BIOPHYSICS AND BIOCHEMISTRY

In Vitro Accumulation of Complexes of Endotoxin and Low-Density Lipoproteins by Macrophages and Arterial Wall

Y. Sh. Schwartz and M. I. Dushkin

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 147, No. 2, pp. 148-151, February, 2009
Original article submitted January 28, 2008

Binding and uptake of complexes of endotoxin and low-density lipoproteins (LPS—LDL) in the arterial wall and mononuclear phagocytes were studied under *in vitro* conditions. Incubation of aortic explants from Wistar rats with complexes of ^{125}I—LDL and *S. minnesota* R595 LPS or ^{125}I--LDL was accompanied by a 6-fold increase in binding (0°C) and 2-fold increase in the uptake (37°C) of LDL—LPS complexes as compared to free LDL. Binding and degradation of ^{125}I—LDL—LPS complexes in the culture of peritoneal macrophages were higher compared to the corresponding parameters for free ^{125}I—LDL. Our results suggest that the formation of LDL—LPS complexes is followed by the increased binding and accumulation of LDL in the arterial wall and macrophages. These changes probably induce the cascade of major atherogenic events in the vascular wall.

Key Words: *bacterial lipopolysaccharides; low-density lipoproteins; LPS--LDL complexes; atherosclerosis*

Systemic endotoxemia (*i.e.*, presence of bacterial lipopolysaccharides (LPS, endotoxins) from gram-negative microorganisms in the circulation) is a common disorder. This process is associated with acute and chronic infections (including latent infections), dysfunction of the intestinal barrier, bacterial translocation, use of antibiotics (particularly of β-lactam antibiotics), gastrointestinal diseases, diffuse liver diseases, traumas, burns, hemorrhages, surgeries, *etc*. [1]. Published data show that 60-80% of circulating LPS are bound to blood proteins. It should be emphasized that up to 50% of these LPS are bound to low-density lipoproteins (LDL) [2,4,6].

Institute of Therapy, Siberian Division of the Russian Academy of Medical Sciences, Novosibirsk, Russia. ***Address for correspondence:*** yshschwartz@mail.ru. Y. Sh. Schwartz

Our previous studies revealed that LDL gain a negative charge during the formation of LPS—LDL complexes. Hence, LDL is modified during this process. A specific feature of modified LDL is the ability to bind to scavenge receptors (ScR) on macrophages, which results in the uncontrolled uptake of these particles and formation of foam cells. Transformation of arterial wall macrophages into foam cells is a key event of atherogenesis. However, little is known about the binding, uptake, and degradation of LPS-modified LDL in macrophages.

LPS—LDL complexes easily enter the subendothelial space and induce the expression of monocyte chemotactic factor on the endothelium and smooth muscle cells. Experiments on the confluent monolayer of endotheliocytes showed that reuptake of acetylated or oxidatively modified LDL

from the intimal surface to the luminal surface is suppressed. It contributes to the accumulation of LDL in the subendothelial space [10]. However, little attention was paid to the binding and accumulation of LPS-modified LDL in the arterial wall. The accumulation of LPS-modified LDL in recruited monocytes and macrophages probably serves as a major mechanism for atherosclerosis.

Here we studied the binding and uptake of LPS—LDL complexes and native LDL in the arterial wall and mononuclear phagocytes.

