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Brief Report

PKCeta Promotes Stress-induced Autophagy and Senescence in Breast Cancer Cells, Presenting A Target for Therapy

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Abstract: The emergence of chemoresistance in neoplastic cells is one of the major obstacles in cancer therapy. Autophagy was recently reported as one of the mechanisms that promote chemoresistance in cancer cells by protecting from apoptosis and driving senescence. Thus, understanding the role of autophagy and its underlying signaling pathways is crucial for the development of new therapeutic strategies to overcome chemoresistance. We have previously reported that PKCη is a stress-induced kinase that confers resistance in breast cancer cells against chemotherapy by inducing senescence. Here we show that PKCη promotes autophagy induced by ER and oxidative stress and facilitates the transition from autophagy to senescence. We demonstrate that PKCη knockdown reduces both the autophagic flux and markers of senescence. Additionally, using autophagy inhibitors, such as chloroquine and 3-methyladenine, we show that PKCη and autophagy are required for establishing senescence in MCF-7 in response to oxidative stress. Different drugs used in the clinic are known to induce autophagy and senescence in breast cancer cells. Our study proposes PKCη as a target for therapeutic intervention, acting in synergy with autophagy-inducing drugs, to overcome resistance and enhance cell death in breast cancer.

Keywords: Protein Kinase C; PKCη, autophagy; senescence; chemoresistance; oxidative stress; ER stress; 3MA; Chloroquine

1. Introduction

Autophagy is regarded as a cellular 'recycling factory', removing non-functional proteins and organelles, generating building blocks necessary for cell survival [1]. Three types of autophagy have been described so far based on the molecular machinery, morphological characteristics and mechanisms by which intracellular components are delivered for degradation: Microautophagy, chaperone mediated autophagy (CMA), and macroautophagy (commonly termed as autophagy) [2, 3]. Autophagy initiates with isolated membranes (phagophores) derived from a lipid bilayer, although their exact membranal origin in mammals is controversial. The phagophore expands to engulf intracellular cargo, such as protein aggregates, organelles, and ribosomes, thereby sequestering the cargo in a double-membrane autophagosome. The loaded autophagosome matures through fusion with the lysosome (autophagolysosome), promoting degradation of the autophagosomal contents by lysosomal acid proteases. The byproducts of degradation are recycled back to the cytoplasm, where they can be reused [1, 4, 5]. During normal cellular homeostasis, autophagy functions as a primary route of degradation for damaged organelles and protein aggregates. As an intracellular self-destructive system, autophagy must be tightly regulated to adapt to different intracellular and extracellular stressors [6]. Interestingly, in cancer, autophagy plays opposing roles— under certain conditions, it has a cytoprotective effect that causes chemotherapy resistance; in

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others, it has a cytotoxic effect, through which some compounds induce autophagy-mediated cell death [2, 3, 7].

Several studies identified autophagy as an effector mechanism of senescence, important for rapid protein remodeling required for an efficient transition from a proliferative to a senescent state (characterized by stable cell cycle arrest with an active metabolism). For example, it was demonstrated that oncogene-induced senescence (OIS) could be dependent on prior induction of autophagy [8]. In agreement, autophagy and senescence appear to be regulated by overlapping signaling pathway involving the generation of ROS, activation of ATM, induction of p53 and p21, and dephosphorylation of pRb [9]. Interestingly, suppression of autophagy induced apoptosis and attenuated senescence, suggesting that autophagy plays an important role in inducing or sustaining senescence [10].

A role for PKC family members in autophagy has recently emerged. PKCδ was demonstrated to activate autophagy by promoting Jun N-terminal kinase 1 (JNK1)-mediated Bcl-2 phosphorylation and dissociation of the Bcl-2/Beclin-1 complex [11, 12]. The pharmacological agents' safingol [13] and oridonin [14] were shown to trigger autophagy via PKC [15, 16]. Activation of PKCθ was required for ER stress-induced autophagy [17]. PKCη expression was reported to promote cellular senescence in MCF-7 cells in response to DNA damage [18]. Here we show a role for PKCη in the induction of autophagy and demonstrate that both PKCη and autophagy are required for establishing senescence in MCF-7 in response to oxidative stress.

