Type II cyclic guanosine monophosphate-dependent protein kinase inhibits epidermal growth factor receptor activation in different cancer cell lines

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Abstract. Previous data has revealed that type II cyclic guanosine monophosphate-dependent protein kinase (PKG II) inhibits epidermal growth factor (EGF)-induced phosphorylation/activation of the epidermal growth factor receptor (EGFR) and mitogen-activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK) in gastric cancer cells. The aim of the present study was to determine whether PKG II inhibited EGF-induced phosphorylation/activation of EGFR and MAPK/ERK in cell lines derived from different cancer tissues. SW480, HepG2, OS-RC-2, A549, MCF-7 and U251 cells were transfected with adenoviral constructs encoding PKG II cDNA (Ad-PKG II) to upregulate the expression of PKG II, and then treated with 8-(4-chlorophenylthio)guanosine 3’,5’-cyclic monophosphate (8-pCPT-cGMP) in order to activate the kinase. Western blot analysis was performed to investigate the phosphorylation of EGFR and MAPK/ERK. The results demonstrated that treatment with 100 ng/ml EGF for 5 min increased the tyrosine (Tyr)1068 phosphorylation of EGFR and the threonine 202/Tyr204 phosphorylation of MAPK/ERK. Transfecting the cells with Ad-PKG II, and stimulating the kinases with 8-pCPT-cGMP efficiently inhibited the EGF-induced phosphorylation of EGFR and MAPK/ERK. The results revealed that PKG II had an inhibitory effect upon EGFR activation and the consequent MAPK/ERK-mediated signaling of cell lines derived from the various cancer tissues.

Introduction

Type II cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG II) is a serine-threonine kinase that was first identified >30 years ago. Novel functions of this kinase have been revealed, including its role in regulating cellular proliferation and apoptosis (1-3). In addition, previous studies have demonstrated that PKG II may be a potential inhibitor of factors involved in the pathogenesis of cancer. It has been reported that PKG II can inhibit the proliferation of human neuroglioma and colonic cancer cells (2,4). In addition, previous data has demonstrated that PKG II inhibits the proliferation, migration and associated signal transduction pathways of gastric cancer cell lines through blocking the phosphorylation/activation of the epidermal growth factor receptor (EGFR) and consequent signaling initiated by the activation of this receptor (5,6). Whether or not PKG II demonstrates similar inhibitory effects in other cancer cell lines deserves further investigation. The present study aimed to elucidate whether PKG II inhibits epidermal growth factor (EGF)-induced phosphorylation/activation of EGFR and signaling mediated by mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) in cell lines derived from different cancer tissues, namely the colon cancer SW480 cell line, the hepatic cancer HepG2 cell line, the kidney cancer OS-RC-2 cell line, the lung cancer A549 cell line, the breast cancer MCF-7 cell line and the neuronal cancer U251 cell line.

Materials and methods

Cell lines and reagents. The SW480, HepG2, OS-RC-2, A549, MCF7 and U251 cancer cell lines were obtained from the Institute of Cell Biology (Shanghai, China). In addition, 293A cells were obtained from the Institute of Cell Biology. The adenoviral vectors encoding the cDNA of β-galactosidase (Ad-LacZ) and PKG II (Ad-PKG II) were kindly provided by Dr Gerry Boss and Dr Renate Pilz from the University of California (San Diego, USA). The Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Carlsbad, CA, USA). Polyclonal rabbit anti-human PKG II antibody (1:200) was obtained from Abgent, Inc. (San Diego, CA, USA), and polyclonal rabbit anti-human EGFR antibody (1:200) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Monoclonal mouse anti-human p-EGFR [tyrosine (Tyr)1068; 1:200], polyclonal rabbit anti-human p-ERK1/2 [threonine (Thr)202/Tyr 204; 1:200] and polyclonal rabbit

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anti-human ERK1/2 antibodies (1:200) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Polyclonal horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG secondary antibodies (1:10,000) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). The cellular permeable cGMP analog, 8-(4-chlorophenylthio)guanosine 3',5'-cyclic monophosphate (8-pCPT-cGMP), was obtained from EMD Millipore (Billerica, MA, USA). The recombinant human EGF was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA) and the electro-chemiluminescence (ECL) reagents were obtained from EMD Millipore. All other reagents used were of analytical grade.

