

# Nitric oxide/cyclic guanosine monophosphate inducers sodium nitroprusside and L-arginine inhibit the proliferation of gastric cancer cells via the activation of type II cyclic guanosine monophosphate-dependent protein kinase

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**Abstract.** Nitric oxide (NO) may activate soluble guanylyl cyclase (sGC), resulting in the increase of intracellular cyclic guanosine monophosphate (cGMP), a key molecule in the activation of type II cGMP-dependent protein kinase (PKG II). In our previous study, the membrane-permeable cGMP analogue 8-pCPT-cGMP was used to activate PKG II. The aim of the present study was to investigate whether NO/sGC-induced endogenous cGMP is able to activate PKG II and induce the corresponding effects. In the AGS gastric cancer cell line, the expression of PKG II was increased by infecting the cells with an adenoviral construct encoding PKG II cDNA (Ad-PKG II) and the activation of PKG II was induced by 8-pCPT-cGMP (positive control), the NO donor sodium nitroprusside (SNP) and the NO precursor L-arginine. ELISA was performed to detect the concentration of cGMP in AGS cells and the Cell Counting Kit-8 was used to analyze the proliferation of differently treated cells. Western blot analysis was used to detect the expression and phosphorylation of associated proteins. The results demonstrated that the level of cGMP was increased in cells treated with the NO donor or precursor. There was an obvious increase of Ser239 phosphorylation of the vasodilator-stimulated phosphoprotein, representing the increase in the activity of PKG II. The epidermal growth factor (EGF)-induced proliferation of AGS cells was inhibited by infection with Ad-PKG II and treatment with SNP or L-arginine. In addition, EGF-induced tyrosine phosphorylation of the EGF receptor (EGFR) and tyrosine/serine phosphorylation of extracellular signal-regulated kinase (ERK) were also inhibited by infection with Ad-PKG II and treatment with the

NO donor or precursor. These data indicated that NO donors and precursors may activate the expression of PKG II, thereby blocking EGF-initiated signaling of the mitogen-activated protein kinase/ERK pathway and inhibiting EGF-induced proliferative activity through preventing the phosphorylation of EGFR at Tyr1068.

## Introduction

Nitric oxide (NO) is synthesized in a variety of tissues and displays various physiological actions via activating soluble guanylyl cyclase (sGC), which converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), thereby leading to an increase in the intracellular concentration of cGMP (1). cGMP is an important secondary messenger and exerts its effects through activating cGMP-dependent protein kinases (PKGs). To date, two types of PKGs have been identified in mammalian cells, namely PKG I and II. The activated PKGs exert their biological effects through phosphorylating target proteins (2,3). The target proteins of PKGs have attracted significant attention and, in particular, the significance of PKG II and its target proteins is currently a major research focus. Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase with a molecular weight of 170 kDa. Activated EGFR may initiate the signal transduction of several pathways, including mitogen-activated protein kinase (MAPK) pathway-mediated proliferation (4,5). Our previous studies demonstrated that, when PKG II is highly expressed through infecting the cells with an adenoviral construct encoding PKG II cDNA (Ad-PKG II), and activated by cGMP, the proliferation of gastric cancer cells is inhibited (6,7). The mechanism of the inhibitory effect of PKG II on the activation of EGFR is through blocking the tyrosine phosphorylation/activation of EGFR. During this process, the cell membrane-permeable cGMP analogue 8-pCPT-cGMP is required to activate PKG II (8,9). NO is synthesized by NO-synthase (NOS) or released by NO donors; increased NO levels may activate sGC, leading to the increase of intracellular cGMP. In the present study, the effect of the NO donor sodium nitroprusside (SNP) and the NOS substrate L-arginine on the activation of PKG II and the proliferative activity and signaling of Ad-PKG II-infected gastric cancer cells was investigated.