2. Materials and Methods

2.1. Cells

MCF-7 and MCF21.5 (MCF-7 cells inducibly-expressing PKCη, previously described [19]), were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 100U/ml penicillin, 0.1mg/ml streptomycin, 2mM L-glutamine, and 10% Fetal Bovine Serum (Biological Industries, Bet Haemek, Israel) in 5% CO_2 humidified atmosphere at 37°C. The medium for MCF21.5 cells additionally included hygromycin B (100μg/ml), G418 sulphate (200μg/ml) (Calbiochem, Merck, MA, USA), and tetracycline (2μg/ml) (Sigma-Aldrich, Israel). The expression of PKCη was induced by the removal of tetracycline from the growth medium.

2.2. Antibodies and reagents

Anti-PKCη (sc-215), p21 (sc-397), and p27 (sc-528) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PARP-1 (9542) was purchased from Cell Signaling Technology (CST, MA, USA). Anti-LC3 (L8918) was purchased from Sigma-Aldrich (Sigma-Aldrich, Israel). Anti-p62 (ab56416) was purchased from Abcam (Abcam, MA, USA). Anti-H3K9meth was purchased from Abcam. Anti-actin was purchased from ICN (691001, ICN Biomedicals, USA). Anti-pPKCηSer675 was specially made from PhosphoSolutions (PhosphoSolutions, CO, USA). iPKCη was purchased from Calbiochem. Horseradish peroxidase conjugated to donkey anti-rabbit (NA934V) or anti-mouse (NA931V) immunoglobulin were from Amersham Biosciences (NJ, USA).

For inhibition of autophagy, 3-Methyladenine (3MA), a PI3K inhibitor, was purchased from Merck (Merck Millipore, MA, USA). Chloroquine (CQ) was purchased from Sigma-Aldrich.

2.3. Cell lysis and Western blot analysis

Cell lysis and western blot analysis was performed as described. Briefly, whole-cell extracts were prepared by lysing cells in RIPA lysis buffer containing 10 mM Tris (pH 8.0), 100 mM NaCl, 5 mM EGTA (pH 8.0), 45 mM 2-mercaptoethanol, 1% NP-40, 10 mM EGTA (pH 8.0), 50 mM NaF, and 0.1% SDS supplemented with protease inhibitors (1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and phosphatase inhibitors (1 mM sodium orthovanadate, 50 mM β-glycerol phosphate, and 5 mM sodium pyrophosphate).

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The lysates were incubated on ice for 30 min, sheared several times through a 21-gauge needle and were centrifuged at 13,000 g for 25 min at 4 °C. The protein concentrations were determined by using the Bio-Rad (Hercules, CA) protein assay and aliquots of 30-60 μg protein were prepared. The samples were resolved by electrophoresis on 10-15% polyacrylamide gels using Bio-Rad Mini-PROTEAN II cells. Proteins from the gel were electroblotted onto PVDF (Bio-Rad) in Bio-Rad Mini Trans-Blot transfer cells followed by 1 h of blocking with 3% BSA in PBS at 37 °C. The PVDF membranes were incubated sequentially with indicated primary antibodies overnight at 4 °C, followed by the HRP-conjugated secondary antibodies. Immunoreactive protein bands were detected using the ECL reagent (Biological Industries) by the GelDoc (Bio-Rad) system.

2.4. SA-β-galactosidase staining

Senescence-associated b-galactosidase (SA-β-gal) activity was determined using a previously described protocol [18]. Briefly, cells were washed once with PBS, fixed with 0.5% glutaraldehyde for 15 minutes followed by 2x PBS wash supplemented with 1mM $MgCl_2$. Cells were stained in X-gal solution (1 mg/ml X-gal, 0.12mM $K_3Fe[CN]_6$, 0.12mM $K_4Fe[CN]_6$ in PBS at pH 6.0) overnight at 37^{O}C. Cells were photographed using an IX70Olympus optical light microscope. To estimate total cell numbers, cell cultures were stained with 10mg/ml Hoechst 333432 (#H6024, Sigma-Aldrich, Israel) for 30 min at 37^{O}C before β-gal staining. Hoechst fluorescence was detected using a light source providing light at 340–380nm, emission was at 465nm. The % of SA-β-gal-positive cells out of Hoechst-stained cells were calculated using Image J software.