Preparation of adenoviral vectors. 293A cells were transfected with an adenoviral vector encoding either PKG II or LacZ. Next, the cells were cultured for up to 10 days until a cytopathic effect was observed. The cells and culture medium were then harvested and subjected to three cycles of freeze-thawing. The supernatants, containing Ad-LacZ and Ad-PKG II, were then used to transfect new 293A cells and amplify the adenoviruses. Finally, the amplified adenoviral preparations were titrated and the number of plaque-forming units/ml was determined. The preparations were then kept at -80°C until use.

Cell culture and infection with adenoviral vectors. The SW480, HepG2, OS-RC-2, A549, MCF7 and U251 cells were cultured in DMEM supplemented with 10% FBS and maintained at 37°C in a humidified incubator with 95% air and 5% CO₂. Fresh medium was supplied every two days, and the cells were sub-cultured upon reaching confluence. On the day prior to transfection, the cells were freshly plated at 70-80% confluence.

Cell transfection. To investigate the inhibitory effect of PKG II on cancer cells, the SW480, HepG2, OS-RC-2, A549, MCF7 and U251 cells were transfected with Ad-PKG II to increase the expression of PKG II or Ad-LacZ as a control, and treated with 8-pCPT-cGMP to activate the kinase and EGF to activate EGFR and ERK. To perform viral transfection, the SW480, HepG2, OS-RC-2, A549, MCF7 and U251 cells (1x10⁶ cells/well) were seeded onto six-well culture plates. After 24 h, the cells were transfected with Ad-LacZ or Ad-PKG II with a multiplicity of infection of 100%. To obtain the control group, cells were transfected with LacZ for 48 h then serum starved overnight. To obtain Ad-LacZ + EGF and PKG II + EGF groups, the cells were transfected with Ad-LacZ and Ad-PKG II, respectively, for 48 h, serum starved overnight and incubated with 100 ng/ml EGF for 5 min. To obtain Ad-LacZ + cGMP + EGF and Ad-PKG II + cGMP + EGF groups, the cells were transfected with Ad-LacZ and Ad-PKG II respectively, for 48 h, starved of serum overnight, treated with 8-pCPT-cGMP (100 μM or 250 μM) for 1 h, then treated with 100 ng/ml EGF for 5 min. Next, the cells were harvested and the protein extracts were prepared.

Western blotting. The whole cell protein extracts were separated using 10% SDS-PAGE and then transferred onto a polyvinylidene fluoride membrane. Primary antibodies were then incubated with the membrane at 4°C overnight. Next, the corresponding secondary antibodies were incubated at room temperature for 1 h. In total, three washes were performed following each incubation. The ECL reagents were used to observe the positive bands on the membrane. To perform densitometry analysis, digital images (gray levels) of the positive bands were obtained using the ChemiDoc XRS acquisition system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and analyzed using Quantity One 1-D Analysis software (Bio-Rad Laboratories, Inc.). The results were expressed as the ratio of gray levels of target protein/loading control protein.

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical analysis was performed using two-tailed analysis of variance. All statistical analyses were performed using SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

EGFR and ERK phosphorylation. The binding of EGF to EGFR causes autophosphorylation of the receptor. A number of autophosphorylation sites exist, which are linked to several signal transduction pathways. Tyr1068 is an EGFR autophosphorylation site that is associated with MAPK/ERK-mediated signaling, and that is closely associated with the proliferation and differentiation of cells (7). ERK is a key component of this pathway, and phosphorylation at the Thr202 and Tyr204 residues of ERK1 and the Thr185 and Tyr187 residues of ERK2 is required for complete activation of the enzyme. The present study used western blot analysis to investigate the inhibitory effect of PKG II on the EGF-induced Tyr1068 phosphorylation of EGFR, and the Thr202 and Tyr204 phosphorylation of ERK1/2 in different cell lines.

