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Anti-Angiogenic and Anti-Cancer Effects by Targeting the Protein Kinase G Type-Iα (PKG-Iα) Signaling Pathway and its Downstream Effects on Expression of Inhibitor of Apoptosis Proteins, C-IAP1, Livin and Survivin

Janica C. Wong, Priyatham Gorjala,
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Additional information is available at the end of the chapter

Abstract

Ovarian cancer is often difficult to treat because of the development of resistance to many of the currently-used therapeutic agents (i.e. chemoresistance). The progression and chemoresistance of ovarian cancer can involve tumor angiogenesis, the development of new blood vessels bringing more blood and nutrients to the growing tumor. Tumor angiogenesis also involves the vascular endothelium-induced stimulation of cancer cell growth (1) and the higher expression levels of certain "cell survival proteins", such as the Inhibitor of Apoptosis Proteins (IAPs, including c-IAP1, Livin and Survivin), which are expressed in both the proliferating cancer cells (2, 3) and the vascular endothelial cells involved in tumor angiogenesis (4).

Keywords: Anti-angiogenesis, cancer treatment, protein kinase G (PKG), chemoresistance, cIAP-1, Livin, Survivin, Inhibitor of Apoptosis Proteins (IAPs)

1. Introduction

Ovarian cancer is often difficult to treat because of the development of resistance to many of the currently-used therapeutic agents (i.e. chemoresistance). The progression and chemore-

sistance of ovarian cancer can involve tumor angiogenesis, the development of new blood vessels bringing more blood and nutrients to the growing tumor. Tumor angiogenesis also involves the vascular endothelium-induced stimulation of cancer cell growth [1] and the higher expression levels of certain "cell survival proteins", such as the Inhibitor of Apoptosis Proteins (IAPs, including c-IAP1, Livin and Survivin), which are expressed in both the proliferating cancer cells [2, 3] and the vascular endothelial cells involved in tumor angiogenesis [4].

Although there are a number of cell signaling pathways that are known to promote angiogenesis and the higher levels of expression of the IAPs, one pathway that has become recognized as an important pro-growth and pro-survival mechanism within both ovarian cancer cells and vascular endothelial cells is the nitric oxide (NO)/cyclic GMP (cGMP)/protein kinase G type-1α (PKG-Iα) signaling pathway. Originally, the NO/cGMP/PKG signaling pathway was recognized to be a key cellular mechanism in regulating the cardiovascular system, specifically involved in promoting vasodilation (relaxation of vascular smooth muscle cells) and in preventing the onset of hypertension and other cardiovascular diseases [5-12]. More recent data from our laboratory have shown that the NO/cGMP/PKG signaling pathway, mediated via one of the isoforms of PKG, i.e. the PKG-Iα splice variant of PKG-I, is involved in promoting cell proliferation and enhanced cell survival (inhibiting the onset of apoptosis) in many types of mammalian cells, including neural cells [3, 6, 10, 13-15], uterine epithelial cells [16], OP9 bone marrow-derived mesenchymal (stromal) stem cells [17], and a number of different type of cancer cells, including ovarian cancer cells [18-22], neuroblastoma cells [15] and lung cancer cells [22].

Our studies have shown that the catalytic/kinase activity of PKG-Iα plays a key role in the phosphorylation of four proteins, BAD, CREB, c-Src and VASP, within mammalian cells, promoting DNA synthesis/cell proliferation and inhibiting the onset of apoptotic cell death, thus enhancing cell survival [22]. We have found that PKG-Iα is hyperactivated in several types of cancer cells, including ovarian cancer cells and lung cancer cells, resulting in abnormally high levels of phosphorylation of BAD, CREB, c-Src and VASP, which contributes to the exaggerated cell proliferation and resistance to certain chemotherapy, such as cisplatin (i.e. platinum resistance) [21]. The key role played by PKG-Iα in promoting DNA synthesis/cell proliferation and chemoresistance has been established by both pharmacological inhibitors and gene knockdown techniques using siRNA and shRNA that target PKG-Iα [21, 22].

