Endogenous cGMP-dependent protein kinase reverses EGF-induced MAPK/ERK signal transduction through phosphorylation of VASP at Ser239

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Abstract. In our previous study, we demonstrated that type II cGMP-dependent protein kinase (PKG II) was expressed at lower levels in different human cancer cell lines and that exogenous PKG II inhibited epidermal growth factor (EGF)-induced MAPK/ERK signaling. In order to investigate its functions further in this signaling pathway, it is necessary to elucidate whether endogenous PKG has the same effect or not. This study aimed to investigate the possible inhibitory effect of endogenous PKG activity on EGF-induced MAPK/ERK signal transduction in human lung cancer cells and its mechanism. Human small cell lung carcinoma cells (SCLCs) were treated with the PKG-selective cGMP analog 8-pCPT-cGMP to activate endogenous PKG, EGF and cGMP followed by EGF, respectively. The results showed that increased endogenous PKG activity inhibited the EGF-induced phosphorylation of the epidermal growth factor receptor (EGFR) and the binding between Sos1 and Grb2. In addition, EGF-triggered Ras activation was reversed by increased endogenous PKG activity. While the EGF-induced phosphorylation of MEK and ERK were inhibited by increased endogenous PKG activity, there was a significant increase of phosphorylated vasodilator-stimulated phosphoprotein (p-VASP) at Ser239. Furthermore, we investigated whether endogenous PKG exerted its effects on EGF-induced MAPK/ERK signaling through phosphorylation of VASP at Ser239. Downregulation of the levels of p-VASP Ser239 by point mutation blocked the effects of endogenous PKG on EGF-induced MAPK/ERK signal transduction. The data shown here suggest that endogenous PKG reverses the EGF-induced MAPK/ERK signaling pathway by phosphorylating VASP at Ser239.

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Introduction

Epidermal growth factor receptor (EGFR) is a 170 kDa transmembrane tyrosine kinase that belongs to the erbB family of receptor tyrosine kinases and contains three domains: an extracellular domain, a cross-membrane domain and an intracellular domain. Further, the intracellular domain can be divided into three sub-domains: the approximate membrane, tyrosine kinase and C-terminal sub-domain (1). EGFR has been strongly implicated in the biology of human epithelial malignancies, with therapeutic applications in cancers of the colon, head and neck, lung and pancreas (2). Its mechanism of activation relies on receptor dimerization and auto-phosphorylation (3). The activation of EGFR triggers several signal transduction pathways, including the MAPK-mediated signaling pathway (4). Activated EGFR promotes the binding between adapter protein Grb2 and Sos1 (5). The activated Sos1 can lead to the activation of small G protein Ras. Ras proteins act as molecular switches that alternate between inactive GDP-bound and active GTP-bound states. The small GTPase Ras has a prominent role in the signaling pathways leading from activated growth factor receptors to ERKs (6-8).

cGMP-dependent protein kinases (PKGs) are serine/threonine kinases. Two main classes of PKG have been indentified: type I PKG (PKG I) and type II PKG (PKG II) (9,10). PKG II is a membrane-bound enzyme primarily found in the epithelial cells of the intestine (11). PKG phosphorylates target effectors, including vasodilator-stimulated phosphoprotein (VASP), inositol-1,4,5-trisphosphate (IP3) receptor-associated cGMP kinase substrate (IRAG) and thromboxane A2 (TXA2) receptor (12-15). Recent research has revealed that PKGs were associated with the proliferation of tumor cells (16). Recently, our experiments indicated that increased exogenous PKG II activity inhibited the proliferation of gastric cancer cells (17). Our further study showed that the inhibitory effects of exogenous PKG II on EGF-triggered proliferation were associated with its effects on the MAPK/ERK signal trans-

duction pathway (18). In light of this, we carried out further experiments to elucidate whether the endogenous PKG activity is able to reverse the EGF-induced MAPK/ERK signaling pathway, and to investigate its possible mechanism.

Materials and methods

Cell line. The human small cell lung carcinoma (SCLC) cell line was provided by the Institute of Cell Biology (Shanghai, China). The study was approved by the ethics committee of Jiangsu University, Jiangsu, China.

