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Inhibition of classical PKC isoenzymes downregulates STAT1 activation and iNOS expression in LPS-treated murine J774 macrophages

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1 Proinflammatory cytokines and bacterial products trigger inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production in inflammatory and tissue cells. In inflammation, NO acts as an important mediator having both proinflammatory and destructive effects.

2 Protein kinase C (PKC) is a family of serine–threonine protein kinase isoenzymes involved in signal transduction pathways related to inflammatory responses. The aim of the present study was to investigate the role of classical PKC (cPKC) isoenzymes in the regulation of iNOS expression and NO production in murine J774 macrophages and the mechanisms involved.

3 RO318220 (inhibits PKCβ, PKCγ and PKCε), GÖ6976 (inhibits cPKC isoenzymes PKCα and PKCβ) and LY333531 (inhibits PKCβ) reduced lipopolysaccharide (LPS)-induced NO production and iNOS expression in a dose-dependent manner as did 6 h pretreatment with 1 μM phorbol 12-myristate 13-acetate (PMA) (which was shown to downregulate PKC expression).

4 PKC inhibitors also reduced LPS-induced iNOS mRNA levels, but they did not affect the half-life of iNOS mRNA. PKC inhibitors did not alter LPS-induced activation of NF-κB as measured by electrophoretic mobility shift assay.

5 All PKC inhibitors used and pretreatment with 1 μM PMA inhibited signal transducer and activator of transcription 1 (STAT1) activation as measured by the translocation of STAT1α from the cytosol to the nucleus by Western blot. In addition, inhibition of STAT1 activation by AG-490, an inhibitor of JAK-2, also reduced NO production.

6 These results suggest that cPKC isoenzymes, especially PKCβ, mediate the upregulation of iNOS expression and NO production in activated macrophages in an NF-κB-independent manner, possibly through the activation of transcription factor STAT1.
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Abbreviations: EMSA, electrophoretic mobility shift assay; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; PDD, phorbol 12,13-didecanoate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; STAT1, signal transducer and activator of transcription 1

Introduction

Nitric oxide (NO) acts as a signalling molecule in, for example, cardiovascular and neuronal systems. In inflammation, NO is an important mediator having both proinflammatory and destructive effects (Moilanen *et al*., 1999; Korhonen *et al*., 2005). High amount of NO is produced by inducible nitric oxide synthase (iNOS) for prolonged time as a response to bacterial products, such as lipopolysaccharide (LPS), and to proinflammatory cytokines (MacMicking *et al*., 1997; Vallance & Leiper, 2002). NO production in activated macrophages is primarily regulated at the level of iNOS expression (Kleinert *et al*., 2003; Korhonen *et al*., 2005).

The protein kinase C (PKC) pathway represents a major signal transduction system in inflammation (Spitaler & Cantrell, 2004). Different tissues seem to have their own characteristic patterns of PKC isoenzyme expression and function (Way *et al*., 2000). The mammalian PKC family comprises of serine–threonine protein kinase isoenzymes, which are divided into three classes based on their structure and ability to bind cofactors (Newton, 2001). The classical PKC (cPKC) isoenzymes (α, γ and the splice variants βI and βII) are activated by diacylglycerol (DAG), Ca^{2+} and phosphatidylserine. These isoenzymes are targets of the tumor-promoting phorbol ester PMA (phorbol 12-myristate 13-acetate, also called TPA), a surrogate of DAG. The novel (nPKC) isoenzymes (δ, ε, η and θ) are Ca^{2+}-independent and activated by DAG and phosphatidylserine. The third group, atypical PKC (aPKC) isoenzymes (ζ and ι/λ), are Ca^{2+}- and DAG-independent kinases. In contrast to the classical and novel isoenzymes, aPKCs do not respond to phorbol esters (Newton, 2001; Spitaler & Cantrell, 2004). In addition, PKCμ

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and PKCν are sometimes regarded to form a fourth class of PKC isoenzymes (Newton, 2001).

A role for PKC has been identified in inflammatory diseases, cancer and heart disease, and PKC inhibitors are under development to treat these diseases (Bowling *et al*., 1999; Chen *et al*., 2001; Goekjian & Jirousek, 2001; Newton, 2001; Tan & Parker, 2003; Aksoy *et al*., 2004). Several lines of evidence suggest that cPKC isoenzymes (Fujihara *et al*., 1994; St-Denis *et al*., 1998; Giroux & Descoteaux, 2000; Molina-Holgado *et al*., 2000; Foey & Brennan, 2004), PKCδ (Chen *et al*., 1998a; Tepperman *et al*., 2000; Carpenter *et al*., 2001), PKCη (Chen *et al*., 1998b; Pham *et al*., 2003) and PKCε (Castrillo *et al*., 2001; Kang *et al*., 2001) are involved in the LPS- and cytokine-induced expression of inflammatory genes including iNOS.

The aim of the present study was to investigate the role of cPKC isoenzymes in the regulation of iNOS expression and NO production in activated macrophages and the mechanisms involved. The results suggest that cPKC isoenzymes, probably PKCβ, mediate the upregulation of iNOS expression and NO production in activated macrophages in an NF-κB-independent manner, possibly through the activation of transcription factor signal transducer and activator of transcription 1 (STAT1).

Methods

Materials

Reagents were purchased as follows: RO318220, phorbol 12,13-didecanoate (PDD) and LY333531 were from Alexis Biochemicals (Lausen, Switzerland); GÖ6976, HBDDE and recombinant PKCγ were from Calbiochem (La Jolla, CA, U.S.A.); LPS (*Escherichia coli* 0111:B4, product number L-4391) was from Sigma Chemical Co. (St Louis, MO, U.S.A.); mouse monoclonal PKCα antibody, rabbit polyclonal iNOS, PKCβI, PKCβII, PKCγ and STAT1α antibodies and goat anti-rabbit HRP-conjugated polyclonal antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.) and goat anti-mouse HRP-conjugated antibody was from Pierce Biotechnology (Rockford, IL, U.S.A.). All other reagents were from Sigma Chemical Co.

