Volume 256, number 1,2, 155–158 FEB 07691 October 1989

Lipopolysaccharide toxin can directly stimulate the intracellular accumulation of lipids and their secretion into medium in the primary culture of rabbit hepatocytes

A.V. Victorov, E.M. Gladkaya, D.K. Novikov*, V.A. Kosykh* and V.A. Yurkiv

Central Research Institute of Epidemiology, USSR Ministry of Public Health, Moscow and **USSR Cardiology Research Center, USSR Academy of Medical Sciences, Moscow, USSR*

Received 27 July 1989

Low doses (0.1–0.3 μg/ml per 10^6 cells) of the lipopolysaccharide toxin (LPS) from *Salmonella typhimurium* were shown to increase (after an 18 h incubation) the intracellular content of free cholesterol (CH), esterified cholesterol (EC) and triglycerides (TG) by 30–40% in the primary culture of rabbit hepatocytes. A similar increase was found for the incorporation of [^{14}C]acetate into these lipids. The concentration of lipids in cultural medium, under these conditions, was also augmented: by 30–40% for CH; by 50–60% for TG and by 60–80% for EC. Higher doses (up to 50 μg/ml) of LPS hardly affected the lipid content in hepatocytes but strongly (by two-fold) inhibited the secretion of lipids. It is suggested that in vivo low concentrations of LPS in bloodstream (in the absence of conspicuous pathology) might induce hyperlipidemia directly influencing on hepatic cells, while, under the higher concentrations of LPS, hyperlipidemia caused by cachectin (or tumor necrosis factor) is probably observed.

Lipopolysaccharide toxin; Hyperlipidemia; (Rabbit hepatocyte)

1. INTRODUCTION

Infection of mammals with Gram-negative bacteria or their lipopolysaccharide toxins (LPS) leads to a noticeable increase in the lipid content in blood plasma, the amount of β-migrating very low density lipoproteins being augmented above all [1,2]. Though the biological grounds for such hyperlipidemia are not presently cleared up, many investigators assume that it does not arise as a direct consequence of the LPS appearance in plasma but rather is due to the action of cachectin (or TNF), a protein which is secreted by macrophages in large quantities in response to LPS [3]. Thus, this protein can increase several-fold the level of TG in plasma of rats which is found to ensue from the stimulation of the biosynthesis and secretion of TG by the liver cells [4,5]. Since the attention of researchers has been basically focused on studying the TNF effects, the direct influence of LPS on the production and secretion of lipids by hepatocytes remains still underinvestigated. It is well known that, on the one hand, liver is the central organ governing the lipid metabolism, but, on the other hand, it greatly contributes to the elimination of various harmful substances from the blood. Despite the fact that in the bloodstream LPS is primarily being attacked by monocytes/macrophages [6], the pronounced quantities of toxin are capable of binding to hepatocytes [7,8]. Application of the primary culture of hepatocytes permits one to elucidate the

Correspondence address: A.V. Victorov, Central Research Institute of Epidemiology, Novogireevskaya str. 3a, Moscow 111123, USSR

Abbreviations: LPS, lipopolysaccharide toxin; NL, neutral lipids; CH, free cholesterol; EC, esterified cholesterol; TG, triglycerides; PL, phospholipids; TNF, tumor necrosis factor; HPTLC, high-performance thin-layer chromatography; HPLC, high-performance liquid chromatography

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

direct effects of LPS on the liver cells ruling out the influence of other factors, e.g. TNF.

Here, we report the ability of low concentrations of LPS to enhance in the primary culture of rabbit hepatocytes both the accumulation of lipids in the cell and their secretion into the outer medium.

2. MATERIALS AND METHODS

Lipopolysaccharide toxin B of *Salmonella typhimurium* was supplied by Difco Laboratories (USA). All lipid standards were purchased from Sigma Chemical Co. (USA). Minimal essential medium with Earle's salts, heat-inactivated fetal calf serum, kanamycin, L-glutamine, trypsin-EDTA, trypan blue and 35-mm culture dishes were the products of Flow Laboratories (USA). Collagenase type IV (125–200 U/mg) was supplied by Sigma Chemical Co. Dispase II (0.5 U/mg) was purchased from Boehringer (FRG). [1-^{14}C]Acetic acid, sodium salt (spec. act. 59 Ci/mol) was supplied by Amersham Corporation (UK). HPTLC plates, Silica Gel 60 (Merck, FRG) were used for lipid analyses. Solvents for HPLC were products of Burdick (USA).

