Published in final edited form as:

Leuk Lymphoma. 2012 September ; 53(9): 1779–1784. doi:10.3109/10428194.2012.668287.

Statin-dependent activation of protein kinase C delta (PKC δ) in APL cells and induction of leukemic cell differentiation

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Abstract

Statins are HMG-CoA reductase inhibitors, which block the conversion of HMG-CoA to mevalonate and have potent cholesterol lowering properties. Beyond their importance in generation of lipid lowering effects, the regulatory effects of statins on the mevalonate pathway have significant impact on multiple other cellular functions. There is now extensive evidence that statins have anti-inflammatory and anti-neoplastic properties, but the precise mechanisms by which such responses are generated are not well understood. In the present study we demonstrate that statins engage a member of the PKC family of proteins, PKC δ , in acute promyelocytic leukemia (APL) cells. Our studies show that atorvastatin and fluvastatin induce proteolytic activation of PKC δ in the APL NB4 cell line which expresses the t(15;17) translocation. Such engagement of PKC δ results in induction of its kinase domain and downstream regulation of pathways important for statin-dependent leukemia cell differentiation. Our studies show that the function of PKC δ is essential for statin-induced leukemic cell differentiation, as demonstrated by studies involving selective targeting of PKC δ using siRNAs. We also demonstrate that the potent enhancing effects of statins on ATRA-induced gene expression for CCL3 and CCL4 requires the function of PKC δ , suggesting a mechanism by which statins may promote ATRA-induced antileukemic responses. Altogether, our data establish a novel function for PKC δ as a mediator of statin-induced differentiation of APL cells and antileukemic effects.

Introduction

There has been accumulating evidence in recent years that statins can interfere in cancer development and progression (1). Several studies have demonstrated distinct antitumor properties and/or protective effects of these HMGCoA inhibitors in the development of different malignancies, including lung cancer, pancreatic cancer and renal cell carcinoma (1-5). By their regulatory effects on the mevalonate pathway, statins control key cellular events for tumorigenesis, as recent studies have established that dysregulation of the mevalonate pathway promotes malignant cell transformation (6). There is evidence that statins are potent inducers of apoptosis (7-9) and can function as enhancers and modulators of the pro-apoptotic properties of chemotherapeutic agents (10). In addition, statins exhibit suppressive effects on cellular cascades critical for malignant cell proliferation and survival, such as the mTOR signaling pathway (5). Statins have also been previously shown to promote differentiation of various cell types (reviewed in 1), underscoring the diversity of the cellular responses and biological effects mediated by these agents.

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In previous work we demonstrated that statins modulate ATRA-dependent transcriptional activation in APL cells and enhance the antileukemic properties of all-trans-retinoic acid (ATRA) (11, 12), raising the possibility that combined use of statins with retinoids may enhance antileukemic responses in APL and, possibly, other hematological malignancies (11, 12). Moreover, as ATRA-resistant APL cells retain statin-sensitivity, it is possible that the use of these agents may provide a novel approach to overcome the development of ATRA-resistance in APL (11-13).

Protein kinase C δ (PKC δ) is a member of the serine/threonine-specific protein kinases C (PKC) family with diverse cellular activities including cell proliferation, differentiation and apoptosis. PKC isozymes are classified into three major groups: the classical cPKCs (α , β I, β II, γ); the novel nPKCs (δ , ϵ , μ , θ); and the atypical aPKCs (ζ , λ) (14-17). PKC δ plays important roles in the regulation of apoptosis in different cellular systems, while there is evidence that its activation can be regulated by the JNK kinase. As there is previous evidence that PKC δ activity lies upstream of JNK activation in DNA damage-induced apoptosis (18, 19), and our previous studies have demonstrated a role for JNK activity in statin-responses (12), we sought to determine whether this kinase is engaged during treatment of APL cells with statins. The studies of the current report establish that PKC δ is activated during statin-treatment of APL cells and that such activation is essential for statin-mediated leukemic cell differentiation. We also identify *CCL3* and *CCL4* as genes whose expression is regulated by PKC δ activity in APL cells, raising the potential of involvement of these chemokines in statin-induced, PKC δ – mediated, antileukemic responses and the enhancing effects of statins on the antileukemic properties of ATRA.

Materials and Methods

Cells lines and reagents

The NB4 human APL cell line was grown in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. Atorvastatin was purchased from 21CEC Pharmaceuticals Ltd. Fluvastatin was provided by Novartis. A polyclonal antibody against phosphorylated PKC δ (Thr⁵⁰⁵) was obtained from Cell Signaling Technology. A polyclonal antibody against PKC δ was purchased from Santa Cruz Biotechnology. An anti- glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody was obtained from Millipore. Silencer validated PKC δ siRNA was purchased from Ambion. Farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) were purchased from Sigma-Aldrich. The anti-CD11b monoclonal antibody and a matched isotype control were purchased from BD Biosciences.

