study was to examine the effect of AgNPs-induced oxidative stress on OGG1 and to elucidate mechanisms underlying AgNPs toxicity. AgNPs decreased OGG1 mRNA and protein expression, resulting in decreased OGG1 activity. Decreased OGG1 activity in AgNPs-treated cells led to increased 8-oxoG levels. The transcription factor NF-E2-related factor 2 (Nrf2) is an important factor in the inducible regulation of OGG1. AgNPs treatment decreased nuclear Nrf2 expression, translocation into nucleus, and transcriptional activity of Nrf2. Extracellular regulated kinase (ERK) and protein kinase B (PKB, AKT), which are upstream of Nrf2, contribute to OGG1 expression. AgNPs attenuated both active forms of ERK and AKT protein expression, resulting in suppression of Nrf2 and decrease of OGG1 expression. These studies demonstrate that down-regulation of Nrf2-mediated OGG1 in exposure to AgNPs occurs through ERK and AKT inactivation.

1. Introduction

Silver nanoparticles (AgNPs) are an important class of nanomaterials for a wide range of industrial and medical applications. Despite their widespread use, AgNPs are toxic to a variety of organs, including the lung, liver, and brain (Ahamed et al., 2010). Possible mechanisms of AgNPs toxicity include induction of reactive oxygen species (ROS), oxidative stress, DNA damage and apoptosis (Ahamed et al., 2010). It has been demonstrated, for example, that AgNPs enter fibroblasts and hepatocytes and cause DNA damage and apoptosis (Arora et al., 2009). We recently reported that AgNPs cause cytotoxicity by oxidative stress-induced apoptosis and damage to DNA, lipid and protein (Piao et al., 2011).

8-Oxoguanine (8-oxoG) is one of the most commonly formed DNA lesions and is considered a cellular marker for both oxidative stress and DNA damage (Dizdaroglu et al., 2002). 8-Oxoguanine DNA glycosylase 1 (OGG1) is the first step and rate-limiting enzyme involved in the removal of 8-oxoG through the base excision repair (BER) pathway (Boiteux and Radicella, 2000; Klungland et al., 1999; de Souza-Pinto et al., 2001). The human OGG1 promoter contains a putative transcription factor NF-E2-related factor 2 (Nrf2) binding site and Nrf2 leads to OGG1 transcription (Merrill et al., 2002; Dhénaut et al., 2000). Mitogen activated protein kinase (MAPK) and protein kinase B (PKB, AKT) are important signaling enzymes involved in the transduction of various signals from the cell surface to the nucleus. MAPK and AKT induce Nrf2 translocation or increased stability, which leads to increased Nrf2 activity (Zipper and Mulcahy, 2003; Wang et al., 2008).

In this study, we investigated whether AgNPs-mediated ROS induction can affect DNA base modification with respect to 8-oxoG and studied the mechanisms by which the 8-oxoG is achieved.

2. Materials and methods

2.1. Reagents

AgNPs were provided by professor Jinhee Choi of Seoul University (Seoul, Republic of Korea) and characterization of AgNPs was conducted as described previously (Eom and Choi, 2010). AgNPs had an even distribution, with individual particle sizes ranging from 5 to 10 nm. AgNPs tend to agglomerate when exposed to cells; the most of the AgNPs in the medium were of about 28–35 nm in size. Ag ions were estimated using a multitype inductively coupled plasma emission spectrometer (ICPE-9000,

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Shimadzu, Tokyo, Japan). The concentrations of AgNPs and Ag ions had equivalent Ag masses and measured prior to exposure of the compounds to cell culture media. The OGG1 promoter-luciferase construct was a generous gift from Professor Ho Jin You of Chosun University (Gwangju, Republic of Korea). OGG1 antibody was purchased from Abcam (Cambridge, MA, USA). ERK2, phospho ERK1/2, Nrf2, and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho AKT (Ser 473) and AKT antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Avidin tetramethylrhodamine isothiocyanate (TRITC) was purchased from Sigma Chemical Company (St. Louis, MO, USA). Other chemicals and reagents were of analytical grade.

2.2. Cell culture

Human Chang liver cells were obtained from the American type culture collection (Rockville, MD, USA) and were maintained in an incubator with a humidified atmosphere of 5% CO_2 at 37 °C. Cells were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml) and penicillin (100 U/ml).

