Involvement of diacylglycerol production in activation of nuclear factor κB by a CD14-mediated lipopolysaccharide stimulus

Hisanori YAMAMOTO, Kentaro HANADA and Masahiro NISHIJIMA*

Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162, Japan

Exposure of Chinese hamster CHO-K1 transfectant cells expressing mouse CD14 (CHO/CD14 cells) to lipopolysaccharide (LPS) induced rapid elevation of the cellular diacylglycerol (DAG) and choline/phosphocholine levels and nuclear translocation of nuclear factor κB (NFκB). When cells were incubated with short-chain DAG analogues or bacterial phospholipase C, NFκB activation occurred even without the LPS stimulus. Treatment of CHO/CD14 cells with tricyclo[5.2.1.$0^{2,6}$]decyl-(9[8])xanthogenate (D609), an inhibitor of phosphatidylcholine-specific phospholipase C and phospholipase D, almost completely inhibited not only the LPS-dependent production of DAG and choline/phosphocholine but also the LPS-dependent NFκB activation. In contrast, treatment of cells with 1-(6-{[3-methoxyoestra-1,3,5(10)-trien-17β-yl]-1*H*-pyrrole-2,5-dione (U73122), an inhibitor of phosphatidylinositol-specific phospholipase C *in vitro*, did not affect the LPS-dependent activation of NFκB. Production of DAG and activation of NFκB after the LPS stimulus were observed in mouse macrophage-like J774.1 cells, and this response to LPS by J774.1 cells was also inhibited by D609. These results suggest that the production of DAG from phosphatidylcholine was upstream of NFκB activation in response to a CD14-mediated LPS stimulus.

INTRODUCTION

Lipopolysaccharide (LPS), which is a component of the cell envelope of Gram-negative bacteria, is the most frequent causative agent of septic shock [1]. Exposure of immune-related cells to LPS results in the intense production of inflammatory cytokines, including tumour necrosis factor α (TNF-α), interleukin 1β and other inflammatory mediators, such as arachidonic acid, thereby leading to septic shock in mammals [2,3]. LPS forms a complex with a serum protein named LPS-binding protein, and the complex binds to its membrane receptor, CD14, on the cell surface [4]. It has been demonstrated that CD14 has an essential role in an efficient response to LPS not only in cultured cells but also in living animals. For example, (1) a mutant J774.1 macrophage-like cell line resistant to LPS was proved to be defective in CD14 expression [5,6], (2) the expression of CD14 in originally CD14-negative cells renders the cells hyper-responsive to LPS [7], and (3) mutant mice deficient in CD14 show no symptom of septic shock after the injection of LPS, even at high doses [8]. In the processes of cellular responses to LPS, an early crucial step is the nuclear translocation of nuclear factor κB (NFκB), which in turn induces the transcriptional activation of the genes for various inflammatory cytokines [3]. However, the signalling pathway(s) from a CD14-mediated LPS stimulus to NFκB activation remains unclear.

Delude et al. [9] previously demonstrated that CHO-K1 transfectants expressing human CD14 show NFκB activation in response to LPS, whereas wild-type CHO-K1 cells, which do not express endogenous CD14, are not responsive to LPS. This previous study indicated that CHO-K1 cells have signalling machinery connecting a CD14-mediated LPS stimulus to NFκB activation, suggesting the usefulness of CD14-expressing CHO cells as a model system for investigating the intermediary signalling pathway without the influence of CD14-independent LPS responses, which occur in various myeloid cell types. In the present study, using CHO-K1 transfectants expressing mouse CD14, we found that the production of *sn*-1,2-diacylglycerol (DAG), a lipidic modulator, participated in NFκB activation by a CD14-mediated LPS stimulus.

MATERIALS AND METHODS

Materials

LPS from the *Escherichia coli* serotype [0111:B4] was purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). *sn*-1,2-Dioctanoylglycerol (C_8-DAG), *sn*-1-oleoyl-2-acetylglycerol (OAG) and 1-(6-{[3-methoxyoestra-1,3,5(10)-trien-17β-yl]-1*H*-pyrrole-2,5-dione (U73122) were purchased from Calbiochem Novabiochem (La Jolla, CA, U.S.A.). Potassium tricyclo-[5.2.1.$0^{2,6}$]decyl-(9[8])xanthogenate (D609) was purchased from Kamiya Biomedical Company (Thousand Oaks, CA, U.S.A.) and *Bacillus cereus* phosphatidylcholine-specific phospholipase C (PC-PLC) from Boehringer Mannheim (Mannheim, Germany).

