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Protein Kinases Type II (PKG II) Combined with L-Arginine Significantly Ameliorated Xenograft Tumor Development: Is L-Arginine a Potential Alternative in PKG II Activation?

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The mammalian cyclic guanosine monophosphate (cGMP)-dependent protein kinases type II (PKG II) plays critical physiological or pathological functions in different tissues. However, the biological effects of PKG II are dependent on cGMP. Published data indicated that L-arginine (L-Arg) promoted NO production, NO can activate soluble guanylate cyclase (sGC), and catalyzes guanosine triphosphate (GTP) into cGMP, which suggested L-Arg could activate PKG II. Therefore, the present work was performed to address: (i) whether L-Arg could be a potential alternative in PKG II activation, and (ii) whether L-Arg also contributes to PKG II against cancer.

Material/Methods: Nude BALB/c mice were inoculated with human MCF-7, HepG2, and SW480 cell lines via subcutaneous (s.c.) injecting. After 7 days of inoculation, Ad-PKG II was injected into the cancer tissues every 4 days, and the next day 10 μmol/mouse L-Arg was administered. Western blotting and immunohistochemistry were used to assess protein expression.

Results: Our results demonstrated that L-Arg significantly activated PKG II and effectively ameliorated xenograft tumor development through inhibiting cancer growth, angiogenesis, and metastasis, which was partially dependent on blocking of epidermal growth factor receptor (EGFR) activity, as well as downstream signaling pathways such as Erk1/2.

Conclusions: Our results provide an exciting new insight: L-Arg is a potential alternative to PKG II activation.

MeSH Keywords: Arginine • Colorectal Neoplasms, Hereditary Nonpolyposis • Inflammatory Breast Neoplasms • Vascular Endothelial Growth Factor, Endocrine-Gland-Derived

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Background

The mammalian cyclic guanosine monophosphate (cGMP)-dependent protein kinases (PKGs) belong to the family of serine/threonine kinases, and have 2 types of isoforms in mammalian cells: PKG I and PKG II [1,2]. PKG I is more extensively expressed [3] and is involved in diverse pathological or physiological processes [4–6]. Membrane-bound PKG II is a crucial regulator of intestinal secretion, bone growth, renin secretion, and circadian rhythms. It is highly expressed in the intestinal mucosa, kidney, chondrocytes, and brain [7]. PKG II can phosphorylate several downstream substrates, including Src homology 2 domain-containing tyrosine phosphatases and cystic fibrosis transmembrane conductance regulator (CFTR). PKG II is also known as a major regulator of CFTR and AMPA receptor trafficking [8–11]. Our previous data also indicated that PKG II could inhibit cancer cell proliferation and migration as well as promote cancer cells apoptosis [12–15]. All the effects of PKG II are dependent on its activation by cGMP or cGMP analog [16]. PKG II activation phosphorylates specific downstream signaling proteins in a cyclic nucleotide-dependent manner [17]. Recently, the presence of noncanonical cyclic nucleotides cCMP and cUMP in eukaryotic cells has been proven, and they can activate PKGs [18]. Whether other agonists or alternatives exist remains unclear.

Nitric oxide (NO) is synthesized by NO-synthase (NOS) in many tissues and plays critical physiological or pathological functions via activating soluble guanylyl cyclases (sGC). NO converts guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP) and then increases the intracellular concentration of cGMP. Furthermore, L-Arg is an NOS substrate, and L-Arg can also activate PKG II as a potential alternative. Therefore, the present work was performed to address: (i) whether L-Arg could be a potential alternative for PKG II activation, and (ii) whether L-Arg also contributes to the effect of PKG II against cancers. Our results demonstrated that L-Arg obviously activated PKG II [19] and effectively ameliorated xenograft tumor development through inhibiting cancer growth and metastasis, which was partially dependent on inhibition of epidermal growth factor receptor (EGFR) activity as well as its mediated-signal pathway. Our results provided an exciting insight: L-Arg is a potential alternative in PKG II activation.