2.3. Transient transfection and OGG1 promoter luciferase assay

Cells were transiently transfected with a plasmid harboring the OGG1 promoter using the transfection reagent DOTAP according to the manufacturer's instructions (Roche, Mannheim, Germany). After overnight transfection, cells were treated with AgNPs for 6–48 h. Cells were then lysed with reporter lysis buffer (Promega, Madison, WI, USA), and the lysate supernatant was mixed with the luciferase assay reagent. The mixture was placed in a luminometer to measure the light produced.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using Trizol (GibcoBRL, Grand Island, NY, USA). PCR conditions for OGG1 and for the housekeeping gene GAPDH were: 35 cycles of 94 °C for 2 min; 94 °C for 20 s; 58 °C for 30 s; 72 °C for 1 min; and 72 °C for 5 min. The primer pairs (Bionics, Seoul, Republic of Korea) were as follows (sense and antisense, respectively): human OGG1-sense 5′-CTGCCTTCTGGACAATCTTT-3′ and human OGG1-antisense 5′-TAGCCCGCCCTGTTCTTC-3′, and human GAPDH sense 5′-GCAGTGAGGGTCTCTCTCCT-3′; and human GAPDH antisense 5′-AAGGTCGGAGTCAACGGATT-3′. Amplified products were resolved by 1% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

2.5. Western blot analysis

Cells were lysed on ice for 30 min in 100 μl of lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP 40] and centrifuged at 13,000 × *g* for 15 min. The supernatants were collected from the lysates and the protein concentrations were determined. Aliquots of the lysates (40 μg of protein) were boiled for 5 min and electrophoresed in a 10% SDS–polyacrylamide gel. The blots in the gels were transferred onto nitrocellulose membranes, and subsequently incubated with primary antibodies. The membranes were further incubated with secondary anti-immunoglobulin-G-horseradish peroxidase conjugates (Pierce, Rockford, IL, USA), followed by exposure to X-ray film. The protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK).

2.6. Transfection of cells with 8-oxoG-containing molecular beacon

Cells were seeded at 1×10^5 cells/well in 24-well plates and treated with 4 μg/ml of AgNPs for 24 h. An 8-oxoG-containing oligonucleotide with the sequence 5′-FAM-GCACTOAAGCGCCGCACGCCATGTCGACGCGCTTCAGTGC-DAB-3′ (where O is 8-oxoG) was synthesized by Bioneer Corporation (Bioneer, Republic of Korea). The 5′-fluorophore (fluorescein amidite, FAM) is in close proximity to the 3′-quencher (4-(4′-dimethylaminophenylazo) benzoic acid, DAB). After being cut the beacon by OGG1, the fluorescent signal is liberated, forming the bases of detection of OGG1 activity. The beacon was dissolved in sodium chloride-tris-EDTA buffer to give a stock solution (100 pmol/μl). The beacon was heated for 5 min at 95 °C, vortexed and then left to cool slowly to room temperature in the dark to permit the adoption of the correct stem-loop conformation of oligonucleotide. Prior to use, the beacon was diluted to 10 pmol/μl in distilled deionized water and 100 pmol beacon and 2 μl Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) were transfected to cells. Cells were then placed in a humidified chamber (37 °C, 5% CO_2) for 6 h. The green foci and fluorescence intensity were examined by both confocal microscopy and flow cytometry, respectively.

2.7. Detection of 8-oxoG

Cells were seeded in a plate at a concentration of 1×10^5 cells/ml and treated with AgNPs at 16 h after plating. After incubation for an additional 24 h at 37 °C, cellular DNA was isolated using DNAzol reagent (Life Technologies, Grand Island, NY, USA) and quantified using a spectrophotometer. The amount of 8-hydroxy-2′-deoxyguanosine (8-OHdG, a nucleoside of 8-oxoG) in DNA was determined using 8-OHdG-EIA™ kit from OXIS Health Products (Portland, OR, USA) according to the manufacturer's instructions. The amount of 8-oxoG was also estimated in a fluorescent binding assay (Struthers et al., 1998). Cells were fixed and permeabilized with ice-cold methanol for 15 min. 8-OxoG was visualized with avidin-conjugated TRITC under a fluorescence microscope.

