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Original Investigations

PKC Alpha Phosphorylates Cytosolic NF-kappaB/p65 and PKC Delta Delays Nuclear Translocation of NF-kappaB/p65 in U1242 Glioblastoma Cells

U1242 Glioblastom Hücresinde PKC Alpha'nın Sitosolik NF-kappaB/p65 Fosforilize Etmedeki ve PKC Delta'nın NF-kappaB/p65 Nukleer Translokasyonundaki Rolü

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ABSTRACT

AIM: Protein kinase-C (PKC) and NF-kappaB are involved in cell survival, proliferation, migration and radioresistance in glioblastoma multiforme (GBM). We sought to determine the interaction between PKC and NF-kappaB pathways.

MATERIAL and METHODS: The activation of NF-kappaB by PKC α and PKC δ was assessed by Western blotting after the stimulation with Phorbol 12-Myristate 13-Acetate (PMA). Gene silencing of PKC α , PKC δ and NF-kappaB/p65 with siRNA interference was utilized to evaluate their roles in NF-kB activation and cell proliferation.

RESULTS: PMA induced the phosphorylation of NF-kappaB/p65 by PKC α. Gene silencing with siRNA against NF-kappaB/p65 inhibited [3H]-thymidine incorporation in U1242 GBM cells. PKC δ decelerated the nuclear translocation of activated NF-kappaB/p65 up to 4 hours after the stimulation. PMA induced death was not observed in PKC δ silenced cells where activated NF-kappaB/p65 was located immediately in the nucleus.

CONCLUSION: NF-kappaB/p65 is pro-survival and proliferative factor in U1242 GBM cells. PKC α is needed to phosphorylate NF-kappaB/p65. PKC δ delays the translocation of active NF-kappaB/p65 into the nucleus. PMA-induced cell death occurred if the phospho-NF-kappaB/p65 was prohibited from entering the nucleus in PKC δ positive cells. Translocation of phosphorylated form of NF-kappaB into the nucleus is critical in GBM cell proliferation.

KEYWORDS: Glioblastoma, NF-kappaB, PKC, Proliferation, Survival

ÖZ

AMAÇ: Protein kinaz C (PKC) ve NF-kappaB sinyal iletim yolları glioblastomanın yaşam, çoğalma, göç ve radyoterapiye dirençte yol alır; bu çalışmada iki yolun etkileşimi çalışılmıştır.

YÖNTEM ve GEREÇ: Phorbol 12-Myristate 13-Acetate (PMA) ile uyarılma sonrası PKC ∝ ve PKC δ ile NF-kappaB /p65 aktivasyonu Western blot tespit edildi. siRNA tekniği ile PKC α ve PKC δ gen sessizleştirilmesi NF-kappaB aktivasyonu ve hücrenin sağkalımı değerlendirildi.

BULGULAR: PKC α NF-kappaB/p65 proteininin fosforlanmasında rol almıştır. PKC δ ise fosforlanmış ve aktif NF-kappaB/p65'in nukleusa girmesini 4 saate kadar geciktirmiştir. U1242 hücrelerinde ölüme yol açan PMA, siRNA ile sessizleştirilen PKC δ yokluğunda NF-kappaB/p65 hemen nukleusa geçmektedir ve PMA ile hücre ölümü görülmemektedir.

SONUÇ: NF-kappaB/p65 glioblastomada bir yaşam faktörüdür ve PKC α fosforlanmsında; PKC δ ise nukleusa geçisinde rol almaktadır. Aktif NF-kappaB/p65'in PKC δ ile hücreye geçişi geciktiğinde PMA hücre ölümüne neden olmaktadır.

ANAHTAR SÖZCÜKLER: Glioblastoma, NF-kappaB, PKC, Çoğalma, Sağkalım

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INTRODUCTION

NF-κB (Nuclear Factor-κB)[1] is known to consist of a family of Rel-domain-containing proteins; Rel A (also called p65), Rel B, c-Rel, p50 (also called NF-κB1), and p52 (also called NF-κB2). Phosphorylation-dependent cleavage of p100 produces p52, whereas p105 is cleaved to form p50 (4, 10,11).

In resting cells, NF-κB is retained in a latent form in the cytosol by I-κB family (I-κBa, I-κBβ and I-κBε), bcl-3, p105, and p100 that mask the nuclear translocation signals on NF-κB. I-κB molecules are rapidly phosphorylated with cell stimulation by antigens, cytokines [tumor necrosis factor a (TNF-α), interleukin 1], bacterial products (lipopolysaccharide), viruses (human T-cell leukemia virus, HIV1), environmental stress.