Material and Methods

Mice and cell lines

Nude BALB/c female mice (6 weeks old) were obtained from the Slac Laboratory Animal House and maintained in the Animal Center of Jiangsu University in compliance with the Guide for the Care and Use of Laboratory Animals [NIH, 76 FR 91 (May 11, 2011)]. The experimental protocol was approved by the Jiangsu University Ethics Committee. The human cancer cell lines MCF-7, HepG2, and SW480 were obtained from the Institute of Cell Biology (Shanghai, China) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) in a humidified atmosphere of 5% CO_2 at 37°C. All DNA profiles for the aforementioned cell lines were counterchecked.

Reagents

Adenoviral vectors encoding β-galactosidase (Ad-LacZ) and PKG II (Ad-PKG II) were kind gifts from Dr Gerry Boss and Dr Renate Pilz, University of California (San Diego, CA, USA). DMEM and FBS were obtained from Gibco (Grand Island, NY, USA). The antibody against PKG II was obtained from Abgent Biotechnology (San Diego, CA). Phospho-antibodies against Erk1/2, EGFR, Akt, and corresponding total antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). The antibody against MMP-2, MMP-7, CD105, and β-actin were obtained from Santa Cruz (Dallas, TX, USA).The electrochemiluminescence (ECL) reagents were purchased from Millipore (Billerica, MA, USA).

Tumor inoculation and treatment

Nude mice were inoculated in the flank with 1×10^7 MCF-7, HepG2, and SW480 cells via subcutaneous (s.c.) injection. After 7 days of inoculation, 1×10^9 pfu Ad-PKG II or Ad-LacZ was injected into the cancer tissues every 4 days, and the next day we injected 10 μmol/mouse L-Arg into the tumors. After 29 days, the cancer-bearing mice were killed.

Immunohistochemistry

Formalin-fixed paraffin-embedded tumor tissue was used for immunohistochemical analysis following a previously published method [20].The antibody against PCNA was obtained from Santa Cruz (Dallas, TX, USA).

Western blotting

Whole-cell lysates were prepared using the Total Protein Extraction Kit (KeyGEN BioTECH, Shanghai, China). Equal amounts of protein were separated by 8–12% SDS-PAGE before being transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked by 5% non-fat dried milk in tris-buffered saline (TBS) for 1 h and incubated with specific primary antibodies against p-EGFR (Y1068), p-Erk1/2 (T202/Y204), CD105, p-Akt (T308), MMP-2, MMP-7, or β-actin overnight at 4°C. After washing, HRP-labeled secondary antibodies were added for 1 h at 37°C. Detection was performed by ECL.

