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ENDOTOXIN ANTAGONISM BY A SYNTHETIC LIPID A ANALOGUE, DT-5461, WITH LOW ENDOTOXICITY IN HUMAN PERIPHERAL BLOOD MONOCYTES

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We examined the molecular mechanism of DT-5461-induced LPS antagonism in human peripheral blood monocytes. Dose-response studies revealed that LPS-induced IL-1 and TNF-α production was apparently totally suppressed in a competitive manner by a 10-fold excess of DT-5461. A 10-fold excess of DT-5461 significantly blocked the binding of FITC-LPS to the monocytes. DT-5461 suppressed IL-1 and TNF-α mRNA expression in LPS-activated monocytes. Western blots showed that DT-5461 suppressed the LPS-induced tyrosine phosphorylation of $p42^{mapk}$/ERK2. These results suggested that the competitive binding inhibition and repression of early intracellular signaling involved in DT-5461-mediated LPS antagonism.

LPS, a major constituent of the outer membrane of gram-negative bacteria, has been implicated as a major factor in the pathogenesis of endotoxemia and a lipid A

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Abbreviations: IL-1, interleukin-1; TNF-α, tumor necrosis factor-α; LBP, LPS-binding protein; FITC-LPS, FITC-conjugated LPS; HRP, horseradish peroxidase; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; HAB, human serum type AB; PE, phycoerythrin; M.F.I., mean fluorescence intensity.

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component represents the active center responsible for most of the pathophysiological effects of LPS (1). The interaction of LPS with cells, especially monocytes/macrophages, causes cellular activation with the subsequent synthesis and secretion of numerous endogenous inflammatory mediators such as IL-1 and TNF-α (1, 2). These mediators are majors effectors in the pathogenesis of septic shock and bacterially mediated local tissue destruction (1, 2). CD14 has been primarily shown to function as a receptor for a complex of LPS and LBP (3). LPS binding to CD14 causes the rapid tyrosine phosphorylation of several intracellular proteins, and this process is essential to LPS-induced cytokine production in monocytes/macrophages (4, 5, 6, 7).

Several lipid A-like compounds act as LPS antagonists in various human and murine cellular systems and reduce LPS-induced lethality in some animal models (8, 9, 10). We showed that a synthetic lipid A analogue, DT-5461, specifically antagonized the LPS activation of monocytes/macrophages to produce IL-1 and TNF-α, and LPS antagonism by DT-5461 conferred protection from LPS-induced lethality in a murine endotoxemia model (11).

In this study we assessed the molecular mechanism involved in LPS antagonism by DT-5461 in human peripheral blood monocytes.

MATERIALS AND METHODS

Reagents. LPS from *E. coli* 0127:B8 and FITC-LPS were purchased from Difco Laboratories (Detroit, MI, U.S.A.). DT-5461 (11) were provided by Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan). HRP coupled-recombinant anti-phosphotyrosine mAb (clone RC-20) was purchased from Transduction Laboratories (Lexington, Kentucky, U.S.A.). Anti-MAPK (ERK2) was purchased from UBI (New York, NY, U.S.A.).

Cells. Human peripheral blood monocytes were separated from leukocyte concentrates from healthy donors by density gradient centrifugation with Ficoll-Hypaque as described preivously (11). The monocytes (1 x 10^6/well) were cultured for 24 h at 37 °C with or without LPS (1 μg/ml) in the presence or absence of DT-5461 (0.1-10 μg/ml) in 1 ml of RPMI-1640 medium containing 7.5 % HAB. Cell-free supernatants were obtained as described previously (11), then stored at -20 °C before use.

Cytokine assay. IL-1 or TNF-α activity in the culture supernatants were measured by bioassay as described previously (11).

Competitive binding assay. The monocytes (1 x 10^6) were mixed with 1 μg

/ml of FITC-LPS and various concentrations of DT-5461 (1-100 μg/ml) in 0.2 ml of PBS in the presence or absence of 7.5 % HAB, and incubated for 30 min at 37 °C. Binding of FITC-LPS to the cells was detected as described previously (11) using a FACS flow cytometer (FACScan; Becton Dickinson Electric Laboratories, Mountain View, CA) and CEllQuest Software.

Nothern blotting. Human peripheral blood monocytes (5 x 10^6/well) were stimulated with or without LPS (1 μg/ml) in the presence or absence of DT-5461 (10 μg/ml) for 4 h at 37 °C. The procedure for the preparation of Nothern blots and the probes used in the detection of human IL-1β, TNF-α and β-actin mRNA species have been described previously (12, 13). β-actin was included as an housekeeping gene to control for RNA loading between lanes, as described previously (12).