Figure 1 shows a cellular model illustrating our findings about the role of NO at low physiological levels, i.e. 10 picomolar (pM) to 1 nanomolar (nM), and its downstream activation of PKG-Iα in promoting increased tumor growth and chemoresistance in cancer cells of epithelial origin, including human ovarian cancer cells [3, 19-22]. The model highlights the recent finding from our laboratory regarding the substrate proteins that can be directly phosphorylated by PKG-Iα, including the apoptosis-regulating protein BAD at serine-155 [15], the transcription factor CREB at serine-133 [3, 6, 22, 23] and the oncogenic tyrosine kinase c-Src at serine-17 [22, 23]. The enhanced phosphorylation of CREB caused by the hyperactivation of PKG-Iα in non-small cell lung cancer (NSCLC) cells was found to be responsible for the maintenance of high levels of expression of several "cell survival proteins", including Mcl-1 and three of the IAPs (c-IAP1, Livin and Survivin) [3]. Gene knockdown of PKG-Iα expression using siRNA

targeting this PKG isoform clearly showed that PKG-Iα plays a critical role in promoting the high-level expression of Mcl-1, c-IAP1, Livin and Survivin in lung cancer cells. Ongoing experiments in the Fiscus Lab in the College of Medicine at Roseman University of Health Sciences are currently determining if a similar relationship between PKG-Iα hyperactivation and the high-level expression of "cell survival protein", such as Mcl-1, c-IAP1, Livin and Survivin, also occurs in human ovarian cancer cells, like in lung cancer cells.

Figure 1. Involvement of picomolar levels of NO and downstream activation of PKG-Iα on promoting tumor angiogenesis and the proliferation and chemoresistance of cancer cells.

Figure 1 also illustrates the role of physiological-level NO and PKG-Iα in mediating the pro-tumor-angiogenesis effects of Vascular Endothelial Growth Factor (VEGF). VEGF is a pro-angiogenesis factor known to be released from many types of cancer cells, including human ovarian cancer cells, and is secreted in especially high amount by higher grade malignancies of the ovary [24]. Interestingly, higher VEGF expression by the ovarian cancer cells of patients was shown to be an independent predictor of poor prognosis of the disease [25].

The model of tumor angiogenesis shown in Figure 1 incorporates the early finding of Hood and Granger published in 1998, showing that VEGF stimulates cell proliferation and tube formation in human umbilical vein endothelial cells (HUVECs), a model of tumor angiogen-

esis, in a manner that was completely dependent on endogenous PKG [26]. Their study did not determined which isoform of PKG was expressed in the HUVECs, however, our laboratory has recently shown that HUVECs express both of the two PKG-I splice variants (i.e. both PKG-Iα and PKG-Iβ) [4], which has been confirmed in the data illustrated below in Figure 3. We have proposed that it is likely that the PKG-Iα isoform is the splice variant that mediates the enhanced cell proliferation of HUVECs, based on our many studies with other cells (e.g. N1E-115 and NG108-15 neural cells) that express exclusively the PKG-Iα splice variant of PKG-I [3, 4, 15, 17, 21-23]. Two key findings by Hood and Granger was that PKG directly interacted with Raf-1 (c-Raf), as assessed by co-immunoprecipitation, and that PKG activity was necessary for VEGF-induced activation of Raf-1 and the subsequent downstream activations of MEK and ERK1/2, regulating in enhanced endothelial cell proliferation [26].

Figure 2 shows a comparison of the two splice variants of PKG-I, illustrating how two very similar protein kinases, identical in their catalytic and regulatory domains, can have very different biological effects. The key difference between these two splice variants is the N-terminal region, the first 100 amino acids, which is encoded by the unique first exon, Iα in the case of PKG-Iα and Iβ in the case of PKG-Iβ. The first 100 amino acids provide the leucine zipper/protein-protein interaction domain, which determines the subcellular localization of these two isoforms and further determines which downstream target protein is phosphorylated by the two PKG-I isoforms within cells. Because of phosphorylating very different subsets of substrate proteins, PKG-Iα and PKG-Iβ are involved in mediating very different biological effects, in some cases, even opposite effects.

The PKG-Iα splice variant promotes cell proliferation and cell survival in both normal non-transformed cells (e.g. neural cells and OP9 bone marrow-derived mesenchymal stem cells) as well as malignant cells (contributing to exaggerated cell proliferation and chemoresistance in lung cancer and ovarian cancer cells) [3, 4, 15, 17, 20-23]. In contrast, the PKG-Iβ splice variant, at least when there is chemically-induced hyperactivation (e.g. using Exisulind to cause large increases in the intracellular cGMP levels) or forced overexpression (e.g. Deguchi et al., 2004), promotes effects that are just opposite of those mediated by PKG-Iα, i.e. inhibition of cell proliferation and induction of cell death mediated by PKG-Iβ [22, 23, 27]. This has led to confusion over the real function of PKG in regulating cell proliferation and cell survival, where PKG appears to have opposite effects in different experiments and/or different laboratories. Our studies have shown that it is critically important to differentiate between the actions of the different splice variants of PKG-I to avoid this confusion and the misunderstanding regarding the functions of PKG.

