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PKC412 (CGP41251) modulates the proliferation and lipopolysaccharide-induced inflammatory responses of RAW 264.7 macrophages

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Abstract

PKC412 (CGP41251) is a multitarget protein kinase inhibitor with anti-tumor activities. Here, we investigated the effects of PKC412 on macrophages. PKC412 inhibited the proliferation of murine RAW 264.7 macrophages through induction of G2/M cell cycle arrest and apoptosis. At non-toxic drug concentrations, PKC412 significantly suppressed the lipopolysaccharide (LPS)-induced release of TNF-α and nitric oxide, while instead enhancing IL-6 secretion. PKC412 attenuated LPS-induced phosphorylations of MKK4 and JNK, as well as AP-1 DNA binding activities. Furthermore, PKC412 suppressed LPS-induced Akt and GSK-3β phosphorylations. These results suggest that the anti-proliferative and immunomodulatory effects of PKC412 are, at least in part, mediated through its interference with the MKK4/JNK/AP-1 and/or Akt/GSK-3β pathways. Since macrophages contribute significantly to the development of both acute and chronic inflammation, PKC412 may have therapeutic potential and applications in treating inflammatory and/or autoimmune diseases.

Keywords: PKC412; RAW 264.7 macrophage; Lipopolysaccharide; TNF-α; IL-6; Nitric oxide

PKC412 (CGP41251), a derivative of the naturally occurring alkaloid staurosporine, was originally identified as a selective inhibitor of the conventional (α, β, and γ) and novel (δ, ε, and η) isoforms of protein kinase C (PKC), and was subsequently shown to inhibit a variety of tyrosine kinases including FLT3, PDGF-α and -β receptors, and the c-kit receptor [1]. Because of its potential to inhibit growth, angiogenesis and P-glycoprotein-mediated multidrug resistance in tumor cells, PKC412 is being developed as a therapeutic agent. Indeed, there is strong preclinical evidence that PKC412, alone or in combination with other anti-cancer agents, can inhibit tumor growth [1,2]. Furthermore, results of phase I/II clinical trials of PKC412 have shown that this orally available agent effectively suppresses cancer cell proliferation, and is relatively safe with minimal toxicity [3–5].

While the kinases inhibited by PKC412 play key roles in cell proliferation and tumorigenesis, they have also been implicated in other cellular processes such as immune responses and neuronal function. For example, several isoforms of PKC are activated by antigen receptors in T- and B-lymphocytes, coupling to signal transduction pathways that regulate their migration, differentiation, and proliferation [6]. Hence, it is reasonable to speculate that PKC412 may potently modulate the activity of lymphocytes and other immune cells, and thus possibly being effective for treating immune-mediated disorders. In fact, a recent study showed that PKC412 effectively inhibits normal human

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T-cell activation, proliferation and TNF-α production in response to mitogenic lectins [7].

Macrophages synthesize a wide variety of immunomodulatory mediators including proinflammatory cytokines and nitric oxide (NO), and serve as an essential interface between innate and adaptive immunity [8]. Their dysregulated activities contribute to the pathogenesis of many chronic inflammatory diseases such as rheumatoid arthritis (RA) and inflammatory bowel disease. In the case of RA, a prominent macrophage infiltrate is commonly found in the inflamed synovial tissues [9]. These cells usually display signs of activation and exhibit widespread proinflammatory, destructive and remodeling capabilities, contributing to both acute and chronic phases of inflammation. Consequently, a number of approaches designed to counter macrophage activities have substantially improved RA treatment [9]. In the present study, we investigated whether PKC412 can modulate macrophage proliferation and production of TNF-α, IL-6 and NO, using murine RAW 264.7 macrophages.

Materials and methods

Reagents and cell culture. PKC412 (CGP41251/4′-*N*-benzoyl staurosporine) was obtained from LC Laboratories, and stored at −80 °C as a 10 mM stock solution in dimethyl-formamide. Lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 was obtained from Calbiochem. All other reagents were of analytical grade, and were purchased from Sigma. RAW 264.7 macrophages were cultured at 37 °C under 5% CO_2 in RPMI1640 (Invitrogen) containing 10% FBS and antibiotics.