Depending on the stimuli, two distinct mechanisms are defined for I-κBα phosphorylation as either serine or tyrosine phosphorylation. The most widely studied pathway is that activated by TNF-α, Interleukin-1, or lipopolysaccharide that is regulated by phosphorylation on serine 32 and 36 residues on I-κBα by the I-κB kinase complex (IKK) (38,41). Serine phosphorylation recruits a specific ubiquitin ligase, which attaches ubiquitin molecules to the N-terminal domain of I-κB targeting the inhibitory subunit for degradation by the 26S proteasome (19,24). The second phosphorylation mechanism is mediated by tyrosine kinases at Tyrosine 42 residue. Tyrosine phosphorylation of I-κBα on Tyr42 is capable of activating NF-κB in the absence of ubiquitin-dependent degradation of I-κBα (17). This pathway is stimulated by H_2O_2, pervanadate, hypoxia, or hypoxia/reoxygenation and gamma irradiation (13,21).

NF-κB is a pro-survival and anti-apoptotic transcription factor in most of tumor types (14,15). In addition, certain members of the NF-κB family are oncogenic. Several oncogenes and growth factors for tumor cells are activated by NF-κB (7,25,32). NF-κB is known to be involved in radioresistance of human malignant glioma cells (12,26,33,39).

Protein kinase C (PKC) represents a major cellular receptor for tumor-promoting phorbol esters (9,20,28). PKC is a family of phospholipid-dependent serine/threonine kinases that play important roles in signal transduction associated with a variety of cellular responses, including cell growth and differentiation, gene expression, hormone secretion, apoptosis and membrane function (5,27,29,30,31,35,37). PKC consists of at least 11 isoforms that show diversity in their structures, cellular distribution, and biological functions and that have been divided into three groups based on their structures and cofactor requirements: conventional, novel and atypical (18). The conventional PKC isoforms α, βI, βII, and γ require phosphatidylserine, and Ca^{2+} for activity. The novel PKC isoforms δ, ε, η and θ do not require Ca^{2+} as a cofactor, but bind to phosphatidylserine when activated. Both conventional and novel PKCs are activated by phorbol 12-myristate 13-acetate (PMA) or 1,2-Diacyl-sn-Glycerol (DAG), The atypical PKC isoforms ζ and λ do not require Ca^{2+} and are not activated by PMA or DAG, but do bind to phosphatidylserine when activated (27,29,31,34).

Glioblastoma multiforme (GBM) is the most malignant and common type of primary brain tumors. PMA induces either apoptosis or proliferative response in different subtypes of GBMs depending on differential expression of PKCs (16). The relationship between PKC activation and NF-κB phosphorylation and its implication in GBM survival is not well understood. The differential expression of PKCs in different subsets of GBMs and its role in NF-κB pathway may play a role in PKC-induced proliferative response in GBM cells.

MATERIALS AND METHOD

Materials

PMA and anti-tubulin antibody (DMA1) were purchased from Sigma Chemical Company (St Louis, MO). The phospho-specific (serine 536) and total antibodies against NF-κB p65 were obtained from Cell Signaling Biotechnology (Boston, MA). The PKC-specific inhibitors Gö 6976, rottlerin and bisindolylmaleimide (BIM) are products of Calbiochem (Cambridge, MA). Ingenol was purchased from RBI (Natick, MA). PKC δ antibody was obtained from Transduction Laboratories (San Diego, CA). PKC α antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Small interfering RNAs (siRNAs) PKC δ, PKC α and scramble sequence (small interfering transfection control) were purchased from Dharmacon, Inc. (Lafayette, CO). NF-κB p65 siRNA was purchased from Cell

Signaling Biotechnology (Boston, MA). The nuclear extract kit was purchased from Active Motif North America (Carlsbad, CA).

Cell Cultures

The human U-1242 Malignant Glioma (MG) cell line was kindly supplied by Dr. A. J. Yates (Ohio State University). The cell lines were originally isolated from astrocytic tumors that were designated as glioblastomas, and their characteristics have been described previously by Hussaini *et al.* (16]. Cell lines were regularly determined to be free of mycoplasma with reagent from Gen-Probe Inc. (San Diego). Cells were grown in modified a-minimal essential medium with 10% defined fetal bovine serum (Hyclone, Logan, UT) and 20 μg/ml bovine zinc insulin (25.7 IU/mg; Sigma). The cells were cultured to 100% confluence, passaged every 4–5 days from an initial concentration of 6–8 x $10^3/cm^2$ in T flasks or 6- or 24-well plates, and cultured in astrocyte growth medium 5% fetal bovine serum at 37°C in 5% CO_2 and 90% relative humidity. Prior to assays, cultures that were 80–100% confluent were washed three times with serum-free medium and kept starved for 24 hr.