Recently, NO has been shown to be a positive regulator of the Warburg effect in ovarian cancer cells, promoting a metabolic switch toward increased glycolysis, with increased glucose consumption, enhanced uptake of glutamine and increased release of lactate [28]. The downstream signaling pathway mediating these metabolic effects of NO in ovarian cancer cells was not reported, but likely involves a mediator role for the different PKG isoforms that are downstream from the NO exposure.

Two splice variants of PKG-I have opposite effects on cell survival and proliferation
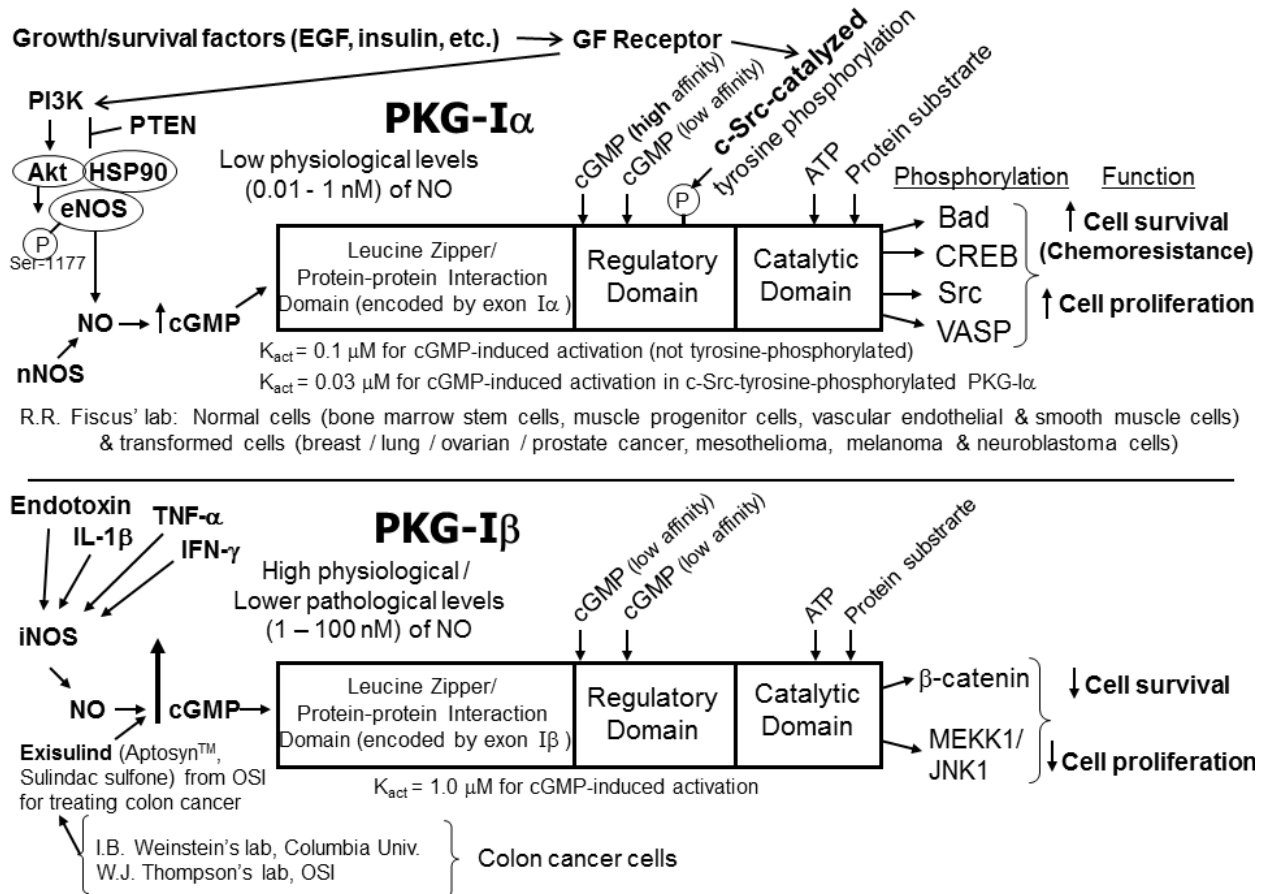

Figure 2. Opposite effects on cell proliferation and cell survival mediated by the two splice variants of PKG-I. The different biological effects of PKG-Iα and PKG-Iβ likely result from the different subcellular distributions of these to isoform (determined by the unique exon, Iα versus Iβ, of the two splice variants), resulting in the phosphorylation and activation of very different downstream substrates proteins, as depicted in the models. Modified from the original model in R.R. Fiscus, E.L. Leung, J.C. Wong and M.G. Johlfs, 2012 [22].

