Modulation of Mouse Endotoxin Shock by Inhibition of Phosphatidylcholine-Specific Phospholipase C

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ABSTRACT

During Gram-negative bacterial infections, lipopolysaccharide (LPS) interacts with monocyte/macrophage receptors, resulting in a host defense response. Activation of intracellular signal transduction pathways implicating various protein kinase and phospholipases is crucial in activating the transcription of genes encoding proinflammatory cytokines and inducible nitric oxide synthase (iNOS). In this article, we demonstrate that in mouse, endotoxin shock activation of phosphatidylcholine-specific phospholipase C (PC-PLC) plays a major role in controlling the inflammatory response. Inhibition of PC-PLC by the specific inhibitor tricyclodecan-9-yl-xanthogenate (D609) before LPS reduced the release of interleukin-1β, interleukin-6 and nitric oxide (NO) in vivo. In contrast, tumor necrosis factor-α serum levels were not altered by the pretreatment with D609. Consequently, survival from endotoxin shock of D609-treated animals was significantly improved compared with control animals (45% vs. 20%). Thus, inhibition of PC-PLC can reduce the inflammatory response to LPS and may serve as a novel approach to therapy of sepsis.

Sepsis due to bacterial infections and experimentally induced endotoxin shock initiate immune responses by activation of intracellular signal transduction cascades resulting in the generation of proinflammatory cytokines such as tumor necrosis factor-α, IL-1β and IL-6 and the synthesis of nitric oxide.

TNF-α and IL-1β in particular have been demonstrated as pivotal mediators of the toxic and lethal effects of LPS determining the outcome of Gram-negative septic shock (Tracey et al., 1986; Shalaby et al., 1991; Dinarello et al., 1993; Pfieffer et al., 1993). Therefore, defining the regulation of the intracellular signal transduction mechanisms of endotoxin and proinflammatory cytokines is crucial for controlling the extent of the immune response to endotoxin and microorganisms. A number of signalling pathways implicating PKC, protein tyrosine kinases, mitogen-activated protein kinases and proline-directed protein kinases have been described in signal transduction of endotoxin and TNF-α (Han et al., 1993; Kolesnick and Golde, 1994; Novogrodsky et al., 1994; Tschaikowsky and Brain, 1994). In addition, activation of membrane-associated phospholipases have been identified as initial events triggering subsequent activation of protein kinases by the release of lipid mediators (Pripic et al., 1987). Especially the activation of a TNF-responsive PC-PLC has been demonstrated as a crucial step in TNF cytotoxicity via a signaling route implicating diacylglycerol, SMase, ceramide and NF-κB activation (Schütze et al., 1992).

By using tricyclodecan-9-yl-xanthogenate (D609), a specific and selective PC-PLC inhibitor that in particular does not interfere with phosphatidylinositol-specific PLC-γ, PKC, protein tyrosine kinase, proline-directed protein kinase and SMase (Müller, 1989; Schütze et al., 1992; Wiegmann et al., 1994; Machleidt et al., 1996), PC-PLC has been demonstrated to be required for the LPS-induced NO release in mononuclear cells (Tschaikowsky et al., 1994).

Recently, inhibition of PC-PLC has been shown to block the cytotoxic and proinflammatory action of TNF-α both in vitro and in vivo (Machleidt et al., 1996). In addition, D609 protects mice from lethal shock induced by TNF-α, LPS or staphylococcal enterotoxin B (Machleidt et al., 1996). However, the mechanisms of the protective effect of D609 in endotoxin or exotoxin shock regarding which alterations in the immune response are achieved by in vivo inhibition of PC-PLC have not been clarified. In particular, it has not been demonstrated whether D609 can directly interfere with the release of inflammatory mediators in response to LPS, or whether it only modulates the biological activity of secondary mediators by interfering with their intracellular signal transduction.

ABBREVIATIONS: D609, tricyclodecan-9-yl-xanthogenate; PC-PLC, phosphatidylcholine-specific phospholipase C; SMase, sphingomyelinase; LPS, lipopolysaccharide; IL-1β, interleukin 1-β; IL-6, interleukin 6; TNF-α, tumor necrosis factor-α; NO, nitric oxide; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-κB.
In this study, we show that inhibition of PC-PLC by D609 in mice reduces the release of IL-1β, IL-6 and nitrite/nitrate in the serum after a lethal challenge with LPS, whereas serum levels of TNF-α were unchanged. Furthermore, pretreatment with a single dose of D609 did reduce but not completely prevent the lethality of endotoxin shock. These results add to the growing evidence that many, but not all, of the pathophysiological effects of LPS are mediated by the activation of PC-PLC.