Reagents. Antibodies against p-MEK (Ser217/221) and p-EGFR (Tyr1068) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against pan-Ras, Sos1, Grb2, VASP and p-VASP (Ser239) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against pERK1/2 (Thr202/Tyr204) and GAPDH were from Bioworld Technology Co., Ltd. (St. Louis Park, MN, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). The cellular permeable cGMP analog 8-pCPT-cGMP was from Calbiochem (San Diego, CA, USA). Electrochemiluminescence (ECL) reagent was from Millipore (Billerica, MA, USA). Dulbecco's modified Eagle's medium (DMEM) and new-born calf serum (NBCS) were from Gibco (Invitrogen Life Technologies, Grand Island, NY, USA).

Cloning and transfection. As described by Geese *et al*, mutagenesis of the VASP phosphorylation site Ser239 (S239A) was performed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions (18). The primers used for pMSCV+EGFP-VASP SAT S239A were CTCAGGAAAGTCGCCAAGCAGGAGGAG-GCC (forward) and GGCCTCCTCCTGCTTGGCGACTTT CCT-GAG (reverse). A *Bam*HI restriction site was introduced into the 5-end of the S239A-S primer, and an *Eco*RI restriction site was introduced into the 5-end of the S239A-A primer. The S239A fragment was then subcloned into lentiviral transfer vector FUGW (F, HIV-1 flap sequence; U, human polyubiquitin promoter; G, green fluorescent protein; W, woodchuck hepatitis virus post-transcriptional regulatory element) by *Bam*HI and *Eco*RI double digestion. Recombinant lentiviral particles were produced in 293T cells by transient cotransfection involving a three-plasmid expression system (20), and cells were transfected as described previously (21). The expression levels of p-VASP Ser239 were verified by western blotting analysis.

Ras activity assay. The activity of Ras was detected with the pull-down method as described previously (22). In brief, cells growing on 100-mm culture plates were washed three times with cold phosphate-buffered saline (PBS) and lysed by adding 400 μl of the lysis buffer [25 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP40, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM Na_3VO_4, 10 μg/ml aprotinin and 10 μg/ml leupeptin]. The sample was collected and centrifuged (14,000 x g, 4˚C, 10 min) to get rid of the debris. The supernatant was incubated with glutathione sepharose beads and glutathione S-transferase-Ras-RBD (GST-RBD) at 4˚C for 1 h. The beads were washed 3 times with lysis buffer and heated in boiled water to release proteins. The protein samples (containing active Ras) were analyzed by western blotting with an antibody against pan-Ras.

Co-immunoprecipitation. The cells growing on the 100-mm culture plate were washed twice with cold PBS and lysed by adding 1 ml RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 1 mM EDTA, 1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM Na_3VO_4) per plate. Antibodies against Grb2 and Sos1 and isotype matched IgG were used for immunoprecipitation.

Western blot assay. Sample proteins were separated on SDS-PAGE gels and blotted onto polyvinyl difluoride (PVDF) membrane. The PVDF membrane was blocked with 3% (w/v) bovine serum albumin (BSA) in TBS-T for 1 h at room temperature. The incubation with the primary antibody was at 4˚C overnight, and with the secondary antibody was 1 h at room temperature, with three washes after each incubation. Electrochemiluminescence reagents were used to show the positive bands on the membrane. The bands were detected by Typhoon 9400 (GE Healthcare, Waukesha, WI, USA).

Results

Increased endogenous PKG activity reverses EGF-induced Tyr1068 phosphorylation of EGFR in SCLC cells. Stimulation of EGFR by its natural ligand EGF causes the dimerization and the auto-phosphorylation of tyrosine of the receptors. Tyrosine 1068 (Y1068) is one of the auto-phosphorylation sites of EGFR. Phosphorylation of this site is associated with MAPK-mediated signaling. In this study, western blot assay was applied to investigate the effect of endogenous PKG activity on the phosphorylation of EGFR (Y1068) in EGF-treated SCLC cells. Results showed that EGF treatment notably increased the EGFR phosphorylation at Y1068. In cells with the treatment of cGMP followed by EGF, this phosphorylation was significantly decreased compared with the EGF treatment alone (Fig. 1). This demonstrates that increased endogenous PKG activity inhibited the Y1068 phosphorylation of EGFR induced by EGF.