Cell lysis and immunoblotting

Cells were treated with different statins for the indicated times at a final concentrations of 10 μ mol/L (unless otherwise indicated). The cells were then lysed in phosphorylation lysis buffer (PBL), as previously described (20-23). Immunoprecipitations and immunoblotting using an enhanced chemiluminescence method were done as in previous studies (24, 25).

In vitro Kinase Assays

NB4 cells were treated with atorvastatin (10 μ mol/L) for the indicated times. The cells were subsequently lysed in phosphorylation lysis buffer and lysates were immunoprecipitated with an antibody against PKC δ , using protein G–sepharose. The immunoprecipitated complexes were subsequently washed three times with phosphorylation lysis buffer containing 0.1% Triton X-100 and twice with kinase buffer (25 mmol/L HEPES, 25 mmol/L MgCl₂, 25 mmol/L β -glycerophosphate, 2 mmol/L DTT, 0.1 mmol/L Na₃VO₄, and 20 μ mol/L ATP) and resuspended in 30 μ L of kinase buffer containing 3 μ g of histone H1

substrate, used as an exogenous substrate. [γ - 32 P]ATP (10 μ Ci) was added to the mixture, and after 30 min of incubation at room temperature, the reaction was terminated by adding SDS buffer. Proteins were resolved by SDS-PAGE, and phosphorylated histone H1 was detected by autoradiography.

Flow Cytometric Analysis

Granulocytic differentiation of APL cells was determined by flow cytometric analysis, as previously described (11, 24, 25). In some experiments, NB4 cells were nucleofected with either control siRNA or siRNA specific for PKC δ . After 24 hours, cells were treated with statin or DMSO as solvent control, were added to the cultures and incubations were carried out for 48 hours. Cell differentiation was determined by flow cytometric analysis after staining with the anti-CD11b monoclonal antibody.

Quantitative Reverse Transcription-PCR (Taqman)

Cells were nucleofected with either control siRNA or siRNA targeting PKC δ , and RNA was isolated using the RNeasy kit from Qiagen. Total cellular RNA (1 μ g) was reverse transcribed into cDNA using the Omniscript reverse transcription kit, and oligo(dT) primer was purchased from Invitrogen. Real-time reverse transcription-PCR (RT-PCR) for *PKC δ* , *CCL3* and *CCL4* genes was carried out by an ABI7900 sequence detection system from Applied Biosystems, using commercially available FAM-labeled probes and primers from Applied Biosystems. Glyceraldehyde-3-phosphate dehydrogenase was used for normalization.

Results

We examined the effects of statin-treatment on the proteolytic cleavage/activation of PKC δ . As shown in Fig. 1A, treatment of NB-4 APL cells with atorvastatin or fluvastatin resulted in proteolytic degradation of PKC δ . The resulting 37-41 kD PKC δ fragment was phosphorylated, indicating that it is the active catalytic PKC δ -fragment (Fig. 1A). To definitively establish whether such proteolytic degradation results in induction of PKC δ kinase activity, immune-complex kinase assays in anti-PKC δ immunoprecipitates from atorvastatin - treated cell lysates were carried out. As shown in Fig. 1B, there was potent activation of the kinase domain of PKC δ in response to atorvastatin-treatment, reflected by the phosphorylation of histone H1, used as an exogenous substrate (Fig. 1B).

In other experiments, we sought to determine whether engagement of PKC δ is involved in statin-mediated cell differentiation. As shown in Fig. 2, using a specific siRNA sequence, we were able to significantly knockdown PKC δ expression in NB4 cells. Such knockdown was seen both at the level of mRNA expression (Fig. 2A) and at the protein level (Fig. 2B). Subsequently, the effects of diminished PKC δ levels on leukemic cell differentiation of NB4 cells were defined by assessing CD11b expression (11, 12, 25). As expected (11, 12), statin-treatment of NB4 cells resulted in high levels of CD11b-expressing cells, consistent with induction of differentiation (Fig. 3A and B). As it is well established that by inhibiting the mevalonate pathway, statins down-regulate farnesyl pyrophosphate (FPP) and geranyl-geranyl pyrophosphate (GGPP) (1), we examined the effects of concomitant treatment with FPP or GGPP on statin-induced differentiation. As shown in Fig. 3A, concomitant treatment with GGPP but not FPP resulted in reversal of atorvastatin-induced differentiation (Fig. 3A), implicating downregulation of GGPP in the process. When the effects of PKC δ knockdown were directly assessed, we found that statin-dependent induction of differentiation was clearly noticeable in cells expressing control siRNA, but was dramatically decreased in cells in which PKC δ -expression was knocked down (Fig. 3B). Thus, statin-induced granulocytic

differentiation is reversed by inhibiting PKC δ expression, establishing that its function is required for granulocytic differentiation of leukemic cells.