2. Hyeractivation of PKG-Iα kinase activity in cancer cells contributes to the higher expression levels of Mcl-1 and certain Inhibitor of Apoptosis Proteins (IAPs), including c-IAP1, Livin and Survivin

Downstream from PKG-Iα hyperactivity in cancer cells is the high levels of expression of "cell survival proteins", including Mcl-1 and certain IAPs, such as c-IAP1, Livin and Survivin [22]. These IAPs have been shown to regulate apoptosis and tumorigenesis [29]. Eight human IAPs have been identified [30] and are known to suppress apoptotic cell death, thus promoting cell survival and, in cancer cells, chemoresistance [31]. c-IAP1 and c-IAP2 possess a caspase recruitment domain [32], and c-IAP1, c-IAP2 and XIAP are known to directly inhibit caspase-3 and caspase-7 activity [33, 34]. Elevated expression of IAP proteins has been shown in almost all types of human cancers and has been implicated as therapeutic targets [35]. Particularly, XIAP was shown to play a predominant role in the inactivation of apoptosome in non-small cell lung cancer NCI-H460 cells [36]. Survivin-specific

siRNA was shown to increase apoptosis and inhibit cell proliferation in A549 lung cancer cells associated with activation of caspase-9 [37].

Although the NF-κB transcription factor is traditionally thought to regulate the expression of the IAPs, another transcription factor, CREB, has also been implicated in regulating some IAPs. For example, CREB phosphorylation at serine-133 and its subsequent activation are thought to be key events in the induction of c-IAP2 and Livin expression, potentially mediated by multiple protein kinases, including PKA, ERK1/2 and p38 MAPK, in colon cancer cells [38, 39].

Recent data from our laboratory have shown that the expression of three of the IAPs, c-IAP1, Livin and Survivin, are dependent on the NO/cGMP/PKG-Iα signaling pathway in non-small cell lung cancer (NSCLC) cells, which appears to involve the exaggerated phosphorylation of CREB at serine-133 catalyzed by the hyperactivated PKG-Iα [3]. We have also shown that some of these same signaling proteins, especially PKG-Iα and downstream elevation of the protein expression of c-IAP1and Livin as well as two other IAPs (c-IAP2 and XIAP) are involved in angiogenesis, using human endothelial cells (the HUVECs) as a model of tumor angiogenesis [4]. Interestingly, we found that resveratrol, a polyphenol from red wine, grapes, berries and peanuts known for its protection against cancers, when added at anti-angiogenesis and anti-cancer concentrations, inhibits the intracellular catalytic/kinase activity of PKG-Iα in the HUVECs, dramatically decreasing protein expression levels of c-IAP1, c-IAP2, Livin and XIAP [4]. Our data suggest that certain naturally-occurring anti-cancer agents, such as resveratrol, may prevent cancers by suppressing the PKG-Iα signaling pathway and lowering the expression levels of the IAPs in the vascular endothelial cells of tumors, thus suppressing tumor angiogenesis.

3. Identification of PKG-I splice variants expressed in human ovarian cancer cells by using a new ultrasensitive advanced nano-proteomics technology, the NanoPro 1000 system

Recent studies from our laboratory have used an ultrasensitive "advanced nano-proteomics" technology, called NanoPro 1000 (ProteinSimple, San Jose, CA, USA), to determine expression and phosphorylation levels of PKG-Iα as well as other protein kinases (especially Akt and c-Src, which interact with PKG-Iα and co-mediate the enhanced cell survival and resistance to apoptosis), and the various IAPs. This technology uses a robotic system for collecting samples and analyzing the samples, involving capillary isoelectricfocusing (cIEF) for separating proteins based on pI values, rather than molecular weight. This has a clear advantage over Western blot analysis when attempting to separate and identify proteins with similar molecular weight, such as isoforms of proteins.