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**Key words:** sodium nitroprusside, L-arginine, type II cyclic guanosine monophosphate-dependent protein kinase, proliferation, gastric cancer cells

## Materials and methods

**Cell lines, antibodies and chemicals.** The AGS human gastric cancer cell line was provided by the Institute of Cell Biology (Shanghai, China). Adenoviral vectors encoding the cDNA of  $\beta$ -galactosidase (Ad-LacZ) and PKG II (Ad-PKG II) were obtained from Dr Gerry Boss and Dr Renate Pilz (University of California, San Diego, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL (Grand Island, NY, USA). The rabbit polyclonal antibody against PKG II was purchased from Abgent Biotechnology (San Diego, CA, USA; catalog no. AP8001a; dilution, 1:200). The goat polyclonal IgG antibody against phosphorylated vasodilator-stimulated phosphoprotein (p-VASP; Ser239; catalog no. sc-23507; dilution, 1:200) and the horseradish peroxidase (HRP)-conjugated mouse monoclonal IgG<sub>1</sub> antibody against  $\beta$ -actin were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA; catalog no. sc-47778; dilution, 1:1,000). Mouse anti-p-EGFR (Tyr1068) monoclonal antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA; catalog no. 2236; dilution, 1:1,000). Rabbit anti-p-ERK (Thr202/Tyr204) polyclonal antibody was obtained from Bioworld Technology (St. Louis Park, MN, USA; catalog no. BS5016; dilution, 1:500). The HRP-conjugated goat anti-mouse, goat anti-rabbit and rabbit anti-goat polyclonal IgG secondary antibodies (catalog nos. 115-035-003, 111-035-003 and 305-035-003, respectively; dilution, 1:10,000) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The cellular membrane-permeable cGMP analog 8-pCPT-cGMP was obtained from Calbiochem (San Diego, CA, USA). EGF and SNP were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-arginine was obtained from the Beyotime Institute of Biotechnology (Shanghai, China). Cell Counting Kit-8 (CCK-8) was obtained from R&S Biotechnology (Shanghai, China). The human cGMP ELISA kit was obtained from Hushang Biotechnology (Shanghai, China) and the electrochemiluminescence (ECL) reagents were purchased from Millipore (Billerica, MA, USA).

**Cell culture and treatment.** The AGS cells were maintained in DMEM supplemented with 10% FBS and penicillin (100 IU/ml)/streptomycin (100 mg/ml) and incubated at 37°C in 5% CO<sub>2</sub>. The medium was changed every 2 days and the cells were subcultured at confluence. The cells were seeded in 6-well plates at a density of 70-80% of confluence and were infected with Ad-LacZ or Ad-PKG II (with a multiplicity of infection of 100%) or mock-infected the following day. At 24 h post-infection, the medium was replaced with serum-free medium and the culture was continued for 12 h. The infected cells were incubated with 250  $\mu$ M 8-pCPT-cGMP for 1 h, the NO donor or precursor (1 mM SNP for 2 h and 2 mM L-arginine for 4 h) and incubated with 100 ng/ml EGF for 5 min.

**CCK-8 assay.** A total of 10,000 cells (in 150  $\mu$ l complete DMEM) were seeded in one well of a 96-well plate. Following attachment, the cells were infected with Ad-LacZ and Ad-PKG II for 24 h. The cells were then washed and serum-starved overnight. Thereafter, the cells were incubated in the presence of 8-pCPT-cGMP (250  $\mu$ M) for 1 h, SNP (1 mM) for 2 h or L-arginine (2 mM) for 4 h, and then treated

with EGF (100 ng/ml) for 24 h. Approximately 10  $\mu$ l of CCK-8 dye was added to each well and the plate was incubated for 30 min. The optical density (OD) at 450 nm was measured using an ELx800 Absorbance Reader (BioTek Instruments, Inc., Winooski, VT, USA). Data are presented as the relative proliferation, calculated by dividing the OD value of each group by the OD value of Ad-LacZ group.