Materials and Methods

Reagents. D609 was kindly provided by Dr. Amtmann (German Cancer Research Center, Heidelberg, Germany). The 5% HA was purchased from Centon (Marburg, Germany). LPS (Escherichia coli, 0111:B4, phenol extraction), culture medium (RPMI-1640 supplemented with 10% heat-inactivated feline serum (endotoxin tested <50 pg/ml), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine) and all other reagents were obtained from Sigma (Deisenhofen, Germany) if not otherwise indicated. For cell culture experiments, D609 was always freshly prepared as a 0.1% stock solution in distilled water and then further diluted in medium at pH 7.0.

Animals and experimental design. Female NMRI mice (28–32 g) were obtained from Charles River Wiga (Sulzfeld, Germany) and given rodent chow and tap water ad libitum. For lethality experiments, animals were randomly assigned to two groups according to the pretreatment they received before LPS challenge. One group (D609, n = 20) was intraperitoneally injected with 1 ml of D609 (150 mg/kg) dissolved in pyrogen-free PBS (5 mg/ml, pH 7.0) supplemented with 5% HA (PBS-5% HA), and the other group (control, n = 20) received 1 ml of PBS-5% HA. Supplementation with 5% HA was used to mitigate the peritoneal irritations caused by intraperitoneal application of D609 (Dr. Amtmann, personal communication). Mice were challenged intraperitoneally 15 min after pretreatment, with LPS (1.5 mg, freshly dissolved with pyrogen-free saline and sonicated 2 min before use). Surviving mice were recorded after 72 hr and monitored for late deaths and signs of toxicity for 14 days. Animals monitored for lethality after LPS injection were not subjected to blood drawings because previous studies have shown that blood withdrawal from mice in endotoxin shock has an impact on outcome. Therefore, animals were studied either for mortality or measurement of inflammatory mediators after administration of LPS.

To determine the release of inflammatory cytokines and NO immediately before (0 hr) and 1.5 and 9 hr after LPS challenge, two additional groups (D609 and control, n = 15/group) were used for each time point. Blood (≈1 ml) was collected by cardiac puncture after sternotomy in pyrogen-free, nonheparinized microtubes. To minimize distress, mice were kept under deep anesthesia breathing an enflurane/oxygen mix delivered from a vaporizer over a nose cone to minimize distress, mice were kept under deep anesthesia breathing an enflurane/oxygen mix delivered from a vaporizer over a nose cone, 2 min before use). Surviving mice were recorded after 72 hr and monitored for late deaths and signs of toxicity for 14 days. Animals monitored for lethality after LPS injection were not subjected to blood drawings because previous studies have shown that blood withdrawal from mice in endotoxin shock has an impact on outcome. Therefore, animals were studied either for mortality or measurement of inflammatory mediators after administration of LPS.

Western blot analysis for iNOS in J774 murine macrophages. iNOS protein expression was determined in J774 murine macrophages (10⁶/ml) that were stimulated with LPS (1 µg/ml) and IFN-γ (200 units/ml) in the presence or absence of D609 at a concentration of 40 and 10 µg/ml, respectively. After 12-hr incubation (37°C, 5% CO₂), cells were washed twice with PBS and lysed by treatment with electrophoresis sample buffer (125 mM Tris-HCl pH 6.8, 2% SDS, 5% glycerol, 0.003% bromophenol blue and 1% β-mercaptoethanol). Then, 15 µg of macrophage lysate was separated by a standard SDS-PAGE (Pharmacia, 7.5 ExcelGel SDS Homogenous) and transferred to 0.45-µm pore size nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The membranes were blocked with 5% nonfat dry milk in PBS for 1 hr at 25°C, washed twice with Tween-PBS (0.05% Tween 20) and incubated overnight at 4°C with anti-iNOS monoclonal antibody (Transduction Laboratories, Lexington, KY) diluted 1:1000 in PBS containing 1% nonfat dry milk. Then, the membranes were washed three times with Tween-PBS, incubated with anti-mouse IgG conjugated to horseradish peroxidase for 1 hr at 25°C and washed twice with Tween-PBS and twice with PBS. Visualization of iNOS was achieved using a lumino-based enhanced chemiluminescence detection kit (ECL Kit; Amersham, Arlington Heights, IL). The membranes were then exposed to Kodak XR film, and the film was developed. Lysates of stimulated RAW 264.7 murine macrophage cell line was used as positive control for iNOS (Transduction Laboratories).