This new NanoPro 1000 technology provides a sensitivity that is >500-times better than conventional Western blot analysis, thus allowing discovery of new signaling proteins that can be used for developing new therapeutic agents. This new technology allows for accurate measurements of lower abundance proteins (undetectable by conventional Western blot analysis), often using fewer than 1,000 mammalian cells for the analysis.
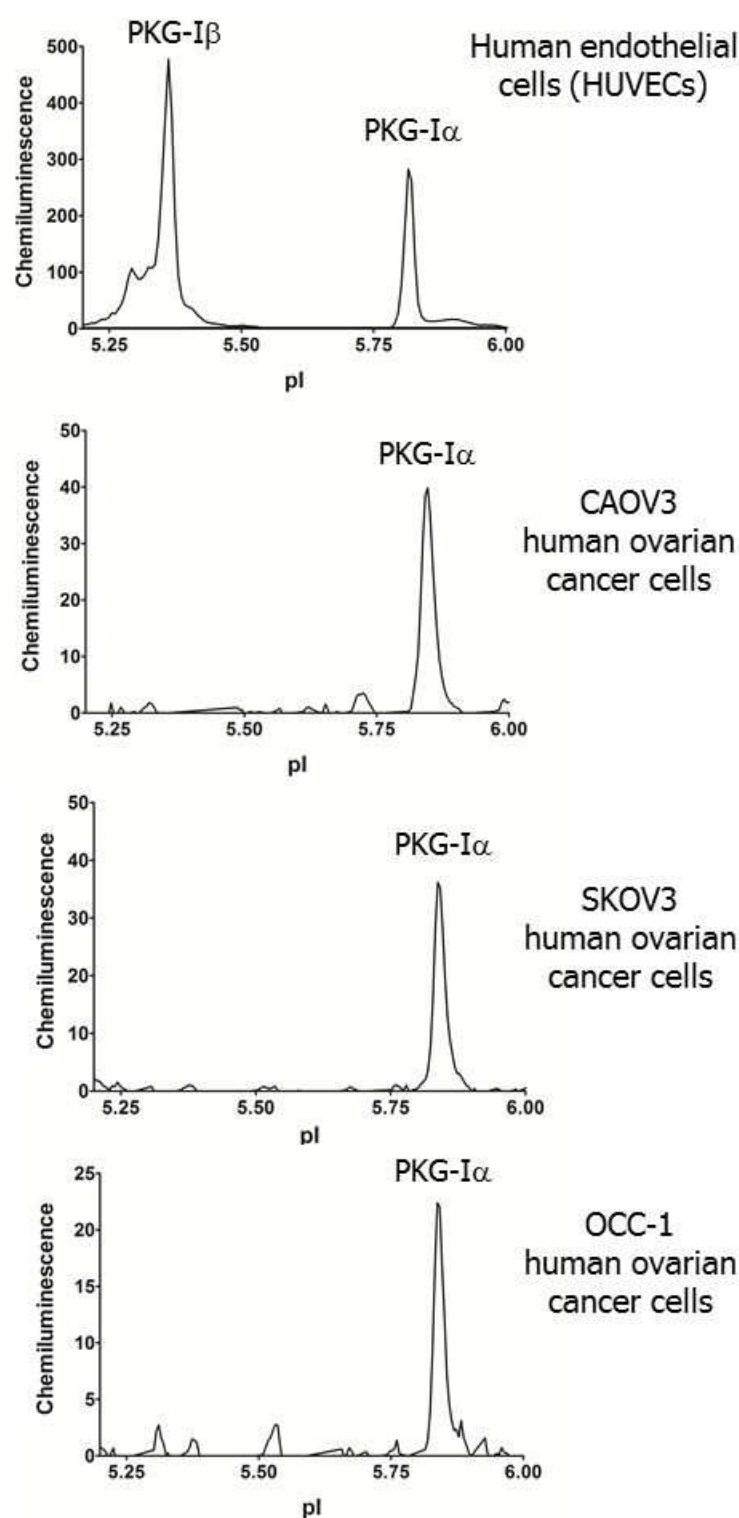
Figure 3. Electropherograms generated by the new technology of NanoPro 1000 system, a capillary isoelectrofocusing (cIEF) instrument for quantifying protein expression levels and phosphorylations levels, with a sensitive >500-times greater than traditional Western blot analysis. The HUVECs are used as positive controls for illustrating the expression of both splice variants of PKG-I in the same cell population. All three of the human ovarian cancer cell lines express only the PKG-Iα splice variant.

Figure 3 illustrates recent experiments showing the expression of PKG-I splice variants in three different ovarian cancer cell lines, CAOV3, SKOV3 and OCC-1 cells, determined by using the NanoPro 1000 technology. The top electropherogram shows that the HUVECs, human endothelial cells, express both PKG-I splice variants, confirming our previous studies of HUVECs using the older, related technology, the NanoPro 100 system, recently published by our laboratory in *Anticancer Research* [4].

Note that all three of the ovarian cancer cell lines shown in Figure 3 express exclusively the PKG-Iα splice variant, in contrast to the expression pattern shown in the human endothelial cells expressing both splice variants of PKG-I. The protein expression levels of PKG-Iα in all of these human ovarian cancer cell lines are typically below the detection limits when analyzed by traditional Western blot analysis, as shown in our previous book chapter on ovarian cancer cells [22]. Thus, the advanced nano-proteomic technology of the robotic cIEF-based NanoPro 1000 system provides a valuable new research tool for studying the expression levels of lower abundance proteins that are undetectable by the 30-year-old technique of Western blot analysis (500-times less sensitive compared to the NanoPro 1000 system).

