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Low density lipoprotein receptor expression and function in human polymorphonuclear leucocytes

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SUMMARY

Low density lipoprotein receptors (LDLR), capable of internalizing LDL, are expressed in polymorphonuclear neutrophils (PMN). The expression was assessed using anti-LDLR antibody by flow cytometry. The internalization of LDL was assessed by: (i) quantification of the uptake of labelled LDL with 1,1′-dioctadecyl-3,3,3′,3′ tetramethyl-indocarboxycyanine perchlorate (DiI) by flow cytometry; and (ii) the binding of LDL-^{125}I. In fresh purified cells, Lineweaver–Burk analysis of LDL binding (LDL-DiI) revealed that the calculated Kd (internalized LDL) for PMN ($15{\cdot}0 \times 10^{-9}$ M) is lower than the Kd for monocytes ($1{\cdot}1 \times 10^{-7}$ M) and the Kd for lymphocytes ($3{\cdot}2 \times 10^{-7}$ M). Scatchard analysis (LDL-^{125}I) revealed 25 000 binding sites and a Kd of $9{\cdot}6 \times 10^{-9}$ M for PMN. The interaction of LDL with its receptor caused a two-fold fast (peak at 1 min) and transient increase in the oxidative burst, measured by the formation of 2′,7′ dicholoflurescein (DCF) by flow cytometry. This effect was not observed in monocytes or lymphocytes, and it was blocked by anti-LDLR antibody. The stimulation of LDL was optimal at 10 μg of protein/ml. LDL was able to suppress DCF formation induced by phorbol myristate acetate (PMA) and PMA was unable to further stimulate LDL-treated cells, suggesting protein kinase-C (PKC) involvement in LDL effects. Using a PKC assay, LDL was shown to induce a two-fold increase in PKC translocation to the membrane. Thus, LDL increases PMN oxidative burst through a PKC-dependent pathway.

Keywords low density lipoprotein LDL receptor oxidative burst polymorphonuclear cells

INTRODUCTION

Low density lipoprotein (LDL) has been implicated in the induction of cell cycle, protein glycosylation and mitochondrial metabolism [1]. LDL contains only one apolipoprotein (apo-B100), and is rich in cholesterol and phospholipids [1–3]. LDL is taken up by the cells through its specific receptor (LDLR), which is internalized along with LDL. Clinical and experimental data suggest that genetic defects in LDLR induce hypercholesterolaemia and eventually atherosclerosis [1–3]. A defect in the expression or internalization of LDLR leads to an increase in circulating plasma LDL, predisposing it to oxidation. These molecules of oxidized LDL, that do not bind to LDLR, have been shown to contribute greatly to the development of the atheroma [3]. The regulation of LDLR gene expression and the function of modified LDL receptors have not been fully elucidated.

Brown & Goldstein [2] showed that macrophages were not able to internalize unmodified LDL, but they were able to internalize modified LDL, leading to the formation of foam cells [2]. However, several reports [4–7] have shown that LDL activated monocyte oxidative metabolism and lysosomal enzyme release. These effects were inhibited by interferon-gamma (IFN-γ) [8]. LDL and modified LDL also altered T lymphocyte proliferative response [1,3] and natural killer (NK) proliferative and cytotoxic responses [9,10].

Polymorphonuclear neutrophils (PMN) are involved indirectly in the formation of the atheroma, since their secretion upon activation of myeloperoxidase (MPO) and free radicals is able to modify LDL, rendering it prone to atheroma formation [3,8,11,12]. In cardiac reperfusion injury, PMN are important in the progress of atherosclerotic lesions observed in these patients [11]. Even though LDL was shown to induce PMN superoxide generation and cell migration [12], there are no reports on LDLR expression in these cells.

The aim of the present study is to demonstrate that polymorphonuclear cells express functional LDLR, and that the interaction ligand–receptor is responsible for an increment in the oxidative burst which is a protein kinase-C (PKC)-dependent process.