2.8. Immunocytochemistry

Cells plated on coverslips were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in PBS for 2.5 min. Cells were then treated with blocking solution (3% bovine serum albumin in PBS) for 1 h and incubated with Nrf2 antibody diluted in blocking solution for 2 h. Immuno reacted primary Nrf2 antibody was detected by a 1:500 dilution of FITC-conjugated secondary antibody (Jackson Immuno Research Laboratories, West Grove, PA, USA) for 1 h. After washing with PBS, stained cells were mounted onto microscope slides in mounting medium with DAPI (Vector, Burlingame, CA, USA). Images were collected using the LSM 510 program on a Zeiss confocal microscope.

2.9. Chromatin immunoprecipitation (ChIP) assay

Cells were processed using the Simple ChIP™ enzymatic chromatin IP kit from Cell Signaling Technology according to the manufacturer's instructions. Briefly, the procedure was initiated by cross-linking the proteins to DNA by adding 1% formaldehyde to the culture dishes for 10 min on a rocking platform at room temperature. The cross-linking was stopped with the addition of glycine solution. Cells were harvested and centrifuged for 10 min at 720 × *g*. Nrf2 antibody (2 μg each) was added to the pre-cleared chromatin in 0.65 ml siliconized tubes and incubated overnight on a rotator at 4 °C. The oligonucleotide containing the Nrf2 binding site within the OGG1 promoter was obtained from Bioneer (Seoul, Republic of Korea). The ChIP procedure was analyzed using PCR with human OGG1 promoter-specific primers sense (−2192) 5′-CCTGGAAGAG-3′ and antisense (−2078) 5′-AATGACTCTGGCG-3′ and Platinum Taq High Fidelity (Invitrogen). The cycle parameters were as follows: first cycle at 95 °C for 5 min; 55 cycles at 95 °C for 30 s; 59 °C for 30 s; 72 °C for 30 s; and a final extension at 72 °C for 7 min. PCR products were resolved on a 1% agarose gel containing ethidium bromide, and visualized under UV illumination (Bartz et al., 2011).

2.10. Statistical analysis

All measurements were made in triplicate and all values are expressed as the means ± standard error of the mean (SEM). The results were subjected to an analysis of variance (ANOVA) using the Tukey test to analyze the significance of the difference. A probability of $p < 0.05$ was considered significant.

3. Results

3.1. Effect of AgNPs on the transcriptional activity of the OGG1 promoter and on OGG1 mRNA and protein expression

The transcriptional activity of the OGG1 promoter was assessed using an OGG1 promoter linked to a luciferase reporter gene. AgNPs treatment (6–48 h) decreased the transcriptional activity of the OGG1 promoter in a time-dependent manner (Fig. 1A). The transcriptional activity of the OGG1 promoter in AgNPs-treated cells was consistent with the expression levels of OGG1 mRNA; AgNPs treatment decreased OGG1 mRNA levels in a time-dependent manner (6–48 h) (Fig. 1B). Similarly, the expression of OGG1 protein decreased in a time-dependent manner (6–48 h) in AgNPs-treated cells (Fig. 1C).

3.2. Effect of AgNPs on OGG1 activity

Confocal microscopic images showed that 8-oxoG-containing beacon-transfected control cells had fluorescence foci, indicating fluorescent cleavage bases containing 8-oxoG (Fig. 2A, right in upper panel). AgNPs treatment showed green fluorescence to be the inherent fluorescence of AgNPs in non-transfected control cells and 8-oxoG-containing beacon-transfected cells (Fig. 2A, left and right in lower panel). However, AgNPs treatment in 8-oxoG-containing beacon-transfected cells decreased the numbers of fluorescence foci compared to 8-oxoG-containing beacon-transfected control cells (Fig. 2A, right in lower and upper panel and histogram graph). The confocal microscopic data were consistent with the flow cytometric data (Fig. 2B).

