The Significance of PKGIB in cGMP Induced Death of Breast Cancer Cells

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Abstract

The cGMP signaling pathway has been shown to be effective in the induction of breast cancer cell death. The mechanism through which cGMP causes cell death remains unknown. However, activation of cGMP-dependent protein kinase (PKG) is thought to play an important role. The following experiments will study the importance of PKG for the anticancer activity of cGMP signaling in breast cancer cells. PKGI-specific siRNA will be used to knockdown the expression of the protein. We have confirmed that three days of treatment with siRNA in MDA-MB-231 breast cancer cells causes a significant decrease in the amount of PKGIβ, the most highly expressed form of this protein in these cells, as determined by a Western blot. Cells expressing PKGIβ and with knockdown of PKGIβ will be treated with sulindac sulfide, MY5445, and NOR-3, compounds known to activate cGMP signaling in breast cancer cells. We will then measure the effects of knockdown on sensitivity of the cells to the anticancer activity of cGMP signaling activation. These studies will determine if PKG is necessary for mediating the death of breast cancer cells in response to cGMP signaling. Understanding how the cGMP/PKG signaling pathway results in breast cancer cell death could play a pivotal role in future drug discovery efforts by identifying novel drug targets.

Introduction

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer related death in women in the United States [1]. Research has shown that one of the strongest risk factors for breast cancer is family history [2]. Cancer is caused by an accumulation of genetic mutations and alterations that allow it to grow, such as evasion of programmed cell death, self-sufficiency in growth signals, and insensitivity to growth-inhibitory signals [1, 3]. Early detection and chemoprevention are widely accepted as the most promising methods through which the impact of breast cancer in terms of death and quality of life will be reduced [1]. Identifying proteins that play an important role in breast cancer development and progression is paramount in the quest for improving detection and preventing the development of breast cancer.

Recent studies have shown that the cGMP/PKG pathway is effective to induce apoptosis of cancer cells. Specifically, the activation of cGMP leads to the activation of PKG, which induces cancer cell death [4]. cGMP signaling has also been found to have negative effects on cell growth and survival depending on the type of cell and type of tissue [5]. There have also been findings that give direct evidence that PKG is adequate to induce cell death and prevent migration of cancer cells [6]. However, the exact mechanism through which cGMP and PKG cause cell death remains unknown [4].

There have also been several studies that have shown that non-steroidal anti-inflammatory drugs (NSAIDs) have an effect on certain pathways and enzymes that are prevalent in cancer cells. NSAIDs have been shown to be effective in inducing apoptosis and inhibiting growth of cancer cells [1, 7]. Drugs such as sulindac sulfide (SS), while helpful in the treatment and prevention of cancer, cause toxicity due to the effects caused by the inhibition of the enzyme cyclooxygenase (COX). This toxicity can ultimately lead to fatal side effects, such as gastrointestinal ulcers, renal toxicity, and increased risk of stroke and heart attack [7]. These side effects have prevented NSAIDs from being used widely for cancer chemoprevention.

The useful nature of NSAIDs stems from its ability to activate the cyclic guanosine monophosphate (cGMP) pathway, which has been shown to be less active in breast cancer cells, along with its inhibition of phosphodiesterase 5 (PDE5) which is over-expressed in breast cancer cells[7]. It has been shown by recent studies that inhibition of PDE5 by SS combined with activation of cGMP signaling results in apoptosis of breast tumor cells [1]. There have also been studies concerning nitric oxide (NO) that show its association with cGMP [8, 9]. While these studies are relevant to the

study of breast cancer, NO can have effects on cancer that are cGMP-dependent or cGMP-independent and at this time, only compounds associated with cGMP are being investigated. [9]. Not only are PDE and NO components of the signaling pathway known to regulate cell survival and cell death in cancer, but also protein kinase G (PKG) plays a major role in this signaling pathway and the mechanism must still be discovered [9].

The current study will deal with this problem and work specifically with PKGIβ, which has recently been shown to be the predominant form of PKG that is expressed in breast cancer cells, to observe its effects on cell growth. To distinguish these effects, small interfering RNA (siRNA) will be used to decrease PKG expression in breast cancer cells. siRNA operates by obtaining an siRNA that is complementary for the mRNA of the protein being studied, in this situation, PKG. The siRNA binds to the mRNA and "silences" the mRNA so that the ribosome cannot translate it into protein. A "knockdown" results, so that the cells have less protein than cells without siRNA [10]. This can be confirmed using techniques of reverse transcription polymerase chain reaction (RT-PCR) and Western blotting. After these processes, if knockdown has been successful, the cells with greatest knockdown will be treated with compounds that are expected to activate cGMP. This study will determine the importance of PKGIβ for mediating the anticancer activity of cGMP signaling in these cells. Understanding how this signaling pathway results in breast cancer cell death could play a pivotal role in future drug discovery efforts by identifying novel drug targets and helping to eliminate potential sources of toxicity.

