The isoform identity of activated protein kinase C (PKC) and its regulation were investigated in bacterial lipopolysaccharide (LPS)-treated human monocytes. Resolution of detergent-soluble lysates prepared from LPS-treated, peripheral blood monocytes using Mono Q anion-exchange chromatography revealed two principal peaks of myelin basic protein kinase activity. Immunoblotting and immunoprecipitation with isoform-specific anti-PKC antibodies showed that the major and latest eluting peak is accounted for by PKC-ζ. In addition to primary monocytes, activation of PKC-ζ in response to LPS was also observed in the human promonocytic cell lines, U937 and THP-1. Consistent with its identity as PKC-ζ, the kininase did not depend upon the presence of lipids, Ca²⁺, or diacylglycerol for activity. In addition, the kinase phosphorylates peptide ε and myelin basic protein with equal efficiency but phosphorylates Kemptide and protamine sulfate poorly. Translocation of PKC-ζ from the cytosolic to the particulate membrane fraction upon exposure of monocytes to LPS provided further evidence for activation of the kinase.

Preincubation of monocytes with the phosphatidylidyinositol 3-kinase (PI 3-kinase) inhibitors, wortmannin or LY294002, abrogated LPS-induced activation of PKC-ζ. Furthermore, activation of PKC-ζ failed to occur in U937 cells transfected with a dominant negative mutant of the p85 subunit of PI 3-kinase. PKC-ζ activity was also observed to be enhanced in vitro by the addition of phosphatidylinositol 3,4,5P3. These findings are consistent with a model in which PKC-ζ is activated downstream of PI 3-kinase in monocytes in response to LPS.

Clear phagocytes. Monocyte activation in response to LPS results in the production of an array of cytokines such as tumor necrosis factor-α, interleukin-1, and interleukin-6, in addition to other inflammatory mediators. In the extreme, the inflammatory response to LPS is an important contributor to septic shock which may occur during infection with Gram-negative bacilli (1, 2).

Although there is an extensive body of knowledge about functional changes in monocytes induced by LPS, there is relatively less known about the signaling pathways used by LPS to bring about these changes. Recently, it has become clear that monocyte responses to LPS involve specific cell surface receptors leading to the activation of pathways containing both tyrosine and serine/threonine protein kinases (3–6). The initial events in at least one dominant LPS signaling pathway are dependent upon the glycosphatidylinositol-linked membrane molecule, CD14 (7). Binding of the complex of LPS and LPS-binding protein to CD14 results in the activation of multiple src family protein tyrosine kinases, and this appears to involve the physical association of p53/p56lyn with the receptor complex (6). It has also been shown that LPS-mediated, CD14-dependent activation of p53/p56lyn leads to its association with an activated form of the lipid kinase, PI 3-kinase (8).

Activation of PI 3-kinase results in the production of PIP₃, which is known to be an activator of the PKC isoforms, ε, and δ (9, 10). This is of interest in the context of LPS signaling since evidence has recently been provided to show that a PKC activity is increased in LPS-treated monocytes (3, 4). Notably, this activity appears to be related to one or more PKC isoforms, the activation of which is sustained in the absence of phosphatidylserine, Ca²⁺, and diacylglycerol (3). This latter finding suggests the possibility that LPS may activate one of the αPKC isoforms, either PKC-ζ, PKC-ε, or both. This subfamily of PKC isoforms differs from cPKC (α, βI, βII, γ) and nPKC (δ, ε, η, θ) subfamily members in that αPKC isoforms are neither receptors for phorbol esters nor are regulated by Ca²⁺ or diacylglycerols (11–14). Rather, αPKCs exhibit activator-independent activity which is increased upon exposure to novel lipids such as PIP₃ and ceramides (9, 15, 16).

In light of the findings indicating that incubation of monocytes with LPS leads to the activation of both PI 3-kinase and a PKC with unusual properties, the objectives of the present study were to identify this PKC isoform and examine its regulation. The results presented show that PKC-ζ is rapidly activated in LPS-treated human monocytes, and this occurs downstream of activated PI 3-kinase. These findings are consistent with a model in which LPS activates p53/p56lyn leading to increased PI 3-kinase activity and activation of PKC-ζ through the production of PIP₃.