4. Human ovarian cancer cells have hyperactivated PKG-Iα, as quantified by a newly- developed ultrasensitive near-infrared-fluorescence (NIRF)-based kinase assay for measuring PKG catalytic/kinase activity in tissue samples and cell lysates

Recently, our laboratory has successfully development a new, ultrasensitive methodology for accurately measuring the catalytic/kinase activity of any protein kinase within biological samples (cell lysates and tissue homogenates) using NIRF-labeled peptide substrates rather than the old technique of using radioactive (^{32}P- or ^{33}P-labeled) ATP. The radioactive protein kinase assays were originally developed in the 1970s and 1980s for measuring the catalytic activity of protein kinase A (PKA) and PKG in freshly-prepared tissue homogenates [5-10, 22, 23]. The new NIRF-based protein kinase assays were developed for improving safety and for lowering the cost of analysis.

Figure 4 illustrates the use of this new methodology, showing that four different ovarian cancer cell lines, CAOV3, OCC1, SKOV3 and A2780cp cells, all possess measureable levels of endogenous PKG-Iα catalytic/kinase activity and that this kinase activity is indeed hyperactivated in all of the ovarian cancer cell lines. The NIRF-labeled peptide substrate used in this assay can also be phosphorylated by eight of the most common isoforms of protein kinase C (PKC), but not by other related protein kinases, such as Akt1, Akt2, p70S6-kinase and RSK2. Thus, the catalytic activities of both PKG and PKC can be measured simultaneously in the same biological sample. To define the component of kinase activity contributed by endogenous PKC, we used a combination of four PKC inhibitors (AEB071, Gö 6976, Gö 6983 and LY333,531), which selectively inhibit the eight isoforms of PKC capable of phosphorylating the NIRF-peptide substrate. This defines the PKC catalytic activity from PKG catalytic activity in complex mixtures of protein kinases, such as tissue homogenates and cell lysates. The kinase

catalytic activity remaining in the presence of the four PKC inhibitors represents only PKG catalytic activity, show in Figure 4.

The percent activation of PKG-Iα in the four human ovarian cancer cell lines was all above 90%, indicating that PKG-Iα is indeed hyperactivated (Figure 4) in these malignant cells. For comparison, homogenates of two normal tissues, vascular smooth muscle (V.S.M.) from rat aorta and human pancreatic islets, were also analyzed. The % activation of PKG in smooth muscle tissue was 21%, similar to the % activation measured by the ^{32}P-ATP-based method-ology developed and used by Dr. R.R. Fiscus in the early and mid-1980s [5, 7-9], and the PKG activation in pancreatic islets was 31%. Thus, compared to normal tissues, all of the ovarian cancer cell lines have highly-activated PKG. This hyperactivation of PKG-Iα in the ovarian cancer cells would result in downstream (hyper)activation of c-Src and CREB, resulting in an exaggerated expression of "cell survival proteins", such as Mcl-1 and certain IAPs, e.g. c-IAP1, Livin and Survivin, likely contributing to the aggressive nature of this form of cancer.
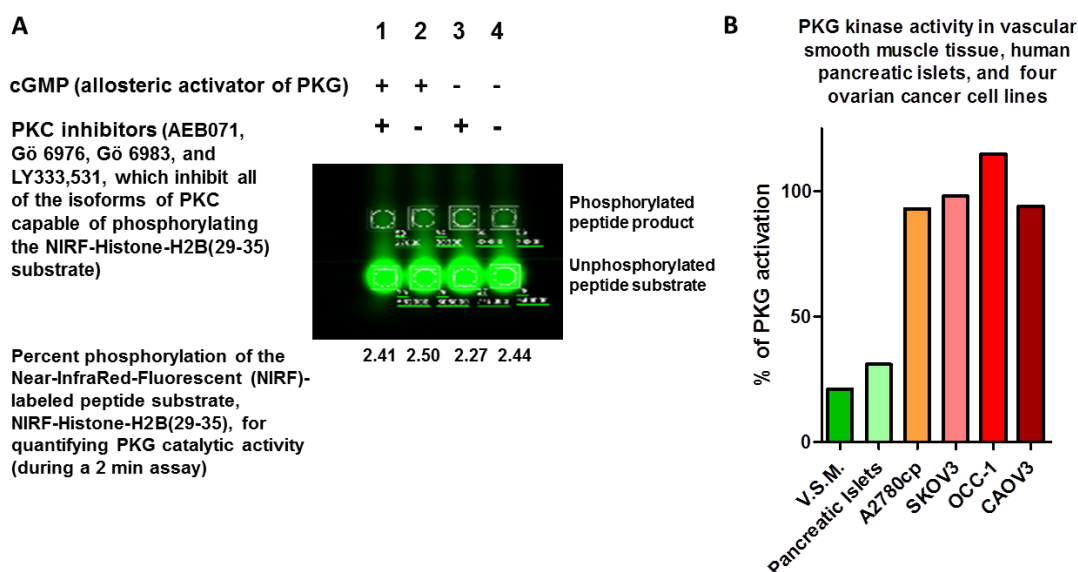