Statistical analysis. All results are expressed as mean ± S.E. The data for determination of cytokines and NO were compared between groups by using the Mann-Whitney U test. Lethality among the groups was compared by using Fisher’s exact probability test. Survival analyses were made using the log-rank test (Kaplan-Meier). Differences were considered as significant at values of P < .05.
Results

Lethality experiments. To examine whether D609 can protect from lethal endotoxin shock, NMRI mice were pretreated with D609 (150 mg/kg i.p.) or PBS-5% HA (control) 15 min. before a challenge with LPS (1.5 mg i.p.). Administration of this high dose of LPS produced 80% lethality of the control animals (fig. 1). In contrast, pretreatment with a single dose of D609 significantly (P < .01) reduced mortality rate to ∼55% but failed to provide full protection from the lethal effects of endotoxin. In both groups, deaths occurred between 12 and 48 hr. There were no signs of intoxication or late deaths up to 14 days after LPS injection. Therefore, animals surviving 72 hr after LPS injection were regarded as survivors. Control mice treated with D609 alone did not show signs of intoxication or pathological findings on organ inspection (liver, lung, intestine) as monitored for up to 1 month after D609 injection. In particular, viability of macrophages harvested from the peritoneal cavity 30 min after intraperitoneal injection of D609 was not affected up to a dose of 150 mg/kg b.wt. compared with control animals (>95%). D609 at a dose of 300 mg/kg intraperitoneal, however, which was not used for further studies, reduced the viability of peritoneal macrophages to 70% as assessed by trypan blue exclusion. Immediately after intraperitoneal injection of D609, the animals seemed to experience some peritoneal irritation for a short period of time, most likely due to the detergent properties of D609. However, this side effect could be attenuated to some extent by the addition of protein (5% HA) to the D609 solution before injection.

Cytokines and NO. Because TNF-α and IL-1β play pivotal roles mediating the lethal effects of sepsis and endotoxin shock, serum concentrations of these proinflammatory cytokines were determined in animal groups not monitored for survival immediately before (0 hr) and at 1.5 and 9 hr after LPS injection. Serum concentrations of IL-1β at 1.5 hr after LPS-injection were significantly lower in animals pretreated with D609 compared with controls (fig. 2). Likewise, IL-6 serum levels were significantly reduced in D609-treated animals at 1.5 and 9 hr after endotoxin challenge (fig. 3). Unexpectedly, amounts and time course of TNF-α found in the serum were almost identical in D609-treated and control animals with a peak concentration at 1.5 hr and no detectable levels at 9 hr after LPS-injection, as shown in figure 3. NO generated by iNOS has also been demonstrated as a chief mediator of severe hypotension and shock observed several hours after endotoxin (Kilbourn et al., 1990; Moncada et al., 1991). Because in mouse macrophage-like J774 cells, LPS-stimulated induction of iNOS activity has been shown to be PC-PLC dependent (Tschaikowsky et al., 1994), we questioned whether inhibition of PC-PLC by D609 can ameliorate the production of NO in mouse endotoxin shock. In agreement with our in vitro findings, pretreatment with D609 resulted in a markedly reduced NO-production in response to LPS as assessed by serum concentrations of nitrite and nitrate at 9 hr after LPS-injection (fig. 2). At 1.5 hr after LPS challenge, however, nitrite/nitrate serum levels were unchanged and within the normal range (<20 μM) regardless of the pretreatment with D609.

Effect of D609 on iNOS expression in vitro. To clarify whether D609 can directly inhibit LPS-stimulated expression of iNOS protein or whether D609 produces this effect by inhibition of other mediators, Western blot analysis were

Fig. 1. D609 protects mice from endotoxin lethality. Mice were either injected intraperitoneally with phosphate-buffered saline/5% human albumin (●) or 150 mg/kg D609 (○) 15 min before endotoxin challenge (1.5 mg LPS i.p.) at 0 hr. Animals were monitored for 1 month; no deaths occurred after 72 hr. Statistical analysis was performed using log-rank test (Kaplan-Meier). **, P < .01 compared with control.