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Bacterial lipopolysaccharide (LPS)1 is one of the most potent agonists known that contributes to the activation of mononu-
Reagents—Anti-PKC-ζ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Upstate Biotechnology (Lake Placid, NY), and Life Technologies, Inc. (Burlington, Ontario, Canada). These antibodies gave similar results and were used interchangeably. Histone III-S, bovine myelin basic protein from bovine brain (MBP), protamine sulfate, protein kinase inhibitor (rabbit sequence), phosphol 12-myristate 13-acetate (PMMA), Q columns and protein G-Sepharose were from Pharmacia Biotech Inc. Horseradish peroxidase-conjugated goat anti-rabbit antibodies, protein A-agarose, and electrophoresis reagents and supplies were purchased from Bio-Rad. U937 and THP-1 cell lines were from the American Type Culture Collection (Rockville, MD). LipofectAMINE was from Life Technologies Inc. Iscove’s methyl cellulose, RPMI 1640, Hank’s balanced salt solution, and penicillin/streptomycin were from Stem Cell Technologies (Vancouver, British Columbia). [γ-32P]ATP, enhanced chemiluminescence reagents, and enhanced chemiluminescence film were from Amersham Int. (Oakville, Ontario, Canada). Lipopolysaccharide (Escherichia coli O127:B8) was from Difco. Human AB⁺ serum was provided by The Canadian Red Cross (Vancouver, British Columbia). Unless specified otherwise, all other reagents were of the highest quality available.

Isolation of Monocytes, Cell Treatment, and Processing—Fractions of peripheral blood enriched in white blood cells were obtained from the Cell Separator Unit (Vancouver Hospital and Health Sciences Center). Monocytes were enriched (85–95% pure) by adherence as described previously (3). Monolayers of adherent cells in RPMI 1640 were treated with LPS (solubilized in RPMI + 10% AB+ human serum, final serum concentration, 0.1%) rinsed with ice-cold phosphate-buffered saline, excited with 95% nitrogen, and stored at −70 °C prior to analysis. Cell lysates for column chromatography were prepared by lysing cells on ice (20 min) in fast performance liquid chromatography (FPLC) extraction buffer (1% Nonidet P-40, 12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophosphate, 2 mM EGTA, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM microcystin, 10 μM aprotinin, 10 μM leupeptin, and 10 μM pepstatin). Lysates were centrifuged at 16,000 × g to remove insoluble material and were filtered through a 0.2-μm filter. Protein concentrations were determined with Bio-Rad protein assay using bovine serum albumin as standard.

Cells lysates for analysis of PI 3-kinase activity were prepared as described previously (8) in 20 mM Tris, pH 8.0, 1% Triton X-100, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM Na3VO4, 5 mM NaF, 100 mM microcystin, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml aprotinin.

Monocyte cell lines were maintained in complete RPMI supplemented with 10% heat-inactivated fetal calf serum (FCS). Twelve to 15 h prior to incubation with LPS, cells were rendered quiescent in RPMI without FCS at a concentration of 5 × 10⁴ cells/ml. Following stimulation with LPS, cells were lysed immediately, and the detergent-soluble material was frozen at −70 °C until further analysis.

Phosphatidylinositol 3-Kinase Assay—Cells were preincubated in FPLC column pre-equilibrated in buffer A (12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophosphate, 2 mM EGTA, and 0.5 mM Na3VO4). Proteins were resolved with a 20-ml linear gradient of 0–0.5 M NaCl in buffer A at a flow rate of 0.5 ml/min. Fractions of 0.25 ml were collected, and aliquots were assayed for protein kinase activity or immunoreactivity as described below.

PKC-ζ Immune Complex Kinase Assay—Quiescent THP-1 cells were either untreated or were incubated with LPS for the times indicated. When the effects of PI 3-kinase inhibitors were being studied, cells were incubated with either 32 μM LY294002 or 100 μM wortmannin for 20 min at 37 °C in the absence of LPS. Following treatment, cells were immediately lysed at 4 °C for 30 min in lysis buffer (1% Triton X-100, 50 mM HEPES, pH 7.4, 150 mM NaCl with protease and phosphatase inhibitors used at the same concentrations as described above). Lysates were precleared with protein A-agarose and PKC-ζ was immunoprecipitated with rabbit polyclonal anti-PKC-ζ (5 μg per sample, Upstate Biotechnology). Kinase activity was measured in the immunoprecipitates using MBP as substrate as described previously (18). Quantitation of kinase activity was done by scintillation counting of the band corresponding to MBP.