Figure 4. Hyperactivation of PKG catalytic/kinase activity in four ovarian cancer cell lines. A, picture representative of the near infrared-fluorescence (NIRF)-based kinase assay, using the CAOV3 ovarian cancer cells as an example. The % of phosphorylation was calculated based on the fluorescence signal measured by LI-COR Odyssey CLx scanner. The activity ratio, activity –cGMP divided by activity +cGMP (i.e. kinase activity with versus without the addition of exoge-nous cGMP (10 μM), an allosteric activator of PKG). All measurements were done in the presence of a combination of select PKC inhibitors (to remove kinase activity contributed by PKC isoforms). The remaining kinase activity reflects the PKG catalytic activity in these cell lines. The data in A., i.e. 2.41, 2.50, 2.27 and 2.44, represent the percent phosphor-ylation of the NIRF-labeled peptide substrate during a 2-min reaction. B, The % of PKG activation (i.e. 100 X the activi-ty ratio) for each sample analyzed is shown in a bar graph. Data was obtained by measuring kinase activity by the new NIRF-based kinase assay method. The four ovarian cancer cell lines, A2780cp, SKOV3, OCC-1 and CAOV3, had % of PKG activation of 93%, 98%, 115% and 94%, respectively, showing that PKG is clearly hyperactivated in all of the ovar-ian cancer cells tested. For comparison, tissue samples of freshly-isolated vascular smooth muscle (V.S.M.) from rat aorta and human pancreatic islets (purchased from Lonza) were used to show PKG catalytic/kinase activity in normal non-cancerous tissues. Note that the % activation of PKG is considerably lower in normal tissue, showing 21% activa-tion of PKG in vascular smooth muscle cells (V.S.M.) and 31% activation of PKG in human pancreatic islets.

5. Future experiments

Our future studies will focus on: 1) determining the expression of PKG isoform and its various phospho-forms in human ovarian cancer cell lines and clinical samples of ovarian tumors using the NanoPro 1000 system, 2) determining the PKG kinase activity of other ovarian cancer cell lines and clinical samples of ovarian tumors using our patented NIRF-based kinase assay, and 3) studying the phosphorylation/activation of the transcription factor CREB and the expression profile of the IAPs using our advanced nano-proteomics technology, with special focus on c-IAP1, c-IAP2, Livin, Survivin and XIAP, in ovarian cancer cells and vascular endothelial cells. The goal of our future experiments is to ultimately develop new therapeutic agents that can target these novel signaling pathways in order to effectively treat chemoresistant ovarian cancer and tumor angiogenesis.

The NanoPro 1000 system is especially useful for studying the multiple phospho-forms of proteins, because of its ability to separate proteins based on pI values rather than molecular weight. Each addition of a phosphate molecule to a protein typically causes a measurable (and resolvable) shift in the pI value, which can be used to determine intracellular activation and/or the catalytic function of a protein kinase within cells. Likewise, our patented technology using NIRF-based kinase assays will be used to determine the effectiveness and potency of protein kinase inhibitors that could potentially be used to treat cancer.

6. Conclusions

Our studies of human ovarian cancer cells as well as other types of cancer cells (e.g. breast cancer, lung cancer, mesothelioma, neuroblastoma and prostate cancer) have identified the NO/cGMP/PKG-Iα signaling pathway as a key cellular mechanism involved in mediating the exaggerated cell proliferation and chemoresistance of these cancers [3, 15, 19-22]. We have shown that the PKG-Iα splice variant of PKG-I, which is expressed in all of these cancer cells, directly phosphorylates important intracellular proteins, including BAD, CREB and c-Src, leading to enahanced cell survival (i.e. chemoresistance) and exaggerated cell proliferation. Specifically, phosphorylation of CREB at serine-133 following activation of PKG-Iα results in increased gene expression of several "cell survival proteins", including Mcl-1 and some of the the IAPs.

