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PKCα Depletion in RAW264.7 Macrophages Following Microbial/IFNγ Stimulation Is PC-PLC-Mediated

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ABSTRACT

Under chronic inflammatory conditions, monocytes/macrophages often exhibit a desensitized phenotype, which is characterized by attenuated reactive oxygen species (ROS) production in close association with depletion of protein kinase Cα (PKCα). This behavior has been observed in monocytes derived from septic blood although the stimulus responsible for initiating these alterations remained obscure. Using RAW264.7 macrophages, we provide evidence that components of neither gram-negative nor gram-positive bacteria deplete PKCα, whereas the T_H1 cytokine interferon-γ (IFNγ) does. As shown by western blot analysis, lipopolysaccharide, as well as lipoteichoic acid, did not alter PKCα expression, but IFNγ dose-dependently decreased PKCα protein level. Taking into consideration that diacylglycerol and Ca^{2+} as established PKCα activators are released in response to phospholipase C activation, we pretreated cells with the phosphatidylcholine-specific phospholipase C (PC-PLC) inhibitor tricyclodecan-9-yl potassium xanthate (D609) and the phosphatidylinositol-specific phospholipase C inhibitor 1-(6-(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1*H*-pyrrole-2,5-dione (U73122). In cells preincubated with D609, IFNγ-mediated PKCα depletion was attenuated, whereas U73122 did not impair this process. Moreover, phorbol 12-myristate 13-acetate-initiated ROS formation, which was attenuated in macrophages pretreated with IFNγ, was restored in the presence of the PC-PLC inhibitor. These results suggest that IFNγ causes PC-PLC stimulation, diacylglycerol release, Ca^{2+} influx, and concomitant PKCα activation, which subsequently depletes PKCα. Strategies to antagonize IFNγ might be helpful to prevent monocyte/macrophage desensitization. *Antioxid. Redox Signal.* 7, 1217–1222.

INTRODUCTION

MONOCYTES/MACROPHAGES are important players of the innate immune system (19) participating in eliminating microbes (1). The contact with pathogens provokes expression of proinflammatory cytokines and mediators that orchestrate pathogen killing and further coordinate immune responses (3). It has been shown that persistent macrophage activation induces a phenotype switch from pro- toward antiinflammatory, as characterized by a hampered proinflammatory response and the expression of antiinflammatory cytokines. This culminates in largely attenuated immune reactions upon secondary infection (7, 13, 25). Therefore, the balance between pro- and antiinflammatory cytokine expression requires fine tuning as any imbalance will provoke pathophysiological settings as observed in the development of, *e.g.,* sepsis (11, 22).

Formation of superoxide (O_2^-) by the NADPH oxidase in monocytes/macrophages is one important mediator to kill pathogens upon first contact (12). Following recognition and uptake, microorganisms are killed by reactive oxygen species (ROS) (8). The constituents of the NADPH oxidase are available preformed in the cytoplasm or linked to the cell membrane (6, 15). Upon activation, some elements translocate to the cell membrane to assemble the functional NADPH oxidase complex, which generates O_2^-. An