Methods

Western Blot Analysis

For detecting phosphorylation, cells were grown in 60 cc dishes and grown to 80–100% confluency, washed three times with serum-free medium and kept starved for 24 hr in serum free α-minimal essential medium, then treated with the pharmacological inhibitors (Gö 6976, BIM). The inhibitors were added to the cells for 30 min before activators (PMA and ingenol). PMA (100 nM) and Ingenol (100 nM) were administered for 30 min. In time dependent experiment, PMA (100 nM) was given 15, 30, 60 and 240 min prior to extraction into separate dishes, respectively.

After the different treatments, the cells were washed with phosphate-buffered saline (PBS; 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, and 1.5 mM KH_2PO_4, pH 7.4) containing 0.2 mM sodium orthovanadate.

For the detection of NF-κB/p65, the rinsed cultured cells were extracted with buffer (50mM Hepes, 100mM NaCl, 2mM EDTA, pH=7.5, 1% NP40, 1μM pepstatin, 1μ/mlM leupeptin, 0.2mM PMSF, 0.2mM Na vanadate, 2μg/ml aprotinin, 40mM PNPP). The extract was centrifuged at 14,000Xg for 15 min, boiled for 5 min in SDS-polyacrylamide gel electrophoresis (PAGE) buffer and separated by SDS-PAGE on 10% polyacrylamide slabs. They were electroblotted onto nitrocellulose-1 (Life Technologies, Inc.) and immunoblotting was performed with a polyclonal antibody specific for phosphorylated or total NF-κB/p65 (1:1.000). The antibodies were detected with anti-rabbit peroxidase-conjugates and final detection was carried out with ECL (Amersham Pharmacia Biotech) as described by the manufacturer.

The nuclear extract kit (Active Motif, CA) was used and manufacturer's instructions were followed for obtaining cytoplasmic and nuclear extraction. Briefly, after starving the cells for 24 hr and administering pharmacological agents adjusted in a time dependent manner as described above, media was aspirated out of dish and washed with 5 ml ice-cold PBS/phosphatase inhibitors. After aspirating rinsing PBS/phosphatase inhibitors, 3 ml of ice-cold PBS/phosphatase inhibitors were added. Cells were scraped from dish gently with cell lifter and centrifuged for 5 min at 500 rpm in a centrifuge pre-cooled at 4ºC. For cytoplasmic fraction collection, cells were resuspended in 250 ml 1X Hypotonic Buffer supplied with the kit and transferred to a pre-chilled microcentrifuge tube, incubated for 15 min on ice. 25 ml detergent was added and vortexed 10 seconds at highest setting. The suspension was centrifuged for 30 seconds at 14,000Xg in a microcentrifuge pre-cooled at 4ºC. Supernatant (cytoplasmic fraction) was transferred into a pre-chilled microcentrifuge tube. For nuclear fraction collection; nuclear pellet in 50 ml complete lysis buffer was re-suspended and vortexed for 10 seconds at highest setting and incubated for 30 min on ice on a rocking platform set at 150 rpm. The suspension was vortexed for 30 seconds at highest setting and centrifuged for 10 min at 14,000Xg in a microcentrifuge pre-cooled at 4ºC. Proteins were boiled for 5 min in SDS-PAGE buffer. Proteins (200 mg/lane) were separated by SDS-PAGE on 10% polyacrylamide gels and electroblotted onto nitrocellulose and reacted with monoclonal and polyclonal antibodies. Immunoblotted proteins were detected using the ECL reagents (Amersham Biosciences) as described by the manufacturer with horseradish peroxidase-conjugated secondary antibodies (Sigma).

siRNA Transfection

siRNAs PKC δ and PKC α were synthesized and purified by Dharmacon, Inc. and NF-κB p65 siRNA by Cell Signaling Biotechnology (Boston MA). PKC δ (200 nM), PKC α (200 nM), and NF-κB (200 nM) siRNAs were transfected separately into U-1242 MG cells using the Amaxa Nucleofector™ (Amaxa, Gaithersburg, MD). Briefly, confluent cells were trypsinized and resuspended in Amaxa Nucleofector solution V at a density of $2X10^6$/100 μl of solution, and either 200 nM PKC δ siRNA or PKC α (200 nM), or NF-κB (200 nM) siRNAs were added. Cells were transfected using the A23 pulsing program. Immediately after electroporation, cells were suspended in 4.9 ml of a-minimal essential medium plus 10% fetal bovine serum and incubated at 37 °C for 48 hr (22). Cells were starved for 24 hr and the siRNA PKC δ or PKC α cells were treated with PMA for 30 min and lysed as described above. The cell and nuclear lysates were separated by SDS-PAGE on 10% polyacrylamide gels. Proteins were electro-blotted onto nitrocellulose and reacted with phosphorylated NF-κB, tubulin antibodies. The blots were stripped and reprobed with PKC δ and PKC α monoclonal antibodies for siRNA PKC δ or PKC α treated cells. The antibodies were detected with peroxidase-conjugated anti-mouse or anti-rabbit antibody, and final detection was carried out using ECL reagents as described by the manufacturer.