MATERIALS AND METHODS

^{125}I—LDL—LPS complexes were obtained as follows. The fraction of LDL (floating density d= 1.019-1.063 g/ml) was isolated from an EDTA-containing (0.1%) combined sample of blood plasma of conventionally healthy volunteers by successive ultracentrifugation in KBr [13]. LDL were concentrated by means of repeated ultracentrifugation and used to obtain LDL—LPS (in combination with lipoprotein-free plasma, d>1.21 g/ml). Centrifugation was performed on a Beckman L8-M ultracentrifuge equipped with a Ti-80 angle rotor at 4°C and 105,000g for 20 h. The fraction of LDL was purified from KBr by gel filtration on a chromatographic column packed with Sephadex G-25. Protein content was measured by the method of Lowry. This fraction was radioiodinated by the iodine chloride method with modifications [3] using Na^{125}I (Amersham Pharmacia Biotech, no carrier, specific activity 100 mCi/ml). After radioiodination, trichloroacetic acid-precipitated radioactivity of labeled LDL was 98%. Specific radioactivity of ^{125}I—LDL was 25 mCi/mg LDL protein. ^{125}I—LDL and lipoprotein-free plasma were sterilized by filtration to obtain ^{125}I—LDL—LPS complexes [16]. Lipoprotein-free plasma (0.5 ml) was added to 1 ml 0.5% aqueous solution of highly purified LPS from *Salmonella minnesota* R595 (gifted by Doctor M. Freudenberg and C. Galanos, Max-Planck Institut fur Immunobiologie, Freiberg). The mixture was preincubated for 30 min. An equal volume of ^{125}I—LDL was added. Incubation was performed at 37°C for 2 h. LPS—LDL were isolated by ultracentrifugation (d=1.063) to remove unbound LPS. The amount of bound LPS was determined after incubation of 25 mg/ml ^{125}I—LPS (1 mCi/mg) with LDL at 37°C for 30 min. Lipoproteins were ultracentrifuged in a KBr density gradient (1.019-1.063 g/ml) at 105,000g for 24 h. Unbound LPS was removed by ultracentrifugation. The amount of LDL-bound LPS was calculated after radioactivity measurement in the fraction of LPS—LDL (4-5 mg LPS/mg LDL protein) [2]. An equal volume of buffer-treated physiological saline was used instead of LPS in control experiments with ^{125}I—LDL.

Binding and incorporation of ^{125}I—LDL and ^{125}I—LDL—LPS in aortic explants were studied as described elsewhere (with small modifications) [6]. Aortic explants were obtained from 20 Wistar rats. The aorta was excised from the arch to bifurcation. The samples were placed in cold Hanks solution. The adventitia was separated, cut along the axis, and divided into fragments (4-5 mm^2). Explants were incubated in triplicate using 35-mm Petri dishes with 2 ml Hanks solution and 10 mM HEPES. Incubation was performed in the presence or absence of ^{125}I—LDL and ^{125}I-LDL—LPS at various concentrations. Incubation was conducted at 5% CO_2, 100% humidity, and 0°C (binding) or 37°C (uptake) for 4 h. After incubation, the explants were washed six times with cold physiological saline and weighted. Radioactivity of the aorta was measured.

Binding and degradation of ^{125}I—LDL and ^{125}I—LDL—LPS in cultured macrophages were studied as described previously (with small modifications) [3]. Peritoneal macrophages of Wistar rats were elicited by intraperitoneal injection of 3 ml 4% starch. The cells were isolated on day 4 and preincubated in 35-mm petri dishes with DMEM medium at 37°C and 5% CO_2 for 2 h. Monolayers were washed three times and incubated in triplicate for 4 h. Incubation was performed using 2 ml RPMI-1640 medium in the presence or absence of 100 mg/ml ^{125}I—LDL or ^{125}I—LDL—LPS. Incubation was conducted in the presence or absence of 5 mg/ml dextran sulfate or 5 mg/ml *E. coli* 0111: B4 LPS. To study the binding of ^{125}I—LDL and ^{125}I—LDL—LPS, incubation was performed at 0°C. Cell monolayers were washed 10 times with 0.2% bovine serum albumin (BSA). The cells were taken with resin policemen. Radioactivity was measured. Degradation of ^{125}I—LDL and ^{125}I—LDL—LPS was studied after incubation of cells at 37°C. An increase in radioactivity in the incubation medium was recorded routinely. The medium was sampled after incubation, treated with 0.1% BSA, precipitated with an equal volume of 25% trichloroacetic acid, and centrifuged at 3000 rpm for 10 min. The supernatants were placed in glass tubes. KI (10 ml, 40%) and H_2O_2 (40 ml, 30%) were added to the supernatant (1 ml). The mixture was washed two times with a 5-fold volume of chloroform to remove free ^{125}I. Radioactivity of the aqueous phase was measured. Radioactivity of protein-unbound ^{125}I in samples was measured on a γ-counter (LKB). The cell-free incubation media with ^{125}I—LDL or ^{125}I—LDL—LPS were treated by the same method and served as a control.

The results were analyzed by standard variational methods. The arithmetic mean and standard error of the mean were calculated. The significance of differences between mean values was evaluated by Student's *t* test.