**ELISA.** The differently treated cells were harvested by phosphate-buffered saline (pH 7.2-7.4) to a concentration of 1 million/ml. The collected cells were subjected to three freeze-thaw cycles to further break the cell membranes. Subsequently, the samples were centrifuged for 20 min at 0.4-0.9  $\times$  g. The collected supernatants were processed using the human cGMP ELISA kit, according to the manufacturer's instructions. The absorbance was determined at 450 nm using the ELx800 Absorbance Reader. The standard curve was constructed by plotting the absorbance for each standard on the x-axis against the concentrations on the y-axis; the sample concentrations were calculated according to the sample OD value and the standard curve.

**Western blotting.** The protein samples were subjected to SDS-PAGE (8-12%) according to the molecular size of the target protein and transferred onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were blocked using 5% non-fat milk in Tris-buffered saline with Tween (TBS-T) for 1 h at room temperature. Incubation with the primary antibodies was performed at 4°C overnight, followed by incubation with the corresponding secondary antibodies at room temperature for 1 h, with three washes in TBS-T (0.1% Tween 20) subsequent to each incubation. The signal was visualized using ECL detection reagents.

**Statistical analysis.** Data are expressed as means  $\pm$  standard deviation. Statistical analysis was performed using a two-tailed analysis of variance with SPSS 15.0 statistical software (SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**SNP and L-arginine inhibit the proliferation of AGS cells expressing high levels of PKG II.** AGS cells were infected with Ad-PKG II to increase the expression of the PKG II protein. SNP or L-arginine was added to the culture medium to activate PKG II. The proliferative activity of the cells was evaluated with CCK-8. The results demonstrated that, in cells infected with Ad-LacZ (control virus) and treated with EGF (100 ng/ml, 24 h), there was a significant increase in proliferative activity compared with cells infected with Ad-LacZ alone ( $P < 0.01$ , Fig. 1). In cells infected with Ad-PKG II, treated with 8-pCPT-cGMP (250  $\mu$ M, 1 h) and then treated with EGF (100 ng/ml, 24 h), the proliferative activity was markedly inhibited. In cells infected with Ad-LacZ, treated with SNP (1 mM, 2 h) or L-arginine (2 mM, 4 h) and then with EGF (100 ng/ml, 24 h), the proliferation was partially inhibited. However, in cells infected with Ad-PKG II, treated with SNP (1 mM, 2 h) or L-arginine (2 mM, 4 h), and then treated with EGF (100 ng/ml, 24 h), significant inhibition of EGF-induced