Incorporation of (^{3}H]- Thymidine into DNA

Relative rates of DNA synthesis were assessed by determination of (^{3}H]-thymidine incorporation. U1242 MG cells transfected with either siRNA directed against PKC δ or NF-κB/p65 were also seeded onto a 24-well plate at a density of 4.5×10^4. Then, the scramble sequence transfected or siRNA PKC δ or NF-κB/p65 transfected cells were stimulated with PMA for 12 hours or 24 hours. The transfected cells were pulsed after 24 hr of starvation. Cells were pulsed with 1 μCi of (^{3}H]-thymidine for 4 hr and washed with PBS. This was followed by 10-min washes with 10% trichloroacetic acid, first at 4 °C and at room temperature. Cells were dissolved overnight in 1 N NaOH, neutralized with an equal volume of 2 N HCl, and placed in scintillation fluid. (^{3}H]-Thymidine incorporation was determined in a Beckman liquid scintillation counter. Thymidine uptake of cells transfected with siRNA against PKC δ or PKC α or NF-κB p65 were compared to that of cells transfected with scramble sequence as their control groups treated in the same way. Cells treated with inhibitors were compared to untreated U1242 MG cells.

Statistical Analysis

One-way analysis of variance was applied to depict the differences between groups and Dunnett's multiple comparison test was applied to compare to control group. A p value less than 0.05 was considered to be significant.

RESULTS

Time-dependent phosphorylation of NF-κB and translocation into the nucleus after PMA stimulation in U1242 MG cells

PMA induced time-dependent phosphorylation of NF-κB at serine 536 position of p65 subunit in GBM cell line. The phosphorylation was observed as early as 15 min and was sustained for 240 min. Translocation of NF-κB into the nucleus was progressively increasing within this time period (Figure 1). Total NF-κB for cytosolic proteins and histone for nuclear proteins were used as controls.

Effect of pharmacological inhibitors on phosphorylation and translocation of NF-κB into the nucleus after PMA stimulation in U1242 MG cells

Bisindolylmaleimide (BIM), an inhibitor of both classical and novel PKCs, was administered 30 min before PMA was added to the cultured cells in serum-free media for 30 min. After PMA stimulation, the phosphorylated form of NF-κB/p65 within the cytosol was significantly reduced by BIM (1 μM) and apparently all phospho- NF-κB/p65 was immediately translocated into the nucleus. However; the classical PKC inhibitor Gö 6976 (10 μM) partially reduced PMA-induced phosphorylation of NF-κB/p65 and caused no significant change at the nuclear fraction of NF-κB/p65 (Figure 2).

Our results suggested that there would be at least 2 different PKCs involved in the PMA-induced activation of NF-κB/p65; one classical PKC mainly involved in the phosphorylation of NF-κB/p65 and one novel PKC involved in the nuclear translocation of NF-κB/p65. Next, we sought to determine which PKC might have been involved. U1242 MG cells are inherently deficiency of PKC η (16). For evaluating role of PKC ε, ingenol at 100 nM, was utilized and there was no change observed at phosphorylated

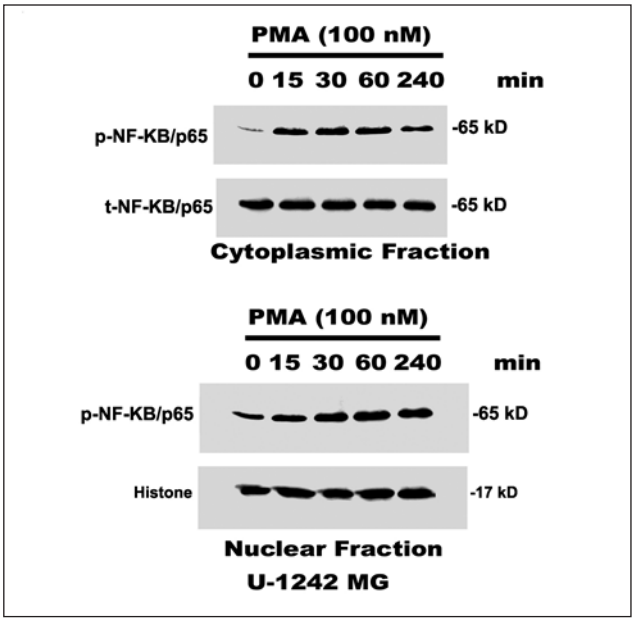

Figure 1: *PMA induced phosphorylation of cytosolic NF-κB at serine 536 residue and nuclear translocation of NF-κB after PMA stimulation.*
U1242 MG cells were serum-starved for 24 h after reaching 80–100% confluence by replacing the medium with serum-free ·- minimal essential medium. The cells were treated with PMA (100nM) at different time points (15, 30, 60 and 240 min). Both cytoplasmic and nuclear fractions were obtained. Protein (200 µg/lane) was fractionated by 10% SDS-PAGE and transferred onto nitrocellulose. The nitrocellulose was reacted with primary antibody against the NF-κB p65 at serine 536 residue. Final detection was carried out using ECL reagents. Blots were stripped and re-probed for total NF-κB and histone. Western blot was representative of 3 different experiments.