The primary culture of rabbit hepatocytes was prepared and maintained as described in detail elsewhere [9]. Before any experiments were started, hepatocytes were cultured for 36 h under standard conditions [9]. Throughout all the experiments more than 85% of cells excluded trypan blue dye, i.e. remained intact. For each experimental point, approx. 8×10^5 cells were taken. LPS was administered into the cultural medium as an aqueous dispersion at final concentration of 0.1–50.0 μg/ml medium per 10^6 cells. For determination of the de novo lipid biosynthesis, [^{14}C]acetate (5 μCi/ml) was added into the medium. Incubation in all cases lasted for 18 h. Then lipids were extracted and analyzed by HPTLC as reported earlier [9]. Briefly, PL were separated in a methyl acetate/*n*-propanol/chloroform/methanol/0.25% KCl (25:25:28:10:7, v/v) system [10]; for measuring NL, a hexane/diethyl ether/acetic acid (15:2:0.1, v/v) system was employed. Application of lipid samples on the HPTLC plate was performed with a special device, Linomat III (Camag, Switzerland), allowing the administration of an exact volume of solution (± 0.1 μl) on the narrow band (<1 mm). PL were also analyzed by HPLC using a DuPont chromatograph with an analytical Zorbax-SIL (5 μm, 250 × 4.6 mm) column and following the procedure described in [11]. Radioactivity was measured with a liquid scintillation counter. Concentrations of PL were determined by phosphorus analysis [12]. Concentrations of NL (and PL) were measured by quantitative densitometry [13] using an HPTLC scanner (Camag) connected with a registrating integrator SP-4100 (Spectra Physics, FRG). Protein was determined according to the method of Lowry [14]. Student's *t*-test was employed for statistical estimates.

3. RESULTS AND DISCUSSION

Data of table 1 demonstrate that incubation of hepatocytes with low concentrations (0.10–0.25 μg/ml per 10^6 cells) of LPS produces a significant increase (by 30–40%) in the level of all major cellular NL, practically no differences between EC, CH and TG being observed. Incorporation of [^{14}C]acetate label characterizing the de novo lipid biosynthesis shows an analogous rise for these cellular NL. When the LPS concentration in the medium amounts to 10–50 μg/ml, the intracellular content of CH, EH and TG returns to normal.

Similar, but less expressed, changes are found for hepatocyte PL (table 2). Namely, the intracellular concentrations of sphingomyelin, phosphatidylcholine and phosphatidylethanolamine, under the action of low doses of LPS, augment by ~20%, while the level of minor negatively charged components (phosphatidylserine, phosphatidylinositol and 'phosphatidic acid + car-

Table 1

Content of major neutral lipids and incorporation of [^{14}C]acetate into NL in rabbit hepatocytes after an 18 h incubation with LPS

LPS (μg/ml per 10^6 cells)	CH		TG		EC	
	μg	cpm × 10^{-3}	μg	cpm × 10^{-3}	μg	cpm × 10^{-3}
0	29.7 ± 3.1	151 ± 17	45.3 ± 5.1	511 ± 48	47.5 ± 5.3	13.1 ± 1.4
0.10	42.5 ± 5.4[a]	222 ± 34[a]	61.6 ± 7.1[a]	807 ± 97[b]	66.5 ± 7.4[a]	20.7 ± 3.0[b]
0.25	40.7 ± 6.1[a]	237 ± 41[a]	57.2 ± 5.0[a]	726 ± 85[b]	61.8 ± 7.7[a]	18.8 ± 2.8[a]
2.0	34.7 ± 4.1	183 ± 22	52.5 ± 6.3	583 ± 65	57.2 ± 6.5	17.7 ± 2.0[a]
10.0	31.5 ± 3.9	143 ± 18	47.1 ± 5.5	491 ± 62	54.4 ± 6.0	14.5 ± 2.1
50.0	32.1 ± 3.5	147 ± 21	46.8 ± 5.3	519 ± 57	55.6 ± 6.2	15.2 ± 2.2

[a] $p \leqslant 0.05$
[b] $p \leqslant 0.01$

All data are given per 1 mg of cellular protein. Presented mean values ± SD for 4–5 experiments