Cell culture

CHO-K1, CHO/CD14 [10] and J774.1 cells were maintained in Ham's F-12 medium supplemented with 10% (v/v) heat-

Abbreviations used: C_8-DAG, *sn*-1,2-dioctanoylglycerol; CHO/CD14 cells, Chinese hamster CHO-K1 transfectant cells expressing mouse CD14; D609, potassium tricyclo[5.2.1.$0^{2,6}$]decyl-(9[8])xanthogenate; DAG, *sn*-1,2-diacylglycerol; EMSA, electrophoretic mobility-shift assay; FCS, fetal calf serum (heat-inactivated); GPI, glycosyl phosphatidylinositol; LPS, lipopolysaccharide; NFκB, nuclear factor κB; OAG, *sn*-1-oleoyl-2-acetylglycerol; PC, phosphatidylcholine; PC-PLC, PC-specific phospholipase C; PI-PLC, phosphatidylinositol-specific phospholipase C; PLD, phospholipase D; TNF, tumour necrosis factor; U73122, 1-(6-{[3-methoxyoestra-1,3,5(10)-trien-17β-yl]-1*H*-pyrrole-2,5-dione.

* To whom correspondence should be addressed.

inactivated (56 °C, 30 min) newborn calf serum (ICN Biomedicals, Costa Mesa, CA, U.S.A.) at 37 °C under a water-saturated air/CO_2 (19:1) atmosphere.

Production of DAG in response to LPS

CHO cells were seeded at 3×10^5 cells per dish (diameter 35 mm), and then cultured in 2 ml of F-12 medium containing 1 % (v/v) heat-inactivated fetal calf serum (FCS) and [^{14}C]myristate (0.5 μCi/ml; Amersham, Little Chalfont, Bucks., U. K.) at 37 °C for 2 days to label cellular lipids metabolically to isotopic equilibrium. After the labelled monolayers had been washed with 1 ml of F-12 medium, the culture medium was changed to F-12 containing 0.1 % FCS. Then the LPS stimulus was started by the addition of LPS to the culture medium at 1 μg/ml, and after various periods of incubation at 37 °C the stimulus was terminated by removing the medium, followed by the addition of 0.8 ml of cold PBS (Mg^{2+}- and Ca^{2+}-free) to the dish. Cells were harvested by scraping for lipid extraction. For controls lacking LPS, only PBS as vehicle was added to the culture medium. In some experiments, after incubation of the cell suspension with D609 (10 μg/ml) at 37 °C for 20 min, the LPS stimulus was started. When J774.1 cells were used, the cells (5×10^5) were cultured in 10 ml of F-12 containing 1 % FCS and [^{14}C]palmitate (0.5 μCi/ml; Amersham) at 37 °C for 2 days, and after washing with 10 ml of F-12 the labelled cells were suspended in 15 ml of F-12 containing 0.1 % FCS. The LPS stimulus was started by the addition of LPS (1 μg/ml); after incubation at 37 °C, 0.8 ml aliquots of the cell suspension were withdrawn for the extraction of lipids.

Lipids were extracted from cells by the method of Bligh and Dyer [11] and separated by TLC on Silica Gel 60 plates (Merck, Darmstadt, Germany) with n-hexane/diethyl ether/acetic acid (80:30:1, by vol.) as the developing solvent. On the basis of co-migration with standard DAG, the bands of radioactive DAG on the silica gel plates were identified and the relative radioactivity of the DAG bands was determined with a Fuji BAS2000 Bio-Imaging analyser.

Production of choline and phosphocholine in response to LPS

CHO cells were seeded at 3×10^5 cells per dish (diameter 35 mm), and then cultured in 2 ml of F-12 medium containing 1 % FCS and [^{14}C]choline (0.5 μCi/ml; Amersham) at 37 °C for 2 days to label cellular lipids metabolically to isotopic equilibrium. After the labelled monolayers had been washed with 1 ml of F-12 medium, the culture medium was changed to F-12 containing 0.1 % FCS. Then the LPS stimulus was started by the addition of LPS to the culture medium at 1 μg/ml and cells were incubated for 3 min at 37 °C; the stimulus was terminated by removing the medium, followed by the addition of 0.8 ml of cold PBS to the dish. Cells were harvested by scraping for lipid extraction. For controls lacking LPS, only PBS as vehicle was added to the culture medium. In some experiments, after incubation of the cell suspension with D609 (10 μg/ml) at 37 °C for 20 min, the LPS stimulus was started. Water-soluble reaction products were separated from lipids by the method of Bligh and Dyer and separated by TLC on Silica Gel 60 plates with methanol/0.5 % NaCl/NH_4OH (100:100:2, by vol.) as the developing solvent. The relative amounts of radioactive choline and phosphocholine were determined with a Fuji BAS2000 Bio-Imaging analyser.