Methods and Materials

Cells and Cell Culture: The MDA-MB-231 and SKBr-3 human breast cancer cell lines were obtained from ATCC and grown in RPMI 1640 media containing antifungal, antibiotic, glutamine, and 5% fetal bovine serum (FBS). Normal human mammary epithelial cells (HMEC) were obtained from Lonza and grown in MEGM media according to manufacturer's protocol. All cells were grown under standard conditions at 37^{o}C, with 5% CO_2 in a humidified atmosphere. Prior to use in any experiment, the cells were counted using a hemacytometer and viability was determined using trypan blue exclusion. Only cultures displaying >95% viability were used for experiments.

RT-PCR: RNA was isolated from MDA-MB-231 cells using Axygen Total RNA Miniprep kit according to manufacturer's specifications. RT-PCR procedure utilized Qiagen OneStep RT-PCR kit according to manufacturer's specifications. Bands were visualized on a 1% agarose gel labeled with ethidium bromide and run for about 30 minutes.

Primers used in solutions for RT-PCR:

Table 1

Name	Sequence
β-actin forward	GGACTTCGAGCAAGAGATGG
β-actin reverse	AGCACTGTGTTGGCGTACAG
PKGIα forward	CGAGTACTTAGCGCCCATTC
PKGIα reverse	CTTCTCTGACAGCCGCTTCT
PKGIβ forward	CACCTTGCGGGATTTACAGT
PKGIβ reverse	ATCACCGAGCGGTACTTGTC
PKGII forward	GCCATTGCTGAACTCACAGA
PKGII reverse	GGAAAATTCAGGGGGTTTGT

Western Blotting: All antibodies were obtained from Cell Signaling Technologies. Protein was isolated using a 1% SDS lysis buffer (1%SDS, 10mM Tris pH 7.5, protease inhibitor cocktail). The concentration of protein was calculated using BCA assay from Pierce following manufacturer's specifications. 15-30μg of obtained protein was loaded per well in a 12% acrylamide gel. Proteins were transferred to nitrocellulose membrane and the membrane was blocked using 5% nonfat milk in TBS containing 1% Tween-20 (TBST) for 1 hour at room temperature. Primary antibodies were diluted 1:1000 in TBST containing 1% nonfat dry milk, except GAPDH antibody, which was diluted 1:5000 in TBST containing 1% nonfat dry milk, and phospho-VASP^{Ser239} which was diluted 1:1000 in TBST containing 1% BSA. The membrane was incubated in primary antibody overnight at 4^{o}C. Secondary anti-rabbit antibody was diluted 1:5000 in TBST containing 1% nonfat milk. Membrane was incubated in secondary antibody at room

temperature for 1 hour. Protein bands were visualized using Millipore Immobilin ECL reagent.

siRNA: All siRNA and transfection reagents were purchased from Santa Cruz Biotechnology. 120,000 cells were plated per well of a 6-well plate. After 48 hours of growth, at approximately 70% confluency, the cells were treated with 40pmol siRNA according to the manufacturer's specifications for 24 hours. After 24 hours, the transfection media was replaced with normal growth media and cells were assayed after an additional 24, 48, or 72 hours. For all transfection experiments, a second set of cells were treated with transfection reagent minus siRNA as a negative control.

Growth Assays: Sulindac sulfide and NOR-3 were purchased from Enzo Life Sciences. Both drugs were reconstituted in DMSO. For evaluation of cell growth, cells were plated and transfected as described above. 48 hours after transfection media was replaced with normal growth media, cells were treated with the specified compound or vehicle (0.2% DMSO) diluted in normal growth media. 72 hours after drug treatment, cells were collected and counted by hemacytometry. Viability of each sample was determined using trypan blue exclusion.

Results

Expression of PKG isoforms in human breast cells MDA-MB-231

Expression of PKG isoforms in human breast cells, both normal and cancerous, was determined by RT-PCR using isoform specific primers. All cells predominantly express PKGIβ, with the highest expression in MDA-MB-231 and normal (HMEC) breast cells as shown in Figure 1A and quantified in Figure 1B. The Western blot for PKGI confirms these findings, showing that both MDA-MB-231 and SKBr-3 cells

express PKGI, but the level of expression is higher in MDA-MB-231 cells as shown in Figure 1C.