Increased endogenous PKG activity prevents binding between adaptor protein Grb2 and Sos1 in SCLC cells. The phosphorylated tyrosines on EGFR provide binding sites for adaptor proteins. Among them, phosphorylated Tyr1068 is associated with the activation of the MAPK-mediated signaling pathway. Grb2 is a growth factor receptor-bound protein and Sos1 is a guanine nucleotide exchange factor (GEF). They play a role as adaptor proteins in the MAPK-mediated signaling pathway. The bind between them activates the downstream signal component Ras. Co-immunoprecipitation assay was applied to detect the binding between Grb2 and Sos1. Results showed that with the treatment of EGF, this binding between Grb2 and Sos1 increased. In the cells stimulated with cGMP followed by EGF, this binding decreased more than in cells with treatment of EGF only (Fig. 2). These results revealed that increased

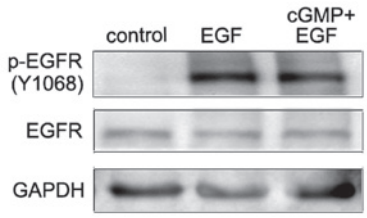

Figure 1. Endogenous PKG inhibits EGF-induced Y1068 phosphorylation of EGFR. Small cell lung cancer cells were serum-starved for 12 h and incubated with EGF (100 ng/ml) for 5 min or treated with 8-pCPT-cGMP (250 μM) for 1 h and then with EGF (100 μg/ml) for 5 min. Cells were harvested and subjected to western blotting. Results showed that EGF treatment induced an increase in the Y1068 phosphorylation of EGFR. cGMP treatment efficiently reversed the EGF-triggered Y1068 phosphorylation of EGFR. Results shown are representative of three separate experiments.

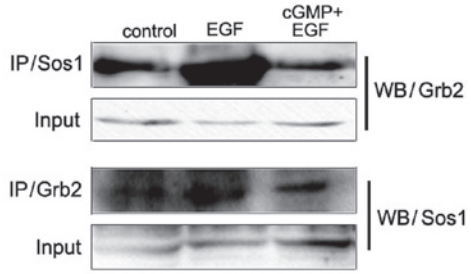

Figure 2. Endogenous PKG prevents EGF-induced binding between Sos1 and Grb2. Small cell lung cancer cells were serum-starved for 12 h and incubated with EGF (100 ng/ml) for 5 min or treated with 8-pCPT-cGMP (250 μM) for 1 h and then with EGF (100 ng/ml) for 5 min. Co-immunoprecipitation was performed to analyze the effect of EGF and endogenous PKG on the binding between Grb2 and Sos1. Results showed that with EGF treatment, the binding level between Grb2 and Sos1 was much higher than that in untreated cells. cGMP treatment significantly inhibited this binding. Results shown are representative of three separate experiments.