Electrophoretic mobility-shift assay (EMSA) for NFκB activation

Synthesized oligonucleotides with a κB site (5′-TTAACAG-AGGGGACTTTCCGAG-3′, 5′-GGCTCGGAAAGTCCCCT-CTGTTAA-3′) (Greiner Japan, Tokyo, Japan) were labelled with [α-^{32}P]dCTP (ICN Biomedicals) at the 3′ end with Klenow fragment and then purified as described previously [12]. Preparation of nuclear extracts of cells and EMSA were performed in accordance with the method of Schreiber et al. [13] with modifications. Briefly, (0.5–1) $\times 10^6$ cells were suspended in 100 μl of 10 mM Hepes/Na, pH 7.9, containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol and 0.1 mM (4-amidinophenyl)methanesulphonyl fluoride (APMSF), and then kept on ice for 15 min. After the addition of NP40 to the cell suspension at 0.6 % (v/v) and vigorous mixing for 10 s, the mixture was centrifuged for 30 s at 15000 ***g***. The pellet was suspended in 20 μl of 20 mM Hepes/Na, pH 7.9, containing 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 0.1 mM APMSF, by vigorous mixing for 15 min at 4 °C. After centrifugation of the suspension of the pellet at 15000 ***g*** for 5 min, the supernatant was used as the nuclear extract. The nuclear extracts (2 μl) were incubated in 10 μl of 10 mM Tris/HCl, pH 7.5, containing 50 mM NaCl, 0.5 mM dithiothreitol, 0.05 % NP40, 10 % (v/v) glycerol, 5 mM $MgCl_2$, 0.5 μg of poly(dI-dC)·poly(dI-dC) and ^{32}P-labelled κB oligonucleotides for 30 min at 25 °C, and then protein–oligonucleotide complexes were separated from free oligonucleotides by electrophoresis in 6 % polyacrylamide gels with Tris/Borate/EDTA buffer at 4 °C. After the gels had been dried, radioactive bands on the gels were revealed with a BAS2000 Bio-Imaging analyser.

RESULTS

Production of DAG caused by a CD14-mediated LPS stimulus in CHO cells

After CHO cells had been metabolically prelabelled with [^{14}C]myristate for 2 days to isotopic equilibrium, the prelabelled cells were incubated with or without LPS for various periods, and then the changes in the relative level of cellular [^{14}C]DAG

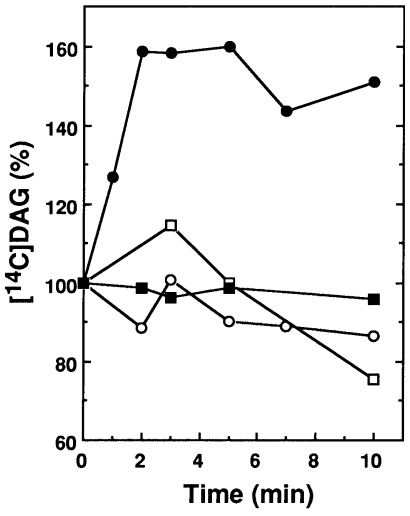

Figure 1 Time course of LPS-induced DAG production in CHO cells

CHO-K1 (□, ■) and CHO/CD14 (○, ●) cells were prelabelled with [^{14}C]myristate for 2 days. After the addition of LPS (■, ●) to a final concentration of 1 μg/ml, or vehicle (PBS) alone (□, ○) to prelabelled cells, the cells were incubated for the indicated periods at 37 °C. Lipids were then extracted from the cells and the relative amounts of radioactive DAG were determined as described in the Materials and methods section. The results shown are means for duplicate experiments and are expressed as the percentages of the initial levels of the respective cell types.