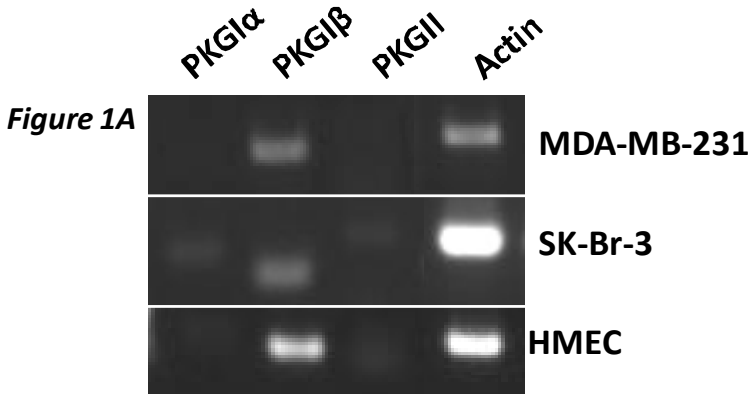
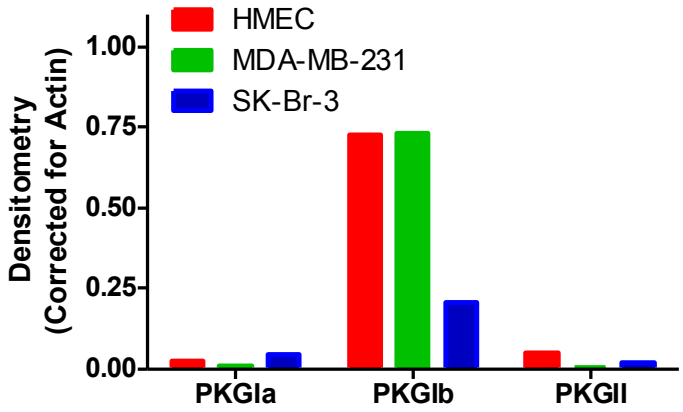


Figure 1A

Figure 1B



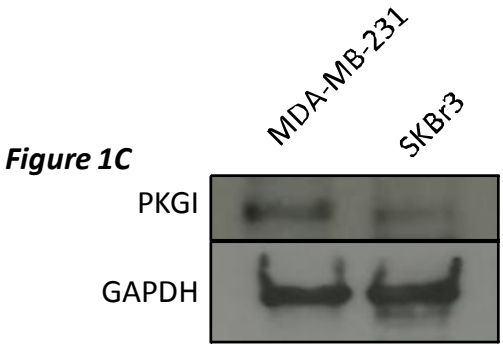


Figure 1C

siRNA knockdown of PKGI in human breast cancer cells

To determine the efficiency of siRNA knockdown of PKGI in MDA-MB-231 cells, protein and RNA were isolated from the cells after 24, 48, and 72 hours of treatment with PKGI specific siRNA.

RT-PCR using PKGIβ-specific primers showed that there was not much break down of mRNA but there was some degradation on day 3 of treatment with siRNA as shown in Figures 2A and 2B.

Figure 2A

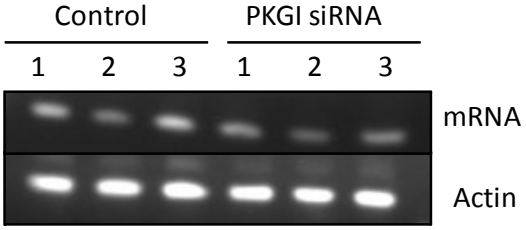
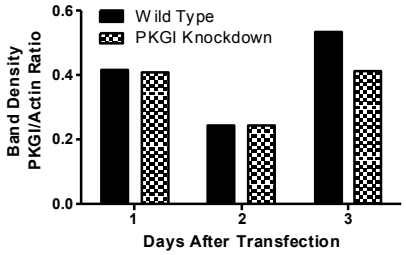


Figure 2B



As shown in Figures 2C and 2D, there were significant decreases in the amount of PKGI in MDA-MB-231 cells treated with siRNA when compared with the untreated controls. Protein expression decreases as the siRNA was prolonged, with 3 days of treatment resulting in levels of protein that were below the level of detection for the Western blot.

