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Modified low density lipoproteins activate human macrophages to secrete immunoreactive endothelin

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This study attempted to determine if low density lipoproteins (LDL) induce the production of endothelins (ET) by human macrophages. Non-protected LDL from macrophage induced oxidation (n-LDL), copper-oxidized LDL (Ox-LDL), acetylated-LDL (Ac-LDL), butylated hydroxytoluene-LDL (BHT-LDL), BHT-Ac-LDL, polyinosinic acid (PiA, 1.5 μg/ml), phorbol myristate acetate (PMA; 0.5 μM) and BHT alone (20 μM) were studied. The different compounds had the following potency to stimulate the ET secretion: PMA>Ox-LDL>Ac-LDL>n-LDL>BHT-LDL>PiA>PiA+Ac-LDL>BHT. In conclusion, modified LDL stimulated ET secretion by human macrophages.

Endothelin; Macrophage; Low density lipoprotein

1. INTRODUCTION

Low density lipoproteins (LDLs) are a well known atherogenic risk factor and may accumulate in vessel walls in hypercholesterolemia [1,2]. Recently, it has become evident that oxidatively modified LDLs (Ox-LDLs) contribute to the development of atherosclerosis [1,2]. The earliest recognized gross lesion in atherogenesis is the fatty streak characterized by an accumulation of foam cells [1,2]. Ox-LDLs cause foam cell formation via the macrophage scavenger receptors [1,2]. Macrophages are a source of growth factors and cytokines [3] such as tumor necrosis factor (TNF) which can cause cell proliferation. Endothelin 1 (ET-1), a recently isolated peptide secreted by endothelial cells, is a very potent vasoconstrictor [4] and presents a mitogenic activity on smooth-muscle cells and fibroblasts [5]. Therefore, ET may stimulate the formation of the atherosclerotic plaques. There is now evidence that Ox-LDLs induce the secretion of ET-1 by endothelial cells [6] and that acetylated-LDLs (Ac-LDLs) induce the secretion of TNF by macrophages [7] via the scavenger receptors. On the other hand, it has been recently published that ET-1 is produced by human macrophages stimulated with phorbol myristate acetate (PMA) [8]. On these grounds, we hypothesized that LDLs may trigger ET production by macrophages. Consequently, the goals of this study were (i) to determine if LDLs activate human macrophages to secrete immunoreactive ET-1 and ET-2 and (ii) to determine the eventual role of the macrophage scavenger receptors.

Abbreviations: ET, endothelin; TNF, tumor necrosis factor; PMA, phorbol 12-myristate 13-acetate; LDL, low-density lipoprotein; Ac-LDL, acetylated LDL; Ox-LDL, oxidized LDL; BHT, butylated hydroxytoluene; n-LDL, native-LDL; BHT-LDL, butylated hydroxytoluene-LDL; BHT-Ac-LDL, butylated hydroxytoluene-Ac-LDL; RIA, radioimmunoassay; PiA, polyinosinic acid; FCS, fetal calf serum; LPDS, lipoprotein deficient serum; MEM, minimal essential serum; PKC, Protein kinase c.

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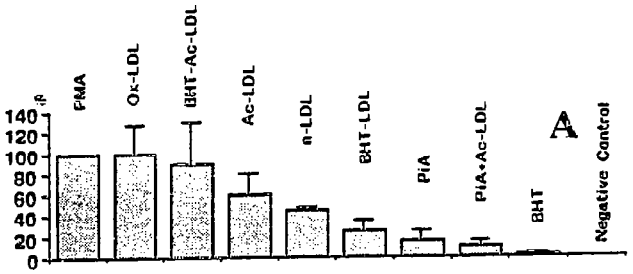
2. MATERIALS AND METHODS