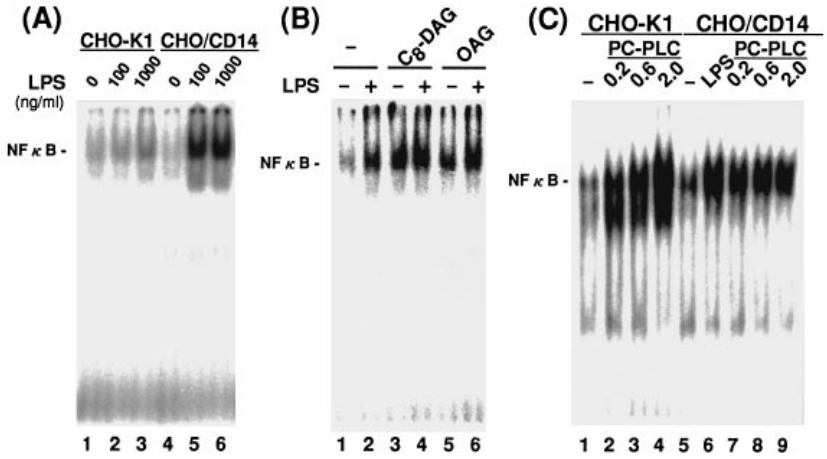

Figure 2 Activation of NFκB by treatment of cells with short-chain analogues of DAG or with bacterial PC-PLC

(**A**) CHO-K1 (lanes 1–3) or CHO/CD14 cells (lanes 4–6) were stimulated with LPS at the indicated concentrations (ng/ml) for 30 min at 37 °C. Nuclear extracts were then prepared from the cells and subjected to EMSA as described in the Materials and methods section. (**B**) CHO/CD14 cells were incubated with 10 μM C_8-DAG (lanes 3 and 4) or 10 μM OAG (lanes 5 and 6) for 30 min at 37 °C in the absence (−) or presence (+) of 1 μg/ml LPS. Nuclear extracts were then prepared from the cells and subjected to EMSA as described in the Materials and methods section. (**C**) CHO-K1 (lanes 1–4) or CHO/CD14 cells (lanes 5–9) were incubated with *B. cereus* PC-PLC (at 0.2, 0.6 and 2.0 units/ml) for 30 min at 37 °C. Then EMSA, with nuclear extracts prepared from these cells, was performed.

were determined. When CHO/CD14 cells were exposed to LPS, the cellular [^{14}C]DAG level increased to approx. 160% of the initial level by 2 min after stimulation with LPS, and then remained at the elevated level for at least 5 min (Figure 1). In contrast, when cells were not exposed to LPS, the [^{14}C]DAG levels of both CHO/CD14 and wild-type CHO-K1 cells gradually decreased (Figure 1), probably because of metabolic conversion of DAG to other compounds. Exposure of wild-type CHO-K1 cells to LPS did not affect the time course of the change in the [^{14}C]DAG level (Figure 1). These results suggest that the CD14-mediated LPS stimulus induced the production of DAG in CHO cells.

Activation of NFκB by exogenous DAG analogues or by treatment with PC-PLC

Exposure of cells to LPS resulted in the activation of NFκB in CHO/CD14 cells but not in CD14-negative wild-type CHO-K1 cells (Figure 2A), which is consistent with the earlier study [9]. To address the question of whether or not the production of DAG caused by the CD14-mediated LPS stimulus has a crucial role in NFκB activation, we examined the effects of C_8-DAG and OAG, short-chain analogues of DAG, on the activation of NFκB in CHO/CD14 cells. We used these short-chain analogues because exogenous DAG with long diacyl chains is very poorly transferred to cells because of its marked hydrophobicity. After pre-incubation of CHO/CD14 cells with C_8-DAG or OAG at 37 °C for 30 min, the cells were incubated with or without LPS, and then nuclear extracts were prepared from the cells for EMSA of NFκB. Activation of NFκB in response to LPS was enhanced by the preincubation of cells with DAG analogues; interestingly, these exogenous DAG analogues activated NFκB even without the LPS stimulus (Figure 2B).

To increase the endogenous DAG level, cells were treated with *Bacillus cereus* PC-PLC, which degrades cellular phosphatidylcholine (PC) to produce endogenous DAG. As shown in Figure 2C, treatment of CHO-K1 and CHO/CD14 cells with PC-PLC resulted in activation of NFκB in these two types of cell to a similar extent to the LPS-induced activation in CHO/CD14 cells. The NFκB activation on treatment of cells with PC-PLC was not due to possible contamination by LPS of the bacterial PC-PLC sample, because the activation occurred even in CHO-K1 cells that were unresponsive to LPS (Figures 2A and 2B). These results indicate that an increase in the cellular DAG level triggered the activation of NFκB, suggesting that DAG production in response to the LPS stimulus was a crucial step for LPS-induced activation of NFκB.