Cell culture

J774 macrophages (American Type Culture Collection) were cultured at 37°C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium with ultraglutamine 1 (Cambrex BioScience, Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (Cambrex BioScience), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 250 ng ml^{-1} amphotericin B (Gibco, Paisley, U.K.) and harvested with trypsin-EDTA (Gibco). Cells were seeded on 24-well plates for nitrite measurements and RT–PCR, on six-well plates for Western blot analysis and on 10 cm dishes for translocation studies, preparation of nuclear extracts and electrophoretic mobility shift assay. Cells were grown to confluence prior to the experiments. Toxicity of the tested compounds was ruled out by measuring cell viability using Cell Proliferation Kit II (Roche Diagnostics, Indianapolis, IN, U.S.A.) according to manufacturer's instructions.

Nitrite assays

Measurement of nitrite accumulation into the culture medium was used to determine NO production. The culture medium was collected at indicated time points and nitrite was measured by Griess reaction (Green *et al*., 1982).

Preparation of cell lysates for Western blotting

At indicated time points, cells were rapidly washed with ice-cold phosphate-buffered saline (PBS) and solubilized in cold lysis buffer containing 10 mM Tris-base, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate, 20 μg ml^{-1} leupeptin, 50 μg ml^{-1} aprotinin, 5 mM NaF, 2 mM sodiumpyrophosphate and 10 μM *n*-octyl-β-D-glucopyranoside. After incubation for 15 min on ice, lysates were centrifuged (13,400 × *g*, 4°C, 10 min), supernatants were collected and stored in SDS sample buffer in −20°C. An aliquot of the supernatant was used to determine protein concentration by the Coomassie blue method (Bradford, 1976).

Preparation of cytosolic and particulate fractions for PKC Western blotting

At indicated time points, cells were rapidly washed with ice-cold PBS and solubilized in cold buffer A (20 mM Tris-base, pH 7.4, 10 mM EDTA, 5 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM sodiumorthovanadate, 10 μg ml^{-1} leupeptin, 25 μg ml^{-1} aprotinin and 1.25 mM NaF). After incubation for 15 min on ice, lysates were centrifuged at 100,000 × *g* for 1 h at 4°C, supernatants were collected and marked as the cytosolic fraction. Pellets were resuspended in cold lysis buffer B (20 mM Tris-base, pH 7.4, 10 mM EDTA, 5 mM EGTA, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM sodiumorthovanadate, 10 μg ml^{-1} leupeptin, 25 μg ml^{-1} aprotinin, 1.25 mM NaF and 10 μM *n*-octyl-β-D-glucopyranoside). After incubation for 2 h on ice, lysates were centrifuged at 100,000 × *g* for 1 h at 4°C, supernatants were collected and marked as the particulate fraction. An aliquot of the supernatant was used to determine protein concentration by the Coomassie blue method (Bradford, 1976).

Preparation of nuclear extracts for electrophoretic mobility shift assay (EMSA) and STAT1α Western blotting

At indicated time points, cells were rapidly washed with ice-cold PBS and solubilized in hypotonic buffer A (10 mM HEPES–KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate, 10 μg ml^{-1} leupeptin, 25 μg ml^{-1} aprotinin, 1 mM NaF and 0.1 mM EGTA). After incubation for 10 min on ice, cells were vortexed for 30 s and the nuclei were separated by centrifugation at 4°C, 21,000 × *g* for 10 s. Nuclei were resuspended in buffer C (20 mM HEPES–KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate, 10 μg ml^{-1} leupeptin, 25 μg ml^{-1} aprotinin, 1 mM NaF and 0.1 mM EGTA) and incubated for 20 min on ice. Nuclei were vortexed for 30 s and nuclear extracts were obtained by centrifugation at 4°C, 21,000 × *g* for 2 min.

Protein contents of the nuclear extracts were measured by the Coomassie blue method (Bradford, 1976).

Western blotting

Prior to Western blotting, proteins were boiled for 10 min with SDS sample buffer and 20 μg of protein was used per lane on 8% (iNOS, STAT1α) or 10% (PKC) SDS–polyacrylamide gel and transferred to Hybond ECL™ nitrocellulose membrane (Amersham Biosciences, U.K., Ltd, Little Chalfont, Buckinghamshire, U.K.). After transfer, the membrane was blocked in TBS-T (20 mM Tris-base, pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% nonfat dry milk for 1 h at room temperature and incubated with primary antibody in the blocking solution at 4°C overnight. The membrane was washed with TBS-T and incubated with the secondary antibody in the blocking solution for 30 min at room temperature and washed. Bound antibody was detected using Super Signal® West Pico chemiluminescent substrate (Pierce, Rockford, IL, U.S.A.) and FluorChem™ 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA, U.S.A.). Super Signal® West Dura and Femto (Pierce) were used for the detection of PKC isoenzymes.

Electrophoretic mobility shift assay

EMSA was performed as described previously (Lahti *et al*., 2002). Briefly, transcription factor consensus oligonucleotides for NF-κB (Promega, Madison, WI, U.S.A.) were 5′-^{32}P-end-labeled with DNA 5′-End Labeling Kit (Roche Diagnostics, Indianapolis, IN, U.S.A.). For binding reactions, 5 μg of nuclear extract was incubated in 20 μl of total reaction volume containing 0.1 mg ml^{-1} (poly)dI–dC, 1 mM dithiothreitol, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 200 mM KCl and 10% glycerol for 20 min in room temperature. ^{32}P-labeled oligonucleotide probe (0.2 ng) was added and the reaction mixture was incubated for 10 min. Protein–DNA complexes were separated from DNA probe by electrophoresis on a native 4% polyacrylamide gel. The gel was dried and autoradiographed using intensifying screen at −70°C. The quantitation of densities of specific bands was carried out using FluorChem™ software version 3.1.

