PKCδ Regulates Death Receptor 5 Expression Induced by PS-341 through ATF4–ATF3/CHOP Axis in Human Lung Cancer Cells

Linyan Xu, Ling Su, and Xiangguo Liu

Abstract

PS-341 (bortezomib), a proteasome inhibitor, has been approved for the treatment of multiple myeloma. Our previous work has shown that PS-341 induces death receptor 5 (DR5)-dependent apoptosis and enhances the TNF-related apoptosis-inducing ligand–induced apoptosis in human non–small cell lung cancer cells. However, the definite mechanism remains undefined. In the present study, we reveal that PKCδ and RSK2 mediate PS-341–induced DR5 upregulation, involving coactivation of endoplasmic reticulum (ER) stress. We discovered that PS-341 activated ER stress through elevating the expression of BiP, p-eIF2α, IRE1α, ATF4, ATF3, and CCAAT/enhancer-binding protein homologous protein (CHOP). Further study showed that DR5 upregulation was dependent on ATF4, ATF3, and CHOP expression. Silencing either one of the ATF4, ATF3, and CHOP decreased DR5 upregulation and subsequent apoptosis. We determined that ATF4 regulated ATF3 and CHOP expression. Thereafter, ATF3 and CHOP formed a complex and regulated DR5 expression. In addition, we discovered that the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 and RSK2 were elevated after PS-341 treatment and inhibition of their phosphorylation using MAP-ERK kinase 1/2 inhibitor decreased the DR5 level, indicating that ERK/RSK2 signaling is involved in DR5 upregulation. Furthermore, we detected the cleavage of PKCδ, and the blockage of PKCδ expression cut down DR5 upregulation and apoptosis. Importantly, knockdown of PKCδ expression decreased the induction of ER stress and the phosphorylation of ERK1/2 and RSK2, suggesting that PKCδ regulates DR5 expression through ERK/RSK2 signaling and ATF4–CHOP/ATF3 axis. Collectively, we show that PS-341 induces PKCδ-dependent DR5 expression through activation of ERK/RSK2 and ER stress signaling pathway. Mol Cancer Ther; 11(10); 1–9. ©2012 AACR.

Introduction

Death receptor 5 (DR5, also named Apo2), one of the TNF-related apoptosis-inducing ligand (TRAIL) receptors, belongs to the TNF receptor superfamily (1). It is located on the cell surface, and becomes trimerized on binding to its ligand TRAIL and recruits adapter proteins, such as Fas-associated death domain, then forms the death inducing signaling complex, and eventually activates caspase cascades (1). Nowadays, many studies have shown that inducing the expression of DR5 contributes to certain cancer therapeutic agents-induced apoptosis and enhances TRAIL-induced apoptotic pathway (1).

CCAAT/enhancer-binding protein homologous protein (CHOP), a downstream component of endoplasmic reticulum (ER) stress pathways, can be mediated by pancreatic ER kinase-like ER kinase-, activating transcription factor 6 (ATF6)-, and inositol-requiring enzyme 1α (IRE1α)-induced signal transduction pathways (2, 3). Many studies have documented that CHOP promotes apoptosis through upregulation of DR5 expression caused by many agents, such as lonafarnib (4), MG132 (5), CDDO-Me (6) in a variety of cancer cells. Activating transcription factor 4 (ATF4) and ATF3 are stress responsive proteins, which can be induced by ER stress (7, 8). Some earlier studies have found that ATF3 can suppress CHOP gene transcription (9), whereas CHOP inhibits ATF3 protein function (10). However, some data show that ATF3 can promote CHOP expression (11).

The protein kinase C (PKC) family is important in regulation of cell proliferation and apoptosis. PKCδ is generally considered to be a proapoptotic protein (12, 13). Studies have revealed that PKCδ can be cleaved between the regulatory domain and catalytic
domain when cells are exposed to diverse stimuli. Consequently, a 41 kDa fragment is generated, which has constitutive bioactivity and is responsible for apoptosis (14–16).