Cytotoxicity and proliferation assays. Cells were seeded at a density of 1×10^4 cells/well in a 96-well plate. The next day, the cells were incubated with various concentrations of PKC412 (0.01–10 μM) for 24, 48 or 72 h, and cell viability was determined thereafter using a colorimetric MTT test (Cell Proliferation Kit I; Roche). The rates of DNA synthesis were measured quantitatively using a BrdU Cell Proliferation Kit (Roche). The 50% inhibitory concentration (IC_{50}, MTT assay) and 50% effective concentration (EC_{50}, BrdU assay) were calculated graphically using a curve-fitting function of the Excel 2003 (Microsoft) software.

Morphological assessment and DNA fragmentation assay. Cells (5×10^5 cells/10-cm dish) were treated with 0.1–2.5 μM of PKC412 for 12 h, and were morphologically assessed by light microscopy, and by fluorescence microscopy using the standard Acridine orange-ethidium bromide (AO–EB) double staining procedure. For DNA fragmentation assay, cellular DNA was isolated by phenol/chloroform extraction and subjected to electrophoresis on EB-stained 1.5% agarose gels.

Flow-cytometric analysis. Following PKC412 treatment, the cells were harvested and fixed with 70% ethanol at −20 °C for 18 h. After washing, 1×10^6 cells were resuspended in a staining solution consisting of 0.2 mg/ml DNase-free RNase and 50 μg/ml propidium iodide (Invitrogen), and incubated for 1 h at room temperature, followed by flow-cytometric analysis using a FACSCalibur (Becton–Dickinson).

Measurement of nitrite production and quantification of cytokine concentrations. Cells were seeded in 96-well plates one day before the experiment at a density of 2×10^5 cells/well. The next day, the cells were preincubated with different concentrations of PKC412 for 1 h, followed by stimulation with 0.5 μg/ml of LPS. After 12 and 24 h, the supernatant medium was collected and the NO_2^- concentration was determined with the Griess Reagent System (Promega). The amounts of TNF-α and IL-6 released into the media were analyzed with ELISA kits (R&D Systems).

RNA isolation and RT-PCR. Total RNA was isolated using an RNeasy kit (Qiagen). First-strand cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen). Semi-quantitative real-time RT-PCR was performed using TaqMan Gene Expression Assays (*Tnfa*, Mm00443258_m1; *Il6*, Mm00446190_m1; *Nos2*, Mm00440485_m1; *Hprt1*, Mm01545399_m1; Applied Biosystems) on an ABI Prism 7900HT system. The comparative C_t method was used to determine the ratio of target to endogenous control gene (*Hprt1*) expression.

Immunoblotting. The primary antibodies used were anti-iNOS (Upstate Biotechnology), anti-GAPDH (Ambion), and anti-Nucleoporin p62 (Nup62; BD Biosciences), as well as antibodies from the Phospho-MAPK, MAPK Family, Phospho-SAPK/JNK and Phospho-Akt Pathway Antibody Sampler Kits (CST), and all were used at the manufacturer's recommended dilutions. Immunoblot analysis was performed according to standard procedures.

Electrophoretic mobility shift assay (EMSA). Subconfluent cells were pretreated with PKC412 for 1 h, followed by stimulation with 0.5 μg/ml of LPS for 30 min. Nuclear extracts were prepared using a Nuclear Extraction Kit (Active Motif), and EMSA was performed using Gel Shift Assay Systems (Promega). Briefly, 1 μg of the nuclear extracts was mixed with 20 pmol of the appropriate [^{32}P]-labeled oligonucleotides (AP-1, NF-κB), then incubated for 30 min at room temperature, and resolved on a 4% polyacrylamide gel, transferred to filter paper, and visualized by autoradiography.

Statistical analysis. The results are presented as means ± SD of four to six samples. Differences between two groups were tested using the unpaired two-tailed Student's t test, and $P < 0.05$ was considered to represent a statistically significant difference.