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important step in NADPH oxidase activation demands protein kinase Cα (PKCα), a classical isoform of the PKC family (17). PKCs have been divided into three subgroups based on their structure and cofactor requirements (18). Conventional PKC (cPKC) isoforms (α, βI, βII, γ), novel PKC (nPKC) isoforms (δ, ϵ, η, θ), and atypical PKC (aPKC) isoforms (ζ, ι/λ) are distinguished. Due to requirements of the protein structure, activation of PKCα is achieved by *sn*-1,2-diacycglycerol (DAG) and/or Ca^{2+}. Once PKCα is activated, it translocates to the cell membrane and concomitantly is depleted in close association with macrophage desensitization. PKC activators originate endogenously from the cell membrane. DAG is released by phospholipase C (PLC) or D (PLD) from phospholipids of the outer cell membrane (9). Within the group of PLC enzymes, two distinct substrate-specific enzyme families, *i.e.,* phosphatidylinositol-specific (PI-PLC) and phosphatidylcholine-specific PLC (PC-PLC), can be distinguished. Activation of either PLC family generates the lipid messenger DAG, whereas PI-PLC activation generates inositol trisphosphate and PC-PLC activation generates choline phosphate. Inositol trisphosphate, in turn, raises intracellular Ca^{2+} by opening stores of the endoplasmic reticulum, which constitutes a major intracellular Ca^{2+}-sequestering organelle (26). Choline phosphate acts mitogenically, provoking DNA synthesis and cell proliferation (21). Activation of PI-PLC and PC-PLC has been described in response to various stimuli, among others, lipopolysaccharide (LPS) and lipoteichoic acid (LTA) (5, 14, 24, 28), which are the most prominent bacterial activators, as well as interferon-γ (IFNγ), a T_H1 cell cytokine, produced during progression of infection (4). However, it remained unclear, whether PI-PLC and/or PC-PLC participate in affecting ROS production in macrophages.

Taking into consideration that PKCα is the most prominent NADPH oxidase activator in monocytes/macrophages, we were interested in studying PKCα expression in RAW264.7 macrophages following treatments with LPS, LTA, or IFNγ. Whereas IFNγ significantly reduced PKCα expression, neither LPS nor LTA shared this behavior. Use of the inhibitor tricyclodecan-9-yl potassium xanthate (D609) points to a role of PC-PLC in facilitating depletion of PKCα in response to IFNγ, as well as blocking ROS formation in response to phorbol 12-myristate 13-acetate (PMA) stimulation. It can be concluded that PKCα down-regulation by IFNγ is mediated via PC-PLC, a signaling pathway with relevance to macrophage desensitization as noticed during sepsis.

MATERIALS AND METHODS

Materials

PMA, LPS (*Escherichia coli,* serotype 0127:B8, trichloroacetic acid extraction, protein 1–10%), and LTA (*Staphylococcus aureus,* phenol extraction, water content 2.08%, alanine/phosphate ratio 0.09) were purchased from Sigma (Deisenhofen, Germany). Hydroethidine (HE) was from Molecular Probes (Leiden, The Netherlands). 1-(6-(17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1*H*-pyrrole-2,5-dione (U73122) and D609 were from Alexis (Grünberg, Germany). Culture supplements and fetal calf serum were ordered from PAA Laboratories (Linz, Austria). Murine recombinant IFNγ was from Roche (Mannheim, Germany). Other chemicals were of the highest grade of purity and commercially available.

Cell culture

The mouse monocyte/macrophage cell line RAW264.7 was maintained in RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (complete RPMI). All experiments were performed using complete RPMI.

Flow cytometry of oxygen-radical production (HE assay)

Following a prestimulation regime, 5×10^5 cells were incubated for 30 min with 1 μ*M* PMA, followed by the addition of 3 μ*M* HE, while incubations continued for 30 min. Cells were harvested, washed with PBS and resuspended in 500 μl of phosphate-buffered saline (PBS). Flow cytometry analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany), and HE was measured through a 630-nm long pass filter (FL3). Data from 10,000 cells were collected to reach significance. All incubations were performed at 37°C.

Western blot analysis

Cell lysis was achieved with lysis buffer (50 m*M* Tris, 5 m*M* EDTA, 150 m*M* NaCl, 0.5% Nonidet P-40, 1 m*M* phenylmethylsulfonyl fluoride, pH 8.0) and sonification (Branson sonifier; 20 s, duty cycle 100%, output control 60%). Whole-cell lysates were cleared by centrifugation (10,000 *g*, 5 min), and protein was determined by the Lowry method. Protein (80 μg) was resolved on 10% polyacrylamide gels and blotted onto nitrocellulose sheets basically following standard methodology. Equal loading and correct protein transfer to nitrocellulose were routinely quantitated by Ponceau S staining. Filters were incubated with the anti-PKCα antibody (1:500; Transduction Laboratories, BD Biosciences) or anti-actin antibody (1:2,000; Amersham Biosciences, Freiburg, Germany) overnight at 4°C. Horseradish peroxidase-conjugated polyclonal antibodies (1:5,000; Amersham Biosciences) were used for enhanced chemiluminescence detection.