In human lung cancer cells, the NO/PKG-Iα signaling pathway represents a major mechanism for the enhanced gene expression of three IAPs, c-IAP1, Livin and Survivin, which corresponds with exaggerated cell proliferation and resistance to cisplatin-induced apoptosis [3]. Blocking the PKG-Iα catalytic activity or knocking-down the gene expression of PKG-Iα using siRNA dramatically sensitizes these chemoresistant cells to the cancer-killing effects of cisplatin. We anticipate that our future studies with ovarian cancer cells will show similar results.

In HUVECs, human endothelial cells used as a model of tumor angiogenesis, the eNOS/NO/cGMP/PKG signaling pathway is now recognized to mediate the pro-angiogenesis effects of

VEGF [4]. VEGF is a factor that is released in large amounts from cancer cells, including human ovarian cancer cells. Our studies with HUVECs show that four IAPs, c-IAP1, c-IAP2, Livin and XIAP, are all downregulated by resveratrol, a polyphenol from grapes, berries, peanuts and red wine, and that this response corresponds to the inhibition of PKG catalytic activity in the HUVECs.

Figure 5 shows a model representing the involvement of the NO/cGMP/PKG-Iα signaling pathway in mediating the pro-angiogenesis effects of VEGF within vascular endothelial cells, promoting the increased expression of the IAPs, c-IAP1, c-IAP2, Livin and XIAP, based on our recent publication [4]. Also shown is the inhibitory action of resveratrol, at anti-angiogenic and anti-cancer concentrations, on the PKG-Iα catalytic actions and downstream expression of c-IAP1, c-IAP-2, Livin and XIAP. We have proposed that the ability of resveratrol to prevent cancers may relate to its ability to inhibit PKG-Iα catalytic activity selectively in tumor endothelial cells involved in tumor angiogenesis, thus suppressing the expression of IAPs in the endothelial cells and the tumor angiogenesis.

Tumor angiogenesis: Involvement of the VEGF/NO/cGMP/PKG-Iα signaling pathway and downstream expression of c-IAP1, c-IAP2, Livin and XIAP, and its inhibition by Resveratrol
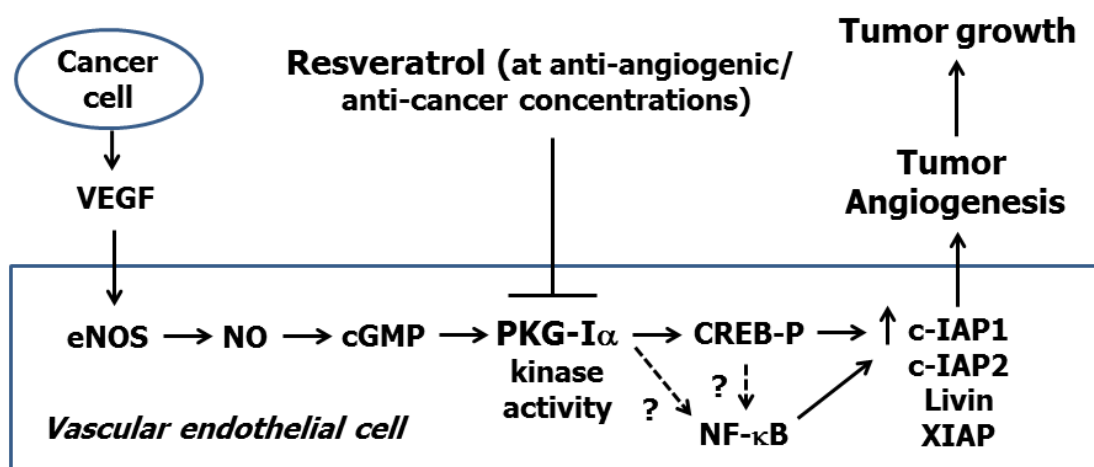
Based on data from: Wong J.C. and R.R. Fiscus (2015) Resveratrol at anti-angiogenic/ anticancer concentrations suppresses protein kinase G signaling and decreases IAPs expression in HUVECs. *Anticancer Research* 35: 273-282.

Figure 5. Involvement of eNOS, NO, cGMP and PKG-Iα in the pro-angiogenesis actions of VEGF in vascular endothelial cells. Enhanced PKG-Iα catalytic activity induced by VEGF can increase CREB phosphorylation and activation, increasing expression of certain IAPs. The traditional transcription factor thought to be involved in promoting the gene expression of the IAPs, i.e. NF-κB, is also shown, although its role in PKG-Iα-mediated increases in the IAPs and tumor angiogenesis is not yet known.

Acknowledgements

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