Many preclinical experiments have shown that the proteasome inhibitor PS-341 (Fig. 1A; ref. 17) alone or combination with other agents have antitumor effect in various cancers, including lung cancer (17, 18). However, the molecular mechanism underlying PS-341 mediated apoptosis is not very clear now. Our previous studies have shown that DR5 expression contributes to PS-341-mediated apoptosis in human non–small cell lung cancer (NSCLC) cells (18). To further characterize the mechanism by which PS-341 upregulates DR5 expression in NSCLC cells, we explored the molecular mechanism of PS-341 on the induction of ER stress and PKCd.

Figure 1. PS-341 (A) enhances CHOP expression in a dose-dependent and a time-dependent manner (B), and inhibiting CHOP expression by siRNA decreases PS-341–induced DR5 upregulation and apoptosis (C and D). A, the chemical structure of PS-341. B, the indicated lung cancer cell lines were treated with 0, 100, 200 nmol/L PS-341 for 24 hours or treated with 50 nmol/L PS-341 for the indicated time. H460, A549, and Calu-1 (C and D) cells were seeded in 6-well plates and on the second day transfected with control (Ctrl) or CHOP siRNA. Forty-eight hours after the transfection, cells were treated with the indicated concentration of PS-341 for 16 hours (C) or 24 hours (D). Then the cells were harvested and prepared for Western blot analysis (C) or for detection of apoptotic cells using Annexin V/7-AAD staining (D). CF, cleaved form.

Materials and Methods

Reagents

The powder of pure PS-341 was purchased from LC Laboratories. DR5 antibody was purchased from ProSci. Caspase-3 antibody was purchased from Imgenex. Caspase-8, caspase-9, PARP, p-ERK, p-RSK2, BiP, IRE1α, p-eIF2α, and eIF2α antibodies were purchased from Cell Signaling Technology. CHOP, ATF3, ATF4, and PKCd antibodies were purchased from Santa Cruz.

Cell culture

The human NSCLC cell lines A549, H1792, H460, H157, and Calu-1 were originally obtained from the American Type Culture Collection. A549 and H1792 cell lines were authenticated in Microrna Gene Technology by short tandem repeat analysis on March 19, 2012. H460, H157, and Calu-1 have not been re-authenticated. The cells were
grown in monolayer culture in RPMI 1640 with 5% FBS at 37°C in a humidified atmosphere consisting of 5% CO2 and 95% air.

**Western blot analysis**
The preparation of whole cell protein lysates and the Western blot analysis processes were described previously (19).

**Silencing of gene expression using small interfering RNA**
The small interfering RNA (siRNA) duplexes for control, ATF4, ATF3, and CHOP genes were described previously (11). PKCδ siRNA duplexes target the sequence of 5'-AAGGCTACAAATGCAGGCAAT-3'. The siRNAs were synthesized by GenePharma. High-Perfect Transfection Reagent (Qiagen) was used to transfect siRNAs into cells following the manufacturer's protocol. Gene silencing effects were evaluated by Western blot analysis.

**Plasmid construction**
CHOP and ATF3 coding regions were amplified by PCR from HCT116 genomic DNA using the following primers:

CHOP sense, 5'-CACTAGTGCCGCCACCATGCGAC- TGAGTCATTGCCTTTC-3';
antisense, 5'-CGGGCCCTCATGCTTGGTGCAGATTCA-CC-3';
ATF3 sense, 5'-CACTAGTGCCGCCACCATGATGCTT-CAACACCCAG-3';
antisense, 5'-CGGGCCCTTAGCTCTGCAATGTTCCTC-3'.

The procedures of plasmid constructions were described previously (20), and the resultant constructs were named pLenti-CHOP and pLenti-ATF3, respectively.

**Plasmid transient transfection and immunoprecipitation**
H157 cells were seeded in 10 cm dishes and transfected with pLenti-CHOP and pLenti-ATF3 plasmids using XtremeGENE HP DNA Transfection Reagent (Roche) following the manufacture's protocol. Then cells were harvested and subjected to immunoprecipitation with CHOP antibody (Santa Cruz Biotechnology Inc., sc-7351). The procedures of immunoprecipitation were described previously (21).