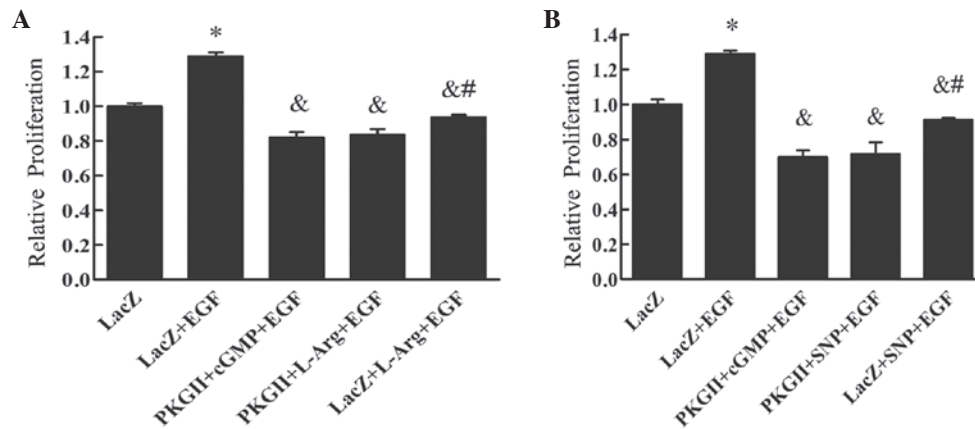


Figure 1. Results of the CCK-8 assay for (A) L-Arg and (B) SNP. AGS cells were infected with either Ad-LacZ or Ad-PKG II, serum-starved for 12 h, then stimulated with 8-pCPT-cGMP (250  $\mu$ M) for 1 h, SNP (1 mM) for 2 h or L-Arg (2 mM) for 4 h, and subsequently treated with EGF (100 ng/ml) for 24 h. The proliferation of the cells was analyzed using the CCK-8 kit and the relative proliferative activities are presented as means  $\pm$  standard deviation of three independent experiments. It was demonstrated that EGF treatment (100 ng/ml for 24 h) promoted cell proliferation (\* $P$ <0.01 compared with the Ad-LacZ group). The proliferative activity of the Ad-PKG II + SNP + EGF and the Ad-PKG II + L-Arg + EGF groups was markedly decreased (\* $P$ <0.01 compared with the Ad-LacZ + EGF group). In addition, the inhibitory effect on proliferation of the Ad-PKG II + SNP + EGF and the Ad-PKG II + L-Arg + EGF groups was more prominent compared with that of the Ad-LacZ + SNP + EGF and Ad-LacZ + L-Arg + EGF groups (\* $P$ <0.05). CCK-8, Cell Counting Kit-8; Ad-LacZ, adenoviral vector encoding  $\beta$ -galactosidase cDNA; Ad-PKG II, adenoviral vector encoding PKG II; L-Arg, L-arginine; SNP, sodium nitroprusside; EGF, epidermal growth factor; cGMP, cyclic guanosine monophosphate; PKG II, type II cGMP-dependent protein kinase.