2.5. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 9 for Windows (GraphPad Software, San Diego, CA). All variables are expressed as means $\pm$ SEM. As indicated in the figure captions, the p-values were calculated using an unpaired Student's t-test or with a one-way ANOVA.

3. Results

3.1. PKCη enhances autophagy induced by ER and Oxidative stress

The novel PKC isoform, PKCη, is an anti-apoptotic stress-induced kinase that was shown to be involved in a variety of cellular responses, such as differentiation, proliferation, and secretion [20, 21]. We have previously reported that PKCη promotes cellular senescence in response to oxidative stress [18]. Our aim here was to determine whether PKCη has a role in oxidative stress-induced autophagy, since autophagy and senescence are indicated to be interconnected.

PKCη is localized in the perinuclear region, the endoplasmic reticulum (ER), and the Golgi apparatus [22], suggesting that it may have a role in ER stress-induced autophagy . ER stress is caused by the accumulation of unfolded and/or misfolded proteins in the ER lumen, resulting in an adaptive signaling pathway termed the unfolded protein response (UPR) and in the degradation of misfolded proteins through ERAD and/or autophagy. As ER stressors we used Tunicamycin (TM) (inhibits N-acetylglucosamine (GlcNAc) phosphotransferase) and Thapsigargin (TG) (inhibits the sarco/endoplasmic reticulum ATPase (SERCA) resulting in increased cytosolic Ca^{2+} concentration).

MCF-7 cells inducibly expressing PKCη under the control of the tetracycline-responsive promoter (MCF21.5, previously described [19]), were treated with H_2O_2 in the presence or absence of chloroquine (CQ), an inhibitor of late-stage lysosomal degradation, causing blockage of the autophagy flux. As shown in Fig.1A, PKCη-expressing cells (-Tet) exhibited higher levels of the autophagic marker LC3-II compared to control cells (+Tet), suggesting that PKCη enhances LC3-II recruitment to autophagosomes, which was evident when lysosomal degradation was blocked by CQ. When cells were treated only with CQ, the level of LC3-II was not altered, implying that basal autophagy in growing and unstressed cells is not affected by the inducible expression of PKCη.

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The effects of PKCη in enhancing autophagy in response to TM and TG were similar to those observed by oxidative stress. As shown in Fig.1B, C, when cells were treated with TM (10mg/ml) and TG (100 nM) for 24 h, LC3-II levels were similar in PKCη-expressing cells (-Tet) compared to control cells (+Tet). However, when the autophagic flux was inhibited by CQ, the levels of LC3-II were higher in PKCη-expressing cells (-Tet) compared to control cells (+Tet), suggesting enhanced autophagic flux by PKCη.

Another marker used to monitor autophagic flux is p62, also called sequestosome 1 (p62/SQSTM1). p62 possesses a short LC3 interaction region (LIR) that facilitates direct interaction with LC3 and GABARAP-family members and causes p62 to be specifically degraded by autophagy. Because its degradation is dependent on autophagy, the level of p62 increases when autophagy is inhibited [23]. As depicted in Fig.1 A, B in response to oxidative stress and ER stress by TM, accumulation of p62 levels was observed in PKCη-expressing cells (-Tet) compared to control cells (+Tet) upon inhibition of the autophagic flux, supporting that autophagy was promoted by overexpression of PKCη.

The activation and stability of PKC family members are dependent on post-translational regulation such as phosphorylation and translocation [24]. PKCη contains three conserved phosphorylation sites: the activation loop (Thr513), turn motif (Thr655), and hydrophobic motif (Ser675) [25, 26]. We have previously shown that phosphorylation on the hydrophobic motif Ser675 of PKCη is increased in response to stress by the chemotherapeutic drug etoposide [27]. As depicted in Fig.1D, E the levels of phosphorylation on Ser675 of PKCη were increased following TM and H_2O_2 treatment demonstrating that PKCη is activated.