LPS-induced Activation of PKC-ζ

Protein Kinase Assays—Aliquots (5 μl) of column fractions were assayed for phosphotransferase activity using various substrates as described previously (3). In brief, assays were performed in a final volume of 25 μl of kinase assay buffer containing 12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophosphate, 2 mM EGTA, 0.5 mM Na3VO4, 2 mM dithiothreitol, 5 mM MgCl2, 4 μM cAMP-dependent protein kinase inhibitor peptide, [γ-32P]ATP (40 μM), and various substrates at the concentrations indicated below. Reactions were allowed to proceed for 10 min at 30 °C at room temperature and were terminated by spotting 23 μl of the mixture on phosphocellulose filter squares. Filters were washed six times in 0.85% (v/v) O-phosphoric acid, and bound radioactivity was determined by scintillation counting.

Immunoadsorption of Kinase Activity—Cell lysates (1 mg), prepared from LPS-treated U937 cells, were incubated with either 7.5 μg of rabbit anti-protein kinase C-ζ antibody or 7.5 μg of monoclonal anti-PKC-ζ antibody for 2–3 h at 4 °C. Immune complexes were then incubated for 1 h with either protein A-agarose or protein G-Sepharose. Solid phase complexes were washed twice in 2 ml with FPLC extraction buffer by centrifugation at 14,000 × g for 1 min, and the supernatant fraction was subjected to a second immunoprecipitation with the same antibodies. Immunoadsorbed supernatants were fractionated by Mono Q chromatography as described above, and fractions were analyzed by immunoblots and protein kinase assays.

Transfection of U937 Cells—pSRa-based mammalian expression plasmids, containing the entire coding regions of either wild-type bo
Fig. 2. LPS-induced activation of MBP kinase activity in promonocytic cell lines. THP-1 or U937 cells were rendered quiescent in serum-free RPMI for 12–15 h and subsequently stimulated (15 min) with 1 μg/ml LPS or medium as described under "Materials and Methods." A, cells were lysed and subjected to Mono Q chromatography. MBP kinase activity in aliquots (5 μl) of each fraction from each cell line was determined as described in Fig. 1. B, detection of PKC-ζ immunoreactivity in fractions corresponding to the major peak was done as described in the legend to Fig. 1. Results are from one of three similar experiments. C, THP-1 cells were treated with LPS for the indicated times, and PKC-ζ was immunoprecipitated with polyclonal anti-PKC-ζ antibodies (Upstate Biotechnology). In vitro kinase activities in the immunoprecipitates were measured using MBP as substrate as described under "Materials and Methods."

RESULTS

Lipopolysaccharide-induced Activation of Protein Kinase C-ζ in Monocytes—Following incubation of peripheral blood monocytes with LPS, cell lysates were fractionated by Mono Q chromatography. As shown in Fig. 1A, one major peak of MBP kinase activity eluted between 370 and 410 mM NaCl. A second, minor peak was also frequently observed eluting at ~300 mM NaCl. This latter peak had previously been identified as p42/p44 mitogen-activated protein kinases (3). Compared with lysates prepared from untreated cells, the mean increase in activity of the major peak was 2.5 ± 0.7 (mean ± S.E., n = 6) fold. Since previous data had suggested that this activity was a lipid-independent isoform of PKC (3), the possible presence of PKC-ζ was analyzed by immunoblotting fractions with an isoform-specific antibody. Immunoreactivity for PKC-ζ was observed in the fractions corresponding to the peak of kinase activity (Fig. 1B). The antibody recognized a protein of ~80–85 kDa which was sometimes resolved into a doublet or triplet of closely migrating proteins. The specificity of antibody reactivity with this ~80-kDa protein complex was confirmed by pep-
of activity (Fig. 2) specifically abrogated recognition of these bands, whereas nonspecific peptide competition. Thus, as shown in Fig. 1B, excess of the PKC-ζ peptide used as immunogen to raise the antibody specifically abrogated recognition of these bands, whereas nonspecific reactivity with other proteins was not eliminated.