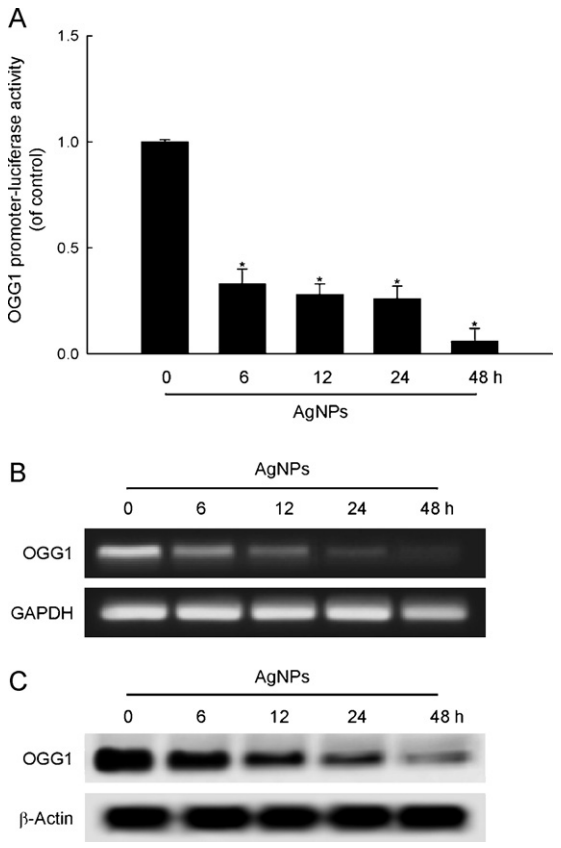

Fig. 1. Effect of AgNPs on transcriptional activity of OGG1 promoter, OGG1 mRNA expression and protein expression. (A) The time course of transcriptional activity of OGG1 promoter was measured after treatment of AgNPs at 4 μg/ml. *Significantly different from control ($p < 0.05$). (B) OGG1 mRNA expression was analyzed by RT-PCR. (C) Cell lysates were electrophoresed and the expression of OGG1 protein was detected using an OGG1 specific antibody.

3.3. Effect of AgNPs on the levels of 8-oxoG

The levels of 8-oxoG, a hallmark of oxidative stress-DNA base damage, were measured using an 8-OHdG detection kit and verified by immunochemistry. As shown in Fig. 3A, AgNPs increased the levels of 8-oxoG in DNA in a time-dependent manner (6–48 h). Condensed staining intensity of 8-oxoG was observed in AgNPs-treated cells (Fig. 3B). These results suggest that AgNPs increase the levels of 8-oxoG.

3.4. Effect of AgNPs on the Nrf2 transcription factor

Nrf2 is an important transcription factor that regulates OGG1 gene expression. AgNPs treatment resulted in decreased nuclear Nrf2 expression and translocation into the nucleus (Fig. 4A and B). As shown in Fig. 4B, AgNPs treatment changed the morphology of cells to round shape compared to control cells. In our previous report, AgNPs showed cytotoxicity via induction of apoptosis (Piao et al., 2011), suggesting that the morphological change in AgNPs-treated cells may be due to induction of apoptosis. Furthermore, AgNPs-treated cells exhibited almost no detectable Nrf2 binding to the sequence in OGG1 promoter (Fig. 4C).

3.5. Effect of AgNPs on AKT and ERK

AKT and ERK1/2 are major signaling enzymes involved in cellular protection against oxidative stress and up-stream regulator of Nrf2. As shown in Fig. 5A and B, exposure to AgNPs caused a decrease in the phosphorylation (active form) of ERK1/2 and AKT in a time-dependent manner.