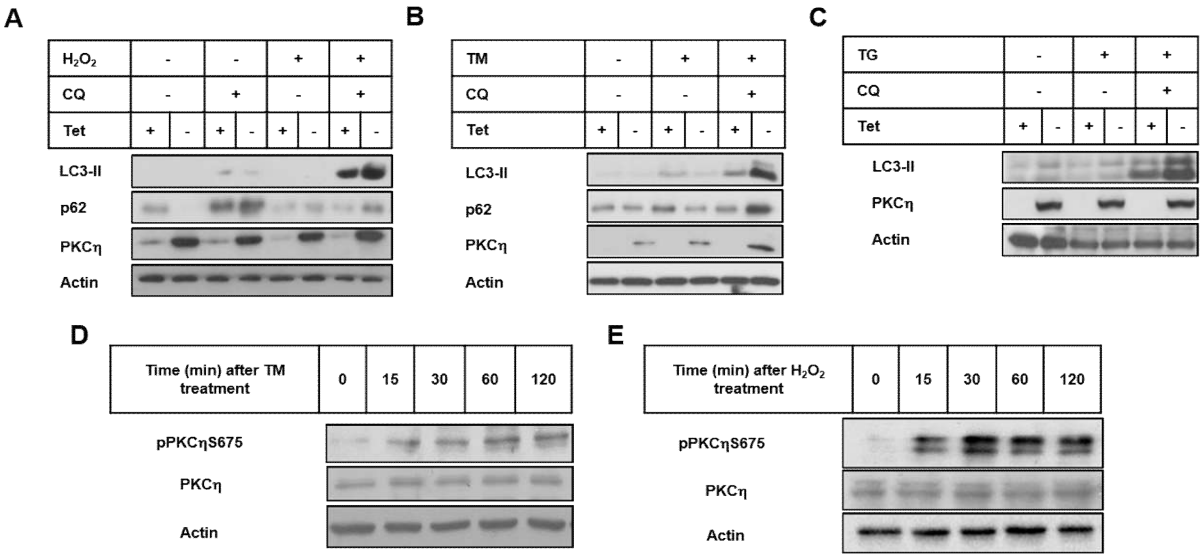

Figure 1. PKCη enhances autophagy induced by ER and Oxidative stress. (A) Sub-confluent MCF21.5 cells (MCF-7 cells inducibly expressing PKCη) were grown in the presence/absence of tetracycline (2 mg/ml) for 48 h. Cells were incubated with CQ (10μM) for 1 h in serum-free medium, followed by H_2O_2 (150μM) treatment for 2 h. Fresh growth medium was replaced for 24 h. (B, C) Sub-confluent MCF21.5 cells were grown as described in (A). After CQ treatment, tunicamycin (TM, 10μg/ml) or thapsigargin (TG, 100nM) were added for 24 h, respectively. Whole-cell lysates were prepared and subjected to immunoblotting with the indicated antibodies. (D) Sub-confluent MCF-7 cells were treated with TM (10μg/ml or (E) H_2O_2 (150μM) for the indicated time points. The phosphorylation of Ser675 on PKCη was detected using a specific antibody. Actin was used as the loading control. Results are representative of three independent experiments.

3.2. PKCη-knockdown reduces autophagy

MCF-7 cells stably transfected with shRNA-PKCη constructs (shPKCη3-5 and shPKCη2-2) and control plasmid (shScr5-3) (previously described [18]), were treated with/without H_2O_2 in the presence/absence of CQ. Treatment with both H_2O_2 and CQ

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resulted in lower levels of LC3-II in PKCη-knockdown cells (shPKCη3-5 and shPKCη2-2) compared to control cells (shScr5-3), suggesting that PKCη-knockdown reduced the autophagic flux in response to oxidative stress.

To confirm that knockdown of PKCη results in inhibition of autophagy, we examined the effects of a PKC-inhibitory peptide (iPKCη), developed to specifically inhibit PKCη kinase activity. The peptide iPKCη itself did not affect LC3-II levels, however, its presence diminished H_2O_2-induced LC3-II levels (Fig.2B). Taken together, our results demonstrate that PKCη enhances autophagy in response to oxidative stress.