RNA extraction and quantitative RT–PCR

Cell homogenization, RNA extraction, reverse transcription of RNA to cDNA and PCR reactions were performed as described previously (Lahti *et al*., 2003), with the exception that in the reverse transcription reaction, the amount of total RNA reverse transcribed was 100 ng and cDNA used in PCR corresponded to approximately 2.5 ng of total RNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control gene.

The relative mRNA levels were quantified and compared using the relative standard curve method as described in Applied Biosystems User Bulletin number 2. Total RNA was isolated from LPS-stimulated J774 macrophages and reverse transcribed. Standard curves for GAPDH and iNOS were created using dilution series of cDNA corresponding to approximately 1 pg to 10 ng of total RNA in PCR. The threshold cycle values obtained were plotted against dilution factor to create a standard curve. Relative mRNA levels in test samples were then calculated using the standard curve. The relative amount of gene transcript present was calculated and normalized by dividing the calculated value of iNOS by the GAPDH value in each sample.

Statistics

Results are expressed as mean ± standard error of mean (s.e.m.). Statistical significance of the results was calculated by the analysis of variances supported by Dunnett adjusted significance levels. Differences were considered significant at $P<0.05$.

Results

Effects of PKC inhibitors on LPS-induced NO production and iNOS protein expression

Bacterial endotoxin LPS induced iNOS protein expression and NO production in J774 macrophages. To determine whether PKC activation participated in the upregulation of NO production by LPS, we measured NO production in the presence of PKC inhibitors. RO318220, an inhibitor of PKC isoenzymes β, γ and ε (Davis *et al*., 1992; Wilkinson *et al*., 1993), and GÖ6976, a selective inhibitor of cPKC isoenzymes (Martiny-Baron *et al*., 1993), both inhibited LPS-induced NO production in a dose-dependent manner (Figure 1a and b). Exposure to increasing concentration of RO318220 resulted in a 34% (0.3 μM) and 74% (1 μM) inhibition of NO production during 24 h incubation. Exposure to GÖ6976 resulted in 59% (0.3 μM) inhibition of NO production and larger doses (1 and 3 μM) inhibited NO production almost completely (91 and 95%). Since the results with GÖ6976 suggest that the effect of PKC on NO production could be mediated by the cPKC isoenzymes, we studied the effects of LY333531, a selective inhibitor of PKCβ (Jirousek *et al*., 1996), and HBDDE, an inhibitor of PKC isoenzymes α and γ (Kashiwada *et al*., 1994), on LPS-induced NO production. LY333531 inhibited NO production in a dose-dependent manner (Figure 1c), but HBDDE did not have any effect on NO production when used up to 100 μM concentrations (at concentrations higher than 100 μM HBDDE started to be toxic to J774 macrophages).

In further studies, we investigated the effects of PKC inhibitors on iNOS expression by Western blot. Cells cultured in the absence of LPS did not contain detectable amounts of iNOS protein. Exposure to LPS enhanced iNOS protein expression markedly. RO318220 (1–3 μM), GÖ6976 (0.1–1 μM) and LY333531 (2.5–7.5 μM) inhibited LPS-induced iNOS expression in a dose-dependent manner (Figure 2a–c).

PKC isoenzyme expression in J774 macrophages and the effect of PMA on PKC isoenzyme translocation

Western blot with antibodies specific for cPKC isoenzymes (α, βI, βII and γ) were carried out. Resting J774 cells expressed three cPKC isoenzymes α, βI and βII, but PKCγ was not found (Figure 3). In the further studies, cells were treated with a PKC activator PMA (100 nM), and after 10 min incubation, all three isoenzymes were activated as measured by isoenzyme translocation from the cytosol to the membrane (Figure 3). In

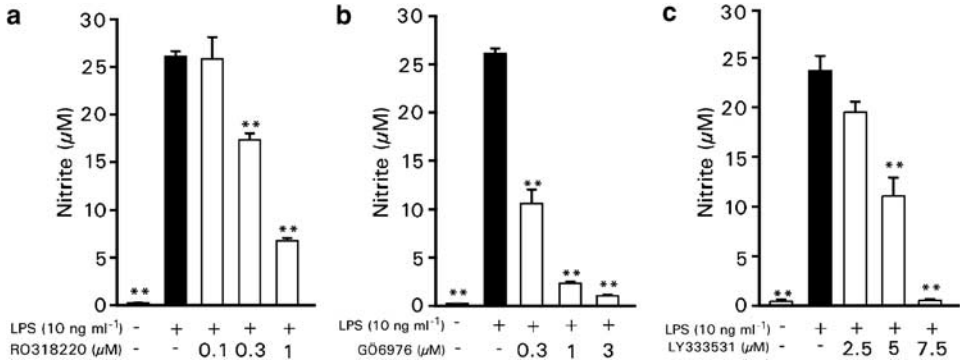

Figure 1 Effects of PKC inhibitors on LPS-induced NO production in J774 cells. J774 cells were stimulated by LPS (10 ng ml^{-1}) and treated with increasing concentrations of RO318220 (a), GÖ6976 (b) or LY333531 (c). After 24 h incubation, nitrite concentrations in the culture medium were measured as a marker of NO production. Values are mean ± s.e.m. ($n = 6$). $^{**}P < 0.01$ as compared with cells treated with LPS only.