Activation of PKC-ζ in response to LPS was also observed in two human, promonocytic cell lines, THP-1 and U937 (Fig. 2A). The MBP kinase activities exhibited similar elution profiles, and immunoreactivity for PKC-ζ corresponded with the peaks of activity (Fig. 2B). The effect of LPS on in vitro PKC-ζ activity was also examined in a parallel system by immune complex kinase assay. PKC-ζ was immunoprecipitated from lysates of control or LPS-treated THP-1 cells, and kinase activity was measured using MBP as substrate. As shown in Fig. 2C, LPS treatment resulted in a rapid and transient increase in kinase activity that was apparent as early as 5 min, was maximal at 10 min, and was nearly back to baseline by 30 min.

Immunodepletion was used to examine further whether the peak of MBP kinase activity was accounted for by PKC-ζ. Prior to fractionation on Mono Q chromatography, MBP kinase activities were determined in peak fractions (5 μl) using various substrates. MBP, protamine sulfate, histone, and Kemptide were used at 0.2 mg/ml. Peptide e was used at 84 μM and S6 peptide at 100 μM. B, kinase activity was measured in the absence (Cont) or presence of various cofactors (PIP3, 0.1 μM; arachidonic acid (AA), 33 μM; PS, 12.5 μM) using MBP as substrate. C, peak kinase activity was determined in the fractions corresponding to both PKC-ζ and cPKC (fractions 15–16) in the presence or absence (Control) of 50 nm PMA, 100 μM free Ca2+, and 25 μM PS. Results are expressed as percent of activity measured in the absence of any factors which was taken as 100%. The values shown are the mean ± S.E. of 3–4 independent experiments. *p = 0.04 (control versus PIP3); **p = 0.04 (control versus PMA), both by Student’s t test.
Biochemical Characterization of PKC-\(\zeta\) — Since PKC-\(\zeta\) is known to behave differently from other PKC family members, experiments were done to biochemically characterize the putative PKC-\(\zeta\). Aliquots of Mono Q fractions containing PKC-\(\zeta\) were analyzed for activity using multiple substrates as shown in Fig. 5A. Of the different substrates tested, the kinase phosphorylated MBP (0.2 mg/ml), peptide e (84 \(\mu\)M), and S6 peptide (0.1 mM) with similar efficiency. In comparison, activities toward Kemptide (0.2 mg/ml), histone (0.2 mg/ml), and protamine sulfate (0.2 mg/ml) were lower. This profile of substrate preferences is consistent with previous reports for PKC-\(\zeta\) (10, 13).

Cofactor requirements for PKC-\(\zeta\) have also been found to be different than those of other PKC isoforms (10, 15). Fig. 5B shows the activity of the kinase toward MBP in the presence or absence of various PKC activators and cofactors. PIP\(_3\), an activator of PKC-\(\zeta\), enhanced the activity of the kinase in Mono Q fractions prepared from control cells (Fig. 5B) but not in those prepared from LPS-treated cells (data not shown). Unlike PIP\(_3\), neither arachidonic acid (alone or with diacylglycerol) nor phosphatidylserine significantly enhanced the activity of the kinase when compared with its activity detected in the absence of added lipids (Fig. 5B). In contrast to these findings with PKC-\(\zeta\), when partially purified cPKC was tested in the presence of phosphatidylserine and diacylglycerol, arachidonic acid (50 \(\mu\)M) was observed to further enhance its activity (data not shown). PMA, a known activator of several PKC isoforms, was also tested in the presence of PS and Ca\(^{2+}\). The addition of PS, Ca\(^{2+}\), and PMA together resulted in an approximate 35% increase in activity when compared with activity in the absence of cofactors (Fig. 5C). In contrast, fractions corresponding to cPKC (i.e. 15–20) showed a robust activation in response to the combination of PMA, PS, and Ca\(^{2+}\).