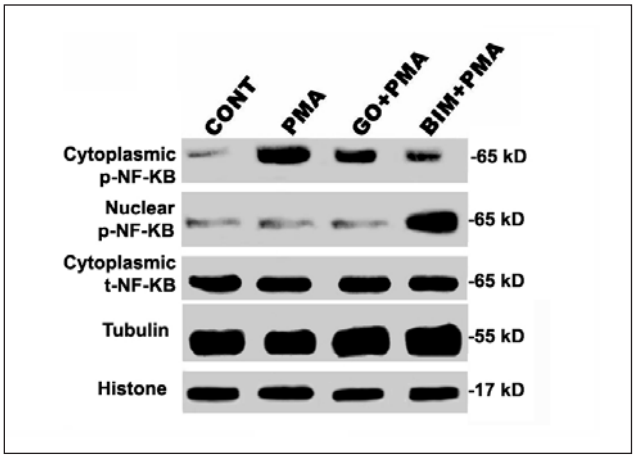

Figure 2: *Effect of inhibitors on phosphorylation of cytosolic NF-κB and translocation of NF-κB into the nucleus after PMA stimulation U1242 MG cells were serum-starved for 24 h after reaching 80–100% confluence by replacing the medium with serum-free α minimal essential medium. The cells were treated with BIM (1 µM) or Gö 6976 (10 µM) for 30 min before administration of PMA. The cells were treated with PMA (100nM) for 30 min. Both cytoplasmic and nuclear fractions were obtained. Protein (200 µg/lane) was fractionated by 10% SDS-PAGE and transferred onto nitrocellulose. The nitrocellulose was reacted with primary antibody against the NF-κB p65 at serine 536 residue. Final detection was carried out using ECL reagents. Blots were stripped and re-probed for total NF-κB p6, histone and tubulin. Western blot was representative of 3 different experiments.*

levels of cytoplasmic or nuclear NF-κB/p65 (data not shown). Neither of PKC ε or η appeared to be involved in NF-κB/p65 activation. In the next step, we determined which PKC isoforms were responsible for NF-κB pathway activation using siRNA gene silencing strategy.

Effect of siRNA PKC α on time-dependent phosphorylation of NF-κB and translocation into the nucleus

U1242 MG cells express PKC α at high levels and PMA stimulation activated PKC α (16). Gö 6976, which is a specific inhibitor of classical PKCs, partially blocked the phosphorylation of NF-κB in several repeated experiments (Figure 2). In order to directly determine the effect of PKC α on NF-κB phosphorylation, and nuclear translocation, PKC α expression was knocked down with specific siRNA interference (Figure 3). Reduction of PKC α levels significantly inhibited the phosphorylation of NF-κB in the cytosol. However, deficiency of PKC α did not have a significant effect on nuclear translocation pattern of NF-κB (Figure 3).

Effect of siRNA PKC δ on time-dependent phosphorylation of NF-κB and translocation into the nucleus

U1242 MG cells express PKC δ at high levels and PMA stimulation activated PKC δ (16). Our results with pharmacological inhibitors and data existing for PKC expression profiling of U1242 MG cells (see above) led us to PKC δ that might have a role for the nuclear translocation of phosphorylated NF-κB/p65. The siRNA-silencing of PKC δ did not have a significant effect on phosphorylation of NF-κB/p65 in the cytosol; however, translocation of phospho-NF-κB/p65 into the nucleus ensued immediately and was substantially increased compared with that of non-transfected cells after PMA stimulation (Figure 4).