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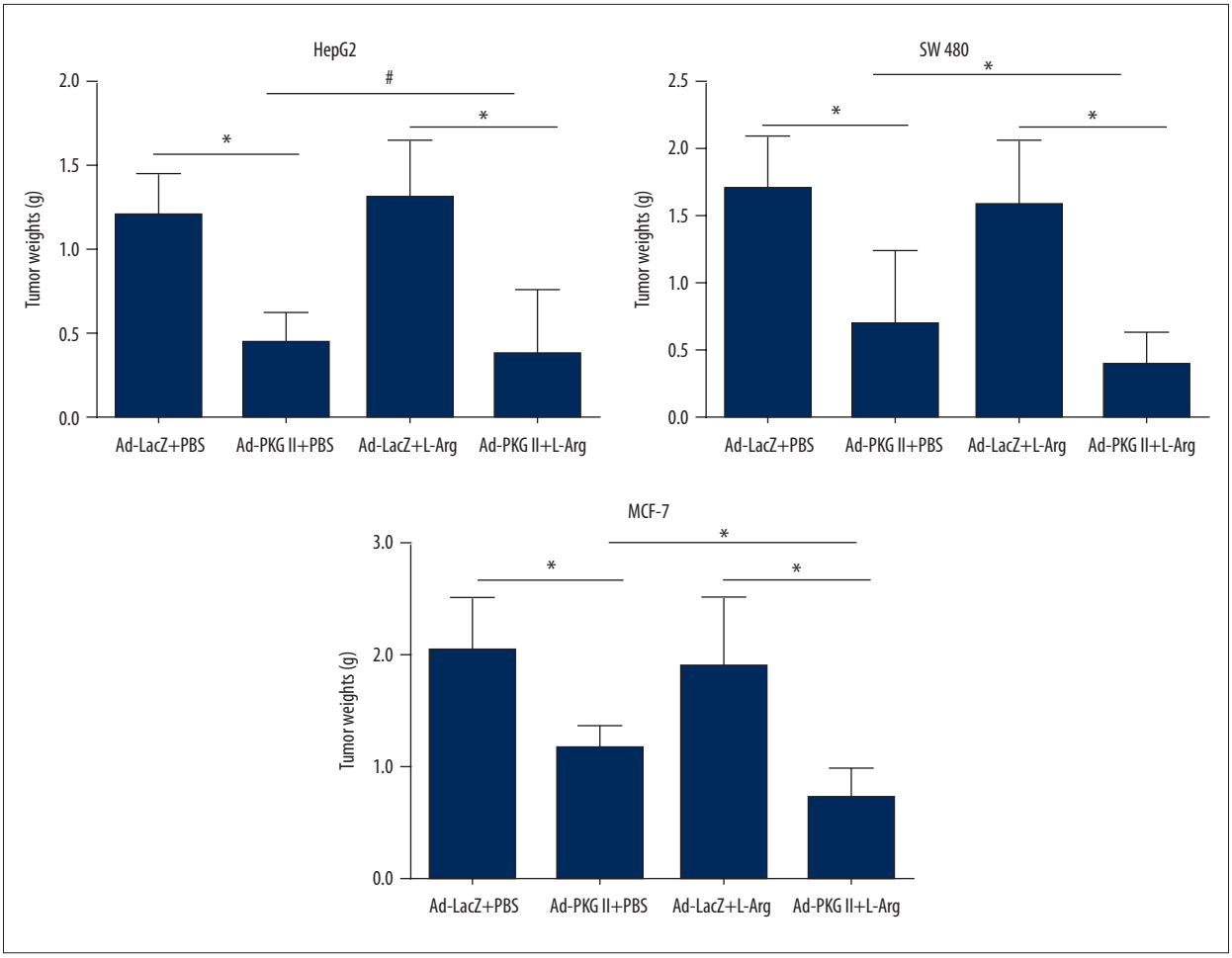

Figure 1. PKG II combined with L-Arg obviously decreased xenograft tumor weights. We injected 100 ml (10^{10} pfu/ml) adenovirus into MCF-7-, SW480-, and HepG2-bearing BALB/c mice via s.c. every 4 days, and the next day we injected 10 μmol/mouse L-Arg into the tumors. After 29 days, the mice were sacrificed by cervical dislocation, and the tumors were rapidly separated and collected. Data are means ± SD from 8 mice. (* $p<0.05$, # $p>0.05$.)

Statistical analysis

All statistical analyses were performed using Graph Pad Prism (GraphPad Software Inc., La Jolla, CA, USA). Data are expressed as the mean ± standard deviation (SD). One-way ANOVA with Bonferroni correction was used to determine statistical significance. For all tests, $p<0.05$ was considered statistically significant.

Results

PKG II combined with L-Arg obviously ameliorated xenograft tumor development

To determine whether L-Arg could activate PKG II and ameliorate the cancer development, cancer-bearing mice models of breast, liver, and colon were constructed. As Figure 1 shown, in the Ad-PKG II+L-Arg group, tumor weights were significantly decreased comparing with the Ad-LacZ+L-Arg group among MCF-7-, HepG2-, and SW480-bearing mice (* $p<0.05$). Similar data were also obtained from the Ad-PKG II+PBS and Ad-LacZ+PBS groups (* $p<0.05$). Furthermore, there were also obvious differences between the Ad-PKG II+L-Arg and Ad-PKG II+PBS groups in MCF-7- and SW480-bearing mice. However, there were no differences between the Ad-PKG II+L-Arg and Ad-PKG II+PBS groups in HepG2-bearing mice (# $p>0.05$). Taken together, the data indicate that the inhibitory effects of PKG II were partially dependent on L-Arg.