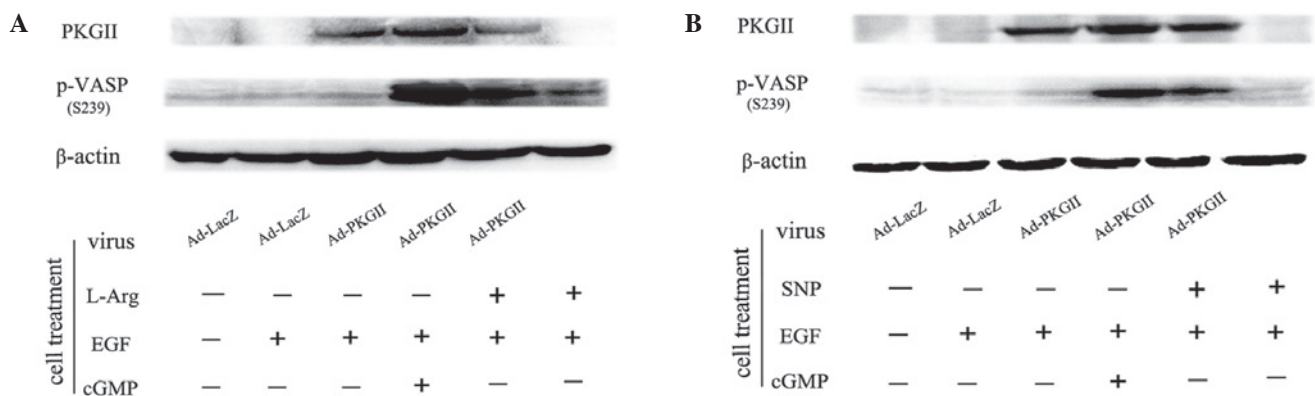


Figure 2. (A) L-Arg and (B) SNP increased the level of p-VASP Ser239. (A) AGS cells were infected with either Ad-LacZ or Ad-PKG II for 24 h and then serum-starved overnight. The Ad-LacZ group received no drug treatment. In the Ad-LacZ + EGF and Ad-PKG II + EGF groups, the cells were treated with EGF (100 ng/ml) for 5 min. In the Ad-PKG II + cGMP + EGF group, the cells were incubated with 8-pCPT-cGMP (250  $\mu$ M) for 1 h and then stimulated with EGF (100 ng/ml) for 5 min. In the Ad-PKG II + L-Arg + EGF and the L-Arg + EGF groups, the cells were treated with L-Arg (2 mM, 4 h) and then with EGF (100 ng/ml) for 5 min. (B) The treatments of the former four groups were same as described in A. In the latter two groups, the cells were incubated with 1 mM SNP for 2 h, followed by EGF (100 ng/ml) for 5 min in the Ad-PKG II + SNP + EGF and the SNP + EGF groups. The cells were harvested and the extracts were subjected to western blotting with the corresponding antibodies, as described in Materials and methods. The results are representative of three independent experiments. L-Arg, L-arginine; p-VASP, phosphorylated vasodilator-stimulated phosphoprotein; Ad-LacZ, adenoviral vector encoding  $\beta$ -galactosidase cDNA; Ad-PKG II, adenoviral vector encoding PKG II; SNP, sodium nitroprusside; PKG II, type II cGMP-dependent protein kinase; EGF, epidermal growth factor; cGMP, cyclic guanosine monophosphate.

proliferation was observed, as was in cells infected with Ad-PKG II, treated with 8-pCPT-cGMP and then with EGF (Fig. 1). These results indicated that, similar to the cGMP analogue 8-pCPT-cGMP, SNP and L-arginine were able to activate PKG II and exert an anti-proliferative effect on AGS cells highly expressing PKG II.

*SNP and L-arginine increase the production of cGMP in AGS cells.* Once NO is released, it activates sGC, thereby increasing the level of cGMP (10,11). In this experiment, ELISA was performed to detect the level of cGMP in differently treated cells. The results demonstrated that, in AGS cells,

the concentration of cGMP was increased by treatment with SNP or L-arginine in a dose- and time-dependent manner, confirming that SNP and L-arginine treatment increases the production of cGMP (Table I).

*SNP and L-arginine increase the level of p-VASP Ser239.* High levels of cGMP may activate PKG II and cause phosphorylation of the substrate protein of this kinase. VASP is a substrate of protein kinases, including PKGs, and its Ser239 residue is a target specific to PKG II (12,13). In this experiment, we analyzed SNP- and L-arginine-induced activation of PKG II by detecting the level of p-VASP Ser239 by western

Table I. SNP and L-arginine treatment increases the production of cGMP in AGS cells.

A, Effect of treatment by dose				
Treatment	Dose (mM)			
	0	1	2	4
SNP (2 h)	18.15±0.3	20.78±0.7 <sup>a</sup>	22.94±0.6 <sup>a</sup>	24.37±0.4 <sup>a</sup>
L-arginine (4 h)	14.97±0.5	22.59±0.8 <sup>a</sup>	26.16±0.8 <sup>a</sup>	28.34±0.4 <sup>a</sup>

B, Effect of treatment by time				
Treatment	Time (h)			
	0	1	2	4
SNP (1 mM)	17.55±0.4	20.93±1.0 <sup>a</sup>	23.26±0.5 <sup>a</sup>	25.10±0.9 <sup>a</sup>
L-arginine (2 mM)	14.06±0.6	20.90±1.1 <sup>a</sup>	24.63±1.0 <sup>a</sup>	27.51±0.6 <sup>a</sup>

Data are presented as means ± standard deviation from three different experiments. <sup>a</sup>P<0.01 vs. 0 mM or 0 h. SNP, sodium nitroprusside; cGMP, cyclic guanosine monophosphate.