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MATERIALS AND METHODS

Chemicals and antibodies

The reagents were obtained from: 1,1′ dioctadecyl-3,3,3,3′ tetramethyl-indocarboxycyanine perchlorate (DiI), 2′,7′ dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Portland, OR); $Na^{125}I$ and γ-^{32}P-ATP (New England Nuclear, Boston, MA); PKC assay kit (GIBCO-BRL, Gaithersburg, MD); and Sigma Chemical Co. (St Louis, MO). The MoAbs were obtained from: anti-CD16-PE (Leu-11a) (Becton Dickinson, Mountain View, CA); anti-CD3-FITC, anti-CD19-FITC, anti-CD14-FITC, mouse IgG1-PE, IgG1-FITC, and IgG2b, and goat anti-mouse IgG-FITC (Coulter Corp., Hialeah, FL); and anti-LDLR (clone 7, IgG2b) (Amersham, Aylesbury, UK). The specificity of anti-LDLR was described previously [13–15].

Lipoprotein purification

LDL was separated from plasma of normolipaemic fasted human donors (approved by local Ethical Committee) according to the method of Havel *et al.* [16]. The plasma was centrifuged twice at 114 000 *g* for 20 h at 16°C (plasma density $d = 1{\cdot}030$) in the presence of inhibitors of lipid oxidation (1 mM butylhydroxytoluene, 2 mM reduced gluthatione, 5 mM ascorbic acid and 5 mM EDTA). The infranatant was adjusted to 1·063 g/ml and centrifuged as described above. LDL was washed using a discontinuous gradient, 0·9% NaCl-KBr ($d = 1{\cdot}063$) at the top, LDL-KBr ($d > 1{\cdot}063$) at the bottom and centrifuged as described above. The only protein content of this fraction was apolipoprotein B as determined by electrophoresis. LDL was endotoxin-free as determined by the timed gel formation kit (Sigma). No oxidative intermediates were detected in the purified LDL fraction using the thiobarbituric acid assay [17]. LDL was used immediately after dialysis and within the first 2 days of purification.

Labelling of lipoproteins with DiI

The labelling of LDL with DiI was performed as previously described [18]. LDL-DiI was dialysed against PBS, adjusted to 2 mg/ml, and filter-sterilized through a 0·45 μm Millipore filter. The labelling efficiency was always close to 50% as determined by measuring the fluorophore at 550 nm. DiI is a hydrolysable and non-toxic fluorophore.

LDL iodination

LDL iodination was performed as described previously by Shepherd *et al.* [19] with minor modifications. Briefly, 100 μl of freshly purified LDL (2 mg/ml of protein), dialysed against PBS, were mixed with 50 μl of Na-^{125}I (1 mCi/μmol) and 50 μl of chloramine T, 0·4% in PBS, vigorously for 45 s at room temperature. The reaction was stopped by adding 40 μl of 0·24% $Na_2S_2O_5$, 50 μl of 1% KI and 1 ml of 0·1 M Tris–HCl–0·1 M NaCl–1% bovine serum albumin (BSA) pH 8·0. Then, LDL-^{125}I was separated from free iodine by passing it through Sephadex G-25. Eighty percent of the label was incorporated in the protein moiety of the lipoprotein.

Cell purification

Total leucocytes and purified PMN were obtained from fasted normal donors with normal lipaemia (approved by local Ethical Committee) as described previously [20,21]. Erythrocytes were lysed with a solution containing 8 g/*l* NH_4Cl, 0·8 g/*l* $NaHCO_3$ and 0·37 g/*l* EDTA. The unlysed cells were washed with PBS-gel (2 mM EDTA, 5 mM glucose, 0·1% gelatin), and resuspended at 1×10^6 cells/ml PBS-gel for LDLR expression and function. The purified cell populations were >95% viable as determined by trypan blue exclusion.

Purified PMN, >95% viable and >95% $CD16^+$, were obtained by the dextran sulphate method described previously by Clarck & Nauseef [21].

LDL receptor assessment

LDL receptor expression determined by anti-LDL receptor. LDLR expression was assessed by indirect labelling with mouse anti-human LDLR MoAb (IgG2b). Briefly, 1×10^6 cells (total leucocytes or purified PMN) were incubated with PBS-gel–0·02% sodium azide at 4°C for 10 min, then labelled with 0·5 μg of anti-LDLR for 30 min at 4°C, washed twice with PBS-gel–azide–0·1% BSA, incubated with 0·5 μg of goat anti-mouse IgG-FITC and finally washed with PBS-gel. Samples incubated with an irrelevant mouse IgG2b plus anti-mouse IgG-FITC were used as controls. Positive cells were assessed in an EPICS 753 cytometer (Coulter). The expression of LDLR on lymphocytes, monocytes