Table 2

Content of phospholipids in rabbit hepatocytes after an 18 h incubation with LPS

LPS (μg/ml per 10^6 cells)	PL					
	SM[a] (μg)	PC[a] (μg)	PS[a] (μg)	PI[a] (μg)	PA+CL[a] (μg)	PE[a] (μg)
0	16.8 ± 1.1	60.5 ± 3.8	8.5 ± 0.7	11.8 ± 1.1	7.3 ± 0.6	37.5 ± 3.1
0.10	20.0 ± 1.6[b]	70.6 ± 5.7[b]	12.1 ± 1.1[c]	17.1 ± 2.0[b]	9.2 ± 0.7[b]	44.8 ± 4.0[b]
0.25	21.1 ± 1.8[b]	68.6 ± 6.1	11.7 ± 1.2[b]	17.2 ± 1.6[c]	9.5 ± 1.0[b]	46.6 ± 4.9[b]
2.0	17.8 ± 1.7	64.2 ± 6.7	7.9 ± 0.8	12.9 ± 1.5	8.7 ± 0.9	39.5 ± 5.0
10.0	15.4 ± 1.5	56.2 ± 5.0	7.4 ± 0.7	12.2 ± 1.3	7.9 ± 0.7	34.2 ± 4.1

[a] SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; CL, cardiolipin; PE, phosphatidylethanolamine
[b] $p \leq 0.05$
[c] $p \leq 0.01$

All data are given per 1 mg of cellular protein. Presented mean values ± SD for 3–4 experiments

diolipin' fraction) increases by 30–40%. At higher doses of LPS, the intracellular content of all PL falls down to control of even lower values. Incorporation of [^{14}C]acetate into cellular PL experiences the same, as for NL, alterations depending on the LPS dose in the outer medium (data not shown). This fact evidences that an increase in the content of PL in the cell is caused by their enhanced de novo biosynthesis.

Further investigation has shown that LPS is capable not only of inducing the accumulation of lipids in the cell but also of stimulating their secretion into cultural medium. As one can see from table 3, low doses of LPS (0.10–0.25 μg/ml) markedly increase the amount of NL detected in the medium, i.e. the content of CH, TG and EC rises by 30–40%, 50–60% and 60–80%, respectively. The very high concentration of LPS (50 μg/ml) strongly reduces (by two-fold) the level of NL in the cultural medium. Since, at such a dose of toxin, the NL content in the cell keeps close to normal, we suppose that it is the lipid secretion that is inhibited by LPS (via suppressing the biosynthesis either of apolipoproteins or NL themselves). It is noteworthy that, in the present work, we have measured the concentrations of NL and PL in cells and medium only after an 18 h incubation with LPS. Though the amount of lipids being secreted in the primary culture of hepatocytes during this period is known to grow monotonously [15], one cannot exclude the possibility that that kinetics might be changed in the presence of LPS.

Obviously, the increase in intracellular content of lipids (attributable to their de novo synthesis) requires the activation of the appropriate enzyme systems. LPS can reportedly elicit in hepatocytes various effects. Thus, interacting with the plasma membrane of the cell, toxin partly destabilizes its organization and alters a microviscosity of the lipid bilayer [8]; it can also perturb the hormone-receptor binding, stimulate the activity of endogenous phospholipase A_2 and aspartate-transaminase; it affects the activity of cytochrome P-450, the synthesis of albumin and numerous proteins appearing in the acute phase of the disease ([16,17] and references cited therein). Moreover, some authors have shown that under the endotoxic

Table 3

Content of major neutral lipids in medium of the primary culture of rabbit hepatocytes after an 18 h incubation with LPS

LPS (μg/ml per 10^6 cells)	CH[a] (μg)	TG[a] (μg)	EC[a] (μg)
0	2.8 ± 0.3	16.8 ± 1.8	4.1 ± 0.5
0.10	3.7 ± 0.4[b]	25.5 ± 3.6[b]	6.7 ± 0.8[b]
0.25	3.8 ± 0.5[b]	24.7 ± 3.3[b]	7.0 ± 0.9[b]
2.0	3.3 ± 0.4	21.8 ± 2.9	5.9 ± 0.7[b]
10.0	2.5 ± 0.3	17.1 ± 2.5	5.1 ± 0.6
50.0	1.3 ± 0.3[b]	7.3 ± 1.4[c]	2.9 ± 0.4[b]