Results

PKC412 inhibits proliferation of RAW 264.7 macrophages through induction of G2/M cell cycle arrest and apoptosis

In the MTT assay, exposure of RAW 264.7 macrophages to 0.01–10 μM PKC412 resulted in a dose- and time-dependent reduction in cell viability (Fig. 1A). The effective PKC412 concentrations for 50% inhibition (IC_{50}) of RAW 264.7 cell growth after 24, 48, and 72 h were 3.82, 1.15, and 0.95 μM, respectively. PKC412 at concentrations below 0.1 μM had no growth inhibitory effect. The DNA synthesis rate measured by BrdU incorporation assay confirmed the anti-proliferative effect of PKC412 (EC_{50}/24 h = 2.67 μM; Supplementary Fig. S1).

Microscopic examination of PKC412-treated RAW 264.7 cells revealed dramatic morphological alterations including irregular shape, heterogeneous size and occasional long projections (Fig. S2A). The AO-EB staining showed changes characteristic of apoptosis (Fig. S2B). As shown in Fig. 1B, the formation of DNA nucleosome ladders was clearly detected in cells treated with 2.5 μM PKC412 for 12 h, while a very faint apoptotic ladder/smear pattern was visible in cells treated with 1.0 μM PKC412. Cell cycle analysis revealed that treatment with PKC412 increased the proportion of cells in the G2/M and sub-G1 (apoptotic cells) phases of the cell cycle in a time-dependent manner, with a corresponding decrease in the numbers of cells in the G0/G1 and S phases (Fig. 1C).

PKC412 differentially modulates the inflammatory response of RAW 264.7 macrophages to LPS

To examine whether PKC412 affects LPS-stimulated productions of TNF-α and IL-6 from RAW 264.7 cells,