Our previous studies have established that atorvastatin-treatment modulates induction of expression of several genes in response to ATRA (12) and promotes induction of ATRA-dependent antileukemic responses. To determine the role of PKC δ - engagement in the process, we examined whether PKC δ - activity modulates expression of genes that may contribute to leukemic cell differentiation, such as macrophage inflammatory protein-1 α (MIP-1 α)/CCL3 and macrophage inflammatory protein-1 β (MIP-1 β)/CCL4 (12). To determine the role of PKC δ in CCL3 or CCL4 gene expression we assessed their induction in response to ATRA or atorvastatin alone, or the combination of ATRA and atorvastatin, in NB4 cells expressing PKC δ or NB4 cells in which PKC δ - expression had been knocked down. As expected (12), the combination of atorvastatin with ATRA resulted in very high levels of expression of CCL3 (Fig. 4A) and CCL4, (Fig. 4B), as compared to ATRA alone. Such potent enhancing effects of the ATRA + atorvastatin combination on the expression of both genes were completely reversed in cells in which PKC δ - expression was knocked down (Figs. 4A and B).

Discussion

In recent years, there have been substantial advances in the treatment of acute promyelocytic leukemia (APL). This subtype of AML is characterized by hematopoietic arrest at the promyelocytic stage; by the presence of the t(15;17) reciprocal chromosomal translocation; and by the resulting fusion genes encoding for PML-RAR α and RAR α -PML proteins (26-29). Such sequence of events results in dominant-negative effects and suppression of the normal functions of PML and RAR, ultimately leading to leukemogenesis (30, 31). The introduction of ATRA and arsenic trioxide in the treatment of this AML subtype has dramatically changed the natural history of the disease and resulted in much improved survival and cure rates among APL patients (32-37). Nevertheless, there are subsets of patients that eventually relapse and/or develop resistance to the antileukemic properties of retinoids and/or arsenic (38, 39). This has prompted extensive efforts over the years to identify other drugs that promote differentiation of APL cells for the treatment of ATRA-refractory APL. We and others have shown that statins are potent differentiation-inducers and agents with suppressive properties on APL cells, as well as other types of AML (8-13, 40-41), including ATRA-resistant primary leukemia blasts (11). There has been also some evidence for statin-induced *in vivo* antileukemic effects in AML patients (41, 42), but a more detailed understanding of the mechanisms of the antileukemic properties of these agents is required in order to design optimal approaches for their use in the treatment of leukemias and to incorporate them in combinations with other antineoplastic agents.

In the current study, we sought to define the role of PKC δ in the generation of statin-responses in APL. This member of the PKC-family of proteins is the only member of the PKC family shown to be activated by proteolytic cleavage (43) and is involved in multiple cellular events and responses that control cell proliferation and survival (15, 44-49). It has been also previously shown to mediate events that promote cell differentiation in other systems (15, 50-55). We found that new generation statins (atorvastatin, fluvastatin) include activation of this PKC-isoform in APL cells and that such engagement is required for differentiation of APL cells in response to statins. It should be noted that our previous studies (11) had demonstrated that combinations of ATRA with atorvastatin or fluvastatin enhance ATRA responses and could reverse ATRA-resistance in certain APL variant cell lines. Moreover, we had shown that statins promote ATRA-dependent expression of many genes with antileukemic properties, suggesting a mechanism by which statins exhibit antileukemic effects and are capable of reversing ATRA-resistance (12). Among genes

dramatically enhanced by the combination of ATRA and atorvastatin were CCL3 (MIP-1 α) and CCL4 (MIP-1 β) mRNA expression (12). MIP-1 proteins belong to the CC subfamily of chemokines, sharing similar structure and patterns of inducibility in most mature hematopoietic cells (56, 57). In the present study, we provide the first evidence that the enhancing effects of statins on the expression of these putative mediators of ATRA-biological responses requires PKC δ activation, suggesting an important functional role for PKC δ in atorvastatin-mediated promotion of ATRA-induced gene transcription and antileukemic effects in APL. In past work we had also shown that ATRA induces the association of PKC δ with RAR α and PKC δ activity, which is required for RARE-driven transcription (25). The results of the present study demonstrating potent proteolytic activation of the kinase in response to statin-treatment of the cells provide evidence for a cellular mechanism that may account for the enhancing effects of statins on retinoid-driven transcription and address a long outstanding issue required to complete our understanding on the mechanisms by which statins promote antileukemic responses.