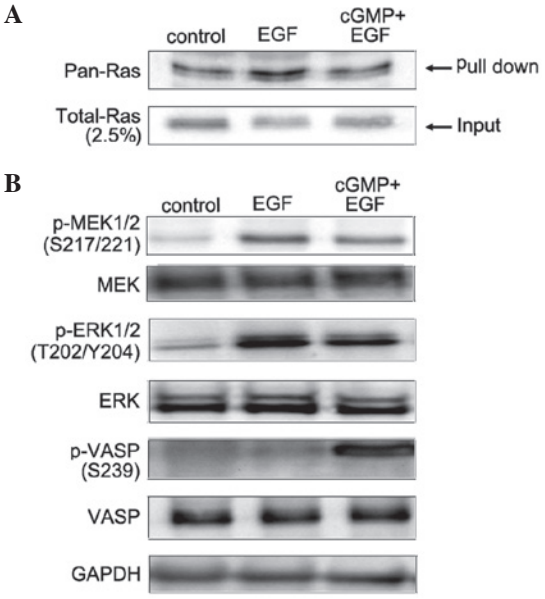

Figure 3. Endogenous PKG inhibits EGF-triggered Ras activation and the phosphorylation of MEK and ERK. Small cell lung cancer cells were serum -starved for 12 h and incubated with EGF (100 ng/ml) for 5 min or treated with 8-pCPT-cGMP (250 μM) for 1 h and then with EGF (100 ng/ml) for 5 min. (A) Pull-down assay was applied to detect the activated Ras. Cell lysate was prepared and equal amounts of protein were incubated with GST-RBD beads as described in Materials and methods. Binding complexes were collected by centrifugation, resolved by SDS-PAGE, and probed with anti-pan Ras antibody. Results showed that EGF treatment caused a notable activation of Ras. cGMP treatment inhibited EGF-induced Ras activation. (B) Cells were harvested and subjected to western blotting. Results showed EGF treatment induced an increase in the phosphorylation of MEK and ERK. cGMP treatment efficiently reversed the EGF-triggered phosphorylation of MEK and ERK, and caused a significant increase of p-VASP Ser239. Results shown are representative of three separate experiments.

endogenous PKG activity inhibited the EGF-induced binding between Grb2 and Sos1.

Increased endogenous PKG activity inhibits EGF-activated Ras and the phosphorylation of MEK and ERK in SCLC cells. Ras is the key component in the MAPK-mediated signaling pathway. Once Ras is in GTP-bound form, it binds and activates Raf-1 and starts the consequent activations of the serine/threonine kinase in the signaling pathway. Raf-1 is a regulator upstream of ERK in MAPK-mediated signal transduction pathway. Activated Raf-1 activates MEK by inducing its phosphorylation of two serine residues at positions 217 and 221. MEK1 and MEK2 are members of the dual specificity protein kinase family, which acts as a MAPK or ERK kinase. Pull-down assay and western blotting were applied to analyze the Ras activity induced by EGF or the combination of cGMP with EGF. Results showed that EGF activates Ras, while the later treatment reverses its effect on Ras activity (Fig. 3A). Furthermore, western blot analysis was applied to detect the phosphorylation of MEK and ERK induced by EGF or the combination of cGMP with EGF. Results indicated that with treatment of EGF alone, the phosphorylation level of both MEK and ERK increased, while with the treatments of cGMP followed by EGF, the phosphorylation level of MEK and ERK notably decreased compared with EGF treatment, and there was a significant increase in the phosphorylation level of VASP at Ser239 (Fig. 3B). These results demonstrate that increased endogenous PKG activity prevents EGF-activated Ras and the phosphorylation of MEK and ERK in SCLC cells.

Downregulating the level of p-VASP Ser239 blocks the effects of endogenous PKG on EGF-induced MAPK/ERK signal transduction. VASP is known to be a substrate for several protein kinases in a variety of cells, including PKG (23). VASP harbors three phosphorylation sites: Ser157, located N-terminally to the central proline-rich region (PRR); and Ser239 and Tyr278, located in the Ena/VASP homology domain 2 (EVH2 domain) (24-26). Since p-VASP Ser239 increased while the phosphorylation level of MEK and ERK decreased in cells treated with cGMP followed by EGF, it was important to investigate whether endogenous PKG exerted its effects on the EGF-induced MAPK/ERK signaling pathway through phosphorylating VASP at Ser239. For the present study, we designed lentivirus-mediated EGFP-VASP Ser239 site mutation (239A) to block its phosphorylation in SCLC cells. The expression of EGFP (green) was used to present the positive infected cells, the infected efficiency was more than 95% (Fig. 4A), and cells stably expressing 239A were named SCLC-239A. Furthermore, western blotting was applied to