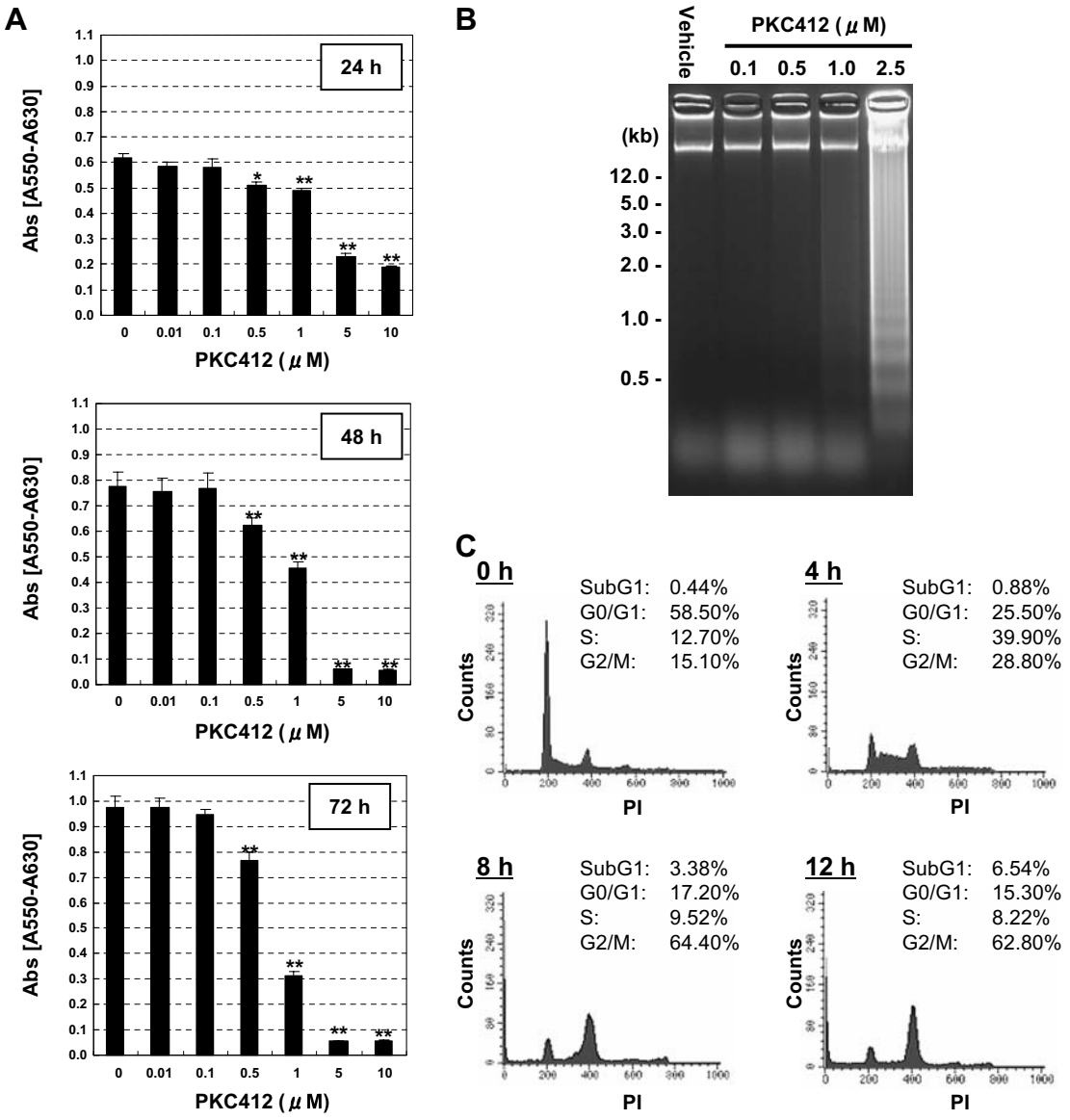

Fig. 1. Effects of PKC412 on RAW 264.7 macrophage viability (A) MTT assay. Cells were treated with increasing concentrations of PKC412 (0–10 μM) for 24, 48 or 72 h. Each bar represents the mean absorbance values (A_{570}–A_{630}) ± SD of four to six determinations. Note that absorbance values are directly proportional to cell viability, and are increased in a time-dependent manner in vehicle-treated cells. $^{*}P < 0.001$, $^{**}P < 0.0001$ versus vehicle-treated control cells (unpaired *t*-test). (B) DNA fragmentation assay. Cells were treated with various concentrations of PKC412 (0–2.5 μM) for 12 h. Genomic DNA was separated on an agarose gel and stained with EB. (C) RAW 264.7 cells were treated with 2.5 μM PKC412 for 4, 8, and 12 h, stained with PI, and the cell cycle distribution was then analyzed by flow cytometry. Representative histograms at each time point of at least three independent experiments are shown.

PKC412 drug concentrations (0.02–0.5 μM) and exposure times (12 or 24 h) were chosen to minimize the influence of its inhibitory effects on cell viability. A 12 h incubation of cells with 0.5 μg/ml of LPS caused a marked increase in TNF-α production as compared to non-stimulated cells (49.8 ± 5.7 versus 0.04 ± 0.06 ng/ml; Fig. 2A), and it was significantly reduced by PKC412 in a dose-dependent manner. Following 24 h LPS-stimulation, there was a decline in LPS-stimulated TNF-α levels in both vehicle- and PKC412-treated cells. This phenomenon has been described in previous reports [10,11], and is presumably attributable to uncharacterized clearance mechanisms (e.g. increased degradation and/or internalization). At this time point, the difference became less clear but a persistent inhibitory effect of PKC412 was observed. In contrast to TNF-α, no significant effects of PKC412 were found in LPS-stimulated IL-6 secretion after a 12 h incubation period (Fig. 2B). In fact, after a 24 h culture, IL-6 production was significantly enhanced by PKC412 at a concentration of $\geqslant$0.1 μM. Basal levels of TNF-α and IL-6 were not affected by treatment with either vehicle or PKC412.