Abrogation of DAG production and NFκB activation by D609

Membrane phosphatidylinositol and PC are two major sources for the production of DAG in response to various stimuli [14–16]. However, we did not observe a significant production of phosphoinositides in CHO/CD14 cells in response to LPS (results not shown). We thus examined the possibility that the production of DAG in response to LPS might result from the hydrolysis of PC by using D609, a potent inhibitor of PC-PLC and phospholipase D (PLD) but not phosphatidylinositol-specific phospholipase C (PI-PLC) *in vitro* [17–19]. Treatment of CHO/CD14 cells with D609 inhibited almost completely the LPS-dependent production of [^{14}C]DAG (Figure 3A).

More importantly, no activation of NFκB in response to LPS was observed under the D609-treated conditions, where LPS-dependent production of DAG was inhibited completely (Figure 3B), although the D609 treatment did not affect cellular viability as judged by Trypan Blue exclusion (results not shown). In contrast, U73122, a potent inhibitor of PI-PLC *in vitro* [20], did not inhibit LPS-induced NFκB activation (Figure 3C). These results suggest that the CD14-mediated LPS stimulus induced the production of DAG from PC in CHO/CD14 cells, and that the DAG production was upstream of the LPS-induced NFκB activation.

Although D609 is known as a potent inhibitor of PC-PLC and PLD, the action of this drug might not be specific to these phospholipases in intact cells. To determine whether DAG

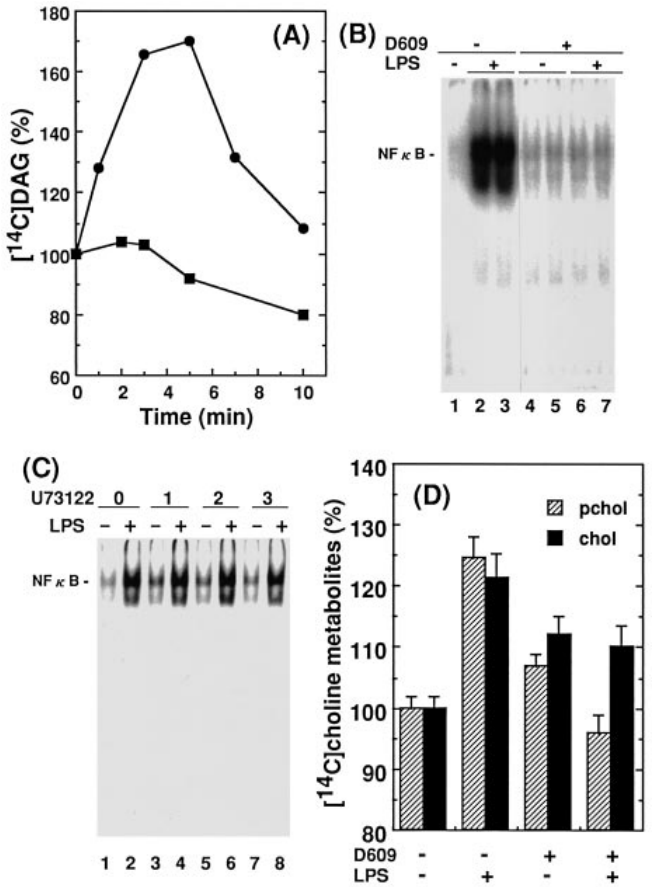

Figure 3 D609 inhibits [^{14}C]DAG production and NFκB activation in response to LPS

(**A**) [^{14}C]Myristate-labelled CHO/CD14 cells were preincubated with (■) or without (●) 10 μg/ml D609 for 20 min at 37 °C, and subsequently stimulated with 1 μg/ml LPS. After the cells had been incubated for the indicated periods at 37 °C, lipids were extracted from the cells and the relative amounts of radioactive DAG were determined. The results shown are means for duplicate experiments and are expressed as percentages of the initial levels. (**B**) CHO/CD14 cells were preincubated with (+) or without (−) D609 at 10 μg/ml for 20 min at 37 °C, and then incubated with (+) 1 μg/ml LPS or without (−) LPS for 30 min at 37 °C. Nuclear extracts were then prepared from the cells and subjected to EMSA. Lanes 2 and 3, 4 and 5, and 6 and 7 are data for duplicate experiments. (**C**) CHO/CD14 cells were preincubated with 0–3 μM U73122 and subsequently incubated without (−) or with (+) 1 μg/ml LPS for 30 min at 37 °C. Then EMSA, with nuclear extracts prepared from these cells, was performed. (**D**) [^{14}C]Choline-labelled CHO/CD14 cells were preincubated with or without D609 (10 μg/ml) for 20 min at 37 °C, and subsequently stimulated with LPS (1 μg/ml). After the cells had been incubated for 3 min at 37 °C, water-soluble reaction products were separated from lipids by the method of Bligh and Dyer, and the relative amounts of radioactive choline (chol) and phosphocholine (pchol) were determined. The amount of [^{14}C]choline and [^{14}C]phosphocholine in the absence of LPS and D609 was taken as 100%; data are shown as means ± S.D. for three independent experiments.