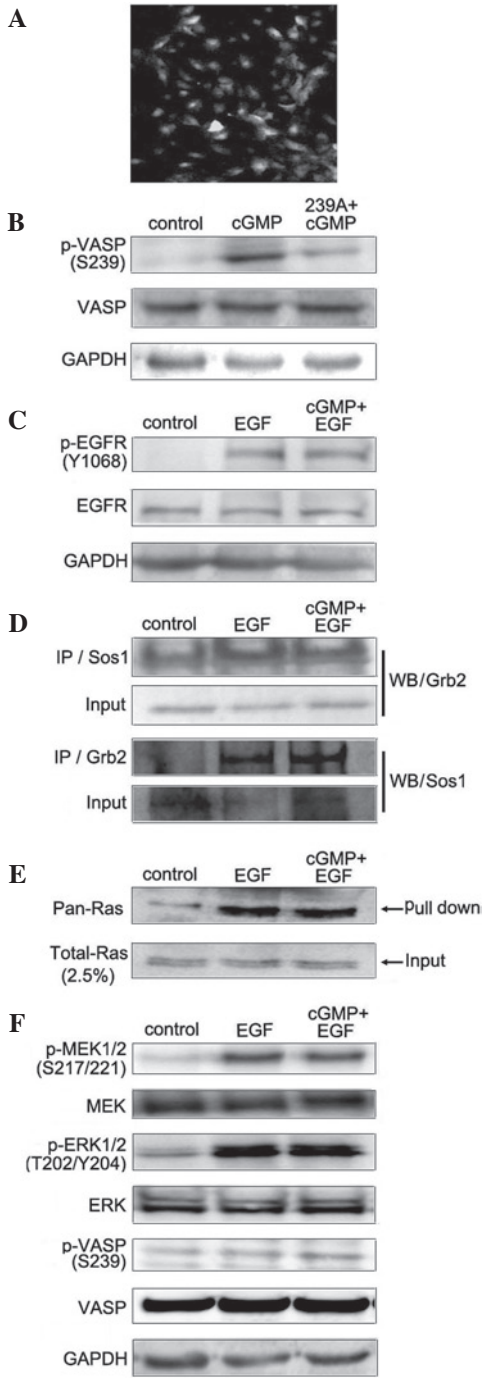

Figure 4. Blocking the phosphorylation of p-VASP S239 attenuates the effects of endogenous PKG on EGF-induced MAPK/ERK signal transduction. Small cell lung cancer cells infected with the lentivirus-mediated EGFP-VASP Ser239 site mutation (239A) were named SCLC-239A. (A) The infected efficiency was indicated by the expression of EGFP (green). (B) SCLC-239A cells were serum-starved for 12 h and treated with 8-pCPT-cGMP (250 μM) for 1 h. Cells were harvested and subjected to western blotting. Results showed that cGMP treatment did not increase p-VASP S239. SCLC-239A were serum-starved for 12 h and incubated with EGF (100 ng/ml) for 5 min or treated with 8-pCPT-cGMP (250 μM) for 1 h and then with EGF (100 ng/ml) for 5 min. Cells were harvested and subjected to western blotting. (C and F) Results showed that EGF treatment induced an increase in the phosphorylation of EGFR, MEK and ERK. cGMP treatment did not reverse the EGF-induced phosphorylation of EGFR, MEK and ERK. (D) Co-immunoprecipitation was used to detect the effect of EGF and endogenous PKG on the binding between Grb2 and Sos1. Results showed that EGF treatment increased the binding level between Grb2 and Sos1. cGMP treatment did not inhibit the EGF-induced binding between Grb2 and Sos1. (E) Pull-down assay was performed to analyze the activated Ras. Results showed that EGF treatment caused a notable activation of Ras. cGMP treatment did not reverse EGF-induced Ras activation.