Production of NO was increased approximately 12-fold after 12 h stimulation with LPS in vehicle-treated cells, and NO production was dose-dependently reduced in PKC412-treated cells (Fig. 2C). Following 24 h stimulation with LPS, NO production was elevated in both vehicle- and

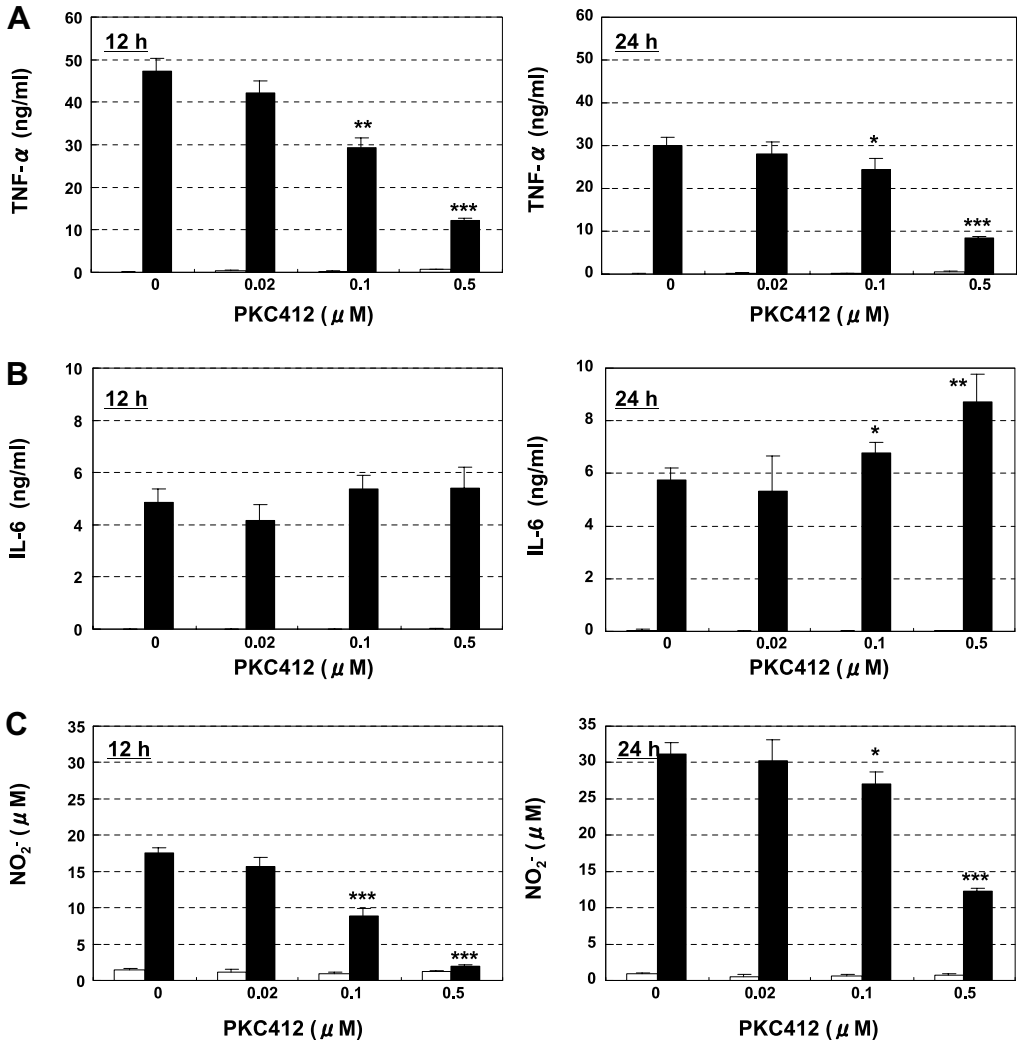

Fig. 2. Effects of PKC412 on LPS-induced inflammatory mediator production. RAW 264.7 macrophages were pretreated with various concentrations of PKC412 for 1 h, and further incubated either in the presence (black bars) or absence of 0.5 μg/ml of LPS (white bars). After 12 or 24 h incubation, the supernatant medium was collected and used to assay for TNF-α (A), IL-6 (B), and NO (C). Values are means ± SD ($n = 4$–6 in each group). Statistical significance (unpaired t-test) is based on the difference as compared to LPS-stimulated, vehicle-treated cells. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0001$.

PKC412-treated cells, but persistent inhibitory effects of PKC412 were observed. Western blot analysis of iNOS protein was consistent with NO production values (Fig. S3): PKC412 significantly attenuated LPS-induced iNOS expression in a parallel concentration-dependent manner.