PKG II inhibits EGF-induced phosphorylation of EGFR and ERK1/2 in SW480 cells. The SW480 cell line was established from a primary adenocarcinoma of the colon from a 50-year-old male (8). In order to investigate the effect of PKG II, the cells were transfected with Ad-PKG II to increase the expression of PKG II, treated with 250 μM 8-pCPT-cGMP for 1 h to activate the kinase, and then treated with 100 ng/ml EGF for 5 min. Next, the cells were harvested and the protein extracts were prepared and subjected to western blotting with antibodies against p-EGFR (Tyr1068) and p-ERK1/2 (Thr202/Tyr204). The results revealed that in the cells transfected with Ad-PKG II and stimulated with 8-pCPT-cGMP, the expression of PKG II was markedly increased compared with the Ad-LacZ control group and the EGF-induced phosphorylation of EGFR and ERK1/2 was significantly decreased compared with the Ad-LacZ + EGF group (Fig. 1). This indicated that PKG II could inhibit the EGF-induced phosphorylation/activation of EGFR and ERK1/2.

PKG II inhibits EGF-induced phosphorylation of EGFR and ERK1/2 in HepG2 cells. This HepG2 cell line was established following explant culture of hepatocellular carcinoma tissue from a 15-year-old male (9). The cells were treated in the same manner as the SW480 cells. The results revealed that transfection with Ad-PKG II caused an evident increase in the expression of PKG II (Ad-PKG II groups) when compared with the Ad-LacZ control group. Treatment with 100 ng/ml EGF for 5 min caused marked increases in the phosphorylation of EGFR and ERK1/2.
compared with the control EGF-negative group. In cells transfected with Ad-PKG II and stimulated with 8-pCPT-cGMP, EGF-induced phosphorylation of EGFR and ERK (Ad-LacZ + EGF group) compared with the control EGF-negative (Ad-LacZ) group. Treatment with cGMP efficiently inhibited the EGF-induced phosphorylation of EGFR and ERK (Ad-PKG II + cGMP + EGF group) compared with the Ad-LacZ + EGF group (C). Ratio of p-EGFR/EGFR gray levels. *P<0.05 vs. Ad-LacZ control group. **P<0.05 vs. Ad-LacZ + EGF, Ad-PKG II + cGMP + EGF and PKG II + EGF groups. The results are representative of three separate experiments. cGMP, cyclic guanosine monophosphate; PKG II, type II cGMP-dependent protein kinase; EGFR, epidermal growth factor; EGFR, EGF receptor; Ad-LacZ/PKG II, adenoviral vectors encoding β-galactosidase/PKG II cDNA.

**PKG II inhibits EGF-induced phosphorylation of EGFR and ERK1/2 in OS-RC-2 cells.** The OS-RC-2 cell line was established from a renal cell carcinoma of a 52-year-old male (10). The inhibitory effect of PKG II on the phosphorylation of EGFR and ERK1/2 was investigated in the OS-RC-2 cells. The results revealed that transfection with Ad-PKG II caused an evident increase in the expression of PKG II (Ad-PKG II groups) compared with the Ad-LacZ control group. Transfection with EGF induced a marked increase in the phosphorylation of EGFR and ERK (Ad-LacZ + EGF group) compared with the control EGF-negative (Ad-LacZ) group. Treatment with cGMP efficiently inhibited the EGF-induced phosphorylation of EGFR and ERK (Ad-PKG II + cGMP + EGF groups) compared with the Ad-LacZ + cGMP group. (D) Ratio of p-EGFR/EGFR gray levels. (E) Ratio of p-ERK/ERK gray levels. *P<0.05 vs. Ad-LacZ control group. **P<0.05 vs. Ad-LacZ + EGF, Ad-PKG II + cGMP + EGF and PKG II + EGF groups. The results are representative of three separate experiments. cGMP, cyclic guanosine monophosphate; PKG II, type II cGMP-dependent protein kinase; EGFR, epidermal growth factor; EGFR, EGF receptor; Ad-LacZ/PKG II, adenoviral vectors encoding β-galactosidase/PKG II cDNA.
**PKG II inhibits EGF-induced phosphorylation of EGFR and ERK1/2 in A549 cells.** The A549 cell line was established in 1972 by Giard et al. (11) through explant culture of lung carcinomatous tissue from a 58-year-old male. The inhibitory effect of PKG II on the phosphorylation of EGFR and ERK (Ad-LacZ + EGF group) compared with the control EGF-negative (Ad-LacZ) group. Transfection with Ad-PKG II + cGMP efficiently inhibited the EGF-induced phosphorylation of EGFR and ERK (Ad-PKG II + cGMP + EGF groups) compared with the Ad-LacZ + EGF group. (B) Ratio of p-EGFR/EGFR gray levels. (C) Ratio of p-ERK/ERK gray levels. *P<0.05 vs. Ad-LacZ control group. †P<0.05 vs. Ad-LacZ + EGF, Ad-PKG II + cGMP + EGF and PKG II + EGF groups. The results are representative of three separate experiments. cGMP, cyclic guanosine monophosphate; PKG II, type II cGMP-dependent protein kinase; EGF, epidermal growth factor; EGFR, EGF receptor; Ad-LacZ/PKG II, adenoviral vectors encoding β-galactosidase/PKG II cDNA.