Immunoprecipitation and Western blotting. The monocytes (5 x 10^6/well) were stimulated with or without LPS (1 μg/ml) in the presence or absence of DT-5461 (10 μg/ml) for 20 min at 37 °C. Cell lysis and immunoprecipitation with anti-MAPK performed exactly as described previously (6). Immunoblotting was carried with HRP-coupled anti-phosphotyrosine mAb (clone RC-20) according to Ref. 7. The immunoreactive proteins were visualized using an ECL detection system (Amersham International, Buckinghamshire, U.K.).

RESULTS

We showed that DT-5461 exhibited low LPS agonistic activity towards IL-1 and TNF-α release but antagonized LPS-mediated increases in this responses in human peripheral blood monocytes (11). We examined the the dose response relation of the antagonistic effect of DT-5461 on LPS-induced these cytokines secretion (Fig. 1). DT-5461 significantly inhibited LPS-induced IL-1 and TNF-α release in a concentration-dependent manner. These results indicated that complete antagonism on LPS-induced IL-1 and TNF-α production were established at a 10-fold excess of DT-5461. We also examined effect of DT-5461 on LPS-induced IL-1β and TNF-α mRNA expression. Figure 2 showes that incubating the monocytes with DT-5461 significantly suppressed the expression of LPS-induced IL-1β and TNF-α mRNA to the control levels.

We investigated the competitive inhibition of DT-5461 on FITC-LPS binding to the monocytes (Table 1). Flow cytometry showed that DT-5461 reduced the FITC-LPS binding to the monocytes in serum-free condition in a concentration dependent manner. The increasingly high concentrations of DT-5461 also caused a

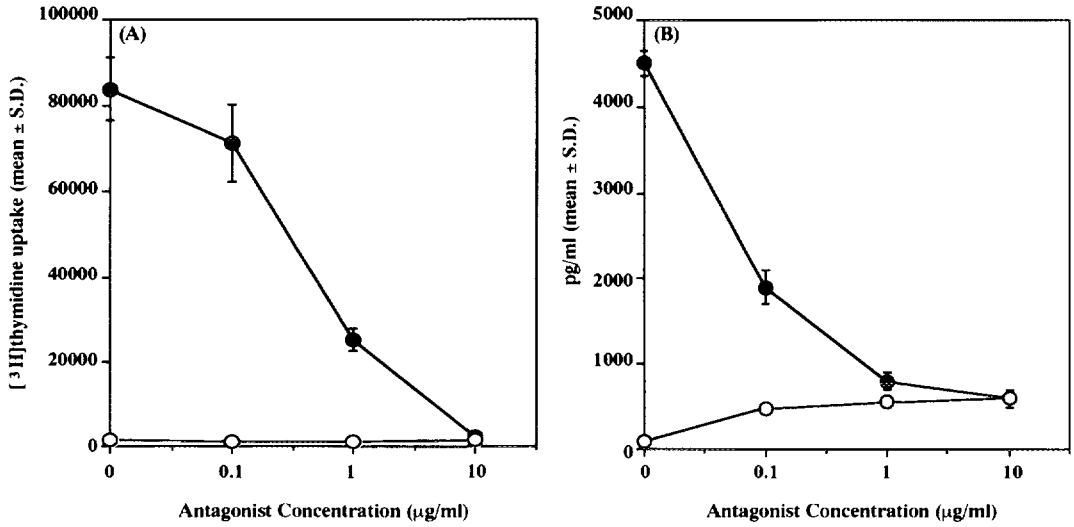

Figure 1. Effect of DT-5461 on LPS-induced production of IL-1 and TNF-α in human peripheral blood monocytes. Human peripheral blood monocytes (1 x 10^6/well) were cultured with or without increasing concentrations of DT-5461 (open circle) or the mixture of DT-5461 and 1 μg/ml LPS (closed circle) for 24 h at 37 °C. The supernatants were collected and assayed for IL-1 (A) or TNF-α (B).