PKG II combined with L-Arg significantly inhibited proliferation- and metastasis-associated proteins expression in cancer-bearing mice

Next, to analyze the potential mechanism of PKG II combined with L-Arg against breast and colon cancers, proliferating cell

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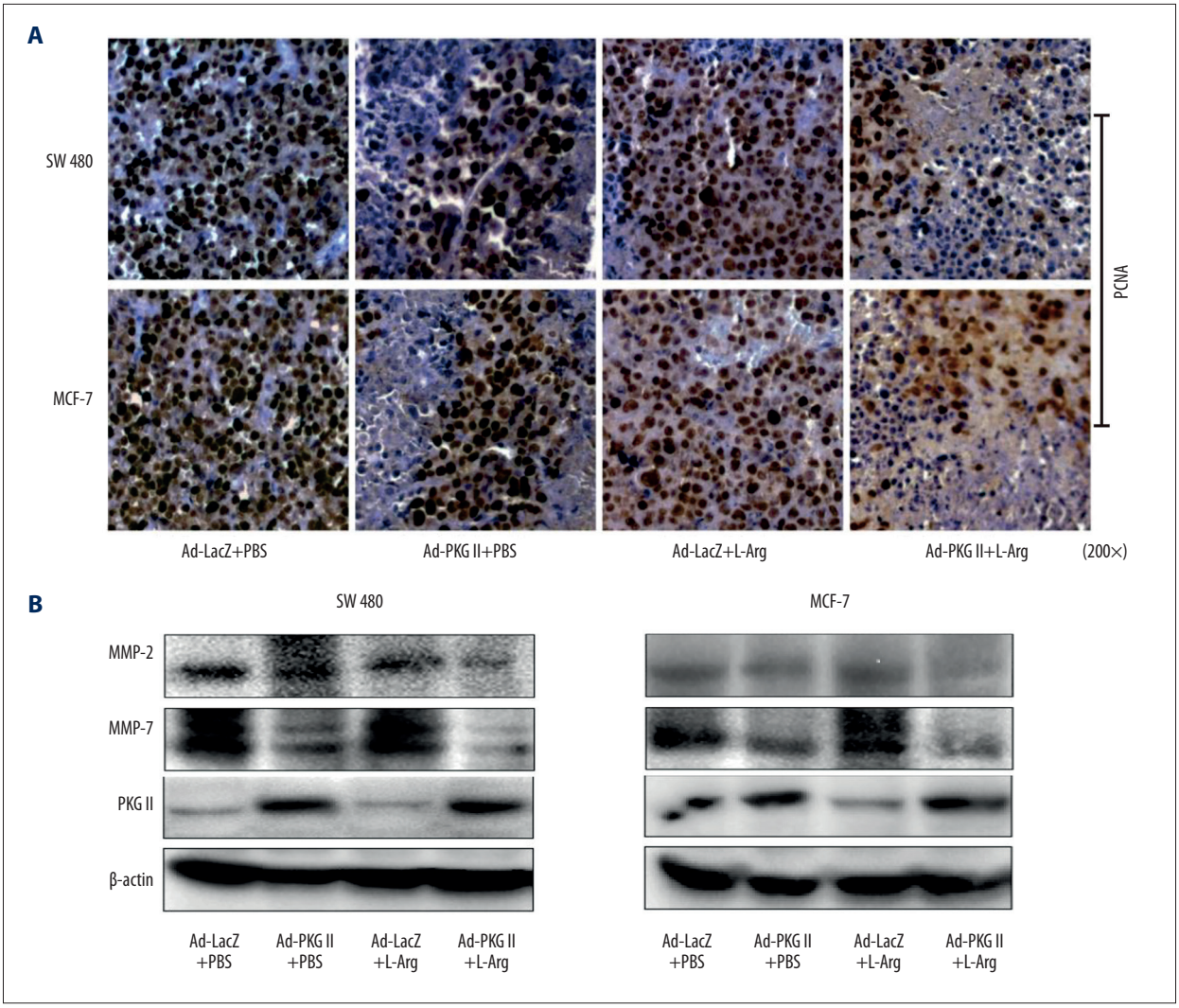