Figure 2C

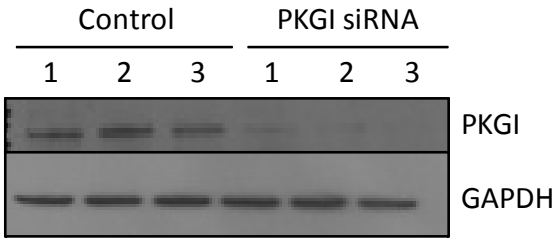
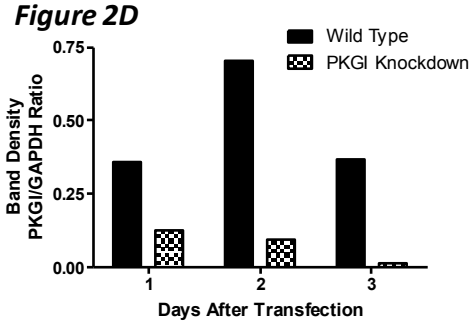


Figure 2D



Importance of PKGI for the growth-inhibitory activity of SS and NOR-3

To determine if PKGI is necessary for the anti-cancer activity of the cGMP signaling pathway, sensitivity of the MDA-MB-231 cell line to two different drugs, SS and NOR-3, in the presence of PKGI siRNA was measured.

To determine the effects of treatment on growth and survival of the cells, cells were counted by hemacytometry. As shown in Figure 3A, there was no significant difference between untreated and PKGI siRNA treated cells for the vehicle. However, there was a difference between untreated and PKGI siRNA treated cells that were also treated with 100μM sulindac sulfide and a significant difference between untreated and PKGI siRNA treated cells that were also treated with 75μM NOR-3. As predicted, decreasing the expression of PKGI caused the cells to be less sensitive to the effects of SS and NOR-3 with a large reduction in the untreated cell number as opposed to the PKGI siRNA treated cells. As predicted because of the differences from the growth assay data, PKGI siRNA treatment decreased the amount of caspase activation that was caused by both SS and NOR-3 treatments as shown in Figures 3B and 3C.

Figure 3A

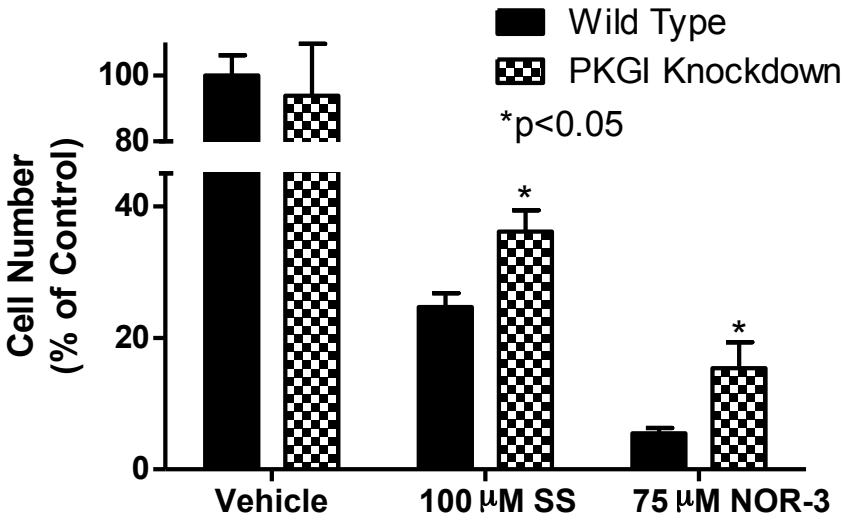


Figure 3B

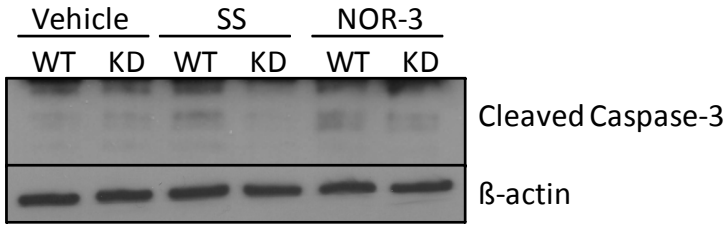
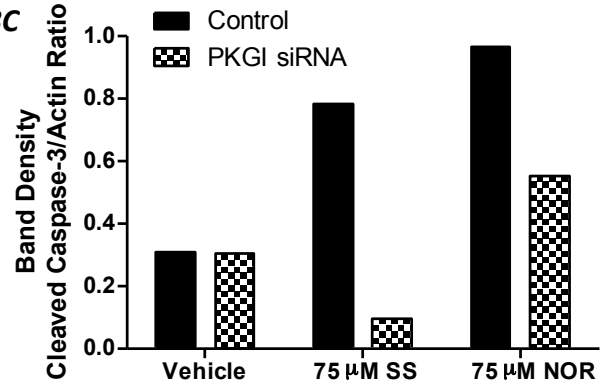