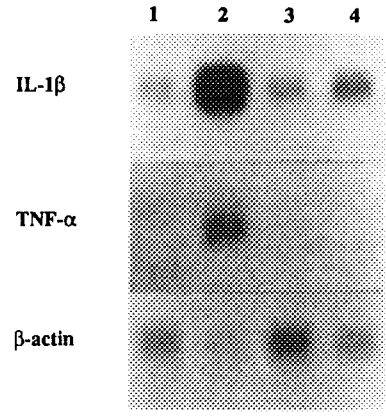

Figure 2. Effect of DT-5461 on LPS-induced IL-1β and TNF-α mRNA expression in human peripheral blood monocytes. Human peripheral blood monocytes (5 x 10^6/well) were cultured with medium alone (lane 1), 1 μg/ml LPS (lane 2), 10 μg/ml DT-5461 (lane 3) or a mixture of LPS and DT-5461 (lane 4) for 4 h at 37 °C. The cells were then lysed, and total RNA extracts prepared. Equivalent amounts of RNA (3 μg/lane) were size fractionated by denaturing agarose gel electrophoresis, then blotted onto Nytran membranes. The blots were then sequentially hybridized with radiolabeled cDNA probes specific for human IL-1β, TNF-α, and β-actin.

Table 1. Influence of DT-5461 on the binding of FITC-LPS to human peripheral blood monocytes

Treatment	Competitor	Concentration (μg/ml)	M.F.I. without serum	M.F.I. with serum
Untreated	---	---	4.80	5.29
FITC-LPS	---	---	17.80	25.12
	DT-5461	1	15.51 (18 %)	20.50 (26 %)
		10	13.60 (32 %)	16.40 (43 %)
		100	8.38 (72 %)	9.49 (79 %)

Thioglycollate-elicited murine peritoneal macrophages (1 x 10^6) were cultured with or without FITC-LPS (1 μg/ml) and the indicated concentrations of unlabeled DT-5461 in the presence or absence of 7.5 % HAB for 30 min at 37 °C. The binding of FITC-LPS to murine peritoneal macrophages was measured by FACS analysis.
The values in parentheses represent % inhibition compared with FITC-LPS treated cells.

marked reduction of the FITC-LPS binding although the binding of FITC-LPS to the cells were significantly enhanced in serum condition.

We examined the role of protein tyrosine phosphorylation in DT-5461-induced antagonism. LPS increased the tyrosine phosphorylation of protein with apparent molecular mass of 42 kDa in monocytes when compared with that of the normal cells or DT-5461-treated cells, and incubating monocytes with DT-5461 repressed increased protein tyrosine phosphorylation in LPS-treated cells (data not shown). We also found that $p42^{mapk}$/ERK2 was phosphorylated on tyrosine in monocytes incubated with LPS (Fig.3), indicating that LPS-induced tyrosine phosphoproteins of 42 kDa is $p42^{mapk}$/ERK2. DT-5461 repressed the LPS-induced tyrosine phosphorylation of $p42^{mapk}$/ERK2 in the monocyte (Fig.3).

DISCUSSION

The results reported here demonstrated the molecular mechanism of DT-5461-mediated LPS antagonism in human peripheral blood monocytes. We showed that IL-1 and TNF-α production were inhibited by DT-5461 not only at the level of IL-1 and TNF-α synthesis, but also at the level of mRNA for IL-1β and TNF-α (Fig.1 and 2). These findings indicated that the antagonistic effect of DT-5461 on LPS-induced cytokine production is regulated at the transcriptional level.

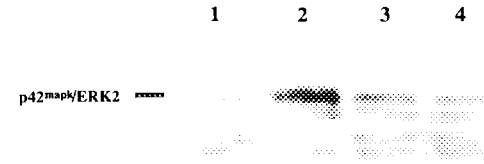

Figure 3. DT-5461 inhibits LPS-induced tyrosine phosphorylation of $p42^{mapk}$/ERK2 in human peripheral blood monocytes. Human peripheral blood monocytes (5×10^6/well) were cultured with medium alone (lane 1), 1 μg/ml LPS (lane 2), 10 μg/ml DT-5461 (lane 3) or a mixture of LPS and DT-5461 (lane 4) for 20 min at 37 °C. The cell lysates were immunoprecipitated with anti-MAPK. The immunoprecipitated fractions were immunoblotted with HRP-conjugated anti-phosphotyrosine mAb, and the tyrosine phosphoproteins were visualized by ECL.

The antagonistic effect of lipid A-related compounds is associated with prevention of the LPS-CD14 interaction by competing with LPS for binding of either LBP or CD14 (9, 10, 12). We showed that the binding of FITC-LPS to the monocytes was suppressed by DT-5461 not only in serum-free but in serum condition. On the other hand, PE-labeled anti-human CD14 mAb binding to the monocytes was significantly inhibited by DT-5461 (data not shown), indicating that the binding of DT-5461 as well as LPS to monocytes is mediated by CD14. These findings support the notion that the LPS antagonism of DT-5461 is partly due to competion with LPS for binding LBP and/or cellular target molecules, including CD14.