Fig. 2. D609 reduces the LPS-induced release of IL-1β and NO. D609 (150 mg/kg) 15 min before LPS (1.5 mg i.p.) inhibits the release of IL-1β at 1.5 hr and NO at 9 hr, measured as nitrite and nitrate, into the serum. Mice were left untreated (N, □), LPS-injected without D609 (P, ■), or LPS-injected after D609 (D, ○). **, P < .01 compared with control.

Fig. 3. D609 inhibits the LPS-induced IL-6 release but leaves the TNF-α response unchanged. D609 (150 mg/kg) 15 min before LPS (1.5 mg i.p.) inhibits the release of IL-6 (●) at 1.5 and 9 hr after LPS compared with LPS-injected animals not treated with D609 (○). In contrast, TNF-α serum concentrations of D609-treated mice (●) are almost identical to those without D609 (○) at 1.5 and 9 hr after a challenge with LPS.
performed with lysate of stimulated J774 murine macrophages. As shown in figure 4, 12-hr stimulation of macrophages (J774 and RAW 264.7) with LPS and interferon-γ resulted in de novo synthesis of iNOS protein (lanes 2 and 6, positive control). D609, simultaneously added to the culture, dose-dependently suppressed iNOS induction in J774 macrophages (lanes 3 and 4).

Discussion

Anticytokine therapies have been promulgated in Gram-negative sepsis as a means of preventing excessive immune reactions. However, the usefulness of antiinflammatory strategies in sepsis aiming to block the activity of a single mediator in the cascade of immune reactions leaving the cellular response and the release of inflammatory mediators unchanged is under discussion. Despite promising experimental data, monoclonal antibodies and receptor antagonists neutralizing a particular inflammatory cytokine, such as TNF-α and IL-1β, have failed so far to improve the clinical outcome of patients with sepsis (Fisher et al., 1996; Opal et al., 1997). Therefore, novel approaches to therapy of sepsis are being investigated that interfere with the intracellular signal transduction to modulate the entire cellular response to endotoxin and inflammatory mediators.

Activation of PC-PLC has been demonstrated as a pivotal step in the signal transduction of LPS and TNF-α. D609, a selective inhibitor of PC-PLC that in particular has no inhibitory effect on phosphatidylinositol-specific PLC-γ, phospholipase A2, PKC and protein tyrosine kinases, has already been shown to block LPS-induced generation of iNOS activity and TNF-mediated-κB activation in mononuclear cells (Schütze et al., 1992; Tshaikowsky et al., 1994). Activation of NF-κB and induction of iNOS, in turn, are regarded as pivotal events for the development of a systemic inflammatory response in vitro by generation of a variety of proinflammatory mediators such as IL-1β, TNF-α and NO. In this study, we investigated whether inhibition of PC-PLC by a pretreatment with D609 can reduce the cytokine response, the NO production, and thereby improve survival in mouse endotoxin shock. To induce lethal shock, we injected 1.5 mg i.p. LPS/mouse corresponding to a dose of 50 mg/kg b.wt. This dose produced 80% mortality within 72 hr, which is in agreement with numerous studies in mice using LPS at doses of 30 to 60 mg/kg i.p., which usually result in mortality rates of >50% (Redmond et al., 1991; Novogrodsky et al., 1994). However, LPS-induced lethality in mice is highly dependent on the LPS preparation used and the age of the mice (Tateda et al., 1996).