Figure 3C



Discussion

As discussed previously, it is known that cGMP signaling is effective to induce apoptosis of cancer cells and has also been shown to have negative effects on cancer cell growth and survival [4, 5]. It has also been shown that PDE5 is over-expressed in breast cancer cells and that activation of cGMP signaling due to inhibition of PDE5 results in apoptosis in tumor cells [1, 7]. However, the exact mechanism responsible, while assumed to involve PKG has yet to be experimentally determined. Through the use of PKGI siRNA and two drugs that act as cGMP signaling activators with known *in vitro* anti-cancer activity (SS, a drug that inhibits PDE5, and NOR-3, which activates GC), the studies presented her demonstrate that PKGIβ is at least partially necessary for the proapoptotic effects of cGMP signaling.

From the data gained from the Western blot of the siRNA PKGIβ knockdown of MDA-MB-231 breast cancer cells it was shown that two to three days of treatment with PKGI-specific siRNA was sufficient to effectively decrease the expression of PKGIβ in these cells. With this data, cancer cells were treated with siRNA for two days in order to knockdown the expression of PKGIβ prior to treatment with SS and NOR-3.

These treatment studies provided some interesting results. First, it was observed that there was no significant difference between control cells and cells treated with only PKGI siRNA. This shows that PKGI is not necessary for these breast cancer cells to grow and survive. Second, there appeared to be large differences between untreated and PKGI siRNA treated cells with SS treatment and also with NOR-3. With a decrease of the expression of PKGI, the cells were less sensitive to the effects of NOR-3 and SS, which suggests that PKGI is necessary for a least a portion of the growth inhibitory

activity of these drugs. However, an alternative mechanism must also be involved since even with substantial PKGI knockdown, cells growth was still inhibited by the compound.

One issue that could be happening is that the growth inhibitory mechanisms for SS and NOR-3 could be different. NOR-3 activates soluble guanylyl cyclase, which is present in the cytoplasm of cells. SS inhibits PDE5, which is present in the cytoplasm but also in very high levels on the nuclear envelope. With the protein targets of the drugs being in separate locations within the cell, even though they both activate cGMP signaling, they may utilize different cGMP pathways depending on which effector proteins are located near them. Additionally, SS is known as a fairly promiscuous drug, having multiple targets. In fact, the primary target of SS is cyclooxygenase, a family of enzymes completely unrelated to cGMP signaling. It is possible that a cGMP-independent target may be compensating for any loss of activity that was afforded by PKGI knockdown. This is supported by previous studies, which showed that SS can inhibit apoptosis while inducing cell cycle arrest, whereas cGMP specific drugs like MY5445 (a PDE inhibitor) were only able to inhibit apoptosis.

The growth data for SS and NOR-3 were conclusive with the hypothesis, but to be more accurate, a Western blot for cleaved caspase 3 was completed. The Western blot analysis for cleaved caspase 3 was strongly indicative of PKGI being vitally important for the induction of apoptosis caused by SS and NOR-3. Caspase is indicative of induction of apoptosis and there was much more cleaved caspase present in the control cells that still expressed PKGI. Therefore, those cells treated with siRNA and SS or NOR-3 were less sensitive to these drugs and apoptosis was not induced as intensely as in

those cells that expressed PKGI. Because of these results it can be concluded that PKGI is very important for the cGMP induced apoptosis of breast cancer cells.

Together, these findings demonstrate that while there still are some unknown parts of the mechanisms that may be affecting the cGMP pathway, PKG is very important for the cGMP induced apoptosis of breast cancer cells. While the growth assays were conclusive, more reliable results were obtained by Western blots done for cleaved caspase 3. The Western blots done for caspase are also conclusive with the hypothesis that PKGI is necessary for cGMP induced cell death to occur. It has been determined that PKGI plays a pivotal role in the cGMP pathway, even though there may be other mechanisms yet to be determined. Further studies should be done to investigate what other mechanisms play a part in the cGMP induced apoptosis of breast cancer cells. However, the findings of this study will be helpful in future drug discovery in that a novel drug target has been identified.

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