2.1. *Lipoproteins*

Human LDL (n-LDL) (density 1.030–1.055 kg/l) was isolated by sequential ultracentrifugation [9] from the blood of normolipidemic donors. Protein content was measured by Peterson's method [11]. LDLs were oxidized (Ox-LDL) by incubating EDTA-free n-LDL (0.1 mg protein/ml) with $CuSO_4$ (5 μM) [11] at 23°C, and acetylated-LDL (Ac-LDL) was obtained with the repeated addition of acetic anhydride according to Basu et al. [12]. The degree of in vitro LDL oxidation was evaluated with three methods [13]: (1) The increased absorption at 234 nm, which indicates the increase in diene formation of fatty acids. The oxidation was stopped by the addition of EDTA when the OD increase flattened; (2) The electrophoretic mobility on cellulose gel (Cellogel; Sebia, Issy-les moulineaux, France). The mean distance of migration was 135 ± 18% in comparison with n-LDL, indicating an enhanced negative charge of Ox-LDL; (3) The TBARS assay, i.e. thiobarbituric acid reactive substances expressed as malonaldehyde (MDA) equivalents. The mean value was 5.2 ± 1.2 nM/ml. The non-lyophylyzed supernatant culture media were concentrated and the degree of oxidation of lipoproteins was evaluated according to method (2). Acetylation of LDL was also controlled by this method. Butylated hydroxytoluene (BHT), an antioxidant compound [11], was incorporated into lipoproteins prior to adding these macromolecules into cell culture medium. Briefly, 40 μg of BHT were solubilized into 10 μl of ethanol



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PMA	PMA							
Ox-LDL	0,35	Ox-LDL						
BHT-Ac-LDL	0,35	0,42	BHT-Ac-LDL					
Ac-LDL	0,10	0,30	0,45	Ac-LDL				
n-LDL	<0,05	0,05	0,35	0,35	n-LDL			
BHT-LDL	<0,05	<0,05	<0,05	0,05	0,35	BHT-LDL		
PIA	<0,05	<0,05	<0,05	<0,05	<0,05	0,65	PIA	
PIA+Ac-LDL	<0,05	<0,05	<0,05	<0,05	<0,05	0,60	0,60	PIA+Ac-LDL
Controls	<0,05	<0,05	<0,05	<0,05	<0,05	<0,05	<0,05	< 0,05

Fig. 1. (A) Relative secretion of immunoreactive ET induced by different compounds, in comparison with the secretion induced by PMA (n=3; mean ± SE). (B) Statistical comparison of the ET secretion in the different groups using the Mann-Whitney test; the differences were considered as significant when P<0.05 (indicated by the shadowed area).

and then added to 3 ml n-LDL or Ac-LDL (1.6 mg protein/ml). This solution was gently mixed during 45 min and then dialyzed against a physiological salt solution gassed with 95% N_2–5% CO_2. BHT-lipoproteins were sterilized by filtration (Millipore: 0.22 μM). An aliquot of this solution was used to evaluate the resistance of BHT-lipoproteins against copper-induced oxidation. Following $CuSO_4$ (5 μM) addition into n-LDL and BHT-LDL solutions, the optical density (OD) was measured each hour for 6 h at 234 nm, and the time to reach the maximum OD was increased by 2 h in the presence of BHT.

2.2. *Cell culture*

Monocytes were obtained from healthy human donors. Blood mononuclear cells were separated on a lymphoprep discontinuous gradient [10] and seeded at a density of 15 × 10^6/35-mm dish. They were incubated in MEM supplemented with 10% (v/v) human serum of blood group AB rhesus-positive and monocytes were allowed to attach for 2 h in 5% CO_2 at 37°C. At the end of the attachment period the medium was replaced with MEM supplemented with 10% FCS for the rest of the experiments. Cells were used between day 15 and 20 after seeding [8]. For each experiment (n=3), only cells derived from one donor were used.

2.3. *Experimental protocol*

Before the assay, cells were pre-incubated in growth medium containing 10% LPDS for 12 h, then incubated with either MEM alone (negative controls), or with PMA 0.5 μM (positive controls) or with different LDLs (100 μg/ml medium): non-protected LDL from macrophage induced oxidation (native-LDL), n-LDL; copper-oxidized LDL (Ox-LDL), acetylated-LDL, (Ac-LDL), butylated hydroxytoluene (an antioxidant compound)-LDL (BHT-LDL) and BHT-Ac-LDL. The effects of polyinosinic acid (PIA, 1.5 μg/ml), a competitor of Ac-LDL for the scavenger receptors [14], and of BHT (20 μM) alone, were also studied. After 20 h of incubation at 37°C, the medium was removed and cells washed twice in phosphate buffered saline. Supernatants were concentrated for radioimmunoassays and cells were released by addition of 500 μl 0.1 N NaOH and processed for protein determination [11]. Data are representative of 3 preparations in triplicate.