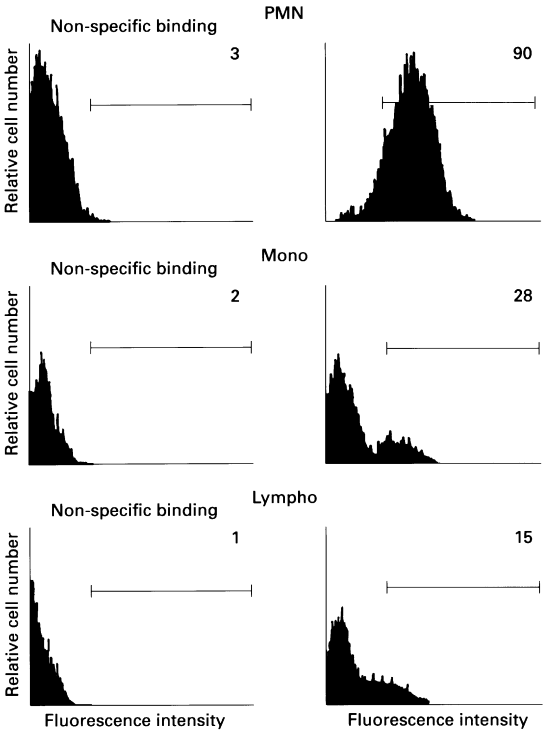

Fig. 1. Flow cytometry assessment of low density lipoprotein (LDL) receptor expression in different leucocytes. Purified leucocytes were incubated in the presence of anti-LDL receptors (LDLR) as described in Materials and Methods, and positiveness was assessed by flow cytometry using goat anti-mouse IgG-FITC and analysed as fluorescence emission at 520 nm (green fluorescence). The figure illustrates the positiveness observed in the different leucocyte populations, PMN, monocytes (Mono) and lymphocytes (Lympho). The numbers on the top right of the histograms represent the positiveness recorded using the cursor represented in the figure. The histograms of the left side correspond to non-specific binding assessed by an irrelevant mouse IgG2b and goat anti-mouse FITC. The histograms of the right side correspond to the expression of LDLR assessed by anti-LDLR plus goat anti-mouse FITC.

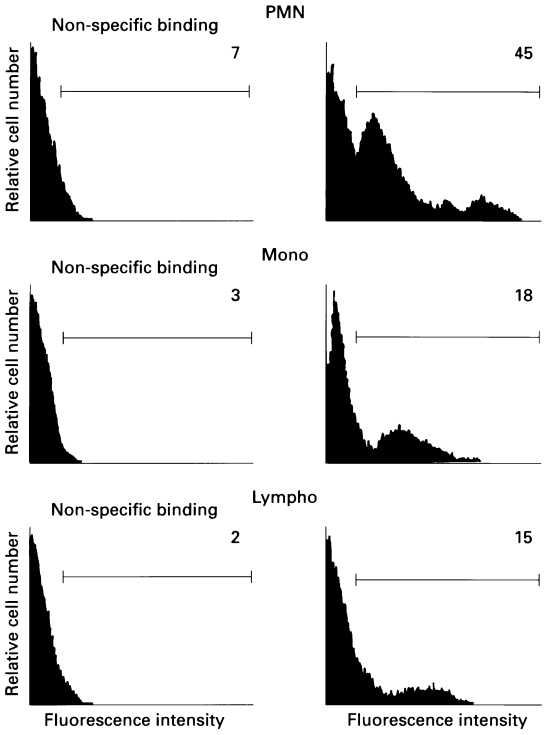

Fig. 2. Low density lipoprotein (LDL)-1,1′ dioctadecyl-3,3,3′,3′ tetramethyl-indocarboxycyanine perchlorate (DiI) internalization by different leucocytes. The figure represents a typical experiment of internalization of LDL-DiI by PMN, momocytes (Mono) and lymphocytes (Lympho) at 37°C using LDL-DiI. The numbers in the top right of the histograms represent the positiveness recorded using the cursor illustrated in the figure (fluorescence >570 nm). The histograms on the left side correspond to the non-specific binding observed when the cells were incubated with LDL-DiI in the presence of 2 mM EDTA. The histograms of the right represent the total uptake of 60 μg/ml LDL-DiI as described in Materials and Methods.