We found that a single injection of D609 15 min before LPS significantly reduces the mortality of endotoxin shock from 80% to 55%. This is in accordance with a previous study demonstrating full protection from lethality by D609 (50 mg/kg) given at 0, 1 and 4 hr after endotoxin (Machleidt et al., 1996). The pathomechanisms responsible for a better survival in D609-treated animals, however, have not been clarified. Because LPS is a major trigger for inflammatory cytokines and NO, which accounts for most of its toxic and lethal effects in vitro and in vivo, we examined the effect of D609 on the release of TNF-α, IL-1β, IL-6 and nitrite/nitrate during endotoxin shock. In a previous study, D609 was not found to inhibit the release of IL-1, TNF-α or IFN-γ in mice treated with Staphylococcus enterotoxin from Gram-positive bacteria, which functions as a T-cell superantigen (Machleidt et al., 1996). In contrast, our findings clearly demonstrate that inhibition of PC-PLC by D609 in mouse endotoxin shock has a profound inhibitory effect on the release of IL-1β at 1.5 hr and IL-6 at 1.5 and 9 hr after LPS-challenge. These are the first data demonstrating a direct inhibitory effect of D609 on the LPS-stimulated cytokine release. Because IL-1β in particular is known to cause lethal shock in various animal models synergistically with TNF-α (Dinarello et al., 1993), the suppression of IL-1β by D609 in this endotoxin model most likely is a major factor for the protection from endotoxin lethality observed in D609-treated mice. The reduced IL-6 release in D609-treated mice may further ameliorate the inflammatory response to LPS (e.g., induction of acute phase proteins), although IL-6 per se does not seem to be a major pathogenic factor for endotoxin lethality (Shalaby et al., 1991). However, IL-6 is a better marker of lethality than TNF-α in endotoxin-treated mice (Kelly and Cross, 1992). In parallel to Staphylococcus enterotoxin, LPS-induced release of TNF-α was not altered by D609 pretreatment in mouse endotoxin shock. These in vivo findings are in agreement with the LPS-stimulated cytokine response that we observed in J774 macrophage-like cells and mouse peritoneal macrophages, showing a decrease of the IL-1β and IL-6 response, and even an increase in TNF-α after pretreatment with D609 (manuscript in preparation). At present, it is not clarified why in mice inhibition of PC-PLC can strongly suppress the LPS-induced release of IL-1β and IL-6 but not the secretion of TNF-α. Although there was no effect on LPS-stimulated release of TNF-α in this study, D609 has previously been shown to block the activity of TNF-α on its target cells (Machleidt et al., 1996). PC-PLC inhibition by D609 protected mice from cytotoxic and lethal effects of TNF-α by blocking the signal transduction of the p55 TNF-α receptor. In addition, PC-PLC has been demonstrated to interfere with the signal transduction of IL-1 and IFN-γ (Schütze and Krönke, 1994). Therefore, D609 can provide protection from endo-
toxin shock not only by suppression of the IL-1β and IL-6 release but also by neutralizing the biologic activity of already released cytokines by interference with their signal transduction in effector cells.

Furthermore, we found that D609 has a strong impact on the LPS-induced production of NO, measured as the stable metabolites nitrite and nitrate in the serum at 9 hr after LPS-injection. Because no increased nitrite/nitrate serum levels were detected at 1.5 hr after LPS injection, this finding is unlikely to account for the NO production observed at 9 hr after LPS. As we have previously shown in J774 murine macrophages, the inhibitory effect of D609 on LPS-stimulated NO production is not due to inhibition of the enzymatic activity of iNOS but due to the suppression of the de novo synthesis of iNOS activity (Tschaikowsky et al., 1994). Here we provided evidence that D609 dose-dependently inhibits expression of iNOS protein, showing complete inhibition at 40 μg/ml. Our data, therefore, confirm a direct effect of D609 on LPS-stimulated generation of iNOS protein. However, inhibition of other mediators by D609 could additionally have contributed to the inhibitory effect of D609 on iNOS expression and the nitrite/nitrate production observed in vivo.

NO, which is massively generated from iNOS in endothelial and smooth vascular muscle cells several hours after a challenge with LPS, is known as an important pathogenic mediator of endotoxin shock (Kilbourn et al., 1990). In our model, lethality due to endotoxin shock occurred later than 12 hr after LPS injection, at a time when markedly increased nitrite/nitrate serum levels were observed due to an extensive generation of NO. We, therefore, assume that in addition to the suppression of the IL-1β and IL-6 release, inhibition of NO synthesis by D609 substantially contributes to the protective effect of D609 observed in endotoxin shock.

Beside the inhibitory effect of D609 on cytokine and NO production as well as on TNF-α activity, there may be further pathogenic mechanisms with which D609 is interfering. For example, D609 dose-dependently prevents TNF-α-induced vascular cell adhesion molecule-1 expression in HUVECs (Weber et al., 1995). D609 has also been shown to interfere with the LPS-induced activation of Raf-1 and mitogen-activated protein kinases, which, in turn, are thought to play an important role in the inflammatory response to LPS (Han et al., 1994; Buscher et al., 1995; Cuenda et al., 1995).

In conclusion, our findings imply PC-PLC as an important mediator of the pathogenicity of LPS by modulating both cytokine response and NO production. D609 may be a useful tool for studying the role of PC-PLC in inflammatory diseases. Further studies are warranted to evaluate whether specific inhibitors of PC-PLC may serve as a novel approach to therapy of sepsis.

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References

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