2.4. *Determination of endothelin*

We used Endothelin 1,2 (high sensitivity) [^{125}I]-RIA system (Amersham, Les Ulis, France) to quantify immunoreactive ET in the cultured media. This RIA system offers a specific, sensitive, reliable and precise quantitative determination of ET-1, ET-2 and Big ET-1. ET-1 can be measured in the range 0.25–32 fmol per tube (0.623–79.74 pg/tube).

3. RESULTS AND DISCUSSION

The ability of human macrophages to secrete ET-1 has been recently documented [8]. This publication has reported that PMA, which is a potent activator of PKC [16], could stimulate a 6- to 10-fold increase in the secretion of this peptide. In our study, we have found that the basal secretion (negative controls) of immunoreactive ET-1-2 was 177 ± 44 fmol/mg cell proteins/20 h and that PMA (positive control) stimulated secretion was 822 ± 45 fmol/mg cell proteins/20 h (P<0.02, Mann-Whitney test). This increase is in agreement with the previously reported data [8]. Our results are presented in Fig. 1 as 'relative secretion of immunoreactive ET-1-2, induced by lipoproteins' in comparison with 'secretion of immunoreactive ET-1-2 induced by PMA'. The following formula was used to calculate this relative secretion:

$$\text{Relative secretion of immunoreactive ET-1-2 induced by lipoproteins} = 100 \times \frac{\left\{\text{Absolute secretion of ET induced by lipoproteins (fmol/mg cell protein/20 h)}\right\} - \left\{\text{Absolute secretion of ET induced by free lipoproteins medium (fmol/mg cell protein/20 h)}\right\}}{\left\{\text{Absolute secretion of ET induced by PMA (fmol/mg cell protein/20 h)}\right\} - \left\{\text{Absolute secretion of ET induced by free lipoproteins medium (fmol/mg cell protein/20 h)}\right\}}$$

Our results show that Ox-LDL induced 100 ± 27% of the ET-1-2 secretion induced by PMA and that n-LDL induced 45 ± 2% of that secretion. On the basis of the previous report on the ability of human macrophages to produce ET [8] we chose a long incubation time for the lipoproteins with the cells (20 h). It has been extensively reported that such a long period of incubation induces LDL oxidation by macrophages [1,2], and it has been shown that BHT delays the cell-induced LDL oxidation [12]. Therefore, in order to evaluate the potency of n-LDL, protected from macrophagic oxidation with BHT, on ET secretion we incubated BHT-LDLs with macrophages and we observed that these protected LDLs induced 25 ± 10% of the PMA-induced secretion. BHT alone did not modify ET secretion. After the 20 h incubation we studied, on cellulose gel, the electrophoretic mobility of the lipoproteins contained in the culture media. Copper Ox-LDLs have been further oxidized during the incubation period, as indicated by a faster mobility (Fig. 2A). Furthermore, in the incubation media, most of the BHT-LDL had the same mobility as n-LDL (Fig. 2B, lane 2 vs. lane 3). Therefore, at the end time of the experiments most of the BHT-LDL