[a] Similar changes were found for ^{14}C-biosynthetically labeled lipid
[b] $p \leq 0.05$
[c] $p \leq 0.01$

Data are given per 1 mg of cellular protein. Presented mean values ± SD for 3–4 experiments

shock caused by LPS, the biosynthesis of many eicosanoids becomes different [18]. We suggest that one of the first events triggering such an 'activation' of hepatocytes might be the LPS-inducible hydrolysis of polyphosphoinositides resulting in the formation of second messengers: inositol phosphates and diacylglycerols. This effect of LPS was already ascertained for other cells, e.g. macrophages [6], B-lymphocytes [19] and blood platelets [20].

Considering hyperlipidemia observed in vivo in the course of LPS infection, one can hypothesize that in the acute period of the disease (at high concentrations of LPS) this phenomenon is likely due to cachectin (or TNF) [3] secreted in this case in large amounts. However, in the absence of conspicuous pathology (i.e. at low concentrations of LPS) when macrophages release negligible quantities of cachectin, hyperlipidemia attributable to the direct action of LPS on the liver cells might occur. Certainly, the magnitude of the effect produced by LPS itself greatly yields to that of cachectin (TNF) [4,5], but, keeping in mind the probably prolonged periods for which low doses of toxin are capable of exerting their influence on hepatic cells, it seems reasonable to propose that it may have far-reaching consequences.

REFERENCES

[1] Rouzer, C.A. and Cerami, A. (1980) Mol. Biochem. Parasitol. 23, 31–38.
[2] Kawakami, M. and Cerami, A. (1981) J. Exp. Med. 154, 631–639.
[3] Beutler, B. and Cerami, A. (1988) Biochemistry 27, 7574–7582.
[4] Chajek-Shaul, T., Friedman, G., Stein, O., Shiloni, E., Etienne, J. and Stein, Y. (1989) Biochim. Biophys. Acta 1001, 316–324.
[5] Feingold, K.R. and Grunfeld, C. (1987) J. Clin. Invest. 80, 184–190.
[6] Hampton, R.Y., Golenbock, D.T. and Raetz, C.R.H. (1988) J. Biol. Chem. 263, 14802–14807.
[7] Pagani, R., Portoles, M.T. and Municio, A.M. (1981) FEBS Lett. 131, 103–107.
[8] Portoles, M.T., Pagani, R., Diaz-Laviada, I. and Municio, A.M. (1987) Cell Biochem. Funct. 5, 54–61.
[9] Kosykh, V.A., Lankin, V.Z., Podrez, E.A., Novikov, D.K., Volgushev, S.A., Victorov, A.V., Repin, V.S. and Smirnov, V.N. (1989) Lipids 24, 109–115.
[10] Vitiello, F. and Zannetta, J.P. (1978) J. Chromatogr. 166, 637–640.
[11] Geurts van Kessel, W.S.M., Hax, W.M.A., Demel, R.A. and De Gier, J. (1977) Biochim. Biophys. Acta 486, 524–530.
[12] Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468.
[13] Schmidtz, G., Assman, G. and Bowyer, D.E. (1984) J. Chromatogr. 307, 65–80.
[14] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[15] Kosykh, V.A., Podrez, E.A., Novikov, D.K., Victorov, A.V., Dolbin, A.G., Repin, V.S. and Smirnov, V.N. (1987) Atherosclerosis 68, 67–76.
[16] Tobias, P.S., Mathison, J.C. and Ulevitch, R.J. (1988) J. Biol. Chem. 263, 13479–13481.
[17] Pagani, R., Portoles, M.T., Bosch, M.A., Diaz-Laviada, I. and Municio, A.M. (1987) Eur. J. Cell Biol. 43, 243–246.
[18] Toth, P.D., Hamburger, S.A. and Judy, U.V. (1984) Circ. Shock 12, 277–286.
[19] Rosoff, P.M. and Cantley, L.C. (1985) J. Biol. Chem. 260, 9209–9215.
[20] Viktorov, A.V., Dank, E.K., Kuznetsov, V.A., Ter-Simonyan, V.G. and Yurkiv, V.A. (1988) Bull. Exp. Biol. Med.-Engl. Tr. 105, 339–342.