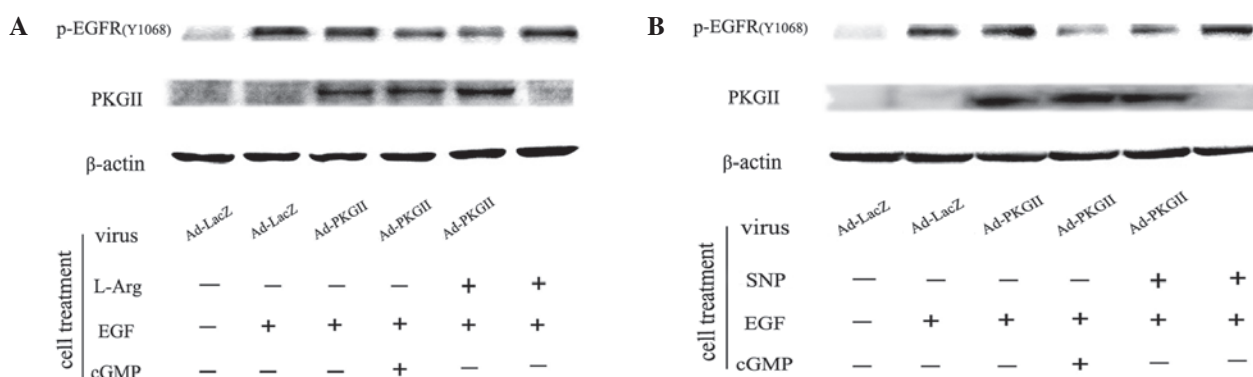


Figure 3. EGF-stimulated Tyr1068 phosphorylation of EGFR is blocked by PKG II activation induced by (A) L-Arg and (B) SNP. The AGS cells were treated as described in Fig. 2. Western blotting with an antibody against p-EGFR (Tyr1068) was used to detect the Tyr1068 phosphorylation of EGFR. The results are representative of three independent experiments. L-Arg, L-arginine; EGF, epidermal growth factor; EGFR, EGF receptor; PKG II, type II cGMP-dependent protein kinase; Ad-LacZ, adenoviral vector encoding  $\beta$ -galactosidase cDNA; Ad-PKG II, adenoviral vector encoding PKG II; SNP, sodium nitroprusside; cGMP, cyclic guanosine monophosphate.

blotting. The results demonstrated that 8-pCPT-cGMP treatment (250  $\mu$ M, 1 h) increased the phosphorylation of VASP at Ser239. Treatment with SNP (1 mM, 2 h) or L-arginine (2 mM, 4 h) also caused a significant increase of Ser239 phosphorylation of VASP (Fig. 2). These results indicated that SNP and L-arginine treatment leads to PKG II activation.

*EGF-induced EGFR Tyr1068 phosphorylation is inhibited by SNP- or L-arginine-induced activation of PKG II.* As a transmembrane tyrosine kinase, EGFR is dimerized and auto-phosphorylated by binding with ligands, such as EGF. Tyr1068 is one of the auto-phosphorylation sites of EGFR associated with the MAPK-mediated signaling pathway (14,15). Our previous results demonstrated that PKG II inhibited EGF-induced Tyr1068 phosphorylation of EGFR (7,8). The present study investigated whether the NO donor/precursor inhibited EGF-induced Tyr1068 phosphorylation of EGFR

through activating exogenous PKG II. The results of the western blotting revealed that, in cells infected with Ad-LacZ and treated with EGF (100 ng/ml, 5 min), the level of Tyr1068 phosphorylation of EGFR increased markedly (Fig. 3). In cells infected with Ad-PKG II and treated with SNP (1 mM, 2 h) or L-arginine (2 mM, 4 h), the EGF-induced increase of Tyr1068 phosphorylation of EGFR was efficiently inhibited. However, in cells without Ad-PKG II infection, treatment with SNP or L-arginine exerted no clear effect on EGF-induced Tyr1068 phosphorylation of EGFR. Therefore, it was confirmed that NO donors and precursors (SNP and L-arginine) inhibit EGF-induced Tyr1068 phosphorylation of EGFR through activating PKG II.

*SNP and L-arginine inhibit EGF-stimulated phosphorylation of ERK1/2 at Thr202/Tyr204.* When EGFR is phosphorylated at Tyr1068, it initiates MAPK/ERK-mediated signaling (16).