had been oxidized, although the intermediate band of the BHT-LDL (Fig. 2B, lane 2), located between native, fresh LDL (Fig. 2B, lane 4) and the major bands of oxidized LDL of the culture medium (Fig. 2B, lanes 2 and 3) was slightly lower than the intermediate band of the non-protected LDL (Fig. 2B, lane 3). This indicates that the oxidation of BHT-LDL was delayed. As we had verified that fresh BHT-LDLs were protected from copper-induced oxidation, we must state that this protection was insufficient to ensure 20 h of macrophage-induced n-LDL oxidation. On the basis of that quoted in the literature [12] and of the intermediate band seen in electrophoresis (Fig. 2B), we may suppose that BHT delayed the LDL oxidation and that the following phase of oxidation processing was accomplished before the end of the incubation period. Therefore, the duration of the contact between oxidized-LDLs and macrophages certainly increased as follows among the 3 groups of LDL: Ox-LDL > n-LDL > BHT-LDL. We remarked that this duration of contact follows the quantity of ET secreted by the macrophages (Fig. 1). It has been reported that Ac-LDLs induce the secretion of TNF, via the stimulation of the scavenger receptors [7]. Therefore, we have formed the hypothesis that such receptors may be involved in modified LDL-induced ET secretion. Ac-LDLs and BHT-Ac-LDLs induced 61 ± 22% and 90 ± 43% of PMA-induced ET secretion, respectively. This secretion is higher than that induced by BHT-LDL, indicating that the scavenger receptors are probably involved. We must note that the protection of the Ac-LDL oxidation by BHT did not reduce the ET secretion. After 20 h incubation Ac-LDLs and BHT-Ac-LDLs of the media had the same mobility as fresh-Ac-LDLs (Fig. 2C). This mobility was the same as that of LDL oxidized by macrophages (Fig. 2B) and did not allow an evaluation of the degree of oxidation of Ac-LDL and BHT-Ac-LDL. In order to confirm the role of the scavenger receptors we chose to incubate the macrophages with polyinosinic acid (PiA), which is known to compete with Ac-LDL for uptake and degradation by the scavenger macrophage receptors [15]. PiA alone induced a moderate secretion of ET (20 ± 15% of the PMA-induced secretion). In the presence of PiA, Ac-LDLs induced 11 ± 5% of the PMA-induced secretion. Therefore, stimulation of the scavenger receptors' pathway with a non-lipoprotein agonist can activate ET secretion. When PiA was bound to the receptors, the addition of Ac-LDL did not enhance ET secretion. This minimizes the potency of lipid–lipid interactions between lipoproteins and cell membranes to induce the peptide secretion, and confirms the importance of the scavenger receptors' pathway in lipoprotein-induced ET secretion. We must emphasize that Ox-LDLs are more potent than Ac-LDLs in inducing ET secretion. This is consistent with the reported data indicating that there is more than one macrophage scavenger receptor for modified LDL [17–19]. Oxidized-LDLs and Ac-

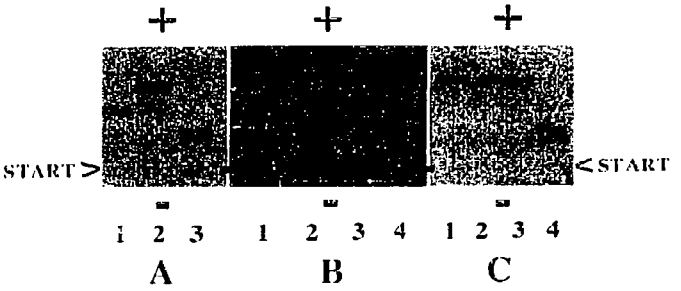

Fig. 2. Electrophoretic mobility of LDLs. (A) lane 1, fresh copper Ox-LDL; lane 2, copper Ox-LDL after 20 h incubation with macrophages; lane 3, fresh n-LDL. (B) lane 1, fresh BHT-LDL; lane 2, BHT-LDL after 20 h incubation with macrophages; lane 3, n-LDL after 20 h incubation with macrophages; lane 4, fresh n-LDL. (C) lane 1, fresh Ac-LDL; lane 2, BHT-Ac-LDL after 20 h incubation with macrophages; lane 3, Ac-LDL after 20 h incubation with macrophages; lane 4, fresh n-LDL.

LDLs are not identical ligands with respect to macrophage recognition and uptake, and there is a class of macrophage receptor that recognizes cell oxidized-LDL but not Ac-LDL [17]. This may partially explain the higher secretion of ET induced by Ox-LDL stimulation rather than by Ac-LDL. The mechanism by which Ox-LDL and Ac-LDL stimulate ET secretion via the scavenger receptors is unknown. ET production in secreting cells is a complex process involving both transcriptional and post-transcriptional controls [20]. Ac-LDL has recently been shown to be a general cellular activator which acts by increasing membrane receptor-mediated phosphatidylinositol turnover [21,22] which in turn secondarily translocates PKC [23]. Thus, modified LDLs could act early in the sequence of activation, like phorbol ester tumor promoters, which are potent activators of PKC [16].