**PKG II inhibits EGF-induced phosphorylation of EGFR and ERK1/2 in MCF7 cells.** The MCF7 cell line was established through explant culture of breast adenocarcinoma tissue from a 69-year-old female (12). The inhibitory effect of PKG II on the phosphorylation of EGFR and ERK (Ad-LacZ + EGF group) compared with the control EGF-negative (Ad-LacZ) group. Transfection with Ad-PKG II + cGMP efficiently inhibited the EGF-induced phosphorylation of EGFR and ERK (Ad-PKG II + cGMP + EGF groups) compared with the Ad-LacZ + EGF group. (B) Ratio of p-EGFR/EGFR gray levels. (C) Ratio of p-ERK/ERK gray levels. *P<0.05 vs. Ad-LacZ control group. †P<0.05 vs. Ad-LacZ + EGF, Ad-PKG II + cGMP + EGF and PKG II + EGF groups. The results are representative of three separate experiments. cGMP, cyclic guanosine monophosphate; PKG II, type II cGMP-dependent protein kinase; EGF, epidermal growth factor; EGFR, EGF receptor; Ad-LacZ/PKG II, adenoviral vectors encoding β-galactosidase/PKG II cDNA.
that transfection with Ad-PKG II (Ad-PKG II group) caused an evident increase in PKG II expression compared with the control EGF-negative group. Transfection with Ad-PKG II + cGMP efficiently inhibited the EGF-induced phosphorylation of EGFR and ERK (Ad-PKG II + cGMP + EGF groups) compared with the Ad-LacZ + EGF group. The results are representative of three separate experiments. cGMP, cyclic guanosine monophosphate; PKG II, type II cGMP-dependent protein kinase; EGF, epidermal growth factor; EGFR, EGF receptor; Ad-LacZ/PKG II, adenoviral vectors encoding β-galactosidase/PKG II cDNA.

PKG II inhibits EGF-induced phosphorylation of EGFR and ERK1/2 in MCF-7 cells. MCF-7 cells were treated as described in the Materials and methods section. (A) Western blotting revealed that treatment with EGF induced a marked increase in the phosphorylation of EGFR and ERK (Ad-LacZ + EGF group) compared with the control EGF-negative group. Transfection with Ad-PKG II + cGMP efficiently inhibited the EGF-induced phosphorylation of EGFR and ERK (Ad-PKG II + cGMP + EGF groups) compared with the Ad-LacZ + EGF group. (B) Ratio of p-EGFR/EGFR gray levels. (C) Ratio of p-ERK/ERK gray levels. *P<0.05 vs. Ad-LacZ control group. **P<0.05 vs. Ad-LacZ + EGF, Ad-PKG II + cGMP + EGF and PKG II + EGF groups. The results are representative of three separate experiments. cGMP, cyclic guanosine monophosphate; PKG II, type II cGMP-dependent protein kinase; EGF, epidermal growth factor; EGFR, EGF receptor; Ad-LacZ/PKG II, adenoviral vectors encoding β-galactosidase/PKG II cDNA.