Statistical analysis

Each experiment was performed at least three times, and representative data are shown.

RESULTS

PKCα depletion in response to IFNγ treatment

The respiratory burst is an early activation marker of macrophages in response to pathogen contact and constitutes a pivotal role in killing microorganisms (8). Taking into consideration that ROS production in monocytes/macrophages is mainly achieved by a PKCα-dependent mechanism (17), we

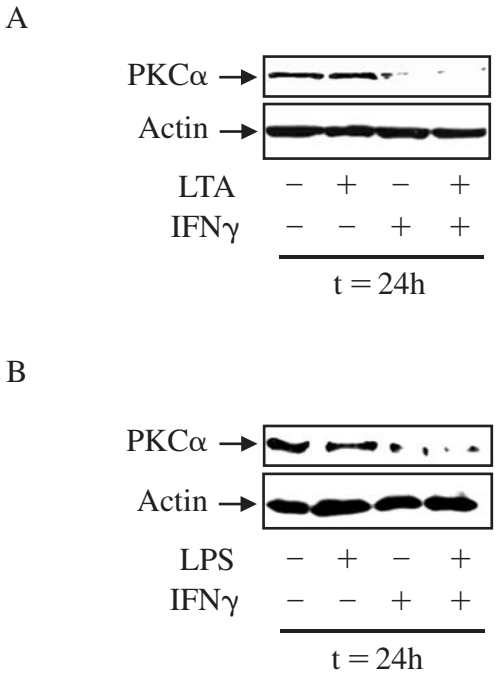


FIG. 1. PKCα expression in activated RAW264.7 macrophages. Cells were harvested 24 h after stimulation, and western blotting was performed using an anti-PKCα antibody as described in Materials and Methods. Blots were normalized by α-actin staining. (**A**) Cells remained as controls (lane 1) or were treated with 10 μg/ml LTA (lane 2), 100 U/ml IFNγ (lane 3), or a combination of both (lane 4). (**B**) Cells remained as controls (lane 1) or were treated with 10 μg/ml LPS (lane 2), 100 U/ml IFNγ (lane 3), or a combination of both (lane 4).

analyzed PKCα expression in RAW264.7 cells in response to immunologically relevant treatments (3, 19). As shown in Fig. 1A, the addition of 10 μg/ml LTA for 24 h did not alter PKCα expression. In contrast, incubations of RAW264.7 cells for 24 h with 100 U/ml IFNγ significantly decreased the PKCα content. Cotreatment of cells with LTA and IFNγ did not alter the response elicited by IFNγ alone. Similar to LTA, the exposure of cells to 10 μg/ml LPS for 24 h did not provoke PKCα down-regulation (Fig. 1B). Addition of LPS and IFNγ in combination did not increase further the impact of IFNγ alone.