produced by LPS stimulus is derived from PC, CHO/CD14 cells were metabolically labelled with [^{14}C]choline, and levels of intracellular [^{14}C]choline and [^{14}C]phosphocholine were measured after LPS stimulus. As shown in Figure 3D, levels of both intracellular [^{14}C]choline and [^{14}C]phosphocholine were increased by LPS stimulus to about 125 % and 120 % respectively. This elevation in the pools of radiolabelled choline and phosphocholine was prevented by pretreatment with D609; in the presence of 10 μg/ml D609, although [^{14}C]choline and [^{14}C]phosphocholine in CHO/CD14 cells were significantly increased without LPS stimulus, no significant changes in [^{14}C]choline and [^{14}C]phosphocholine levels were caused by LPS stimulus. These results provide additional evidence that PC is the source of DAG accumulated by LPS treatment. However, it remains undetermined what kind of phospholipase is responsible for the initial reaction of this DAG formation, because the levels of both [^{14}C]choline and [^{14}C]phosphocholine were increased by LPS stimulus.

Involvement of DAG production from PC in LPS-responsive activation of NFκB in mouse macrophage-like J774.1 cells

We next examined whether the production of DAG from PC might participate in the LPS-responsive activation of NFκB in mouse macrophage-like J774.1 cells, which endogenously express CD14. J774.1 cells were metabolically prelabelled with [^{14}C]palmitate to isotopic equilibrium and then exposed to LPS. A transient increase in the cellular [^{14}C]DAG level was observed in an LPS-dependent manner (Figure 4A), which was similar to what occurred in CHO/CD14 cells (Figure 1). Treatment of J774.1 cells with D609 inhibited almost completely the LPS-induced increase in the [^{14}C]DAG level (Figure 4B). Moreover treatment with D609 also inhibited the LPS-responsive activation of NFκB (Figure 4C). These results suggest that the production of DAG from PC participated in the LPS-responsive activation of NFκB in J774.1 cells as well as in CHO/CD14 cells.

DISCUSSION

In this study we examined the possibility that the production of DAG, a lipidic modulator, might be involved in the activation of NFκB in response to a CD14-dependent LPS stimulus. Exposure of CHO/CD14 cells to LPS induced a rapid increase in the cellular DAG level and activation of NFκB. When cells were incubated with short-chain DAG analogues or with bacterial PC-PLC, NFκB activation occurred even without the LPS stimulus. Treatment of CHO/CD14 cells with D609, a potent inhibitor of PC-PLC and PLD [17–19], inhibited almost completely the LPS-dependent production of DAG and also the LPS-dependent activation of NFκB, whereas treatment of cells with U73122, a potent inhibitor of PI-PLC [20], did not affect the LPS-dependent activation of NFκB. The production of DAG and activation of NFκB after the LPS stimulus were observed in macrophage-like J774.1 cells; these LPS responses of J774.1 cells were also inhibited by D609. These findings suggest that the production of DAG from PC was upstream of NFκB activation in response to a CD14-mediated LPS stimulus.

We found that the [^{14}C]DAG level increased to approx. 160 % of the initial level by 2 min after LPS-stimulation in cells labelled with [^{14}C]myristate (Figure 1), and that intracellular [^{14}C]choline and [^{14}C]phosphocholine levels also increased to approx. 125 % and 120 % respectively in response to LPS stimulus in [^{14}C]choline-labelled cells (Figure 3D). However, we did not detect a significant change in DAG mass in CHO/CD14 cells in response to LPS (results not shown). It is well known that the apparent activities of phospholipases or the amounts of their reaction products vary when they are determined with cells prelabelled with different kinds of phospholipid precursor. It has been also reported that v-Src activates PKC-independent PLD, which specifically hydrolyses PC labelled with [^{3}H]myristate but not that labelled with [^{3}H]arachidonate [21], and that a basal PLD activity is detectable in [^{14}C]linoleate-labelled CHO cells but not in cells labelled with [^{14}C]arachidonate [22]. Thus the LPS-induced DAG production from the PC pool might occur at