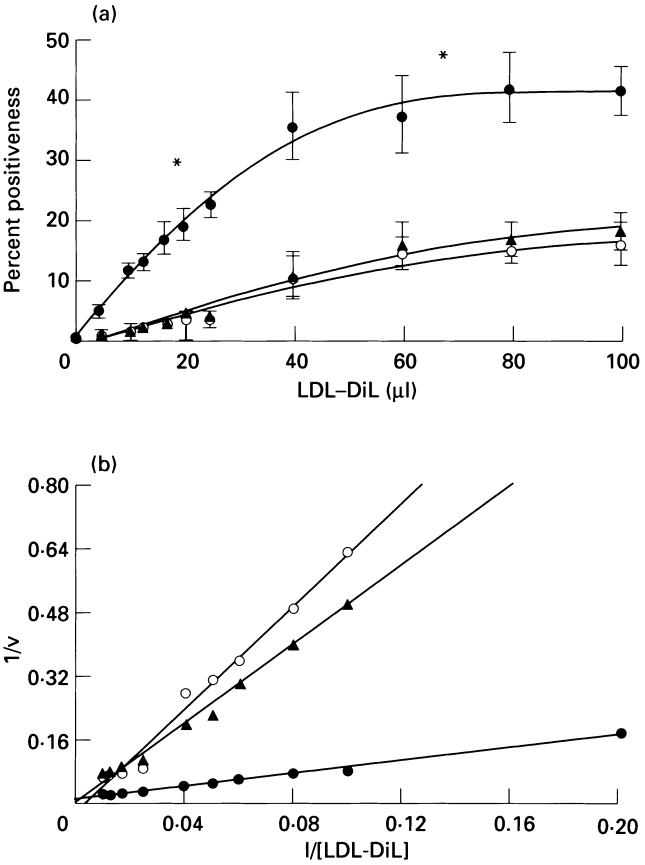

Fig. 3. Internalization of low density lipoprotein (LDL)-1,1′ dioctadecyl-3,3,3′,3′ tetramethyl-indocarboxycyanine perchlorate (DiI) by different leucocytes and Lineweaver–Burk plot. (a) Uptake of LDL-DiI by different leucocyte populations. LDL-DiI was incubated with the cells, at 37°C, as described in Materials and Methods and in Fig. 2. The results represent the mean and s.e.m. of five different experiments. Significant differences were observed when the values of PMN were compared with either lymphocytes or monocytes (*$P < 0{\cdot}05$). (b) Lineweaver–Burk plot of LDL-DiI binding to the different leucocyte populations. The data represented in (a) were analysed with the following equation: $1/(\%\ \text{positiveness}) = 1/(\text{LDL-DiI}) \times 1/(\text{Kd}) + 1/(V_{max})$. The calculated Kd for LDL was $1{\cdot}1 \times 10^{-7}$ M for monocytes (▲), $3{\cdot}2 \times 10^{-7}$ M for lymphocytes (○) and 15×10^{-9} M for PMN (●). This equation is generally used to calculate the apparent Km of enzyme kinetics and therefore represents only an approximation of the real Kd.

and polymorphonuclear cells was performed using bitmaps in which each cell population was defined by granularity and by antigen expression (CD3, CD19, CD14 and CD16).

LDL receptor function determined by the uptake of LDL-DiI. Total leucocytes (1×10^6) or purified PMN were incubated with PBS–0·1% BSA (fatty acid-free) with different concentrations of LDL-DiI for 4 h (the time in which maximum uptake was observed, results not shown) at 37°C in a humidified incubator with 5% CO_2–95% air mixture (cell viability >90%). Then the cells were washed twice with PBS-gel and analysed by flow cytometry. Fluorescence (>570 nm) signal from the accumulated LDL-DiI in the cells was collected by the red photomultiplier (using a 600 nm dichroic short pass filter and a 645 nm band pass filter), following the method of Suzuki *et al.* [22]. The specificity of LDL-DiI binding was assessed by incubating the cells with 100 μg/ml unlabelled LDL 1 h before incubation with LDL-DiI. The maximum non-specific binding was close to 7% for all cell types. In addition, the Kd for LDL-DiI binding for each leucocyte population was determined using the Lineweaver–Burk equation as described previously [1,14]. This equation is generally used to calculate the apparent Km of enzyme kinetics and therefore represents only an approximation of the real Kd; a more specific assay for ligand–receptor interaction is the Scatchard equation.