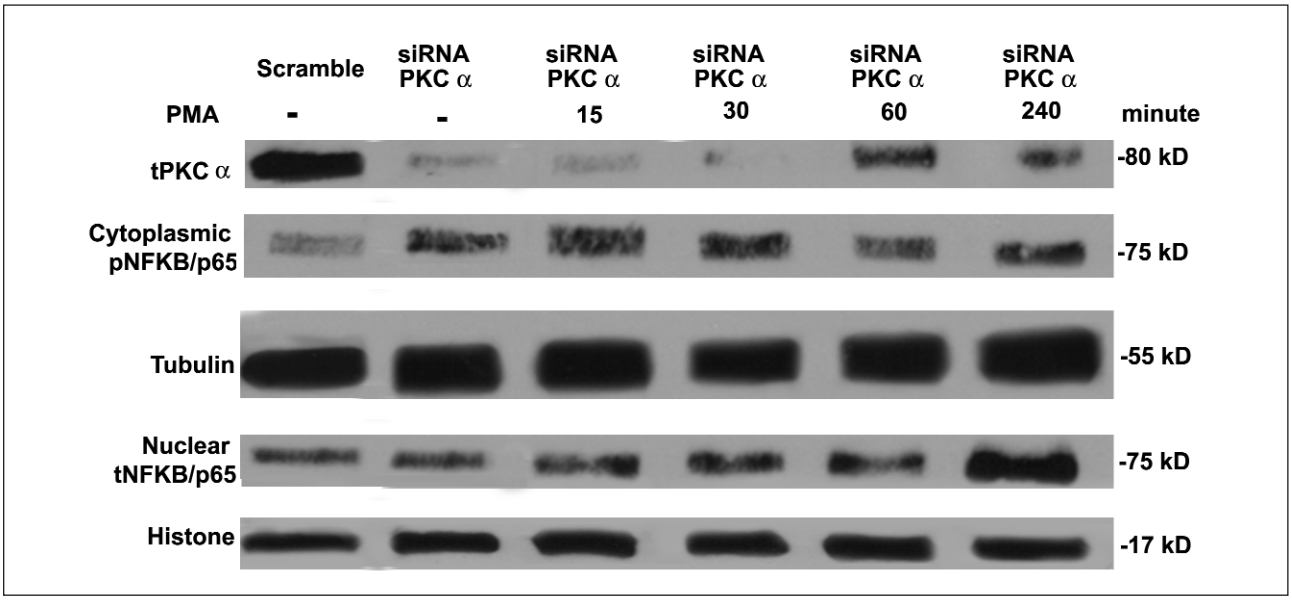

Figure 3: *siRNA silencing of PKC α decrease phosphorylation of NF-κB p65 U1242 MG cells were transfected with siRNA PKC α or scramble control as described under "Methods." Cytoplasmic and nuclear fractions were obtained after the cells were treated with PMA for 15, 30, 60 and 240 minutes. Protein (200 µg/lane) was fractionated by 10% SDS-PAGE and transferred onto nitrocellulose. The nitrocellulose was reacted with primary antibody against total PKC α, NF-κB, tubulin and histone. Final detection was carried out using ECL reagents. Western blot was representative of 2 different experiments.*