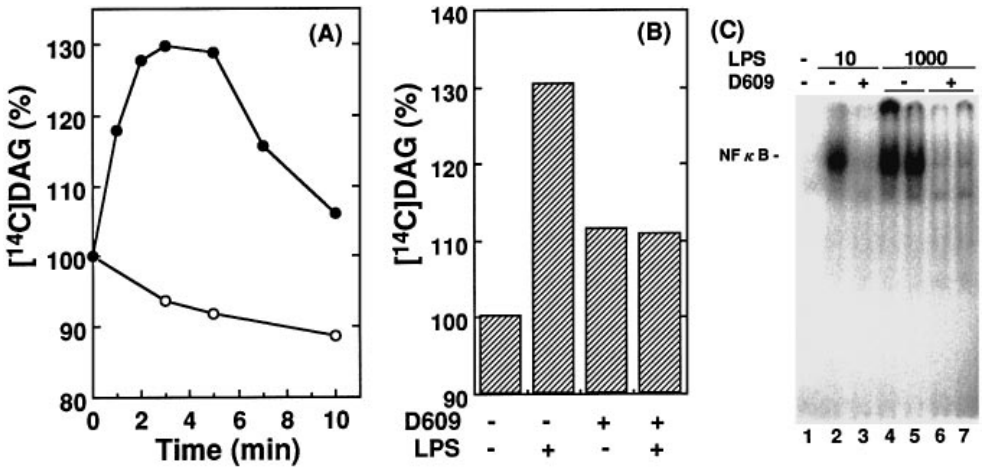

Figure 4 LPS-induced production of [^{14}C]DAG and activation of NFκB in J774.1 cells

(**A**) J774.1 cells labelled with [^{14}C]palmitate for 2 days were incubated with (●) or without (○) 1 μg/ml LPS for the indicated periods; changes in the cellular [^{14}C]DAG level were then determined as described in the Materials and methods section. (**B**) J774.1 cells labelled with [^{14}C]palmitate for 2 days were preincubated with (+) or without (−) 10 μg/ml D609 for 20 min and then incubated with (+) or without (−) 1 μg/ml LPS for 3 min. The relative levels of cellular [^{14}C]DAG were determined. (**C**) Inhibition of LPS-induced NFκB activation by D609. J774.1 cells were preincubated with (lanes 3, 6 and 7) or without (lanes 1, 2, 4 and 5) 10 μg/ml D609 for 20 min, and then the cells were incubated with or without LPS for 30 min. LPS concentrations: lane 1, none added; lanes 2 and 3, 10 ng/ml; lanes 4–7, 1 μg/ml.

putative membrane subdomains where LPS-signalling apparatus including CD14 and PC-PLC/PLD might be localized, and the change in DAG mass in response to LPS might be too small to be detected.

As well as LPS, various inflammatory cytokines, including TNF-α and interleukin 1β, strongly activate NFκB [23]. Exposure of cells to TNF-α results in transient elevation of the cellular DAG level, probably via degradation of PC, and DAG production seems to be crucial for the activation of NFκB in response to TNF-α [24]. Our present findings and these previous results might show a similarity between the LPS and TNF-α signalling systems; however, these two types of stimulus seem to involve distinct mechanisms for transmembrane signalling. In TNF-α signalling, a 55 kDa transmembrane protein serves as the receptor for TNF-α [25]. After ligation of the extracellular domain of the transmembrane receptor by TNF-α, the cytoplasmic domain of the receptor mediates the signal, recruiting an adaptor protein to the cytoplasmic domain [26]. Enigmatically, in LPS signalling, the known membrane receptor, CD14, should be incapable of transducing an extracellular LPS stimulus directly to cytoplasmic machineries because CD14 is a glycosyl phosphatidylinositol (GPI)-anchored protein that is intercalated in the outer lipid layer of the plasma membrane and thus has no cytoplasmic region [27].

It was recently demonstrated that glial cell line-derived neurotropic factor uses a GPI-anchored protein as its high-affinity receptor and that, after ligation, the GPI-anchored receptor forms a specific complex with c-Ret, a transmembrane tyrosine kinase, thereby activating this kinase [28,29]. Complex formation between a GPI-anchored receptor and another transmembrane protein, gp130, has also been shown in the signalling of the ciliary neurotropic factor [30]. These findings led to the hypothesis that one or more transmembrane proteins, currently unidentified intermediary molecules, might also participate in CD14-dependent LPS signalling. The fact that DAG production in response to LPS occurs in CHO/CD14 cells indicates that CHO cells potentially have signalling machineries connecting a CD14-dependent LPS stimulus and activation of NFκB. Genetic approaches with the CHO cell line, which is a good tool for the isolation of recessive mutants, could be useful for elucidating the LPS-signalling mechanism.