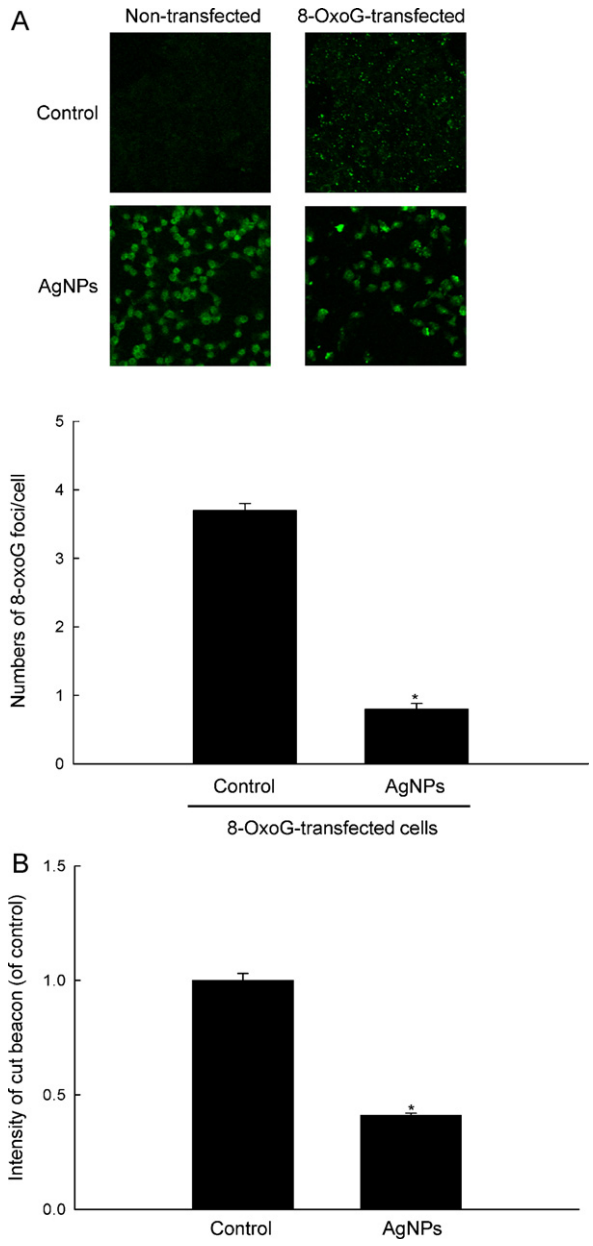

Fig. 2. Effect of AgNPs on OGG1 activity. The 8-oxoG-containing beacon was transfected into cells. (A) The fluorescence foci of the cleaved bases containing 8-oxoG were detected by confocal microscopy and counted. (B) The fluorescence foci were detected by flow cytometry. *Significantly different from control ($p < 0.05$).

4. Discussion

Oxidative stress is caused by an imbalance between oxidants and antioxidants, and results in damage to membrane lipids, proteins and DNA, which leads to cell death (Toyokuni, 1999; Zimmerman, 1998; Ercal et al., 2001). Oxidative stress is an important mechanism mediating the toxicity of nanoparticles (Nel et al., 2006). For example, we recently reported that AgNPs induced ROS and damaged DNA, leading to apoptosis (Piao et al., 2011). 8-OxoG is known to be a sensitive marker of oxidative DNA damage (Shtarkman et al., 2008; Vadim et al., 2002), and is involved in mutagenesis, tissue/cell damage, and apoptosis (Hyun et al., 2000, 2003; Tsuzuki et al., 2007; Tsuruya et al., 2003). In the present study,

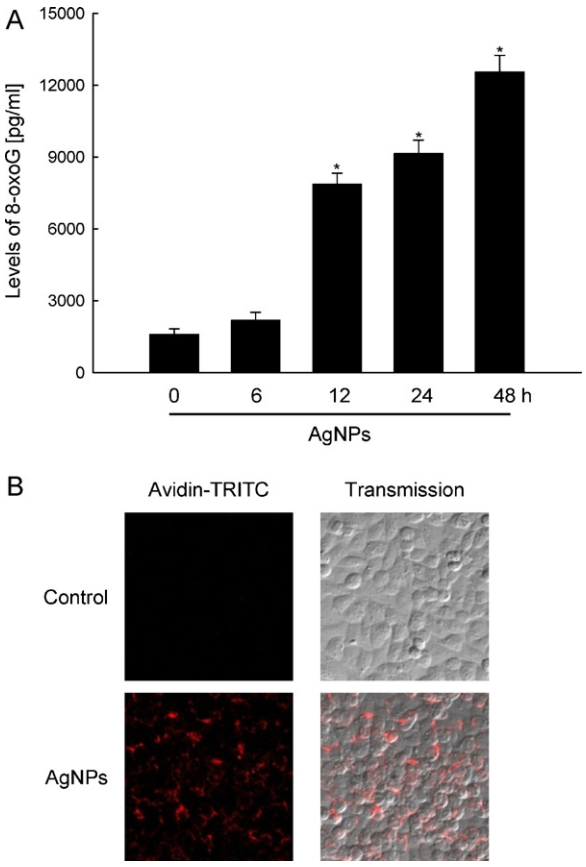

Fig. 3. Effect of AgNPs on 8-oxoG levels. (A) Cellular DNA was isolated using DNAzol reagent and the amount of 8-oxoG in DNA quantified using the Bioxytech 8-OHdG-ELISA kit. *Significantly different from control ($p < 0.05$). (B) 8-OxoG detected by the binding of avidin-TRITC was visualized by fluorescence microscope.