**Apoptosis assays**
Apoptosis was evaluated by Annexin V-PE/7-AAD Apoptosis Detection Kit purchased from BD Biosciences following the manufacture's protocol. Caspase activation was detected by Western blot analysis.

**Results**
**CHOP contributes to PS-341–induced DR5 upregulation and apoptosis**
Studies have revealed that some antitumor agents can upregulate CHOP expression to induce cell apoptosis (4–6). Consistently, we found PS-341 strongly promoted
CHOP expression in NSCLC cells in a dose-dependent fashion (Fig. 1B). In the time course assay, we detected CHOP expression occurred at 4 hours, reached a peak at 12 hours (H460 and H157 cells) or 24 hours (Calu-1 cells; Fig. 1B). Besides, we noticed that CHOP expression was always earlier than DR5 upregulation (Fig. 1B), so we questioned whether CHOP would play an important role in PS-341–induced DR5 upregulation. We used siRNA to silence CHOP expression and found that PS-341–induced DR5 levels were dramatically decreased (Fig. 1C). Moreover, we examined weaker cleavage of caspase-8, caspase-9, caspase-3, and PARP in CHOP knockdown cells after exposure to PS-341 (Fig. 1C). Consistently, the early apoptosis was detected in CHOP siRNA-transfected cells by Annexin V staining–flow cytometry (Fig. 1D). Taken together, we conclude that CHOP expression contributes to PS-341–induced DR5 upregulation and apoptosis.

**PS-341 activates ER stress**

Because CHOP is the key member of ER stress (2, 3), we wondered whether PS-341 could activate ER stress in human NSCLC cells. We examined the expression of ATF4, ATF3, BiP, p-eIF2α, and IRE1α, which were regarded as key protein markers of ER stress and found that their expression were all elevated in a dose-dependent manner (Supplementary Fig. S1B), indicating PS-341 activates ER stress. Studies have shown that p53 regulates ATF3 expression (22), but we found that the levels of ATF3 were increased in all tested cells no matter the p53 status (Supplementary Fig. S1), indicating that PS-341–induced ATF3 expression is p53-independent.

**ATF3 expression is required for PS-341–induced apoptosis**

Because PS-341 upregulated ATF3 expression (Supplementary Fig. S1), we then questioned whether ATF3 had an effect on DR5 induction and apoptosis. Western blot analysis showed that the levels of DR5 and the cleaved forms of caspase-8, caspase-9, caspase-3, and PARP were all decreased in ATF3 siRNA-transfected cells (Fig. 2A). By Annexin V staining–flow cytometry, the percentage of apoptotic cells in control siRNA-transfected cells was 41%, whereas only 26% in ATF3 siRNA-transfected cells (Fig. 2B). So, we come to a conclusion that ATF3 expression is important for PS-341–induced DR5 upregulation and apoptosis.

**Inhibition of ATF4 expression decreases DR5 expression and PS-341–induced apoptosis**

It is known that ATF4 could enhance CHOP expression (2, 3), so we explored whether PS-341–induced ATF4
upregulation would contribute to DR5 expression. To do this, we successfully blocked PS-341–induced ATF4 expression using ATF4 siRNA in H460, A549, and H157 cells (Fig. 3A). We found that the inhibition of ATF4 upregulation decreased the expression of DR5 and CHOP and the cleavage of caspase-8, caspase-9, caspase-3, and PARP after exposure to PS-341 (Fig. 3A). Besides, we noticed that after abrogating ATF4 expression, PS-341–induced ATF3 expression was also reduced (Fig. 3A). Accordingly, the percentage of apoptotic cells decreased from 29% to 19% (Fig. 3B). Collectively, these results indicate that ATF4 expression contributes to DR5 upregulation and is involved in PS-341–mediated apoptosis.