detect the level of p-VASP Ser239 following treatment with cGMP in SCLC-239A cells. Results showed that increased endogenous PKG activity by cGMP treatment did not trigger the phosphorylation of VASP at Ser239 in SCLC-239A cells (Fig. 4B). Finally, we analyzed the role of p-VASP Ser239 in endogenous PKG activity in the EGF-induced MAPK/ERK signaling pathway. The SCLC-239A cells were applied and the effects of cGMP on EGF-induced MAPK/ERK signaling were measured. Western blotting was applied to detect the effect of endogenous PKG activity on the phosphorylation of EGFR (Y1068) in EGF-treated SCLC-239A cells. The results showed that treatment with cGMP did not inhibit the EGF-induced phosphorylation of EGFR (Y1068; Fig. 4C). In order to analyze the binding between Grb2 and Sos1 in SCLC-239A cells, co-immunoprecipitation assay was applied. Results showed that, compared with EGF treatment alone, there was no detectable alteration on this binding with the treatment of cGMP (Fig. 4D). Pull-down assay and western blotting were applied to detect the Ras activity in SCLC-239A cells. Results showed that cGMP treatment did not inhibit Ras activation induced by EGF (Fig. 4E). Furthermore, western blot analysis was applied to investigate the effects of cGMP on EGF-induced phosphorylation of MEK and ERK in SCLC-239A cells. Results showed that with the treatment of cGMP, there was no detectable decrease in MEK and ERK phosphorylation (Fig. 4F). Together, these data demonstrate that p-VASP Ser239 is critical for endogenous PKG activity in the EGF-induced MAPK/ERK signal transduction pathway.

Discussion

EGFR, a transmembrane tyrosine kinase, can be selectively activated through the binding with ligands belonging to the EGF family of peptide growth factors. Its mechanism of activation depends on the receptor dimerization and auto-phosphorylation. The adaptor proteins bind EGFR at phospho-Tyr1068 and phospho-Tyr1086 and activate the MAP kinase and PI3K/Akt pathways (27,28). The majority of human epithelial cancers are marked by the activation of EGFR, and it was the first growth factor receptor to be proposed as a target for cancer therapy. Anti-EGFR therapies have been recently developed for the treatment of multiple cancer types.

PKG II is membrane-anchored, with high levels in brain and intestinal mucosa and low levels in certain human cancer cell lines (29). Previous research indicated that PKG II was associated with proliferation and apoptosis in certain cells (30,31). Our previous study revealed that exogenous PKG II suppressed EGF-induced MAPK/ERK-mediated signal transduction in gastric cancer cells (18). However, the mechanisms involved remained unknown. In order to investigate its functions further, we focused on the endogenous PKG as well as exogenous PKG. In this study, we aimed to investigate the role of endogenous PKG on EGF-triggered MAPK/ERK signaling, and further focused on its possible mechanism in this signaling pathway.

In the present study, PKG-selective cGMP analog 8-pCPT-cGMP was applied to increase the endogenous PKG activity. Through confirming the effect of EGF on the MAPK/ERK signaling pathway, we analyzed the role of endogenous PKG on the EGF-triggered MAPK/ERK signal transduction pathway. Compared with the EGF treatment

alone, 8-pCPT-cGMP treatment prior to EGF treatment notably blocked EGF-induced alteration, including the phosphorylation of EGFR, MEK and ERK, the binding between Sos1 and Grb2, and the Ras activity. Notably, with the treatment of cGMP followed by EGF, the phosphorylation of EGFR, MEK and ERK decreased, while the phosphorylation of VASP at Ser239 increased. VASP is a major substrate of PKG. In order to test the role of p-VASP Ser239 during this process, we designed lentivirus-mediated EGFP-VASP Ser239 site mutation (239A) to block its phosphorylation in SCLC cells. The results showed that the effects of endogenous PKG activity on EGF-induced MAPK/ERK signaling were notably attenuated. In SCLC-239A cells, with treatment of cGMP to increase the endogenous PKG activity, there was no increase in p-VASP Ser239, and compared with EGF treatment alone, increased endogenous PKG activity did not notably block the effect of EGF on MAPK/ERK signaling.

Our interpretation of the data presented in this study is that increased endogenous PKG activity inhibits the EGF-triggered MAPK/ERK signal transduction pathway, and p-VASP Ser239 plays a critical role. However, the exact role of p-VASP Ser239 in PKG/MAPK/ERK signal transduction requires further investigation.

Acknowledgements

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