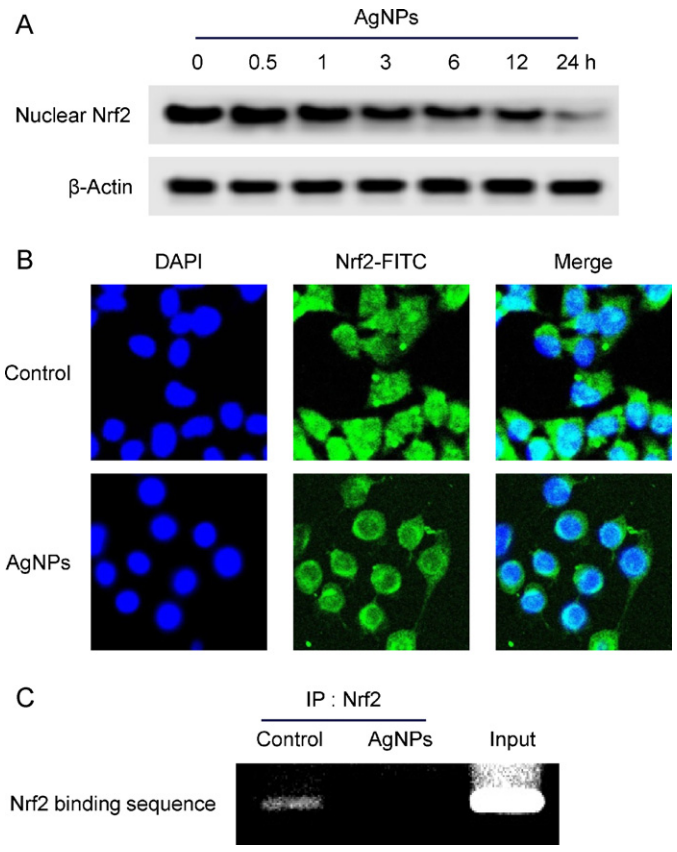

Fig. 4. Effects of AgNPs on Nrf2 expression, translocalization into the nucleus, and binding to OGG1 promoter. (A) Nuclear lysates were electrophoresed and the time-course of the effects of AgNPs on the expression of Nrf2 protein was detected using an Nrf2-specific antibody. (B) FITC-conjugated secondary antibody staining indicates the location of Nrf2 (green) by anti-Nrf2 antibody; DAPI staining indicates the location of the nucleus (blue); and the merged image indicates the nuclear location of Nrf2 protein. (C) Nrf2 binding sequence in OGG1 promoter obtained by the ChIP procedure was analyzed using PCR.

AgNPs increased the levels of 8-oxoG in a time-dependent manner. This result demonstrated that oxidative stress induced by AgNPs causes DNA modification and leads to the accumulation of 8-oxoG in DNA.