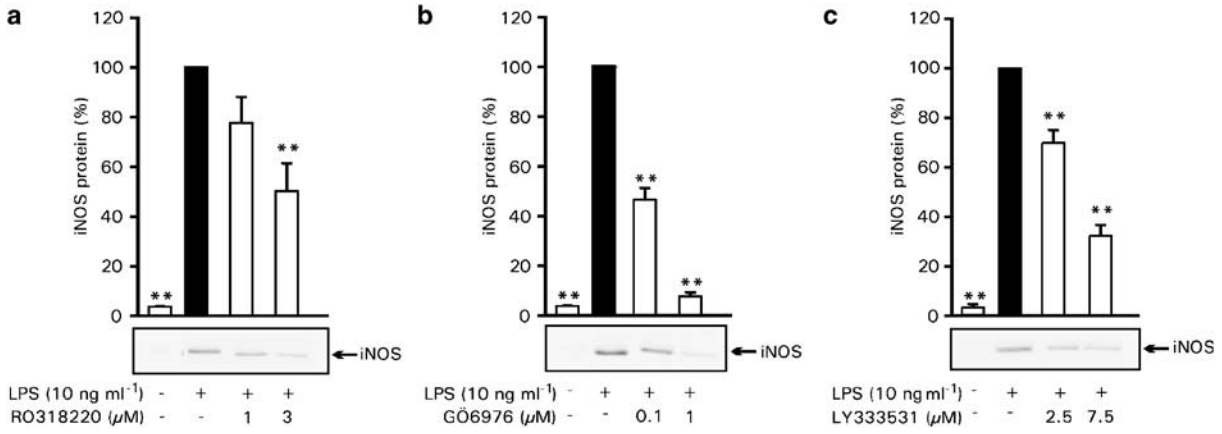

Figure 2 Effects of PKC inhibitors on LPS-induced iNOS protein expression in J774 cells. J774 cells were stimulated by LPS (10 ng ml^{-1}) and treated with increasing concentrations of RO318220 (a), GÖ6976 (b) or LY333531 (c). After 24 h, incubations were terminated and immunoblots were run using antibody against iNOS. Chemiluminescent signal was quantified as described under the Methods section. Values are mean ± s.e.m. ($n = 3$). $^{**}P < 0.01$ as compared with cells treated with LPS only.

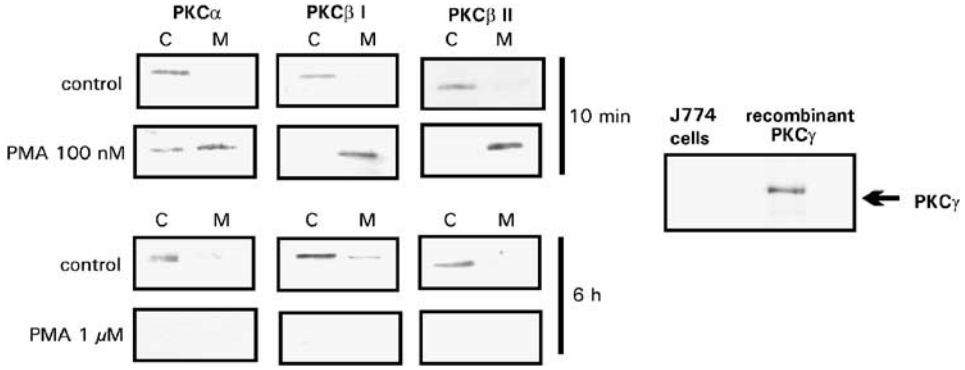

Figure 3 cPKC expression in J774 macrophages and the effects of PMA on PKC isoenzyme translocation. J774 cells were treated with 100 nM PMA or 1 μM PMA as indicated for 10 min or 6 h, respectively. Subsequent to preparation of cell lysates, the expression of individual PKC isoenzymes was assessed by immunoblotting with isoenzyme specific antibodies as outlined in the Methods section. Each experiment is a representative of three others with similar results. C = cytosolic fraction; M = membrane fraction. The expression of PKCγ in resting J774 macrophages was tested by Western blotting using recombinant human PKCγ as a positive control.

addition, incubation with a high concentration of PMA (1 μM) for 6 h resulted in the downregulation of all three PKC isoenzymes (Figure 3). Prolonged exposure to higher concentrations of phorbol esters, such as PMA, is known to cause almost complete downregulation of cPKCs and nPKCs, presumably as a result of proteolysis and it can be used as another means to downregulate PKC activity (Huang *et al*., 1989; Liu & Heckman, 1998).

Effects of phorbol esters on LPS-induced NO production and iNOS protein expression

To further determine the participation of PKC in LPS-induced NO production and iNOS expression, we measured the effects of PMA on NO production and iNOS protein expression. When PMA was used at concentrations (100 nM) that activate PKC (Figure 3), it enhanced LPS-induced NO production and iNOS protein expression as shown in Figure 4a and b. Another phorbol ester, PDD, also enhanced iNOS protein expression, when it was used at 100 nM concentration (Figure 4b).

When the cells were pretreated with 1 μM PMA for 6 h before LPS addition (which was shown to downregulate PKC expression, see Figure 3), both LPS-induced NO production and iNOS protein expression were inhibited similarly to the effects of PKC inhibitors (Figure 5a and b). In addition, 6 h pretreatment with PDD (1 μM) had a similar suppressive effect on iNOS expression as 1 μM PMA (Figure 5b). These results