Effects of PKC412 on LPS-induced gene expressions

To gain greater insight into the mechanisms by which PKC412 modulates productions of TNF-α, IL-6, and NO, we examined the effects of PKC412 on the expressions of the corresponding mRNAs, *Tnfa*, *Il6,* and *Nos2* (the gene encoding murine iNOS protein) (Fig. S4). Transcripts for these genes were markedly induced following LPS stimulation, although they exhibited distinct temporal differences in peak expression levels. Specifically, the induction of *Tnfa* mRNA was the fastest, peaking at 2 h after stimulation, with mRNA levels subsequently decreasing. Strong induction of *Il6* was also observed in the relatively early period of LPS treatment: however, the induction kinetics was delayed compared to *Tnfa*, with peak values achieved at 4 h. *Nos2* expression levels demonstrated a time-dependent increase over a 24 h culture period.

At a concentration of 0.5 μM, PKC412 significantly attenuated LPS-induced *Tnfa* expression over a 24 h time course (Fig. S4A). A similar inhibitory effect of PKC412 on *Il6* expression was observed at 4 h after LPS treatment: however, following longer exposures (16–24 h), PKC412 appeared to enhance *Il6* expression as compared to the vehicle, although these differences were not statistically significant (Fig. S4B). Effects of PKC412 on LPS-induced *Nos2* expression were complex and a time-dependent biphasic effect was observed (Fig. S4C). That is, *Nos2* expression was enhanced by PKC412 at early time points (⩽4 h), while later being markedly suppressed.

Effects of PKC412 on macrophage signaling pathways

Our preliminary study showed that, in RAW 264.7 cells, LPS induced rapid phosphorylation of all three MAPKs (ERK, p38, and JNK), with peak activation at approximately 15 min after stimulation (data not shown). To determine if PKC412 blocked the activation of MAPKs, cells were stimulated with LPS in the presence of PKC412 (0–2 μM). As shown in Fig. 3A, PKC412 treatment dose-dependently blocked LPS-induced JNK phosphorylation without affecting the total level of protein, whereas phosphorylations of ERK and p38 were unaffected. Activation of JNK in response to LPS may be mediated through MKK4. Once activated, JNK phosphorylates and activates c-Jun and ATF-2, the major components of AP-1 and AP-1-like transcription factors, and, in turn, controls the expression of a number of genes whose promoter regions contain AP-1 binding sites [12]. We found that PKC412 concentration-dependently attenuated the phosphorylation of MKK4 in response to LPS (Fig. 3A). The blockade of the MKK4/JNK signaling pathway by PKC412 was further confirmed because, within the same concentration range, it also inhibited the LPS-induced phosphorylation of c-Jun and, to a lesser degree, that of ATF-2 (Fig. 3B).

PKC412 also markedly inhibited LPS-stimulated phosphorylation of Akt on both Ser473 and Thr308, and its downstream molecule GSK-3β (Fig. 3C). These inhibitory effects were evident even at a low drug concentration of 0.25 μM. PDK1 is an upstream Akt kinase phosphorylating Thr308, and is activated mainly by PI3K-generated lipids [13]. We observed a high steady-state level of phosphorylated PDK1 in non-LPS-treated RAW 264.7 cells, which was only marginally suppressed by PKC412 (Fig. 3C).

Consistent with the observed inhibitory effects on MKK4/JNK/c-Jun activation, the EMSA results showed PKC412 to significantly inhibit LPS-induced AP-1 DNA binding activity (Fig. S5). LPS stimulation also resulted in a significant increase in the DNA binding activity of NF-κB in RAW 264.7 macrophages, while PKC412 had no significant effects (Fig. S5). Consistently, PKC412 had no effects on the kinetics of IκB-α and -β in response to LPS, the phosphorylation and degradation of which are responsible for the activation of NF-κB (data not shown).