Ox-LDLs and macrophages are involved in atherogenesis. Our study suggests one potentially important link between the two, through the secretion of ET. The role of ET in atherogenesis has not, however, been established. Nevertheless, its mitogenic action could facilitate the transformation of fatty streaks into atheroma.

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REFERENCES

[1] Ross, R. (1986) New Engl. J. Med. 320, 915–924.
[2] Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C. and Witztum, J.L. (1989) New Engl. J. Med. 320, 915–924.
[3] Nathan, C.F. (1987) J. Clin. Invest. 79, 319–326.
[4] Yanagisawa, M., Durchara, H., Migake, S., Kimura, S., Tomobe, Y., Kobayashi, M., Mitoui, Y., Yazaki, Y., Gotto, K. and Masaki, T. (1988) Nature 332, 411–415.
[5] Weissberg, P.L., Witchell, C., Davenport, A.P., Hesketh, T.R. and Metcalfe, J.C. (1990) Atherosclerosis 85, 257–262.
[6] Boulanger, C., Hahn, A.W.A. and Lüscher, T.F. (1990) Circula-

tion 82, 4 III, Abstr. 0891, p. 225.
[7] Barath, P., Cao, J. and Forrester, J.S. (1990) FEBS Lett. 277, 180–184.
[8] Ehrerreich, H., Anderson, R.W., Fox, C.H., Riekmann, P., Hoffman, G.S., Travis, W.D., Coligan, J.E., Kehrl, J.H. and Fauci, A.S. (1990) J. Exp. Med. 172, 1741–1748.
[9] Havel, R.J., Eder, H.S. and Bradgon, J.H. (1955) J. Clin. Invest. 3, 1345–1353.
[10] Boyüm, A.J. (1968) Scand. J. Clin. Lab. Invest. 21, Suppl. 97, 77–99.
[11] Petersen, G. (1977) Anal. Biochem. 3, 346–356.
[12] Esterbauer, H., Dieber-Rotheneder, M., Waeg, G., Strongl, G. and Jürgens, G. (1990) Chem. Res. Toxicol. 3, 77–92.
[13] Basu, S.K., Goldstein, J.M., Anderson, R.G.W. and Brown, M.S. (1976) Proc. Natl. Acad. Sci. USA 73, 3178–3182.
[14] Esterbauer, H., Striegel, G., Puhl, H. and Rotheneder, M. (1989) Free Radical Res. Commun. 6, 67–75.
[15] Brown, M.S. and Goldstein, J.L. (1983) Annu. Rev. Biochem. 52, 223–261.
[16] Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmssdorf, H.J., Jonat, C., Herrlich, P. and Karin, M. (1987) Cell 49, 729–739.
[17] Sparrow, C.P., Parthasarathy, S. and Steinberg, D. (1989) J. Biol. Chem. 264, 2599–2604.
[18] Kodoma, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaina, P. and Krieger, M. (1990) Nature 343, 570–572.
[19] Rohrer, L., Freeman, M., Kodoma, T., Penman, M. and Krieger, M. (1990) 343, 570–572.
[20] Yanagisawa, M. and Masaki, T. (1989) Trends Pharmacol. Sci. 10, 374–378.
[21] Block, L.H., Knorr, M., Vogt, E., Lochner, R., Vetter, W., Geroscurth, P., Qiao, B.Z., Pometta, D., James, R., Regenass, M. and Pletscher, A. (1988) Proc. Natl. Acad. Sci. USA 85, 885–889.
[22] Isikawa, Y., Asaoka, Y., Taniguchi, T., Tsunemitsu, M. and Fukuzaki, H. (1989) FEBS Lett. 246, 35–38.
[23] Scott-Burden, T., Resink, T.J., Haln, A.W.A., Bauer, U., Box, R. and Buhler, F.R. (1989) J. Biol. Chem. 264, 12582–12589.