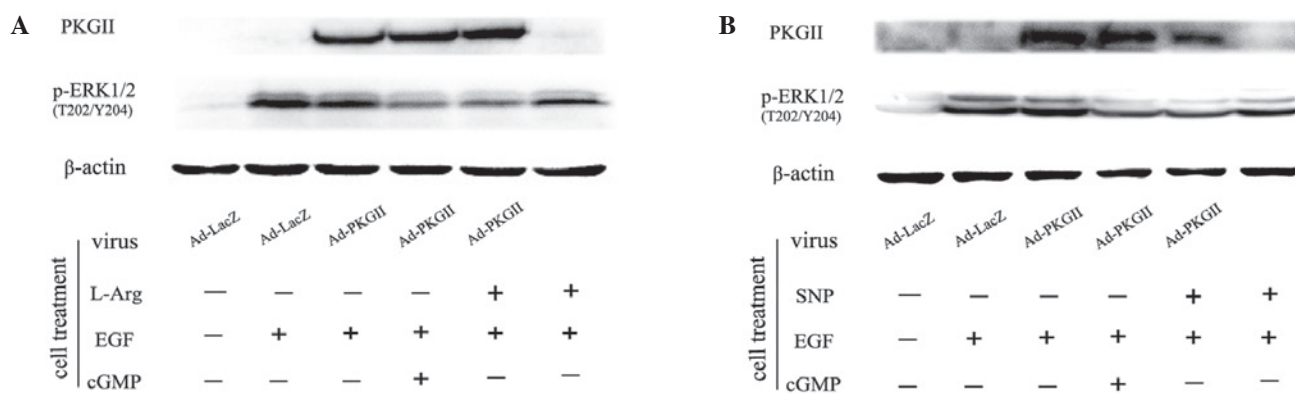


Figure 4. (A) L-Arg and (B) SNP inhibit EGF-stimulated phosphorylation of ERK1/2 at Thr202/Tyr204. The AGS cells were treated as described in Fig. 2. Western blotting with an antibody against p-ERK1/2 (Thr202/Tyr204) was applied to detect the phosphorylation of p-ERK1/2. The results are representative of three independent experiments. L-Arg, L-arginine; EGF, epidermal growth factor; PKG II, type II cGMP-dependent protein kinase; Ad-LacZ, adenoviral vector encoding  $\beta$ -galactosidase cDNA; Ad-PKG II, adenoviral vector encoding PKG II; SNP, sodium nitroprusside; PKG II, type II cGMP-dependent protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; cGMP, cyclic guanosine monophosphate.

Phosphorylation of ERK1/2 at the Thr202/Tyr204 residues is one of the key events of the signaling process of this pathway (17,18). Western blotting with an antibody against p-ERK1/2 (Thr202/Tyr204) was performed to detect the dual phosphorylation of ERK. The results demonstrated that EGF treatment (100 ng/ml, 5 min) caused an obvious increase in the level of p-ERK1/2 (Thr202/Tyr204). However, the phosphorylation was efficiently inhibited by pre-infecting cells with Ad-PKG II and activating the enzyme by treatment with SNP (1 mM, 2 h) or L-arginine (2 mM, 4 h). Furthermore, in cells without Ad-PKG II infection, SNP (1 mM, 2 h) or L-arginine (2 mM, 4 h) treatment also exerted a mild inhibitory effect on EGF-induced p-ERK1/2 (Fig. 4). Therefore, treatment with SNP or L-arginine alone has a mild inhibitory effect on p-ERK1/2. However, when the expression of PKG II was increased by Ad-PKG II infection, SNP and L-arginine treatment completely inhibited EGF-induced phosphorylation of p-ERK1/2 through the activation of PKG II.