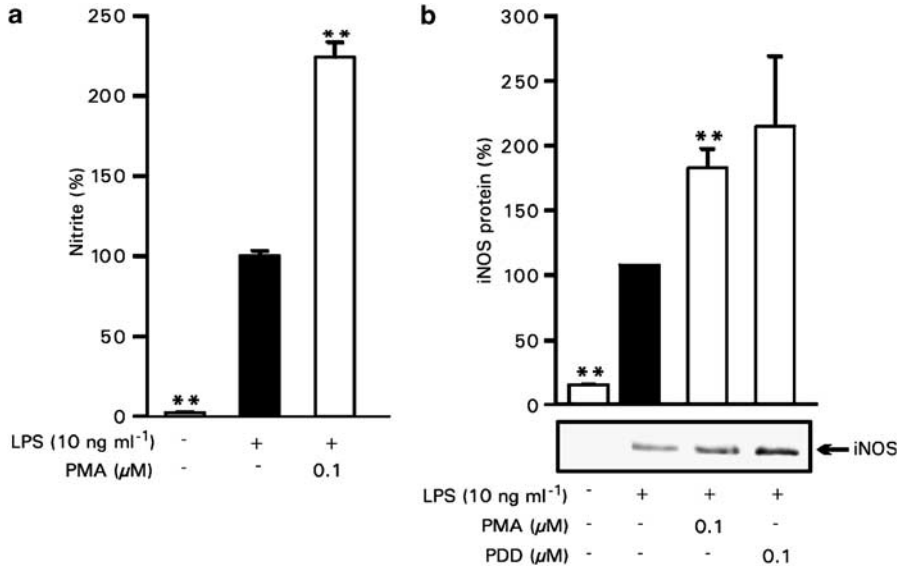

Figure 4 Activation of PKC by phorbol esters induces iNOS protein expression and NO production in J774 cells. (a) J774 cells were stimulated by LPS (10 ng ml^{-1}) and treated with PMA (100 nM) or vehicle (DMSO). After 24 h incubation, nitrite concentrations in the culture medium were measured as a marker of NO production. Values are mean ± s.e.m. ($n = 6$). (b) J774 cells were stimulated by LPS (10 ng ml^{-1}) and treated with PMA (100 nM), PDD (100 nM) or vehicle. After 24 h, incubations were terminated and immunoblots were run using antibody against iNOS. Chemiluminescent signal was quantified as described under the Methods section. Values are mean ± s.e.m. ($n = 3$). $^{**}P < 0.01$ as compared with cells treated with LPS.

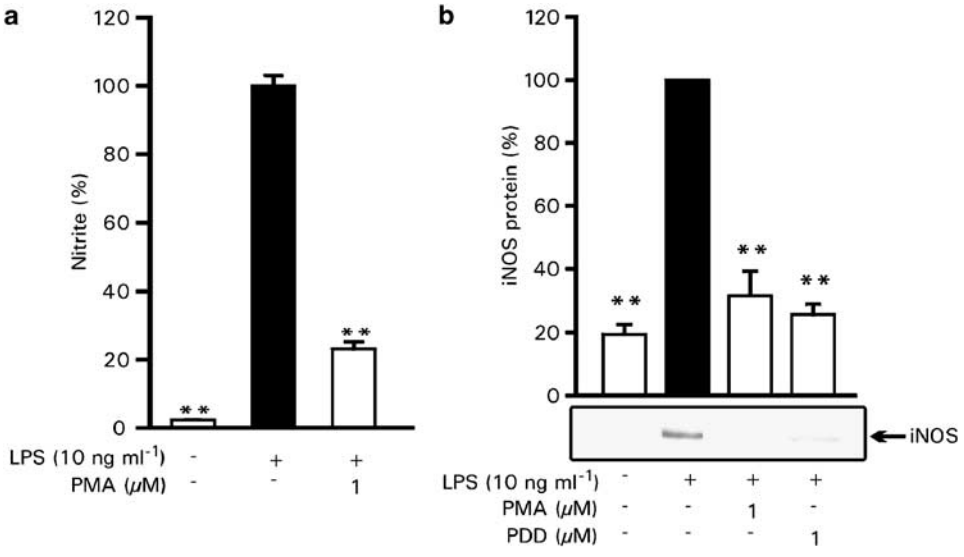

Figure 5 PKC downregulation by 6 h pretreatment with phorbol esters inhibits iNOS protein expression and NO production in J774 cells. (a) J774 cells were pretreated with PMA (1 μM) or vehicle for 6 h before stimulation by LPS (10 ng ml^{-1}). After 24 h incubation, nitrite concentrations in the culture medium were measured as a marker of NO production. Values are mean ± s.e.m. ($n = 6$). (b) J774 cells were pretreated with PMA (1 μM), PDD (1 μM) or vehicle for 6 h before stimulation by LPS (10 ng ml^{-1}). After 24 h, incubations were terminated and immunoblots were run using antibody against iNOS. Chemiluminescent signal was quantified as described under the Methods section. Values are mean ± s.e.m. ($n = 3$). $^{**}P < 0.01$ as compared with cells treated with LPS.

further suggest that PKC is involved in the signalling mechanisms mediating LPS-induced iNOS expression and NO production.

Effects of PKC inhibitors on iNOS mRNA expression

We used RT–PCR to investigate the effects of RO318220 and GÖ6976 on LPS-induced iNOS mRNA expression. In J774 macrophages, LPS-induced transient iNOS mRNA expression which had a 2 h lag phase and peaked at 6 h after the addition of LPS. We chose our time points 2 h before and after the 6 h peak to investigate the effects of PKC inhibitors on iNOS mRNA expression. At all measured time points, 4, 6 and 8 h after LPS induction, the iNOS mRNA levels were reduced in both RO318220- and GÖ6976-treated cells (Figure 6a). To determine whether PKC inhibitors reduce the half-life of iNOS mRNA, the cells were treated with LPS and the tested drugs, and after 6 h, transcription inhibitor actinomycin D ($0.1\,\mu g\,ml^{-1}$) was added into the culture. Cells were then further incubated for 0, 2, 4, 6 or 8 h before total RNA was extracted. As shown in Figure 6b, neither of the PKC inhibitors seemed to have any effect on iNOS mRNA half-life, nor did 100 nM PMA or pretreatment with 1 μM PMA (Figure 6c). These results suggest that the effect of cPKC isoenzymes on LPS-induced iNOS protein expression is mediated at the level of iNOS induction rather than at the level of post-transcriptional events.

Effects of PKC inhibitors on transcription factors NF-κB and STAT1

To evaluate whether the effect of PKC inhibitors on iNOS mRNA expression levels could be a consequence of their effects on transcription factors, we measured the effects of RO318220 and GÖ6976 on the activation of NF-κB and STAT1, which are essential transcription factors for LPS-induced iNOS expression. The activation of NF-κB was measured by EMSA. RO318220 or GÖ6976 had no effect on NF-κB activation or binding activity (Figure 7). In contrast, when we investigated the effects of PKC inhibitors on STAT1 activation, as measured by the translocation of STAT1α from the cytosol to the nuclei by Western blot, both RO318220 and GÖ6976 inhibited STAT1α translocation (Figure 8a). In addition, the PKCβ-selective inhibitor LY333531 (5 μM), as well as pretreatment for 6 h with 1 μM PMA inhibited STAT1α translocation to the nuclei (Figure 8b and c). These data suggest that the effects of cPKC isoenzymes on LPS-induced iNOS protein expression are NF-κB-independent, but may well be mediated through the activation of transcription factor STAT1.