The relationship among ATF4, ATF3, and CHOP

We have shown that ATF4, ATF3, and CHOP contributed to DR5 expression, but the relationship among them is not clarified. In our experiments, we showed that ATF4 expression stimulated CHOP and ATF3 expression, as their expression was reduced in ATF4-inhibited cells (Fig. 3A). Then, we considered the relationship between CHOP and ATF3. In H157 and Calu-1 cells, when ATF3 expression was suppressed, CHOP expression was weakly inhibited, whereas ATF4 expression was unaltered (Fig. 4A); when CHOP was knocked down, we detected the lightly depressed level in ATF3 expression, whereas ATF4 was unaffected (Fig. 4B). To further investigate the physical relationship between ATF3 and CHOP, we conducted immunoprecipitation experiment (Fig. 4C). The lentiviral plasmids containing CHOP and ATF3 were cotransfected in H157 cells and subjected to immunoprecipitation using CHOP antibody and detected ATF3 by Western blot analysis. We found that ATF3 was associated with CHOP (Fig. 4C). Taken together, we speculate that PS-341 treatment induces ATF4 upregulation, and subsequently as a transcriptional factor, promotes ATF3 and CHOP expression. While ATF3 and CHOP form a complex, and perhaps they together regulate DR5 expression, as DR5 promoter contains a functional CHOP binding site (4, 23).

ERK/RSK2 regulates DR5 expression in PS-341–induced apoptosis

Given that ERK/RSK2 signaling promotes apoptotic induction in some experiments (23, 24), we further addressed their roles in cells exposed to PS-341. Along with the increased dose and time, the phosphorylation of ERK1/2 and RSK2 was all augmented (Fig. 5A and B). When using MAP–ERK kinase inhibitor, U0126, to suppress p-ERK and p-RSK2 phosphorylation, we found that DR5 induction was diminished (Fig. 5C), indicating that ERK/RSK2 contributes to PS-341–mediated DR5 expression.

PKCδ contributes to PS-341–induced DR5 expression and apoptosis

Because PKCδ is conducive to apoptosis, we investigated the expression of PKCδ in PS-341–treated cells. Using Western blot analysis, the 41 kDa fragment of PKCδ was detected in all tested cells, and the levels were increased along with the enhanced concentration and time (Fig. 6A), suggesting PS-341 activates PKCδ. Then, we explored the role of PKCδ on DR5 expression when cells were exposed to PS-341. After inhibition of PKCδ expression by siRNA, we found that DR5 expression was decreased (Fig. 6B), suggesting PKCδ contributes to DR5 induction. Furthermore, we found the cleaved forms of caspase-8, caspase-9, caspase-3, and PARP were all reduced (Fig. 6C), and the percentage of apoptotic cells decreased from 41% to 23% (Fig. 6D). Therefore, we concluded that PKCδ conducted a markedly function in PS-341–mediated apoptosis. In the meantime, we noted that the phosphorylation of ERK1/2 and RSK2 in PKCδ siRNA-transfected cells was also suppressed (Fig. 6B), suggesting that PKCδ

---

**Table:**

<table>
<thead>
<tr>
<th>PS-341 (nmol/L):</th>
<th>Ctrl</th>
<th>ATFF3</th>
<th>Ctrl</th>
<th>ATFF3</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3PDH</td>
<td>0 100</td>
<td>0 100</td>
<td>0 100</td>
<td>0 100</td>
</tr>
<tr>
<td>CHOP</td>
<td>0 100</td>
<td>0 100</td>
<td>0 100</td>
<td>0 100</td>
</tr>
</tbody>
</table>