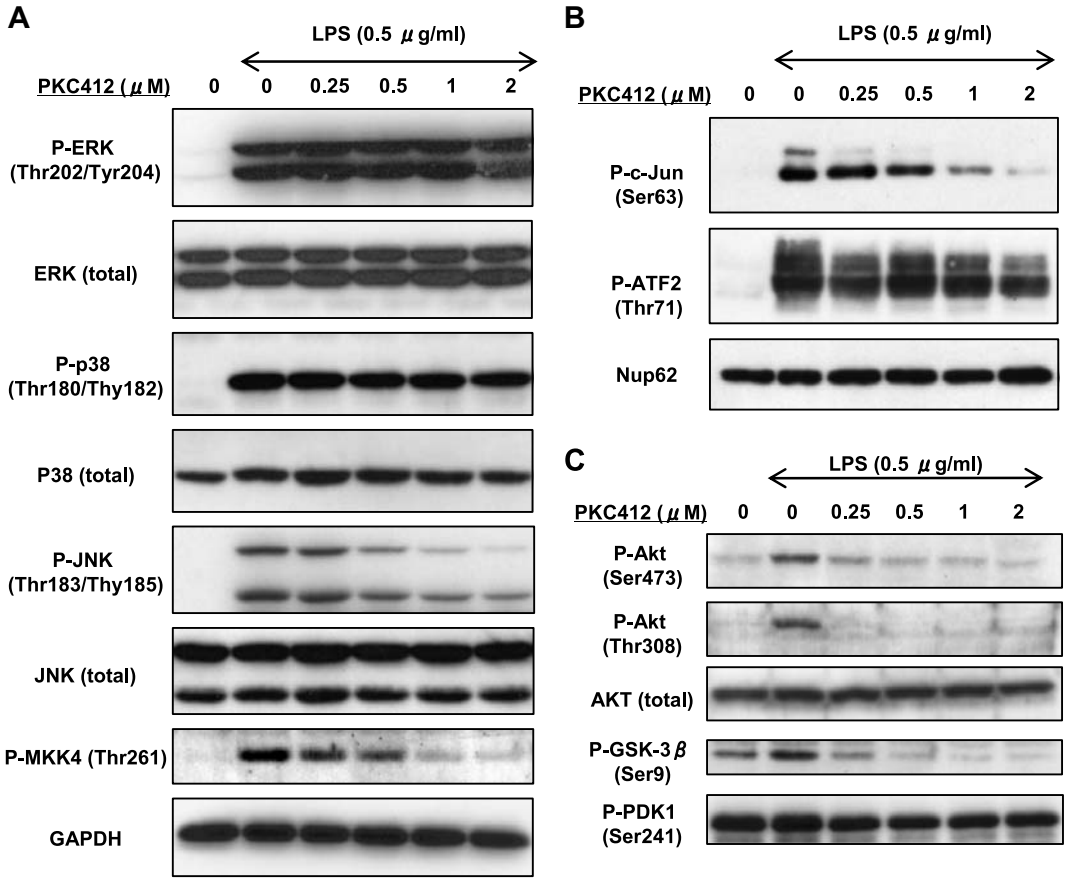

Fig. 3. Effects of PKC412 on LPS-induced signaling pathways. (A) RAW 264.7 cells were pretreated with the indicated concentrations of PKC412, followed by 15 min stimulation with LPS (0.5 μg/ml). Activations of ERK, p38, JNK and MKK4 were assessed by immunoblotting using phospho-specific anti-active antibodies. The blot was reprobed with antibodies against corresponding total-proteins and GAPDH. (B) Cells were treated with PKC412 as described above, and stimulated with LPS for 30 min. The nuclear extracts were subjected to immunoblotting using phospho-specific antibodies for c-Jun and ATF-2, and anti-Nup62 antibody (loading control). (C) Activations of Akt, GSK-3β and PDK1 were assessed using phospho-specific antibodies, and the blot was reprobed with anti-total-Akt and GAPDH antibodies.

Discussion

PKC412 inhibited cell proliferation of RAW 264.7 macrophages in a dose- and time-dependent manner, with IC_{50} values (3.82–0.95 μM for 24–72 h) slightly higher than those reported for various tumor and normal cell lines, as well as in xenograft models (approximately 0.2–1 μM). The anti-proliferative properties of PKC412 in RAW 264.7 cells were associated with induction of cell cycle arrest in the G2/M phase and apoptosis. These findings are consistent with previous studies in other cell types showing exposure of cells to PKC412 to result in an increase in the G2/M phase of the cell cycle concomitant with increased polyploidy, apoptosis and enhanced sensitivity to ionizing radiation [1].