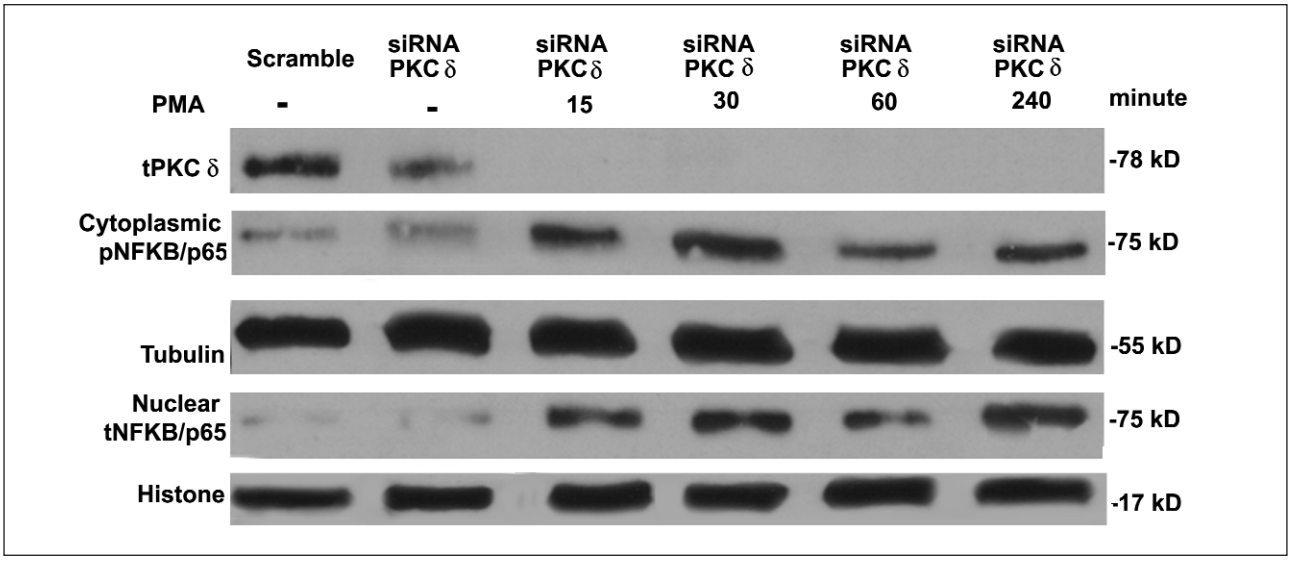

Figure 4: *siRNA silencing of PKC δ increase early translocation of NF-κB/p65 U1242 MG cells were transfected with siRNA PKC δ or scramble control as described under "Methods." Cytoplasmic and nuclear fractions were obtained after the cells were treated with PMA for 15, 30, 60 and 240 minutes. Protein (200 µg/lane) was fractionated by 10% SDS-PAGE and transferred onto nitrocellulose. The nitrocellulose was reacted with primary antibody against total PKC δ, NF-κB, tubulin and histone. Final detection was carried out using ECL reagents. Western blot was representative of 2 different experiments.*

Effects of deficiency of NF-κB after PMA stimulation on cellular proliferation in U1242 MG cells

NF-kB has been reported to be either pro-apoptotic or pro-survival transcription factor in a number of cell types. The nuclear translocation is crucial for NF-κB/p65 to exert its effects either in the direction of survival or apoptosis (7,14,15,25,26,32,39) and we sought to determine the effects of deficiency of NF-κB/p65 and how PKCs regulated its phosphorylation on cell proliferation. (^{3}H]-thymidine incorporation was used as a measure of

cell proliferation. We have previously shown that PMA treatment of U-1242 MG cells resulted in growth arrest of the cells (16). As expected, PMA decreased cell proliferation in U1242 MG cells by 65%. Only silencing with siRNA against NF-κB produced 60% inhibition of U-1242 MG cells proliferation; however, PMA stimulation did not further decrease the proliferation in NF-κB/p65 deficient cells after stimulation for 12 and 24 hours (Figure 5). Similarly, when PKC δ was knocked down, PMA was unable to induce death (Figure 6). These data suggested that NF-κB/p65 presence was essential for survival and PMA induced cell death was conducted through PKC/ NF-κB/p65 pathway only if activated NF-κB/p65 was prohibited to enter to the nucleus by PKC δ.

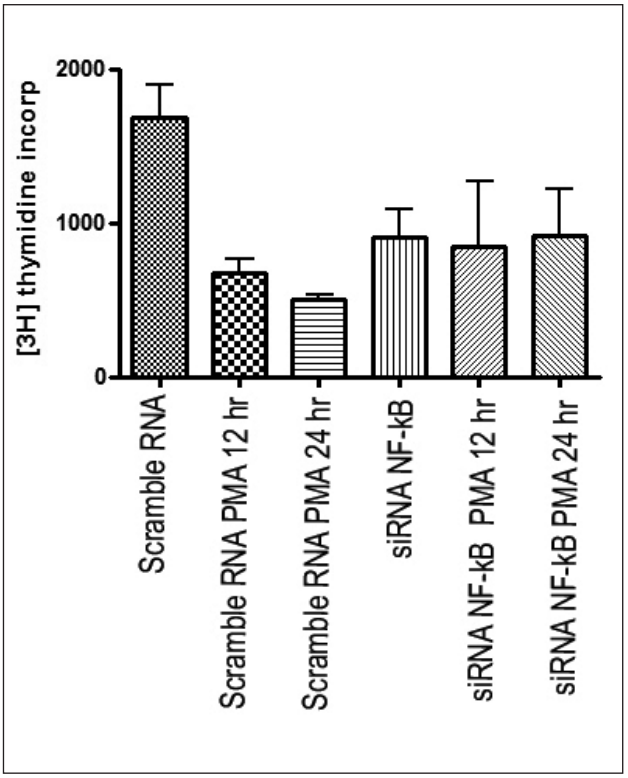

Figure 5: Deficiency of NF-κB/p65 decreases cell proliferation and blunts response to PMA induced cell death in U1242 MG cells U1242 MG cells were transfected with siRNA NF-κB/p65 and stimulated with PMA for 12 and 24 hours as described under "Methods" Cells were pulse-labeled with (^{3}H]thymidine for 4 hr, and thymidine incorporation was measured using a Beckman scintillation counter. Results were from the quantitative analysis two independent experiments (means ± S.E.), with each experiment performed in quadruplicate. Cell proliferation was calculated as percent of control.