**Figure 4.** ATF3 and CHOP minimally modulate each other (A and B) while physically interact with each other (C). H157 and Calu-1 cells were seeded in 6-well plates and on the second day transfected with control (Ctrl) or ATF3 (A) or CHOP (B) siRNA. Forty-eight hours after the transfection, cells were treated with 100 nmol/L PS-341 for 16 hours, and then the cells were harvested and prepared for Western blot analysis (A and B). H157 cells were seeded in 10 cm dishes and on the second day cotransfected with pLenti-CHOP and pLenti-ATF3 plasmids. Forty-eight hours later, cells were harvested and subjected to immunoprecipitation using CHOP antibody (Santa Cruz Biotechnology Inc., cat#: sc-7351). Bound protein were eluted and prepared for Western blot analysis with ATF3 (Santa Cruz Biotechnology Inc., cat#: sc-188) and CHOP antibody (C).
regulates DR5 expression maybe through ERK/RSK2 signaling. In addition, we studied the effect of PKC\(\delta\) on ER stress. As compared with cells expressing PKC\(\delta\), the expression of ATF4, ATF3, and CHOP were all attenuated in PKC\(\delta\)-abolished cells (Fig. 6B), manifesting that the regulation of PKC\(\delta\) on DR5 expression is through ER stress pathway. Taken together, we speculate that PKC\(\delta\) regulates DR5 expression and apoptosis via coactivation of ERK/RSK2 signaling and subsequent ER stress.

Discussion

PS-341 has shown to induce apoptosis in various kinds of cancer cells, but the molecular mechanism is not very clear (17, 18). Our previous studies have shown that DR5 contributes to PS-341-mediated apoptosis in human NSCLC cells (18). In this study, we further show that PKC\(\delta\) regulates DR5 expression through ERK/RSK2 signaling and ATF4–CHOP/ATF3 axis. First, PS-341 induced CHOP upregulation, and CHOP was required for DR5 expression. Second, PS-341 activated ER stress, and both ATF3 and ATF4 contributed to DR5 induction and apoptotic sensitivity to PS-341. Third, p-ERK and p-RSK2 expressions were enhanced by PS-341, and ERK/RSK2 signaling was conducive to DR5 induction. Finally, PS-341 cleaved PKC\(\delta\), and PKC\(\delta\) regulated DR5 expression through ERK/RSK2 signaling and ER stress.

Studies have shown that ATF4 facilitates DR5 expression in sorafenib and salermide-mediated cell death (11, 25). Consistently, we discovered that abolished ATF4 expression effectively cut down DR5 upregulation and the cleavage of caspase-8, caspase-9, caspase-3, and PARP and the apoptosis detected was inhibited (Fig. 3). Obviously, ATF4 is a key component of DR5 induction and apoptosis. Data have shown that ATF3 plays a dichotomous role in regulating gene transcription, apoptosis, and cell cycle (26). In our experiments, silenced ATF3 expression decreased the apoptosis induced by PS-341, and the levels of DR5 were lower in ATF3-inhibited cells (Fig. 2). This was also in agreement with our previous finding that ATF3 exerts positive function in salermide-induced DR5 expression and apoptosis (11).

Su and Kilberg (27) have shown that ATF4 interacts with CHOP \textit{in vivo} by yeast 2-hybrid screen and immunoprecipitation. Wang has shown that ATF3 can also form complex with ATF4 \textit{in vivo} (28). Because our results showed that ATF4, ATF3, and CHOP all contributed to DR5 expression, we examined the relationship among ATF4, ATF3, and CHOP in PS-341–induced DR5 upregulation in NSCLC cells. In the present study, we found that ATF3 and CHOP were the downstream of ATF4 (Fig. 3A). However, ATF3 and CHOP minimally modulated each other (Fig. 4A and B). By immunoprecipitation, we found that ATF3 and CHOP had physically interacted with each other.
PKCδ Regulates DR5 via ER Stress Pathway

Figure 6. PS-341 stimulates the cleavage of PKCδ in a dose-dependent and time-dependent manner (A), and silencing of PKCδ expression by siRNA attenuates DR5 induction (B) and apoptosis induced by PS-341 (C and D). The indicated lung cancer cell lines were treated with 0, 100, 200 nmol/L PS-341 for 24 hours or treated with 50 nmol/L PS-341 for the indicated time (A). H157 (B and C) and Calu-1 (B-D) cells were seeded in 6-well plates and on the second day transfected with control (Ctrl) or PKCδ siRNA. Forty-eight hours after the transfection, cells were treated with 100 nmol/L PS-341 for 16 hours (B and C) or 24 hours (D). Then the cells were harvested and prepared for Western blot analysis (B and C) or for detection of apoptotic cells using Annexin V/7-AAD staining (D). CF, cleaved form.