Figure 2. PKG II combined with L-Arg significantly inhibited the expression of proliferation and metastasis associated proteins in cancer-bearing mice. The interventions of cancer bearing mice and the operations are shown in Figure 1. (**A**) Representative photomicrographs of immunohistochemistry detection of PCNA in tumor tissues. PCNA protein was mainly shown in nuclei. Brown, PCNA staining; blue, nuclear staining. (**B**) MMP-2 and 7 levels in tumor. The tumor tissue was extracted. Western blotting was used to analyze the MMP-2 and -7 expressions. β-actin was examined as a loading control. The experiment was repeated 3 times and similar data were obtained. Representative blots are shown.

nuclear antigen (PCNA), a hallmark of representative cancer proliferation [21], was detected. Immunohistochemistry showed that Ad-PKG II combined with/without L-Arg both down-regulated PCNA expressions compared with their correspondence control groups (Ad-LacZ as well as Ad-LacZ+L-Arg group, respectively); of course, the L-Arg treated group more effectively down-regulated PCNA expression compared to the untreated group (Figure 2A).

The metastasis-associated proteins matrix metalloproteinase (MMP)-2 and MMP-7 were also detected. As Figure 2B shows, Ad-PKG II combined with/without L-Arg both decreased MMP-2 and -7 expressions compared with their corresponding control groups (the Ad-LacZ and Ad-LacZ+L-Arg group, respectively). However, there was no obvious decrease of MMP-2 and -7 expressions in the L-Arg-treated group compared with the untreated group (Ad-LacZ+PBS & Ad-LacZ+L-Arg; Ad-PKG II+PBS & Ad-PKG II+L-Arg) in MCF-7 and SW480-bearing mice. Our results show that PKG II combined with L-Arg effectively ameliorated breast and colon cancers development through inhibiting the proliferation and metastasis of cancer cells.

PKG II combined with L-Arg significantly inhibited angiogenesis

We next detected CD105 as a marker of neovascularization. As shown in Figure 3, PKG II combined with L-Arg obviously

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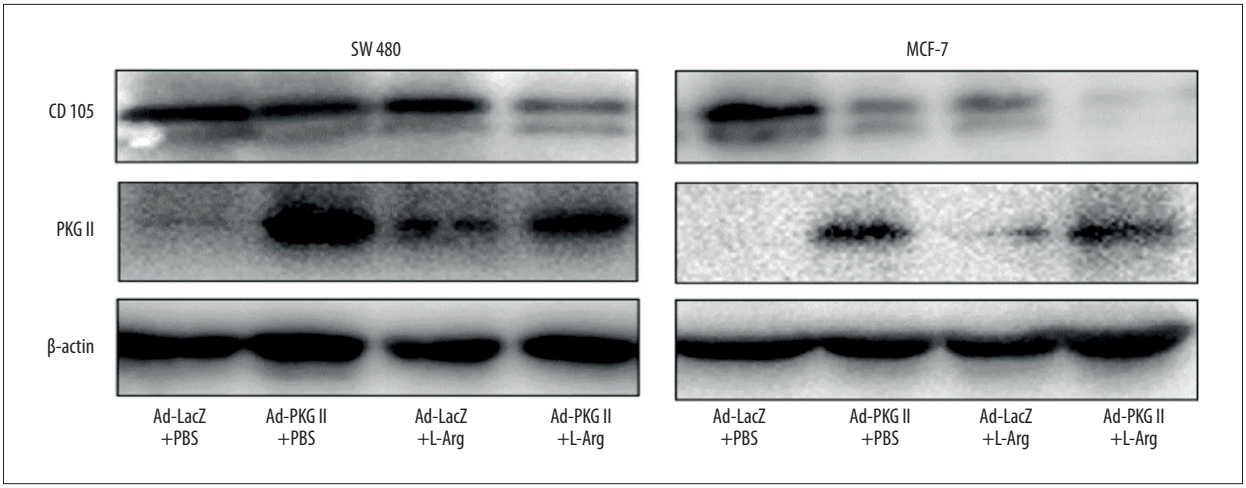

Figure 3. PKG II combined with L-Arg significantly inhibited CD105 expression. The intervention of cancer-bearing mice and the operation are shown in Figure 1. CD105 expression was detected by Western blotting. β-actin was also examined as a loading control. The experiment was repeated 3 times and similar data were obtained. Representative blots are shown.