The functional antagonism may not be strictly correlated with competition of deacylated LPS or lipid A-related compounds with LPS for cell-surface binding (10). We found that DT-5461 completely antagonized LPS-induced cytokine release when LPS binding was prevented by approximately 40 % (Table 1). The suppression of LPS activity indicated that receptor antagonism cannot explain the mechanism of DT-5461-mediated LPS antagonism. Early intracellular signaling involved in lipid A-based compounds-mediated endotoxin antagonism has not been still clarified. We found that incubating monocytes with DT-5461 prevented the LPS-induced tyrosine phosphorylation of $p42^{mapk}$/ERK2 (Fig. 3). Novogrodsky *et al.* (14) have reported that the tyrphostin AG 126 family suppressed the LPS-induced production of TNF-α in murine peritoneal macrophages, and that this suppression correlates with the ability of these agents to block LPS-induced protein tyrosine phosphorylation. DT-5461-mediated repression of LPS-induced

signal transduction pathway, including MAPK cascade, leading to subsequent gene expression is partially responsible for endotoxin antagonism. The mechanism by which DT-5461 suppressed the LPS-induced tyrosine phosphorylation of $p42^{mapk}$/ERK2 remains unclear. The signal transduction elicited by ligating DT-5461 to an appropriate cellular receptor including CD14 may negatively regulate the LPS-elicited intracellular signaling, such as MAPK cascade. Further study is required to examine this possibility.

The present study regarding the molecular mechanism of endotoxin antagonism should deepen the understanding of LPS-elicited signal transduction pathways and the potential use of lipid A-based LPS antagonists in the therapy of endotoxemia and septicemia.

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REFERENCES

1. Morison, D. C., and Ryan, J. L. (1987) Annu. Rev. Med. 38, 417-432.
2. Billiau, A., and Vandekerckhove, F. (1991) Eur. J. Clin. Invest. 21, 559-573.
3. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990) Science. 249, 1431-1433.
4. Stefanova, I., Corcoran, M. L., Horak, E. M., Wahl, L. M., Bolen, J. B., and Horak, I. D. (1993) J. Biol. Chem. 268, 20725-20728.
5. Shapira, L., Takashiba, S., Champagne, C., Amar, S., and Dyke, T. E. 1994. J. Immunol. 153, 1818-1824.
6. Weinstein, S. L., Sanghera, J. S., Lemke, K., Defranco, A. L., and Pelech, S. L. (1992) J. Biol. Chem. 267, 14955-14962.
7. Dong, Z., Qi, X., and Fidler, I. J. (1993) J. Exp. Med. 177, 1071-1077.

8. Ulmer, J. A., Werner, F., Heine, H., Kirikae, T., Kirikae, F., Kusumoto, S., Kusama, T., Brade, H., Schade, U., Rietschel, E. T., and Flad, H-D. (1992) Infect. Immun. 60, 5145-5152.
9. Christ, J. W., Asano, O, Robidoux, A. L. C., Peter, M., Wang, Y., Dubuc, G. R., Gavin, W. E., Hawkins, L. D., McGuinness, P. D., Mullarkey, M. A., Lewis, M. D., KIshi, Y., Kawata,T., Bristol, J. R., Rose, J. R., Rossignol, D. P., Kobayashi, S., Hishinuma, I., Kimura, A., Asanuma, N., Katayama, K., and Yamatsu., I. (1995) Science 268, 80-83.
10. Kitchens, L. R., and Munford, R. S. (1995) J. Biol. Chem. 270, 9904-9910.
11. Sato, K., Yoo, Y. C., Fukushima, A., Saiki, I., Takahashi, T. A., Fujihara, M., Tono-oka, S., and Azuma. I. (1995) Infect. Immun. 63, 2859-2866.
12. Fujihara, M., Ito, N., Pace, J. L., Watanabe, Y., Russell, S. W., and Suzuki, T. (1994) J. Immunol. 269, 12733-12778.
13. Huang, J. J., Newton, R. C., Rutledge, S. J., Horuk, R., Matthew, J. B., Covington, M., and Lin, Y. (1988) J. Immunol. 140, 3838-3843.
14. Novogrodsky, A., Vanichkin, A., Patya, M., Gazit, A., Osherov, N., and Levitzki, A. (1994) Science 264, 1319-1322.