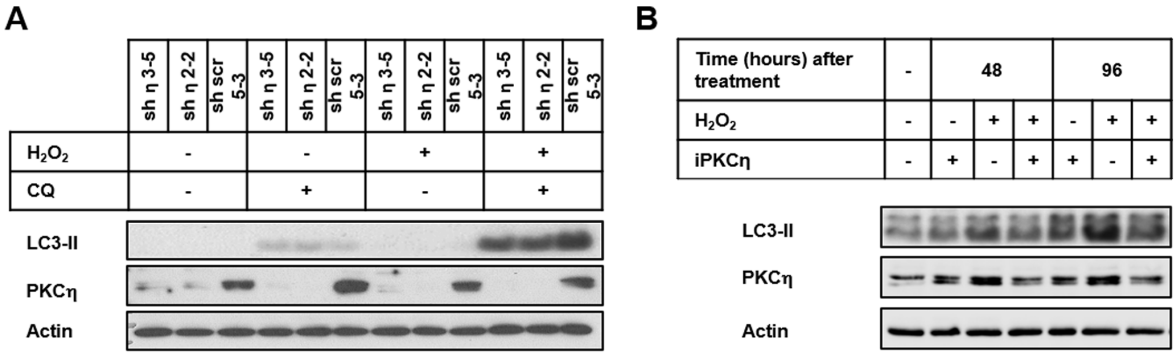

Figure 2. PKCη-knockdown reduces oxidative stress-induced autophagy. (A) Sub-confluent MCF-7 stably transfected with shRNA constructs (shPKCη3-5, shPKCη2-2) or scrambled control plasmid (shScr5-3) were treated with CQ (10μM) for 1 h in serum-free medium, followed by H_2O_2 (150μM) for 2 h. Fresh growth media was replaced for 24 h. (B) Sub-confluent MCF-7 cells were treated with an inhibitory peptide for PKCη (iPKCη) for 4 h in serum-free medium, followed by treatment with H_2O_2 (150μM) for 2 h. Fresh growth medium was replaced for 48 h and 96 h. At the end of the experiments, whole-cell lysates were prepared and subjected to immunoblotting with indicated antibodies. Actin was used as the loading control. Results are representative of three independent experiments.

3.3. . Inhibition of autophagy attenuates the induction of senescence by PKCη

Our current study shows a role for PKCη in promoting autophagy under oxidative stress. To investigate the transition from autophagy to senescence, we inhibited autophagy using 3MA, under conditions of oxidative stress, followed by assessing SA-β-gal and other senescence markers. 3MA is an inhibitor of the activity of class III phosphatidylinositol 3-kinase (PI3K type III) that prevents the formation of autophagosomes, thereby blocking the autophagic process [28] [28]. As shown in Fig. 3A, B the addition of 3MA to the H_2O_2-treated MCF-7 cells decreased SA-b-gal staining. The expression levels of the senescence markers; the cell cycle inhibitor p21, and the histone H3K9me2/3 were reduced in H_2O_2-treated cells upon 3MA application (Fig. 3C). Furthermore, inhibition of autophagy by 3MA promoted apoptotic cell death in H_2O_2-treated cells as indicated by the increased levels of cleaved PARP-1 (Fig. 3D). In CQ treated cells, PARP-1 cleavage was increased under basal condition, however, H_2O_2 treatment did not alter PARP-1 cleavage (Fig. 3D). Our data suggests that autophagy is required to protect MCF-7 cells from oxidative stress induced cell death.

We have previously demonstrated a role for PKCη in promoting senescence [18]. To investigate the role of PKCη in regulating the transition from autophagy to senescence, we employed the PKCη-knockdown cells described above. As shown in Fig. 3E, F, the percentage of SA-b-gal positive cells following oxidative stress were lower in PKCη-knockdown cells (shPKCη2-2) compared to control cells (shScr5-3). Inhibition of autophagy using CQ significantly reduced SA-b-gal positive cells in PKCη expressing MCF-7 cells (shScr5-3) (Fig. 3E, F). The oxidative stress-induced autophagic flux was reduced in PKCη-knockdown cells as indicated by lower levels of LC3-II compared to scrambled control cells (Fig. 3G). Taken together, our results suggest that PKCη promotes oxidative stress-induced senescence via the upregulation of autophagy.

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4. Discussion

One of the major obstacles in cancer therapy is the emergence of chemoresistance. Recently, autophagy has been reported as one of the mechanisms that promote resistance in cancer cells (e.g., in HER2-positive breast cancer) [29]. Autophagy functions to remove oxidized proteins and damaged mitochondria accumulating in cells [30], and is frequently linked to the induction of cellular senescence [9]. In this study, we show that PKCη promotes autophagy in response to ER and oxidative stress. Moreover, we also show a role for PKCη in the transition from autophagy to senescence in stress-induced cells by demonstrating that its knockdown reduces the autophagic flux and induction of senescence.