### Activation of PKC-\(\zeta\) Is Phosphatidylinositol 3-Kinase-dependent

Recent evidence has suggested a role for PI 3-kinase metabolites in a signaling cascade leading to the activation of PKC (9). To examine the potential involvement of PI 3-kinase in LPS-induced activation of PKC-\(\zeta\), cells were incubated with the PI 3-kinase inhibitors, wortmannin or LY294002, prior to the addition of LPS. As shown in Fig. 6, when used at concentrations known to be relatively selective for inhibition of PI 3-kinase, both inhibitors markedly attenuated activation of PKC-\(\zeta\) induced by LPS.

The requirement for PI 3-kinase for activation of PKC-\(\zeta\) was
also examined in cells transfected with a dominant negative mutant of p85 (Δp85). Stable transfection with Δp85 resulted in a significant reduction in both basal and LPS-activated PI 3-kinase activity (Fig. 7, A and B). In contrast, cells transfected with wild-type p85 showed increased PI 3-kinase activity in response to LPS stimulation. To assess the effects of Δp85 on activation of PKC-ζ by LPS, lysates of transfected cells were analyzed using Mono Q chromatography. As shown in Fig. 8, activation of PKC-ζ by LPS was abrogated in cells transfected with Δp85, and cells expressing wild-type p85 showed enhanced PKC-ζ activity in response to LPS. Fig. 8C demonstrates that PKC-ζ was expressed in Δp85 transfected cells and PKC-ζ immunoreactivity correlated with the peak kinase activity.

**DISCUSSION**

Previous studies demonstrated that a PKC with unusual properties is activated in human monocytes following exposure to LPS (3, 4). Important questions arising from these studies are the identity of this PKC isoform and its mechanism of activation. Several considerations suggested the possibility that this LPS-activated isoform may be PKC-ζ. PKC-ζ is a member of a subfamily of atypical PKCs that is not activated by diacylglycerols, and it does not require Ca²⁺ activation. Rather, recent findings indicate that the activity of PKC-ζ is enhanced in vitro by PIP₃. This, together with the previous observations that LPS induces the accumulation of PIP₃ in monocytes (8), suggested the hypothesis that LPS may activate PKC-ζ in a PI 3-kinase-dependent manner. Multiple lines of evidence presented in this paper support this argument. Analysis of extracts of LPS-treated peripheral blood monocytes, U937 cells, and THP-1 cells showed principal peaks of enhanced MBP kinase activity eluting from Mono Q between 370 and 410 mM NaCl (Figs. 1A and 2A). These peaks of kinase activity were detected in the absence of exogenous lipids and were also independent of Ca²⁺. These characteristics are consistent with PKC-ζ, and immunoblotting of Mono Q fractions indicated the presence of PKC-ζ in these samples (Figs. 1B and 2B). In addition, immunoprecipitation of PKC-ζ directly from lysates of LPS-treated cells showed that the activity of the enzyme was increased when compared with the activity observed in immunoprecipitates obtained from control cells (Fig. 2C). Furthermore, the LPS-enhanced MBP kinase activity could be removed by immunoadsorption with anti-PKC-ζ (Fig. 3). Prior analyses of PKC expression in blood monocytes, U937 cells, and HL-60 cells indicate the presence of PKC-α, -β₁, -β₁l, -β₁l-ζ, and ζ isoforms (13, 21–23). In U937 cells, PKC-α, -β, and -ζ elute from Mono Q at or below 320 mM NaCl, whereas PKC-ζ elutes at ~460 mM NaCl (13). The somewhat earlier elution of PKC-ζ (370–410 mM NaCl) observed in the present study most likely reflects procedural differences related to detergent solubilization and variations in the elution buffers.

Two of the anti-PKC-ζ antibodies (Santa Cruz Biotechnology and Life Technologies, Inc.) used in this study were raised against a peptide (SEFEGFYINPLLWAEESV) corresponding to amino acids 573–592 present in the COOH terminus of the kinase. This exact sequence is not found in any of the other known PKC isoforms (24). The closely related PKC-ι contains a similar COOH-terminal sequence differing at only two amino acids (SEFEGFYINPLLSAEESV) and can be detected with antibodies to the COOH terminus of PKC-ζ. However, it is unlikely that the major immunoreactive band detected in this study is PKC-ι since this isoform is known to migrate at 65 kDa (12), consistently lower than the ~80–85-kDa protein observed in this study. The third antibody (Upstate Biotechnology) used in the present study is reported not to cross-react with either PKC-α, PKC-ι, or PKC-λ (according to information provided by the manufacturer). It has also been reported that antibodies directed against the COOH terminus of PKC-ζ react with a Ca²⁺ and phorbol ester-sensitive PKC isoform (25). However, the kinase that is the subject of this report is only weakly activated by a combination of PMA, Ca²⁺, and PS and has sustained activity in the absence of exogenous lipids. These findings preclude the notion that it may be a member of either the cPKC or nPKC subfamilies.