PKG II inhibits EGF-induced phosphorylation of EGFR and ERK1/2 in U251 cells. The U251 cell line was established through explant culture of glioblastoma (astrocytoma) tissue from a 75 year-old male (13). The inhibitory effect of PKG II on the phosphorylation of EGFR and ERK1/2 in the U251 cells was investigated by western blotting. The results revealed that transfection with Ad-PKG II (Ad-PKG II group) caused an evident increase in the phosphorylation of EGFR and ERK (Ad-LacZ + EGF group) compared with the control EGF-negative group. Transfection with Ad-PKG II + cGMP efficiently inhibited the EGF-induced phosphorylation of EGFR and ERK (Ad-PKG II + cGMP + EGF groups) compared with the Ad-LacZ + EGF group. (A) Western blotting revealed that treatment with EGF induced an evident increase in the phosphorylation of EGFR and ERK (Ad-LacZ + EGF group) compared with the control EGF-negative group. Transfection with Ad-PKG II + cGMP efficiently inhibited the EGF-induced phosphorylation of EGFR and ERK (Ad-PKG II + cGMP + EGF groups) compared with the Ad-LacZ + EGF group. (B) Ratio of p-EGFR/EGFR gray levels. (C) Ratio of p-ERK/ERK gray levels. *P<0.05 vs. Ad-LacZ control group. **P<0.05 vs. Ad-LacZ + EGF, Ad-PKG II + cGMP + EGF and PKG II + EGF groups. The results are representative of three separate experiments. cGMP, cyclic guanosine monophosphate; PKG II, type II cGMP-dependent protein kinase; EGF, epidermal growth factor; EGFR, EGF receptor; Ad-LacZ/PKG II, adenoviral vectors encoding β-galactosidase/PKG II cDNA.
and incubated with EGF (Ad-PKG II + cGMP + EGFR group), the EGF-induced phosphorylation of EGFR and ERK1/2 was significantly decreased compared with the Ad-LacZ + EGFR group (P<0.05; Fig. 6). This indicated that PKG II inhibited the EGF-induced phosphorylation/activation of EGFR and ERK1/2 of glioblastoma cells.

Discussion

EGFR is a member of the ErbB receptor tyrosine kinase family, which includes ErbB1 (EGFR), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4). EGFR is a 170-kDa protein composed of extracellular, transmembrane and cytoplasmic domains (14). The activation of EGFR depends upon ligand binding and the subsequent dimerization and autophosphorylation of the receptor. Following activation, EGFR recruits effector proteins to its phosphorylated C-terminal sub-domain and initiates effector protein-mediated signaling mechanisms. Previous data indicate that EGFR is closely associated with tumorigenesis, and that overexpression and mutation of EGFR occurs in the majority of cancers (15). In vitro experiments have confirmed that preventing the activation of EGFR can inhibit the proliferation of certain tumor cells (16). In addition, a previous clinical study revealed that cancer patients with overexpressed EGFR often exhibit poorer prognoses (17). EGFR is therefore a potential therapeutic target for the treatment of cancer. Furthermore, techniques that inhibit EGFR activity and its associated signal transduction pathways have been extensively researched, including the use of specific antibodies against EGFR, and the application of agents that inhibit the function of EGFR (18).

PKG II was at first believed to be implicated in certain physiological functions, including learning and memory, intestinal secretion and bone growth (19). However, novel functions of PKG II have been identified, including an involvement in mechano-signal transduction in osteoblasts, and in the regulation of sodium channels (20,21). In addition, previous studies have indicated that PKG II may be a potential cancer suppressor due to its role in the regulation of proliferation and apoptosis in cancer cells (22,23). Previous studies have demonstrated that PKG II inhibits the EGF-induced Tyr1068 phosphorylation of EGFR and the Thr202/Tyr204 phosphorylation of ERK1/2 in gastric cancer cells (22,24). The present study aimed to determine whether PKG II inhibits the EGF-receptor kinase activity and its associated signal transduction pathways have been extensively researched, including the use of specific antibodies against EGFR, and the application of agents that inhibit the function of EGFR (18).

PKG II is a type II cGMP-dependent protein kinase that regulates proliferation and differentiation in the colonic mucosa. Am J Physiol Gastrointest Liver Physiol 303: G209-G219, 2012.

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