PKCδ can be cleaved by caspase-3 and generates a catalytic fragment, which is necessary for apoptosis (14–16). In the present study, we determined that PS-341 induced the cleavage of PKCδ in a dose- and time-dependent manner (Fig. 6A), suggesting that PKCδ was activated by PS-341. Work of others has shown that PKCδ causes the activation of death receptor ligands and activates the extrinsic apoptotic pathway (30). Studies also show that PKCδ contributes to ursodeoxycholic acid–induced DR5 expression in human gastric cancer cells (31). In the present study, we showed inhibition of PKCδ resulted in the decrement of DR5 expression (Fig. 6B), proving DR5 induction is dependent on PKCδ in lung cancer cells. Furthermore, PKCδ knockdown decreased the apoptosis induced by PS-341 (Fig. 6C and D), indicating that PKCδ functions as a proapoptotic protein in PS-341–induced apoptosis.

Some data show that PKCδ contributes to ER stress induction, and inhibited PKCδ expression decreases thapsigargin-induced CHOP expression (32). Consistently, we detected that the upregulation of ATF4, ATF3, and CHOP were markedly suppressed in PKCδ siRNA-transfected cells (Fig. 6B), suggesting that PKCδ is the upstream of ER stress. Then, we inferred that PKCδ regulated DR5 expression relies on ER stress activation. In addition, we found that the phosphorylation of ERK1/2 and RSK2 were decreased when PKCδ expression was inhibited (Fig. 6B), suggesting that ERK/RSK2 signaling is associated with PKCδ-dependent DR5 upregulation. This result is consistent with the fact that PKCδ is involved in the activation of ERK (33). Several studies have suggested that PKCδ activates ERK...
dependently of Raf (34). Recent studies have shown that Ras and B-Raf induce DR5 expression in an ERK/RSK2-dependent manner (35). So, it is necessary to investigate whether PKCδ regulates ERK/RSK2 expression through activation of Ras and Raf.

In summary, we showed that PS-341 activates PKCδ, and thereafter regulates ERK/RSK2 pathway and ER stress, which contributes to DR5 induction via ATF4–ATF3/CHOP axis in human NSCLC cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: L. Su, X. Liu
Development of methodology: L. Xu, L. Su, X. Liu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Su
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Xu, L. Su, X. Liu
Writing, review, and/or revision of the manuscript: L. Xu, L. Su, X. Liu

Acknowledgments

The authors thank Dr. D. Zhong for helpful technical support.

Grant Support

This work was supported by grants from the National Natural Science Foundation of China (30971479, 31071215 to X. Liu and 81000947 to L. Su), the Doctoral Fund of Education of China (20090131110002, X. Liu), the Program for New Century Excellent Talents in University (NCET-10-0521, X. Liu), the Promotive Research Fund for Excellent Young and Middle-aged Scientists of Shandong Province (BS200901004, X. Liu), and the Shandong Provincial Natural Science Foundation (2010GSF10218 and JQ201007, X. Liu).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 15, 2012; revised July 25, 2012; accepted July 25, 2012; published OnlineFirst July 30, 2012.

References


Molecular Cancer Therapeutics

PKCδ Regulates Death Receptor 5 Expression Induced by PS-341 through ATF4–ATF3/CHOP Axis in Human Lung Cancer Cells

Linyan Xu, Ling Su and Xiangguo Liu

Mol Cancer Ther  Published OnlineFirst July 30, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-12-0602

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2012/07/30/1535-7163.MCT-12-0602.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://mct.aacrjournals.org/content/early/2012/10/01/1535-7163.MCT-12-0602. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.