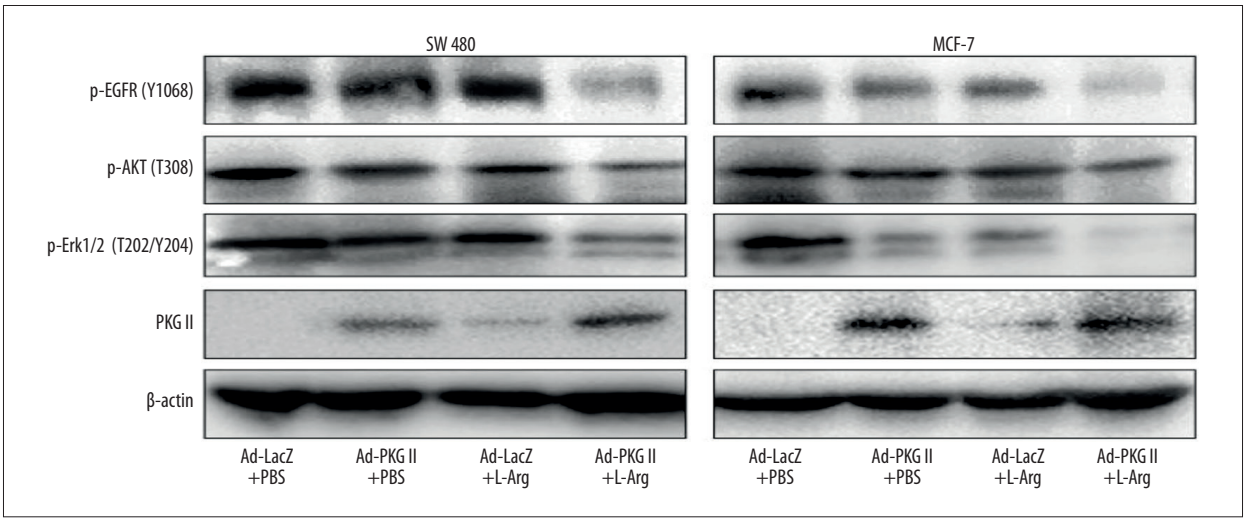

Figure 4. PKG II combined with L-Arg effectively ameliorated the development of breast and colon cancers via inhibition of EGF/EGFR activation inducing Erk1/2 or Akt pathways. The interventions of cancer bearing mice and the operation are shown in Figure 1. The lysates from tumor tissues were used to analysis the levels of p-EGFR, p-ERK1/2, and p-Akt. β-actin was also examined as a loading control. The experiment was repeated 3 times and similar data were obtained. Representative blots are shown.

inhibited CD105 expression compared with the Ad-LacZ+PBS, Ad-LacZ+L-Arg, and PKG II+PBS groups in MCF-7 and SW480-bearing mice. Furthermore, there were no obvious differences among Ad-LacZ+PBS, Ad-LacZ+L-Arg, and PKG II+PBS groups in SW480-bearing mice. However, CD105 expression was slightly decreased in the Ad-LacZ+L-Arg and PKG II+PBS groups compared with the Ad-LacZ+PBS group of MCF-7-bearing mice. Taken together, our results show that PKG II combined with L-Arg significantly inhibited angiogenesis.