We confirmed that non-toxic doses of PKC412 modulate inflammatory responses of RAW 264.7 macrophages. PKC412 (0.1–0.5 μM) dose-dependently inhibited LPS-induced enhancement of TNF-α and NO releases. These inhibitory effects were more significant for relatively short exposure times (12 h), and tended to diminish over longer incubation periods. This observation might relate to the biological half-life of PKC412, or might be attributable to our use of a relatively high (sub-maximal) concentration of LPS (0.5 μg/ml) for stimulation. Alternatively, an autocrine feedback mechanism might be involved, as it has been proposed that macrophage-derived TNF-α or NO can activate macrophages themselves [14,15]. In contrast to TNF-α and NO, IL-6 production was not inhibited, but rather was enhanced by PKC412 following 24 h LPS-stimulation. Previous studies have demonstrated that IL-6 exerts pro- as well as anti-inflammatory activities during acute inflammatory responses [16,17]. Therefore, how IL-6 enhancement by PKC412 is associated with the eventual outcome of LPS-induced inflammation in macrophages remains unclear.

Overall, our results on the production of inflammatory mediators are consistent with the earlier findings of Tremblay et al. [18], who treated RAW 264.7 cells with a single high dose of PKC412 (1 μM) under serum-free conditions and measured LPS-induced cytokine productions using bioassay-based methods instead of ELISA. Our experiments were conducted in the presence of serum in the medium, since, without serum, the RAW 264.7 macrophages rapidly became apoptotic (data not shown). A similar observation was reported previously, and an apoptotic mechanism involving autocrine secretion of IFN-α and -β has been suggested [19]. Yet, under our experimental conditions, it is possible that serum protein binding of PKC412 interferes with its pharmacological activity or distribution: however, it has been shown that oral administration of PKC412 at 150–300 mg/day to cancer patients resulted in steady-state PKC412 plasma levels in the micromolar range [3], suggesting that the drug concentrations used in this study are at least physiologically relevant.

Gene expression analyses revealed different temporal induction profiles for *Tnfa*, *Il6,* and *Nos2* genes with LPS stimulation. Interestingly, the effects of PKC412 on transcription levels were also variable among the genes investigated, and did not necessarily correspond to the results of production/secretion. These observations suggested that PKC412 affects macrophage production of inflammatory mediators by interfering at not only the transcriptional but also other levels, presumably translational and/or post-translational regulation.

Our results showed that PKC412 inhibited LPS-induced JNK phosphorylation and AP-1 activation in RAW 264.7 cells. As phosphorylation of MKK4 was also inhibited by PKC412, it is likely that PKC412 acts proximal to MKK4, which is regulated by a complex and inter-related group of MAPK kinase kinases (MAPKKKs). PKC412 also markedly suppressed LPS-stimulated phosphorylation of both Akt and its downstream molecule GSK-3β, yet exerted no effects on NF-κB activities, another downstream target of Akt. Although it has been well accepted that Akt is a downstream target of PI3K [13], the role of the PI3K/Akt signal pathway in LPS signaling is controversial. There are several reports describing a positive role for PI3K/Akt in LPS-induced inflammatory responses, while several others have demonstrated a negative role with special emphasis on its protective effects from prolonged inflammatory responses [20–22]. In addition, one report suggested the PI3K-independent pathway to be involved in Akt activation in LPS-stimulated RAW 264.7 cells [23]. Further studies are necessary to elucidate the significance of Akt signaling inhibition by PKC412 in relation to macrophage functions.

In summary, we have shown that PKC412 exerts both anti-proliferative/apoptosis-inducing and immunomodulatory activities in RAW 264.7 macrophages, and that these activities are likely to be mediated, at least in part, by inhibition of the MKK4/JNK/AP-1 and/or Akt/GSK-3β signaling pathways. Since macrophages contribute to both acute and chronic inflammatory diseases and their function is a potential target for novel therapeutic intervention, our results suggest that PKC412 has potential clinical utility as it can counter macrophage activities.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.06.009.

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