The upstream effector signals and regulatory mechanisms by which statins engage PKC δ and potentially other PKC isoforms remain to be precisely defined in future work. It is likely that such effects on the activation of PKC δ occur downstream of inhibition of geranyl-geranylation by statins, as by inhibiting the mevalonate pathway, statins down-regulate farnesyl pyrophosphate (FPP) and geranyl-geranyl pyrophosphate (GGPP) (1, 58, 59). Moreover, previous work has implicated inhibition of farnesylation and/or geranyl-geranylation in the generation of their pro-apoptotic effects in malignant hematopoietic cells (40, 60, 61) and our studies here demonstrated that GGPP suppression is required for atorvastatin-induced leukemic cell differentiation. Nevertheless, additional regulatory mechanisms may also be involved and contribute to PKC δ engagement in APL cells. Independently of the precise upstream and downstream cellular signals and events that account for statin-dependent engagement of PKC δ , the results of this work may prove of translational value in the long run. The demonstration that PKC δ activity is essential for generation of the differentiating effects of statins and the enhancing effects of statins on ATRA-responses in APL raises the potential for future development of PKC δ activator molecules that may promote leukemic cell differentiation and ATRA-responses in APL in more potent and specific ways than statins.

Acknowledgments

This work was supported by a Merit Review grant from the Department of Veterans Affairs and by NIH grant CA121192.

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Figure 1.

Effects of statin-treatment of APL cells on the proteolytic cleavage/activation of PKCδ. **A**, *Top*, NB4 cells were incubated in the presence or absence of atorvastatin or fluvastatin (10 μmol/L), for the indicated times. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against the phosphorylated form of PKCδ. *Middle*, the same blot was then stripped and re-probed with an anti-PKCδ antibody to confirm the specificity of the detected active fragment. *Bottom*, the same blot was re-probed with an anti-GAPDH antibody to control for protein loading. **B**, NB4 cells were treated in the presence or absence of atorvastatin (10 μmol/L) for the indicated times. Cell lysates were immunoprecipitated (*IP*) with an antibody against PKCδ or control non-immune rabbit immunoglobulin (*RlgG*). The immunoprecipitates were then subjected to *in vitro* kinase assays, using histone H1 as an exogenous substrate. Proteins were analyzed by SDS-PAGE, and the phosphorylated form of histone H1 was detected by autoradiography. *Bottom*, the blot was subsequently immunoblotted with an anti-PKCδ antibody

Figure 2.

Targeting of PKC δ in APL cells. **A**, NB4 cells were nucleofected with either control siRNA or siRNA targeting PKC δ . Expression of PKC δ mRNA was then evaluated after 72 hrs from nucleofection by quantitative real time RT-PCR (TaqMan). GAPDH was used for normalization. Data are expressed as fold increase over control samples and represent means $\pm$ S.E. of seven independent experiments. **B**, NB4 cells were nucleofected with either control siRNA or siRNA targeting PKC δ and treated with atorvastatin (2 μ mol/L), for 48 hrs. Total cell lysates were resolved by SDS-PAGE and immunoblotted with an antibody against PKC δ . The blot was subsequently immunoblotted with an anti-GAPDH antibody to control for protein loading.

A **B** **Figure 3.**

Reversal of statin-induced differentiation of APL cells by PKC δ - knockdown. **A**, NB4 cells were treated with atorvastatin (2 μ mol/L) for 48hrs as indicated, in the presence or absence of FPP (10 μ M) or GGPP (10 μ M), and induction of CD11b expression was assessed by flow cytometry. Means + SE of three experiments are shown. **B**, NB4 cells were incubated for 48 hours in the presence or absence of DMSO (solvent control) or fluvastatin (3 μ mol/L). The cells were subsequently stained with a phycoerythrin-conjugated anti-CD11b monoclonal antibody and analyzed by flow cytometry. Data are expressed as cells positive for CD11B and represent means + SE of 3 independent experiments.

Figure 4.

Effects of PKC δ knockdown on *CCL3* and *CCL4* gene expression. NB4 cells nucleofected as described in material and methods with control siRNA or PKC δ – siRNA were treated for 48 hours with DMSO (solvent control), ATRA (0.5 μ M), atorvastatin (2 μ M), or the combination of ATRA and atorvastatin. Expression of mRNAs for *CCL3* (A) or *CCL4* (B) was evaluated by quantitative real time RT-PCR (TaqMan), using GAPDH for normalization. Data are expressed as fold increase over control samples and represent means $\pm$ S.E. of 3 independent experiments for panel A and 2 independent experiments for panel B.