Using both PKCη-overexpressing MCF-7 cells and cells with endogenously reduced PKCη expression by shRNA knockdown, we demonstrate that PKCη enhances the autophagic flux upon stress. Additionally, inhibition of PKCη kinase activity in MCF-7 cells using a specific PKCη inhibitor (iPKCη) resulted in lower levels of LC3-II, suggesting that PKCη activity is required for enhancing autophagy.

Autophagy is currently considered an important mechanism for intervention in anticancer therapy to abrogate cancer resistance [2, 3, 7, 29]. Thus, to design new antitumoral therapies targeting stress-induced autophagy, it is important to understand the regulatory pathways modulating autophagy. Our results, demonstrating that PKCη promotes autophagy induced by ER stress and oxidative stress in MCF-7 cells, may be of therapeutic relevance in breast tumors expressing high levels of PKCη. Targeting this signaling pathway could reduce resistance to anti-cancer therapy since we have already established that PKCη promotes chemotherapeutic resistance in breast cancer cells treated with DNA damaging drugs [18].

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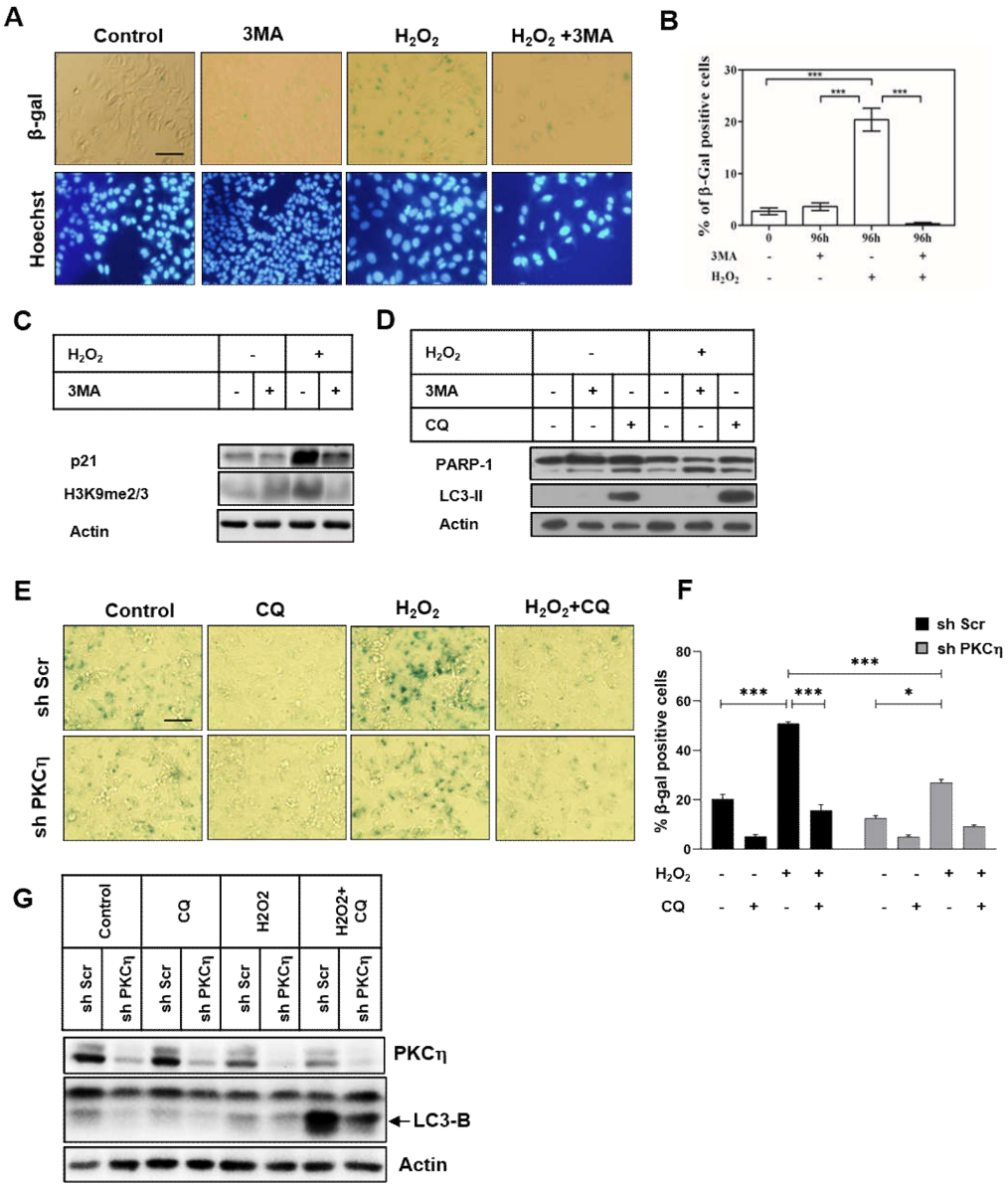