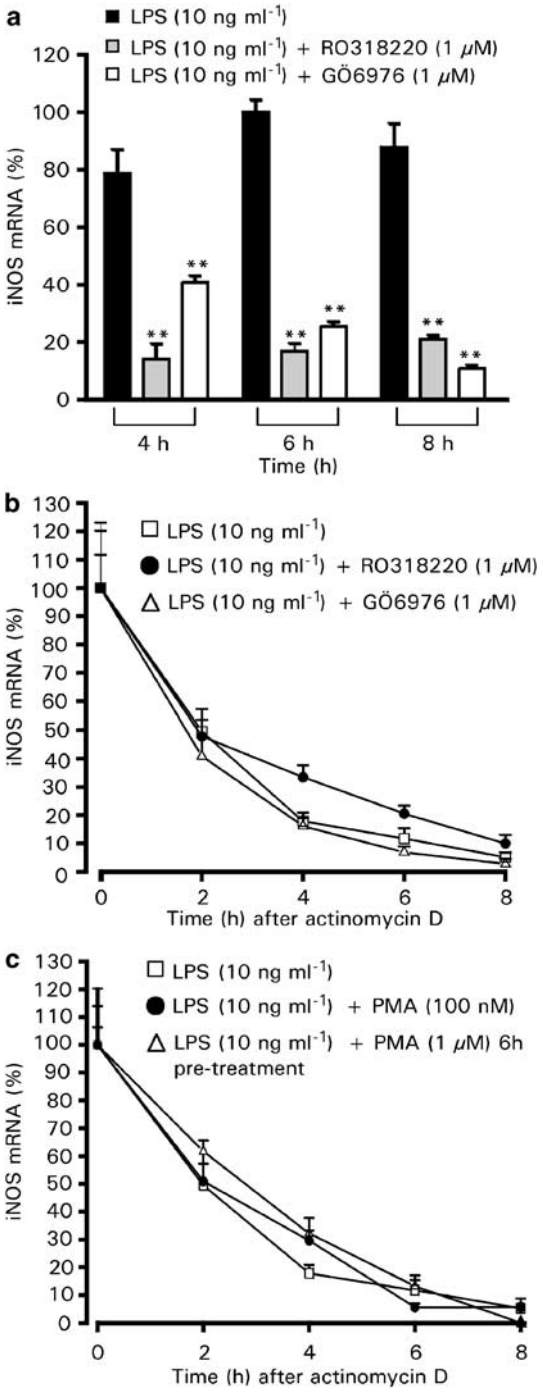

Figure 6 Effects of PKC inhibitors on iNOS mRNA expression and stability in J774 cells. (a) J774 cells were stimulated by LPS ($10\,ng\,ml^{-1}$) and treated with RO318220 (1 μM) or GÖ6976 (1 μM) for 4, 6 or 8 h. At indicated time points, the incubations were terminated and extracted total RNA was subjected to RT–PCR. iNOS mRNA levels were normalized against GAPDH mRNA. (b) Effect of PKC inhibitors on iNOS mRNA degradation. Cells were stimulated by LPS ($10\,ng\,ml^{-1}$) and treated with RO318220 (1 μM) or GÖ6976 (1 μM) for 6 h before the addition of actinomycin D ($0.1\,\mu g\,ml^{-1}$) to inhibit transcription. Incubations were terminated at indicated time points after actinomycin D and extracted total RNA was subjected to RT–PCR. iNOS mRNA levels were normalized against GAPDH mRNA. (c) Effect of PMA on iNOS mRNA degradation. Cells were treated with LPS ($10\,ng\,ml^{-1}$), with LPS ($10\,ng\,ml^{-1}$) and PMA (100 nM) to activate PKC or pretreated with PMA (1 μM) for 6 h to downregulate PKC before the addition of LPS ($10\,ng\,ml^{-1}$). After 6 h incubation with LPS, actinomycin D ($0.1\,\mu g\,ml^{-1}$) was added to inhibit transcription. Incubations were terminated at indicated time points after actinomycin D and extracted total RNA was subjected to RT–PCR. iNOS mRNA levels were normalized against GAPDH mRNA. Values are mean ± s.e.m. ($n=3$). $^{**}P<0.01$ as compared with cells treated with LPS only.

Effects of JAK-2 inhibitor AG-490 on LPS-induced NO production and STAT1 activation

To further investigate the role of STAT1 on LPS-induced NO production, we used JAK-2 inhibitor AG-490. JAK-2 (Janus kinase-2) is an upstream kinase of STAT1 and inhibition of JAK-2 leads to the inhibition of STAT1 (Shuai & Liu, 2003). AG-490 inhibited LPS-induced NO production in a dose-dependent manner (Figure 9a). Same concentrations that downregulated LPS-induced NO production also inhibited LPS-induced STAT1 activation, as measured by the translocation of STAT1α from the cytosol to the nuclei by Western blot (Figure 9b). These results further suggest that the effects of cPKC isoenzymes on iNOS expression and NO production could be mediated through the activation of STAT1.