## Discussion

NO may be synthesized by three isoforms of NOS, namely neuronal, inducible (in macrophages) and endothelial NOS. Once released, NO diffuses through the cell membrane and enters the cytosol, where it meets the target protein, such as sGC (19,20). NO may bind to the protoporphyrin ring of sGC, causing activation of the enzyme and leading to the formation of cGMP (21,22). As an intracellular signaling molecule, cGMP plays an important role in the regulation of various cellular events and may exert its effects through PKGs, which catalyze the phosphorylation at serine/threonine residues on substrate proteins, thereby altering their activities (23).

In our previous experiments, it was demonstrated that PKG II blocked the EGF-induced activation of EGFR and inhibited EGFR-initiated signal transduction and the proliferation of gastric cancer cells (6,7). To obtain a high activity of PKG II in cancer cells, which usually express this enzyme at a low level, Ad-PKG II was used to increase the expression of PKG II in these cells. The membrane-permeable cGMP analogue 8-pCPT-cGMP was required to activate PKG II. The

present study was designed to identify a reagent to replace 8-pCPT-cGMP.

Since NO induces cGMP production, a NO donor and a NO precursor were selected to increase the content of NO in AGS cells infected with Ad-PKG II, with the intention of using endogenous cGMP to activate PKG II. The results of the ELISA revealed that the NO donor SNP and the NO precursor L-arginine caused a dose- and time-dependent increase of intracellular cGMP in the cells. The effect of SNP and L-arginine on PKG II activity was confirmed by detecting the phosphorylation of VASP, the serine/threonine residues of which are substrates for the PKA and PKG (24). There are three phosphorylation sites of VASP, namely Ser157, Ser239 and Tyr278 (13,25-27). Tao *et al* (13) reported that Ser239 was a key phosphorylation site of VASP for PKG II activation. Therefore, the level of p-VASP Ser239 was detected to reflect PKG II activity. The results demonstrated that, under treatment with cGMP, the level of p-VASP Ser239 was markedly increased in cells pre-infected with Ad-PKG II. Similar to cGMP, SNP and L-arginine also increased the level of p-VASP Ser239, causing PKG II activation in these cells.

In the present study, the inhibitory effects of SNP and L-arginine on EGF-induced proliferative signaling; the proliferation of gastric cancer cells infected with Ad-PKG II was also confirmed. When combined with EGF, EGFR is activated, which then recruits the effectors to its phosphorylated intracellular domain and initiates the downstream protein-mediated signaling. Among the phosphorylation sites, Tyr1068 is associated with the MAPK/ERK pathway (28). It was revealed that cGMP-induced PKG II activation blocks the EGF-induced phosphorylation of EGFR at Tyr1068. The present study revealed that treatment with SNP or L-arginine alone did not cause a distinct inhibition of EGF-induced Tyr1068 phosphorylation of EGFR in cells without Ad-PKG II infection. However, when PKG II was highly expressed following Ad-PKG II infection, SNP or L-arginine were able to efficiently inhibit EGF-induced Tyr1068 phosphorylation of EGFR. The effect of SNP and L-arginine on EGF/EGFR-induced signaling of the MAPK/ERK pathway was then further investigated. The results demonstrated that treatment with SNP or L-arginine

alone exerted a mild inhibitory effect on the EGF-induced Thr202/Tyr204 phosphorylation of ERK1/2, which is a key signaling event of the MAPK/ERK pathway. This was also observed by Sang *et al* (29). However, when combined with Ad-PKG II infection, SNP and L-arginine treatment markedly inhibited the EGF-induced activation of p-ERK1/2, suggesting that SNP and L-arginine-induced NO/cGMP production exerts an effect on the activation of ERK, but not EGFR. However, through the activation of PKG II, SNP and L-arginine exerted inhibitory effects on EGFR and ERK activation and, therefore, exerted more distinct inhibitory effects on proliferative signaling. In conclusion, a NO donor and a NOS substrate may replace 8-pCPT-cGMP and activate PKG II by increasing the level of endogenous cGMP, providing an alternative method of activating this potential cancer inhibitory factor.

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