OGG1 is a specific repair enzyme involved in the removal of 8-oxoG from DNA via the BER pathway in mammals, and plays an integral role in genomic maintenance. AgNPs decreased OGG1 activity, suggesting that inability of OGG1 to repair 8-oxoG leads to an increase of 8-oxoG in DNA. The inhibition of OGG1 activity by AgNPs could be the result of changes in gene transcription or translation. The transcriptional activity of OGG1 was decreased in AgNPs-treated cells and this led to a decrease in OGG1 mRNA expression. Similarly, OGG1 protein expression was decreased in AgNPs-treated cells. It has been suggested that the expression of OGG1 is affected by the redox status of the cell because a putative Nrf2 transcription factor binding site is present in the promoter of the human OGG1 (Dhénaut et al., 2000). In the present study, AgNPs down-regulated OGG1 expression by decreasing Nrf2 expression and Nrf2 binding to sequences present in the OGG1 gene promoter. Nrf2 is involved in the regulation of phase II xenobiotic metabolism and antioxidant genes, and is also an important factor in the inducible regulation of OGG1 (Cox, 2007). Structurally, Nrf2 is a basic leucine zipper transcription factor that under normal conditions remains sequestered in the cytosol (Moi et al., 1994; Venugopal and Jaiswal, 1998). Sequestration of Nrf2 is facilitated by interaction with its inhibitory partner, kelch-like protein 1 (Keap1) (Itoh et al., 1999; Dinkova-Kostova et al., 2002). Under basal conditions, Nrf2-Keap1 complexes are maintained in the cytosol tethered to the actin cytoskeleton where Nrf2 is continually targeted to the proteasome via an ubiquitin-dependent pathway (Nguyen et al., 2003). However, in the presence of ROS, xenobiotics or enzymatic activation, Nrf2 is released from Keap1 and translocates to the nucleus where it interacts with small Maf binding proteins. The final function of active Nrf2 is to interact with its cognate binding sequence in promoters and induce transcription (Itoh et al., 2004; Lee and Johnson, 2004; Nguyen et al., 2004). AgNPs decreases the nuclear level of Nrf2, its binding to its cognate sequence in the OGG1 promoter, and its transcriptional activity. The mechanisms leading

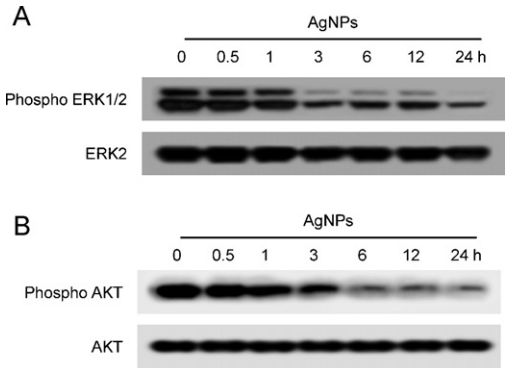

Fig. 5. Effect of AgNPs on the phosphorylation of AKT and ERK. Cell lysates were electrophoresed, and (A) phospho ERK1/2 and ERK2, (B) phospho AKT and AKT were detected by using their respective specific antibodies.

to nuclear translocation of Nrf2 include its release from Keap1 in the cytosol. ERK and AKT phosphorylates Nrf2, which may facilitate the release of Nrf2 from the Keap1-Nrf2 complex, allowing activated Nrf2 to translocate into the nucleus where it forms a heterodimer with small Maf protein (Li et al., 2007; Chan et al., 2001; Kim et al., 2001; Kwak et al., 2002). Our data indicate that AgNPs act through down-regulation of ERK and AKT to down-regulate OGG1, resulting in the accumulation of oxidized DNA bases. The OGG1 promoter contains Nrf2 and stimulation protein-1 (SP-1) binding sites (Dhénaut et al., 2000). The AKT pathway regulates Nrf2 and SP-1 (Piantadosi et al., 2008; Sroka et al., 2007), and AKT up-regulation is involved in the resistance of cancer cells to gamma-ray via OGG1 induction (Ueta et al., 2008). ERK signaling regulates Nrf2 activation and ERK up-regulation involved in up-regulation of OGG1 against hyperoxic cytotoxicity (Papaiahgari et al., 2004; Kannan et al., 2006). Thus, the AKT and ERK pathways mediate up-regulation of OGG1. AKT and ERK have been implicated in the regulation of a variety of signal transduction pathways that mediate gene transcription, cell cycle events, cell proliferation, DNA repair, and cell survival (Wanzel et al., 2005; Gao et al., 2004; Santos et al., 2001; Kandel et al., 2002; Yu et al., 2003; Ueta et al., 2008; Kannan et al., 2006).

In summary, 8-oxoG accumulation in AgNPs-treated cells occurs through decreased activities of ERK and AKT, decreased nuclear translocation of Nrf2, decreased Nrf2 binding to the OGG1 promoter, and down-regulation of OGG1 gene expression.

Conflict of interest statement

There are no conflicts of interest for any of the authors.

Acknowledgements

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