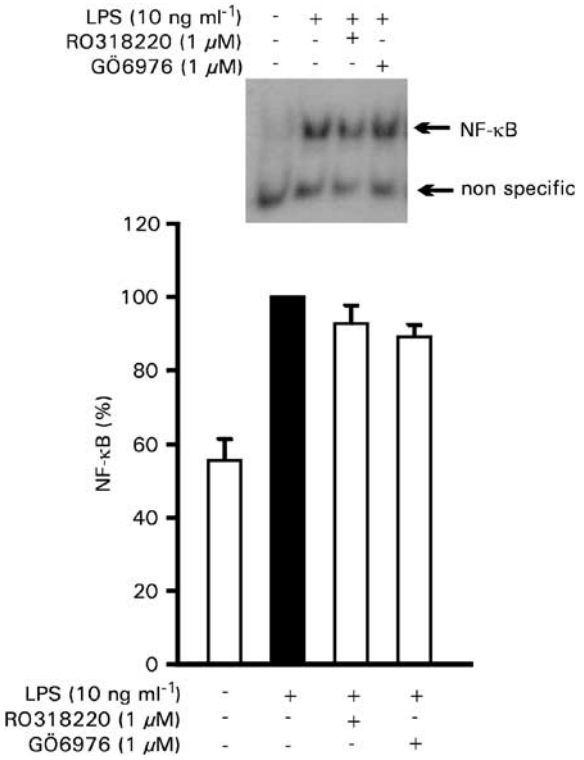

Figure 7 Effect of PKC inhibitors on NF-κB activity. J774 cells were stimulated by LPS (10 ng ml^{-1}) and treated with RO318220 (1 μM) or GÖ6976 (1 μM) for 30 min before the preparation of nuclear extracts. NF-κB DNA binding activity was analyzed by EMSA. Densities of specific bands were quantified as described under the Methods section. Values are mean ± s.e.m. ($n=3$).

Discussion

In the present study, we show that inhibition of classical isoenzymes, especially PKCβ, inhibits LPS-induced iNOS expression and NO production in activated J774 macrophages, and that this effect is possibly mediated through the inhibition of transcription factor STAT1.

Distribution of PKC isoenzymes is cell type- and tissue-specific. PKCα, βI, βII, δ, ε and ζ seem to be ubiquitous isoenzymes, and are found in most tissues (Liu & Heckman, 1998). Classical isoenzyme PKCγ is largely restricted to the central nervous system and spinal cord (Liu & Heckman, 1998; Way *et al*., 2000). In the present study, we focused on cPKCs and found that PKCα, PKCβI and PKCβII are expressed in macrophage cultures used. PKC regulates various inflammatory functions in an isoenzyme-specific manner (Tan & Parker, 2003). The regulation of cell signalling events by single PKC isoenzymes have also been shown to differ between cell types (Paul *et al*., 1997).

In the present study, four PKC inhibitors with different PKC isoenzyme profiles were used to study the role of PKC and its classical isoenzymes in LPS-induced iNOS protein expression and NO production in macrophages. PKC inhibitors RO318220, GÖ6976 and LY333531 inhibited LPS-induced iNOS expression and NO production in a dose-dependent manner. RO318220 is a PKC inhibitor, which inhibits isoenzymes β, γ and ε (Davis *et al*., 1992; Wilkinson

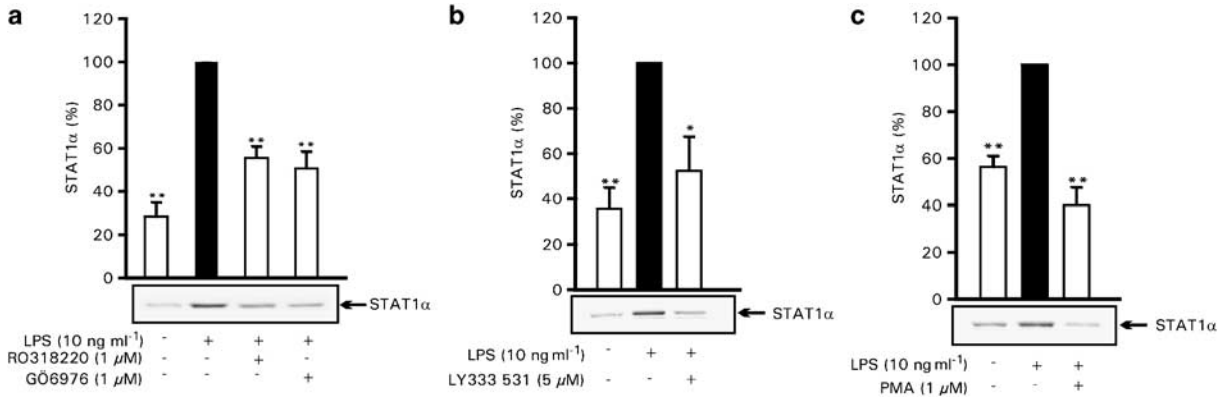

Figure 8 The effect of PKC inhibitors and PMA pretreatment on STAT1α translocation. J774 cells were stimulated by LPS (10 ng ml^{-1}) and treated with RO318220 (1 μM), GÖ6976 (1 μM) (a) or LY333531 (5 μM) (b) for 6 h before the preparation of nuclear extracts. STAT1α translocation to the nuclei was determined by Western blotting using specific antibody against STAT1α. (c) Cells were pretreated with PMA (1 μM) or vehicle (DMSO) for 6 h before stimulation with LPS (10 ng ml^{-1}). Cells were further incubated for 6 h before the nuclear extracts were prepared. STAT1α translocation to the nuclei was determined by Western blotting using specific antibody against STAT1α. Chemiluminescent signal was quantified as described under the Methods section. Values are mean ± s.e.m. ($n=3$). *$P<0.05$, **$P<0.01$ as compared with cells treated with LPS only.