RESULTS

Binding and uptake of ^{125}I—LDL and ^{125}I—LDL—LPS in aortic tissue were studied under *in vitro* conditions. Incorporation of labeled LDL and LDL—LPS into vascular tissue depended on the temperature (Table 1). Therefore, this process was partially mediated by the energy-dependent transport mechanisms. Our results agree with published data that the incorporation of LPS—LDL into vascular tissue is not related to the impairment of endothelial integrity. Binding of free LDL particles (0°C) was 7.3-fold lower than their uptake (37°C). This ratio was lower for LDL—LPS complexes (1:2.7). Binding of LDL—LPS complexes was much greater than that of free LDL (by more than 6 times). The uptake of complexes in the aortic wall increased by 2.3 times. Increasing the concentration of LDL and LDL—LPS in the incubation medium was followed by an increase in their uptake. It should be emphasized that an increase in the uptake was proportional to the concentration of particles. The LDL/LDL—LPS uptake ratio remained unchanged at various concentrations (Fig. 1).

A sharp increase in the binding of LDL—LPS complexes (as compared to free LDL) is probably associated with a greater number of binding sites for these complexes. They may include apoB,E receptors and various ScR that have affinity for LPS and are expressed by endotheliocytes, macrophages, smooth muscle cells, fibroblasts, and other cells of vascular tissue. The increased uptake of these complexes probably results from a strong retention and accumulation in the subendothelial matrix and intimal cells, which is typical of modified LDL [10,11].

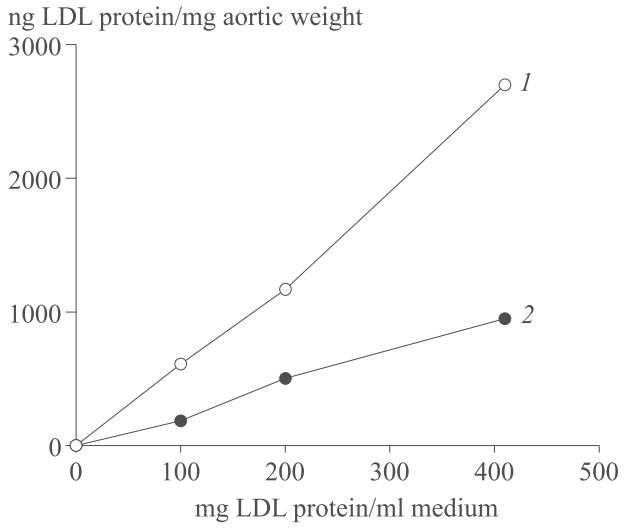

Fig. 1. Effect of the concentration of ^{125}I—LDL and ^{125}I—LDL—LPS in the incubation medium on their accumulation in rat aortic tissue. ^{125}I—LDL—LPS (*1*) and ^{125}I—LDL (*2*).

TABLE 1. Accumulation of Complexes of ^{125}I—LDL and ^{125}I—LDL—LPS (200 mg/ml medium) in Rat Aortic Tissue during *in Vitro* Incubation (*M*±*m*)

Incubation conditions	Lipoproteins	Lipoprotein accumulation, ng LDL/mg aortic weight
At 0°C	^{125}I—LDL	69±13
	^{125}I—LDL—LPS	438±66**
At 37°C	^{125}I—LDL	503±87
	^{125}I—LDL—LPS	1169±154*

Note. *p<0.01 and **p<0.001 compared to ^{125}I—LDL.

Macrophages of the vascular wall may engulf modified LDL and undergo transformation into foam cells. We evaluated the possibility for increased binding and degradation of LDL—LPS in mononuclear phagocytes.

Binding and degradation of ^{125}I-LDL—LPS in macrophages were much greater than those of free

TABLE 2. Binding of Complexes of ^{125}I—LDL and ^{125}I—LDL—LPS by Rat Peritoneal Macrophages at 0°C (*M*±*m*)

Incubation conditions	Binding of LDL, ng ^{125}I—LDL/mg cell protein
^{125}I—LDL	24.00±0.81
^{125}I—LDL—LPS	39.8±4.2*
^{125}I—LDL+LPS	63.51±7.20**
^{125}I—LDL—LPS+ dextran sulfate	170.4±29.0**

Note. *p<0.01 and **p<0.001 compared to ^{125}I—LDL.