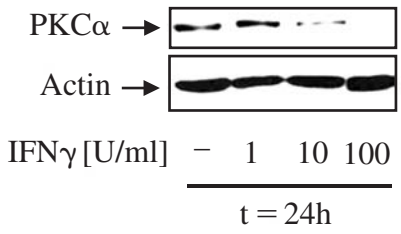


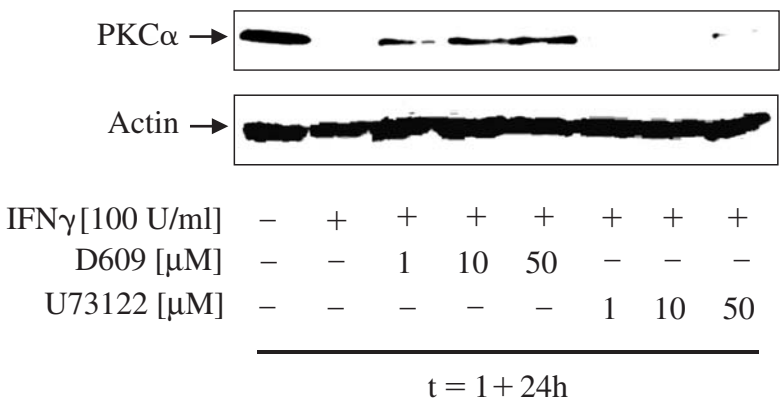
FIG. 2. PKCα expression in IFNγ-activated RAW264.7 macrophages. Cells were harvested 24 h after stimulation. Western analysis was performed using an anti-PKCα antibody as described in Materials and Methods. Blots were normalized by α-actin staining. Cells remained as controls (lane 1) or were treated with 1 U/ml (lane 2), 10 U/ml (lane 3), or 100 U/ml IFNγ (lane 4).

In a second set of experiments, we determined concentrations of IFNγ that are necessary to lower PKCα expression in RAW264.7 macrophages. Therefore, we treated RAW264.7 cells for 24 h with 1, 10, and 100 U/ml IFNγ and analyzed PKCα expression by western blotting (Fig. 2). Whereas 1 U/ml IFNγ did not alter PKCα expression, PKCα was significantly down-regulated in response to 10 U/ml IFNγ and completely depleted after 100 U/ml IFNγ. We conclude from these data that efficient down-regulation of PKCα is only achieved by the T_H1 cytokine IFNγ, but not by bacterial cell wall components such as LPS and LTA.

PC-PLC-dependent PKCα depletion

Considering PI-PLC and PC-PLC as potential upstream signaling components involved in PKCα activation, we performed inhibitor studies using D609 to inhibit PC-PLC and U73122 to repress PI-PLC activity. To this end, RAW264.7 macrophages were exposed to increasing concentrations of inhibitors for 1 h and stimulated with IFNγ for 24 h prior to western analysis of PKCα (Fig. 3). D609 in part reversed IFNγ-evoked PKCα depletion. This was partially obvious with 1 μ*M* D609. The effect became more pronounced at 10 μ*M* D609 and reached its maximal effect at 50 μ*M* of the inhibitor when IFNγ-mediated PKCα depletion was reversed to a very large extent. In contrast, pretreatment of RAW264.7 cells with U73122, at 1–50 μ*M*, did not antagonize PKCα down-regulation provoked by IFNγ treatment. These results imply that PKCα depletion in response to IFNγ stimulation is transmitted via PC-PLC activation.

FIG. 3. PKCα expression in IFNγ-activated RAW264.7 macrophages under the influence of PLC inhibitors. Cells were pretreated for 1 h with inhibitors as indicated and subsequently stimulated for 24 h with 100 U/ml IFNγ. Western analysis was performed to follow PKCα expression as described in Materials and Methods. Blots were normalized by α-actin staining. Cells remained as controls (lane 1) or were treated with 100 U/ml IFNγ (lane 2), or prestimulated with 1 μ*M* (lane 3), 10 μ*M* (lane 4), or 50 μ*M* (lane 5) of the PC-PLC inhibitor D609 or 1 μ*M* (lane 6), 10 μ*M* (lane 7), or 50 μ*M* (lane 8) of the PI-PLC antagonist U73122 prior to the addition of 100 U/ml IFNγ.