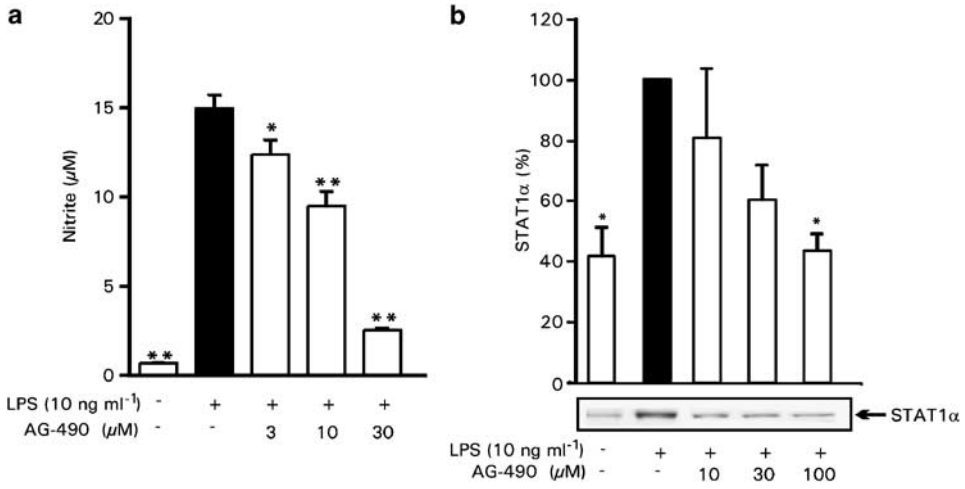

Figure 9 Effect of JAK-2 inhibitor AG-490 on LPS-induced NO production in J774 cells. J774 cells were stimulated by LPS (10 ng ml^{-1}) and treated with increasing concentrations of AG-490 (a). After 24 h incubation, nitrite concentrations in the culture medium were measured as a marker of NO production. Values are mean ± s.e.m. ($n=6$). (b) The effect of JAK-2 inhibitor AG-490 on STAT1α nuclear translocation. J774 cells were stimulated by LPS (10 ng ml^{-1}) and treated with increasing concentrations of AG-490 for 4 h before the preparation of nuclear extracts. STAT1α translocation to the nucleus was determined by Western blotting using specific antibody against STAT1α. Chemiluminescent signal was quantified as described under the Methods section. Values are mean ± s.e.m. ($n=3$). *$P<0.05$, **$P<0.01$ as compared with cells treated with LPS only.

et al., 1993), GÖ6976 is more selective to cPKC isoenzymes (Martiny-Baron *et al*., 1993) and LY333531 is a selective inhibitor of PKCβ (Jirousek *et al*., 1996). HBDDE, which is a relatively selective inhibitor of PKCα and PKCγ (Kashiwada *et al*., 1994), did not have any effect on LPS-induced NO production. These results suggest that PKC, probably its isoenzymes PKCβI and PKCβII, mediate the LPS-induced upregulation of iNOS expression and NO production in macrophages.

The results may be complicated by the fact that RO318220 and GÖ6976 have been reported to inhibit some other kinases in addition to PKC (Davies *et al*., 2000). Therefore, we also studied the effects of phorbol esters PMA and PDD on LPS-induced iNOS expression and NO production. Phorbol esters are known to activate cPKC isoenzymes (Castagna *et al*., 1982), whereas a longer pretreatment with a higher concentration of phorbol esters have been shown to result in the downregulation of cPKCs, presumably due to proteolysis (Huang *et al*., 1989; Liu & Heckman, 1998). The bidirectional effect of PMA and PDD on cPKCs was seen also in the present study. PMA and PDD at 100 nM concentration enhanced cPKC activation, while 6 h pretreatment with 1 μM concentration of PMA or PDD suppressed cPKC expression. When used at cPKC-activating concentrations, PMA and PDD enhanced iNOS expression and NO production. In contrast, cPKC downregulation due to 6 h pretreatment with PMA or PDD (1 μM) resulted in the suppression of iNOS expression and NO production, similarly as inhibition of cPKCs by pharmacological means. These results further support the role of cPKC isoenzymes in the regulation of iNOS expression and NO production in macrophages.

To determine the mechanisms by which the regulation by PKC is mediated, we studied the effects of PKC inhibitors RO318220 and GÖ6976, and PMA on LPS-induced iNOS mRNA expression and mRNA stability. Our results show that inhibition of cPKCs does not effect the stability of LPS-induced iNOS mRNA. The effect of PKC isoenzymes is rather at the level of iNOS transcription, since PKC inhibitors decreased the expression of iNOS mRNA already at the early time points after addition of LPS.

NF-κB and STAT1 appear to be important transcription factors for the enhanced iNOS gene expression in macrophages exposed to LPS (Lowenstein *et al*., 1993; Chartrain *et al*., 1994; Xie *et al*., 1994; Gao *et al*., 1998; Jacobs & Ignarro, 2001). In the present study, all three PKC inhibitors used (RO318220, GÖ6976 and LY333531) inhibited STAT1 activation as measured by translocation of the transcription factor from the cytosol to nuclei as did pretreatment with 1 μM PMA. In contrast, none of the treatments did inhibit NF-κB activation as measured by EMSA. These results suggest that the regulation of LPS-induced iNOS protein expression by PKC is NF-κB-independent and is most likely mediated through the activation of transcription factor STAT1.

RO318220 and GÖ6976 have earlier been reported to inhibit LPS and IFN-γ-induced NO production in macrophages (Paul *et al*., 1997; Chen, B.C. *et al*., 1998; Chen *et al*., 1998a). In addition, PKCδ and PKCη have been suggested to regulate NO production and iNOS expression in activated macrophages and some other cell types (Chen *et al*., 1998a, b; Carpenter *et al*., 2001; Banan *et al*., 2003; Pham *et al*., 2003). The present study extends the earlier data by providing a cellular mechanism for the inhibitory effects of RO318220 and GÖ6976 on LPS-induced iNOS expression and NO production in macrophages. Our results show that inhibition of cPKC isoenzymes results in the suppression of STAT1 activation, which may well explain the inhibitory effect on iNOS expression and NO production. In addition, we were able to show that LY333531, a selective inhibitor of PKCβ, also suppressed STAT1 activation and iNOS expression, supporting the role of PKCβ in the regulation of iNOS expression in activated macrophages.

In conclusion, the present results show that inhibition of cPKC isoenzymes, especially PKCβ, inhibits the LPS-induced activation of transcription factor STAT1, iNOS expression

and NO production in macrophages. The results suggest that inhibition of cPKC isoenzymes provides a way to prevent iNOS protein expression and NO production in inflammation, offering a novel target for the development of anti-inflammatory drugs.

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