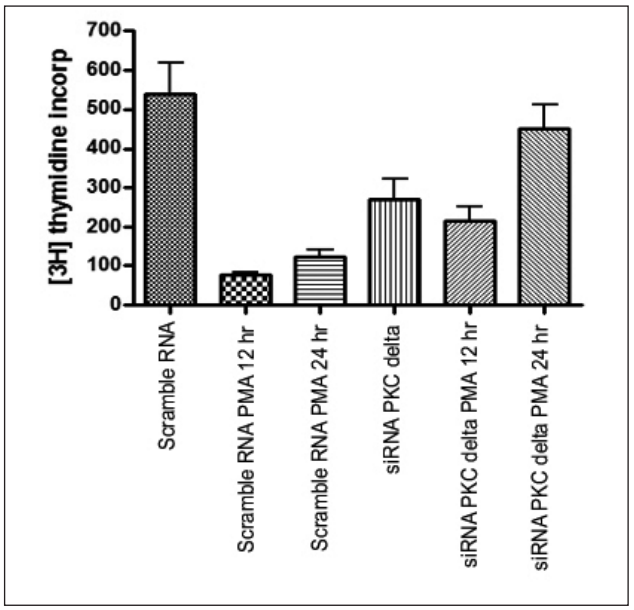

Figure 6: Deficiency of PKC δ decreases cell proliferation and reverses the response to PMA induced cell death in U1242 MG cells U1242 MG cells were transfected with siRNA PKC δ and stimulated with PMA for 12 and 24 hours as described under "Methods" Cells were pulse-labeled with (^{3}H]thymidine for 4 hr, and thymidine incorporation was measured using a Beckman scintillation counter. Results were from the quantitative analysis two independent experiments (means ± S.E.), with each experiment performed in quadruplicate. Cell proliferation was calculated as percent of control.

DISCUSSION

NF-kB proteins can form multiple homo- and hetero-dimers, each of which has distinct DNA binding site specificities and affinities resulting in activation and expression of over 100 genes, which coordinate immune, inflammatory, proliferative or apoptotic response (3,4,10,11). Whether NF-κB acts as an inhibitor or activator of apoptosis depends on the relative levels of Rel A and c-Rel subunits (11). The pathways leading to NF-kB activation are uniquely stimulus and cell-type specific. NF-κB can be inducible or constitutively active in different cell types. The tumor-promoting phorbol ester, PMA can induce NF-κB activity and prevent apoptosis in pancreatic carcinoma and leukemia (8,36). NF-κB is constitutively active in most tumor cells (hematopoietic, prostate, breast cancers), rarely found to be constitutively active in normal cells except for proliferating T cells, B cells, thymocytes, monocytes, and astrocytes. Suppression of NF-κB in tumors inhibits proliferation, causes cell cycle arrest, and leads to apoptosis indicating the crucial role of

NF-κB in cell proliferation and survival (1,6). NF-κB is suggested to be a pro-survival and proliferative transcriptional factor in GBMs (26,39); our results supported those observations. Silencing NF-κB expression with NF-κB siRNA would reduce proliferation of GBM cells (Figure 5).

Glioblastomas represent a diverse group of tumors with several different phenotypes and genotypes; and with highly variable, differential expression profiles of PKCs (16). We demonstrate in this study that PMA-induced NF-κB phosphorylation was mediated by PKC α, in addition, PKC δ seemed to delay nuclear translocation of NF-κB/p65 because silencing this PKC isoform facilitated entry of phosphorylated NF-κB/p65 into the nucleus (Figure 3-4). The effect of PMA on cell proliferation and survival might be regulated by differential expression of PKC in different subsets of GBMs and our results showed that NF-κB pathway might play a role in GBM proliferation and growth.

Among PKC isoforms, PKC δ and PKC α are suggested to have opposite effects in gliomas. PKC δ is generally decreased in GBMs and it is associated with apoptosis in response to gamma-irradiation, ultraviolet radiation (UV), Fas ligation and etoposide. PKC α is found to be responsible for the resistance to apoptosis in response to gamma irradiation and chemotherapy (23,40). On the other hand, in a recent study, PMA was shown to transactivate the EGFR and increases cell proliferation by activating the PKC δ/c-Src pathway in GBMs (2). In U1242 MG cells where PKC δ was expressed, PMA stimulation led to decreased proliferation and decreased survival. Once PKC δ was knocked down, PMA stimulation resulted in proliferative response in 24 hours.

Our study demonstrates that NF-κB is a pro-survival and proliferative transcription factor in U1242 MG cells. PMA activation of NF-κB pathway involves at least two PKCs. In cells like U1242 MG cells, which express predominantly PKC α and PKC δ, the former is primarily responsible for phosphorylation and activation of NF-κB/p65 in the cytosol. However, the final transcriptional activation of NF-κB/p65 is prevented by PKC δ via delaying translocation of activated NF-κB/p65 into the nucleus. The mechanism needs to be elucidated further for the purpose of developing a new target in GBM treatment.

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