This work was supported in part by Grants-in-Aid for General Scientific Research and for Creative Basic Research from the Ministry of Education, Science and Culture of Japan, by the Human Sciences Basic Research Project and the Integrated Study Projects in Drug Innovation Science of the Japan Health Sciences Foundation, and by CREST of Japan Science and Technology Corporation.

REFERENCES

1 Glauser, M. P., Zanetti, G., Baumgartner, J.-D. and Cohen, J. (1991) Lancet **338**, 732–736
2 Sweet, M. J. and Hume, D. A. (1996) J. Leukocyte Biol. **60**, 8–26
3 Müller, J. M., Ziegler-Heitbrock, H. W. L. and Baeuerle, P. A. (1993) Immunobiology **187**, 233–256
4 Tobias, P. S., Soldau, K. and Ulevitch, R. C. (1986) J. Exp. Med. **164**, 777–793
5 Hara-Kuge, S., Amano, F., Nishijima, M. and Akamatsu, Y. (1990) J. Biol. Chem. **265**, 6606–6610
6 Nishijima, M., Hara-Kuge, S., Takasuka, N., Akagawa, K., Setouchi, M., Matsuura, K., Yamamoto, S. and Akamatsu, Y. (1994) J. Biochem. (Tokyo) **116**, 1082–1087
7 Wright, S. D., Ramos, R. A., Hermanowski-Vosatka, A., Rockwell, P. and Detmers, P. A. (1991) J. Exp. Med. **173**, 1281–1286
8 Haziot, A., Ferrero, E., Köntgen, F., Hijiya, N., Yamamoto, S., Silver, S., Stewart, C. L. and Goyert, S. M. (1996) Immunity **4**, 407–414
9 Delude, R. L., Fenton, M. J., Savedra, R., Perera, P. Y., Vogel, S. N., Thieringer, R. and Golenbock, D. T. (1994) J. Biol. Chem. **269**, 22253–22260
10 Hanada, K., Izawa, K., Nishijima, M. and Akamatsu, Y. (1993) J. Biol. Chem. **268**, 13820–13823
11 Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. **37**, 911–917
12 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
13 Schreiber, E., Matthias, P., Müller, M. M. and Schaffner, W. (1989) Nucleic Acids Res. **17**, 6419
14 Nishizuka, Y. (1992) Science **258**, 607–614
15 Exton, J. H. (1994) Biochim. Biophys. Acta **1212**, 26–42
16 Divecha, N. and Irvine, R. F. (1995) Cell **80**, 269–278
17 Müller-Decker, K. (1989) Biochem. Biophys. Res. Commun. **162**, 198–205
18 Schütze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K. and Krönke, M. (1992) Cell **71**, 765–776

19 Kiss, Z. and Tomono, M. (1995) Biochim. Biophys. Acta **1259**, 105–108
20 Thompson, A. K., Mostafapour, S. P., Denlinger, L. C., Bleasdale, J. E. and Fisher, S. K. (1991) J. Biol. Chem. **266**, 23856–23862
21 Song, J. and Foster, D. A. (1993) Biochem. J. **294**, 711–717
22 Dunlop, M. and Clark, S. (1993) Biochim. Biophys. Acta **1220**, 43–48
23 Schütze, S., Machleidt, T. and Krönke, M. (1994) J. Leukocyte Biol. **56**, 533–541
24 Schütze, S., Berkovic, D., Tomsing, O., Unger, C. and Krönke, M. (1991) J. Exp. Med. **174**, 975–988
25 Vandenabeele, P., Declercq, W., Beyaert, R. and Fiers, W. (1995) Trends Cell Biol. **5**, 392–399
26 Hsu, H., Xiong, J. and Goeddel, D. V. (1995) Cell **81**, 495–504
27 Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J. and Mathison, J. C. (1990) Science **249**, 1431–1433
28 Treanor, J. J., Goodman, L., de Sauvage, F., Stone, D. M., Poulsen, K. T., Beck, C. D., Gray, C., Armanini, M. P., Pollock, R. A., Hefti, F. et al. (1996) Nature (London) **382**, 80–83
29 Jing, S., Wen, D., Yu, Y., Holst, P. L., Luo, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R. et al. (1996) Cell **85**, 1113–1124
30 Davis, S., Aldrich, T. H., Stahl, N., Pan, L., Taga, T., Kishimoto, T., Ip, N. Y. and Yancopoulos, G. D. (1993) Science **260**, 1805–1808

Received 17 February 1997; accepted 18 March 1997