Figure 3. **Inhibition of autophagy attenuates the induction of senescence by PKCη.** (A) Sub-confluent MCF-7 cells were treated with 3MA (1mM) for 1 h in serum-free medium , followed by the addition of H_2O_2 (150μM) for 2 h. Fresh growth medium was added for 96 h. Cells were stained for SA-β-gal and the nuclei were stained with Hoechst. Images were taken by fluorescence inverted microscope. Scale bar equals 50μm. (B) Quantification of SA-β-gal positive cells was performed as described in Materials and methods. (C, D) Sub-confluent MCF-7 were grown and treated as described in (A). Whole-cell lysates were prepared and subjected to immunoblotting with indicated antibodies. (E) Sub-confluent MCF-7 stably transfected with shRNA constructs (shPKCη2-2) or control plasmid (shScr5-3) were grown for 48 h and were treated with CQ (10μM) for 1 hour, followed by H_2O_2 (150μM) in serum-free medium for 2 h. Fresh growth medium was added for 96 h. Cells were stained for SA-β-gal and images were taken by fluorescence inverted microscope. (F) Quantification of SA-β-gal positive cells was determined as described in Materials and Methods. (G) Whole-cell lysates were subjected to immunoblotting with indicated antibodies. Actin was used as the loading control Results are representative of three independent experiments. Statistical analysis was performed using one-way ANOVA; *p<0.05, ***p<0.001.

Several studies have demonstrated that autophagy is followed by senescence in response to oxidative stress [31, 32]. PKCη was previously shown to promote cellular senescence induced by DNA damaging agents (H_2O_2 or Etoposide) as demonstrated by increased expression of senescence markers (p21 and pRb) and elevated senescence-associated secretory phenotype (SASP) [18]. Another study presented evidence

that the induction of autophagy by p38α protects U2OS cancer cells from doxorubicin-induced apoptosis by promoting senescence [33]. Here we show that PKCη expression promotes autophagy and senescence induced by oxidative stress and that the inhibition of either autophagy or PKCη expression reduces cellular senescence. Several studies have demonstrated that interference/inhibition of autophagy compromises the generation of senescent cells; however, definitive proof that senescence is entirely dependent on prior autophagy is still under debate. Our study supports the role of autophagy as a pro-senescence mechanism, required to induce senescence in MCF-7 breast cancer cells.

Taken together, we show that PKCη is involved in the cellular response to oxidative stress through its role in autophagy regulation and subsequent induction of senescence. Upregulated PKCη expression in stressed cells [34], exhibiting activated survival pathways such as autophagy and senescence, implies that targeting PKCη could act in synergy with drugs used in breast cancer therapy, inducing oxidative stress and autophagy, such as Lapatinib, Gemcitabine, Tamoxifen, Trastuzumab etc. [3, 29, 35]. Thus, targeted therapy against PKCη could restrict breast cancer cell survival and chemoresistance in response to drug-induced autophagy.

Author Contributions: E.L. conceived the study and supervised the project; N.R.D, A.M, and E.L. designed the experiments; N.R.D. and A.M. carried out the experiments and analysis; N.R.D and A.M. wrote the original manuscript. A.M and E.L reviewed and edited the manuscript.

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Data Availability Statement: The data that support the findings of this study is available from the corresponding author on reasonable request

Conflicts of Interest: Authors declare no competing interest.

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