PKG II combined with L-Arg effectively ameliorated the development of breast and colon cancers via inhibition of EGF/EGFR activation inducing Erk1/2 or Akt pathways

EGF/EGFR has been extensively implicated in cancer development, signal transduction, angiogenesis, metastasis, and membrane trafficking [22,23]. We sought to determine whether PKG II combined with L-Arg ameliorated breast and colon cancer development via inhibition of EGF/EGFR mediated-signal cascades. As Figure 4 shows, PKG II combined with L-Arg obviously inhibited phosphorylation of EGFR at tyrosine 1068 compared with the other 3 groups in MCF-7 and SW480-bearing

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mice, and EGFR phosphorylation inhibitory effects obviously blocked Erk1/2 and Akt activation. In the PKG II infection group without L-Arg, there were no obvious decreases in the activation of Erk1/2 and Akt. Taken together, we found that PKG II combined with L-Arg more effectively ameliorated tumor development via blockade of EGF/EGFR activation and its downstream signal cascades in breast and colon cancer-bearing mice.

Discussion

PKG II, a membrane-anchored and tissue-specific enzyme, is expressed mainly in intestinal mucosa and the brain. PKG II is involved in different physiological and pathological processes such as intestinal secretion, hypertrophic differentiation of chondrocytes, and endochondral ossification [9,24]. Recently, our data and the other reports indicated that up-regulation of PKG II decreased colony formation and inhibited cell proliferation, tumorigenesis, and metastasis [14,25,26] in a cGMP- or 8-pCPT-cGMP-dependent manner [27]. Of course, it was an effective method to up-regulate PKG II by adenovirus infection. However, for animal experiments, cGMP or cGMP analog need repeated injections to activate the kinase and this is expensive. Therefore, we sought an economic, effective, and convenient method to activate PKG II. Published data indicated that cellular cGMP levels are increased by the activity of sGC, which are activated by NO or small peptides, respectively [28]. NO is synthesized by NOS, whereas L-Arg acts as an NOS substrate [29]. Therefore, we speculated that L-Arg may replace cGMP or cGMP analog to activate PKG II.

L-Arg, as an α-amino acid, is mainly used in the biosynthesis of proteins and is classified as a semi-essential or even essential amino acid in some conditions. L-Arg, a precursor of NO, acts as a free radical scavenger and pro-oxidant enzyme inhibitor; these roles of L-Arg are attributed to NO production [30,31]. NO increases guanosine monophosphate production by activating sGC, which in turn increases sperm viability and motility as well as being involved in mitochondrial biogenesis, respiration, and reconstruction [32,33]. In the present work, our results showed that PKG II infection combined with L-Arg effectively decreased the weights of tumors compared with Ad-LacZ+PBS and Ad-LacZ+L-Arg groups in MCF-7- and SW480-bearing mice, which may be dependent on inhibiting cancer cell proliferation and angiogenesis through blockage of EGF/EGFR activation and ERK1/2 or Akt pathways. Conversely, there was no obvious difference in HepG2-bearing mice (data not shown). In angiogenesis, CD105 is known as a marker for neovascularization and can be divided into 2 isoforms – (long isoform (L-CD105) and short isoform (S-CD105) – according to the length of the intracellular domain. Normally, they differ in tissue distribution and degree of phosphorylation. Furthermore, different isoforms are involved in various biological processes [34,35]. Interestingly, our data showed that the 2 isoforms were both expressed, which indicates that the 2 isoforms might be regulated by PKG II and may be involved in cancer development.

Remarkably, our present data showed that only up-regulation of PKG II also inhibited the tumor growth, indicating that exogenous PKG II might be activated *in vivo,* possibly through endogenous cGMP or other molecules. In addition, in the absence of PKG II infection, L-Arg did not obviously inhibit the growth of breast, liver, and colon tumor; but in mice bearing MCF-7 cells, L-Arg administration decreased CD105, p-EGFR, and p-Erk1/2, which suggests that L-Arg might regulate the above proteins through a PKG II-independent pathway or other target proteins such as PKG I. In other words, we cannot completely exclude the effects of L-Arg against cancer by self- or indirectly-activating PKG I [29], and the antitumor effect of PKG II and L-Arg might differ in different tumor tissues. To confirm all the above speculations or questions, more work is needed in the future on questions such as whether PKG II infection combined with L-Arg regulates the tumor microenvironment, changes cell mitochondrial biogenesis, respiration, and reconstruction, and whether it affects energy metabolism.

Conclusions

Our present work indicates that L-Arg is a potential alternative PKG II activation.

Conflict of interest

None.

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