Inhibition of PC-PLC restores ROS formation that is attenuated by IFNγ pretreatment

To support our hypothesis that IFNγ-evoked down-regulation of PKCα affects ROS production in RAW264.7 macrophages, we followed O_2^- formation by flow cytometry using HE as the redox-sensitive dye in response to the DAG homologue PMA. In control cells, PMA provoked ROS production as shown by the rightward shift of the trace indicating HE oxidation compared with untreated cells (Fig. 4A). Prestimulation of macrophages for 24 h with IFNγ attenuated O_2^- generation in response to PMA (Fig. 4B). As expected, pretreatment of RAW264.7 macrophages with the PI-PLC inhibitor U73122 did not reverse attenuated O_2^- synthesis (Fig. 4C). However, PC-PLC inhibition with D609 fully restored ROS production, because PMA elicited O_2^- formation although cells had been prestimulated with IFNγ (Fig. 4D). These observations emphasize the role of IFNγ in desensitizing RAW264.7 macrophages, suggesting a similar role under septic conditions.

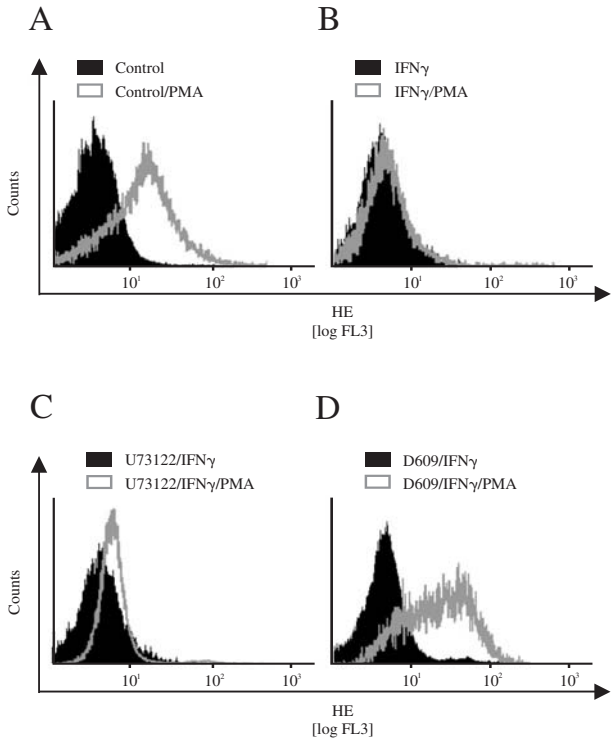


FIG. 4. Inhibition of PC-PLC restores IFNγ-evoked desensitization. ROS production in response to 1 μ*M* PMA was analyzed by flow cytometry using 3 μ*M* HE as the redox-sensitive dye. Cells were pretreated for 1 h with inhibitors as indicated and subsequently stimulated for 24 h with 100 U/ml IFNγ. Afterwards 1 μ*M* PMA was added for 30 min as indicated, followed by incubations with 3 μ*M* HE for an additional 30 min. Thereafter, cells were harvested, resuspended in PBS, and analyzed by flow cytomtery. (**A**) Cells remained as controls (solid filled trace), or ROS production was initiated with PMA (gray line). (**B**) Cells were treated for 24 h with IFNγ (solid filled trace), and ROS production was initiated with PMA (gray line). (**C**) Cells were pretreated for 1 h with 50 μ*M* of the PI-PLC inhibitor U73122, followed by the addition of IFNγ for 24 h (solid filled trace). Subsequent ROS production was initiated by PMA (gray line). (**D**) Cells were preincubated for 1 h with 50 μ*M* of the PC-PLC inhibitor D609, followed by the addition of IFNγ for 24 h (solid filled trace). Subsequent ROS production was initiated by PMA (gray line).