TABLE 3. Degradation of Complexes of ^{125}I—LDL and ^{125}I—LDL—LPS in Rat Peritoneal Macrophages at 37°C (*M*±*m*)

Incubation conditions	Degradation of LDL (mg ^{125}I—LDL/mg cell protein over 4-h incubation)
^{125}I—LDL	0.748±0.080
^{125}I—LDL+ dextran sulfate	9.51±0.84*
^{125}I—LDL—LPS	5.26±0.64*
^{125}I—LDL—LPS+ dextran sulfate	8.20±0.86*

Note. *p<0.001 compared to ^{125}I—LDL.

^{125}I—LDL (Tables 2 and 3). Previous experiments were performed with triglyceride-rich lipoproteins [9]. Endotoxemia is accompanied by significant changes in the lipoprotein composition of blood. The content of high-density lipoproteins decreases or remains unchanged under these conditions. By contrast, the amount of apoB-containing lipoproteins increases by many times [7]. Increasing the concentration of apoB-containing lipoproteins in the blood is accompanied by the increased formation of LPS—LDL complexes [2]. It may be suggested that *in vivo* endotoxemia contributes to the binding and accumulation of LDL—LPS complexes in macrophages.

An increase in the binding and degradation of LDL—LPS is probably related to the interaction of these complexes with ScR on macrophages and involves the CD14-dependent uptake. Addition of dextran sulfate at high concentration (5 mg/ml) to the incubation medium was followed by a significant increase in binding (Table 2) and degradation of complexes (Table 3). Probably, this polyanion promotes the modification of LDL and involvement of ScR in endocytosis of particles.

Independently on the mechanism for an increase in binding and uptake of LDL—LPS complexes, these changes probably have the proatherogenic effect and contribute to macrophage transformation into foam cells. The increased binding and accumulation of LDL in the arterial wall and macrophages indicate that detoxification of LPS during the interaction of endotoxin with blood lipoproteins is a major cause of atherosclerosis [1,2,15].

REFERENCES

1. Y. Sh. Schwartz and M. I. Dushkin, *Ros. Kardiol. Zh*., **30**, 83-92 (2001).
2. Y. Sh. Schwartz and M. I. Dushkin, *Biokhimiya*, **67**, 901-908 (2002).
3. D. W. Bilheimer, S. Eisenberg, and R. I. Levy, *Biochim. Biophys. Acta*, **260**, No. 2, 212-221 (1972).
4. M. I. Dushkin, A. F. Safina, E. I. Vereschagin, and Y. Sh. Schwartz, *Cell Biochem. Funct.*, **14**, No. 3, 209-217 (1996).
5. K. R. Feingold, J. L. Funk, A. H. Moser, *et al*., *Infect. Immun*., **63**, No. 5, 2041-2046 (1995).
6. R. Fumagalli, E. Csonka, G. C. Ghiselli, *et al*., *Pharm. Res. Commun*., **11**, No. 4, 323-339 (1979).
7. I. Hardardottir, C. Grunfeld, and K. R. Feingold, *Curr. Opin. Lipidol*., **5**, No. 3, 207-215 (1994).
8. H. W. Harris, J. A. Johnson, and S. J. Wigmore, *Crit. Care Med*., **30**, No. 1, 23-31 (2002).
9. H. W. Harris, D. C. Rockey, and P. Chau, *Hepatology*, **27**, No. 5, 1341-1348 (1998).
10. S. Jimi, N. Sakata, A. Matunaga, and S. Takebayashi, *Atherosclerosis*, **107**, No. 1, 109-116 (1994).
11. K. Juul, L. B. Nielsen, K. Munkholm, *et al*., *Circulation*, **94**, No. 7, 1698-1704 (1996).
12. M. J. Kim, J. Dawes, and W. Jessup, *Atherosclerosis*, **108**, No. 1, 5-17 (1994).
13. F. T. Lindgren, *Analysis of Lipids and Lipoproteins*, Ed. E. G. Perkins, American Oil Chemists Soc., Champaign, SL., USA (1975), pp. 204-223.
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, No. 1, 265-275 (1951).
15. Y. Sh. Schwartz, M. I. Dushkin, and I. S. Kuznetsova, *J. Endotoxin Res*., **6**, 113-114 (2000).