Membrane translocation of PKC has been used extensively as a measure of its activation (14). The finding that PKC-ζ translocates to the membrane following exposure to LPS provides additional evidence for its activation. Given that PIP₃ increases the activity of PKC-ζ in vitro (Ref. 9 and Fig. 5) and that activation of PKC-ζ in vivo requires activation of PI 3-kinase (Figs. 6 and 8), a possible mechanism for redistribution of PKC-ζ is through direct binding to PIP₃ in the membrane compartment. Although membrane translocation of PKC-ζ induced by PIP₃ in vivo has not been reported, it has been shown that PKC-ζ translocates to the membrane transiently following

**FIG. 8.** Attenuation of LPS-induced activation of PKC-ζ by a dominant negative mutant of the p85 subunit (Δp85) of PI 3-kinase. Quiescent transfected U937 cells were treated either with LPS (1 μg/ml, 15 min) or medium alone and lysed in FPLC extraction buffer. Detergent lysates were fractionated by Mono Q chromatography as described under “Materials and Methods.” Aliquots (5 μl) of each fraction from cells transfected with Δp85 (A) or with wild-type p85 (Wp85) (B) were assayed for MBP kinase activity in triplicate. The standard deviations of each data point were <20% of the mean. C, aliquots of fractions from LPS-treated cells were analyzed by immunoblotting with anti-PKC-ζ antibodies. The results shown are from one of four experiments with similar results.
teterization of hippocampal slices (26). This translocation correlated with the cytosolic accumulation of PKM-ζ, a 51-kDa catalytic subunit of the holoenzyme which suggested activation-induced proteolysis. These findings raise the interesting possibility that PKM-ζ may be generated by LPS in monocytes and that this may be responsible for the increased kinase activity detected. However, two lines of evidence argue against LPS-induced generation of PKM-ζ. First, levels of the ~80–85-kDa PKC-ζ were not observed to be decreased in Mono Q fractions prepared from LPS-treated cells. Second, smaller anti-PKC-ζ immunoreactive proteins were not observed in response to LPS (data not shown).

PKC isoforms have been observed to display different substrate specificity profiles, and to some extent the results obtained are influenced by the specific assay conditions (13, 15, 27). In the present study, it was found that MBP, peptide ε, and S6 peptide were equally efficient substrates for monocyte thi-PKC-ζ fractions prepared from LPS-treated cells. Second, smaller and PKC-ζ ally unrelated PI 3-kinase inhibitors. LPS-induced activation of approaches. The first approach involved the use of two structur- logues display activator- and cofactor-independent activity (28). Moreover, in the present study it was observed that the addition of PI 3-kinase in vitro to specific Mono Q fractions from control cells led to enhanced enzyme activity (Fig. 5B). This observation is consistent with a previous report showing direct activation of purified bovine PKC-ζ by PI 3-kinase (9). The related finding that PKC-ζ from LPS-treated cells could not be further activated in vitro by PI 3-kinase most likely reflects the fact that the enzyme was maximally activated in vivo in response to LPS. An important question arising from these observations is how PKC-ζ maintains its activation following exposure to PI 3-kinase. One possibility is that PI 3-kinase induces a change in the phosphorylation state of the PKC-ζ which sustains its activity until it becomes dephosphorylated by a cellular phosphatase.

In summary, this report provides evidence indicating that LPS activates PKC-ζ in primary human monocytes and in human promonocytic cell lines. This activation requires LPS-induced activation of PI 3-kinase and is sustained in the absence of exogenous lipids. Thus, the results are consistent with a model in which LPS activates p53/p56

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LPS-induced Activation of PKC-ζ

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