DISCUSSION

The balance of pro- versus antiinflammatory immune responses constitutes a prominent role in regulating innate immunity. This is achieved, among other mechanisms, by switching the macrophage phenotype from a proinflammatory toward an antiinflammatory behavior. Overproduction of proinflammatory cytokines may cause severe tissue damage, organ failure, and/or death (11). In contrast, an antiinflammatory, *i.e.,* desensitized macrophage phenotype, causes an inadequate immune response toward secondary infection (7, 13, 25). However, the understanding of the molecular switch toward this antiinflammatory appearance remains unclear. Recently, we provided evidence that, in monocytes from septic patients, PKCα is down-regulated and concomitant ROS production in these cells is attenuated (unpublished observations). To determine underlying signaling pathways in a cell culture model, we used macrophages exposed to gram-negative and gram-positive cell wall components such as LPS and LTA, as well as the T_H1 cytokine IFNγ. By using this approach, it was expected to gain insights into mechanisms of PKCα depletion and to establish a correlation toward attenuated ROS production in desensitized macrophages.

Here we provide evidence that prolonged treatment with neither the gram-negative cell wall constituent LPS nor the gram-positive cell wall component LTA altered PKCα expression in RAW264.7 macrophages. Taking into account that PKCα depletion requires its prior activation (20), our results imply that in RAW264.7 macrophages both LTA and LPS failed to activate PKCα.

It is now appreciated that several markers of inflammation demand PKCα activation with the further notion that the T_H1 cytokine IFNγ gained potential interest as an activating compound. Along that line, PKCα is needed to facilitate IFNγ-induced MHC class II expression in macrophages (10) or ICAM-1 expression in epithelial cells (4). In both systems, the participating role of PKCα was verified by transfecting a catalytically inactive mutant that eliminated signal transmission. In some homology, in our system IFNγ provoked PKCα activation in RAW264.7 macrophages as determined by its subsequent protein depletion, as well as attenuated ROS formation. As an initiating and upstream signaling event, we propose IFNγ-mediated PC-PLC activation. In turn, active PC-PLC, by liberating DAG and increasing intracellular Ca^{2+}, will stimulate PKCα, followed by its subsequent down-regulation. This model is supported by observations of Yamauchi *et al.* showing that apolipoprotein A-I activates PKCα by PC-PLC-mediated generation of DAG (27). As seen in our experiment with IFNγ also, apolipoprotein A-I-evoked activation of PKCα was attenuated by D609, but not U73122. Furthermore, Ca^{2+} release in response to PC-PLC activation by a still unclear mechanism has been described in ATP-

treated monocytes (2), confirming our results. It is of interest that also in the case of inducible nitric oxide synthase (iNOS) expression in murine macrophages following IFNγ application, the activation of PC-PLC rather than PI-PLC is required, whereas LPS uses both PLC isoenzymes for iNOS induction (23). We conclude that PC-PLC is a target of IFNγ that not only is involved in ROS formation, but helps to coordinate macrophage activation in general. Besides, PC-PLC activation in response to LPS and LTA has been reported as well (14, 16), among other systems also in RAW264.7 macrophages (5, 28). Assuming that this might also be the case in our experimental system, we postulate that activation of PC-PLC is not strong enough compared with IFNγ to accomplish activation of PKCα that is associated with its depletion.

Consistent with the idea that PKCα is needed to assemble NADPH oxidase and thus to stimulate ROS production in macrophages, we observed that depletion of PKCα by IFNγ attenuated O_2^- formation. A cause–effect relation was established when D609 allowed ROS generation to be reestablished in response to IFNγ. Further experiments will prove the significance of these data in primary human monocytes/macrophages and address whether IFNγ indeed plays a fundamental role in macrophage desensitization, *i.e.,* decreased ROS formation under conditions of sepsis. One may speculate whether antagonizing IFNγ signaling may help to improve an appropriate immune response during prolonged periods of infection.

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ABBREVIATIONS

DAG, diacylglycerol; D609, tricyclodecan-9-yl potassium xanthate; HE, hydroethidine; IFNγ, interferon-γ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; LTA, lipoteichoic acid; O_2^-, superoxide; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PI, phosphatidylinositol; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; U73122, 1-(6-(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1*H*-pyrrole-2,5-dione.

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