LDL-^{125}I binding to purified PMN. Purified PMN (1×10^6) were mixed with different concentrations of LDL-^{125}I and the assay was performed at 4°C for 1 h. After incubation, the cells were washed with PBS-gel in plastic radioimmunoassay (RIA) tubes and the cell pellet was counted in the gamma counter (LKB Bromma, Sweden). Non-specific binding was assessed by incubating the cells with 100 μg/ml unlabelled LDL 1 h before addition of different concentrations of LDL-^{125}I. The non-specific binding was lower than 30% of the total bound LDL-^{125}I.

The percent specific binding was calculated according to the following formula:

$$\text{percent specific binding} = \frac{\text{total ct/min incorporated} - \text{non-specific bound LDL-}^{125}\text{I}}{\text{total ct/min added}}$$

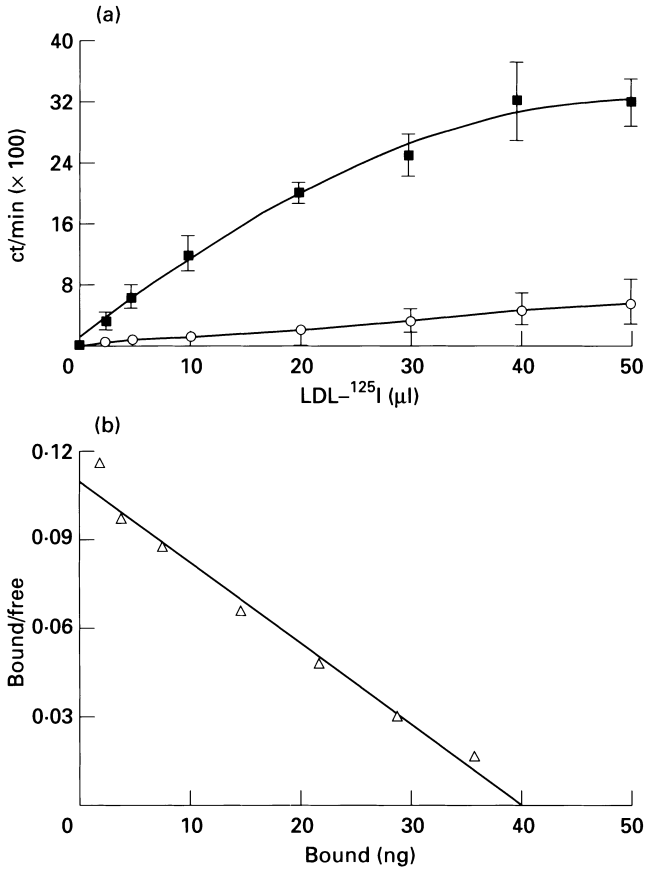

Fig. 4. Binding of low density lipoprotein (LDL)-^{125}I to purified PMN and Scatchard analysis. (a) Binding of LDL-^{125}I by purified PMN. PMN were incubated with LDL-^{125}I for 1 h at 4°C, and cell binding was determined using the gamma counter as described in Materials and Methods. Non-specific binding was assessed by incubating the cells with 100 μg/ml of unlabelled LDL 1 h before the addition of LDL-^{125}I. The results represent the mean and s.d. of five different experiments. ■, Specific; ○, unspecific. (b) Scatchard analysis of the data presented in (a). The number of binding sites was calculated to be 25 000 and the Kd $9{\cdot}6 \times 10^{-9}$ M.

Scatchard analysis was performed using a computerized program developed by Munson & Robbard [23]. The value of Kd obtained in the Scatchard analysis was compared with the value obtained with the Lineweaver–Burk equation using LDL-DiI.

Oxidative burst assessment by flow cytometry

Peroxide production was determined by flow cytometry using the intracellular oxidation of DCFH-DA to generate DCF, as described previously [24]. The cells were labelled for 15 min at 37°C with 1 μl of 20 mM DCFH-DA. Then, the cells were stimulated either with 150×10^{-9} M phorbol myristate acetate (PMA), 10 μg/ml LDL or 10 μg/ml LDL plus 150×10^{-9} M PMA, and a time kinetic assay was performed. The reactions were stopped by placing the tubes on ice. Mean channel green fluorescence intensity (which represents fluorescence intensity in logarithmic units) was reported as DCF production. The specificity of the assay was confirmed by incubating the cells with different concentrations of mannose (1, 5, 10 and 20 mM) or maleimide (0·5, 1, 2 mM) before the kinetic studies. Mannose [25] and maleimide [26] are specific inhibitors of the oxygen burst since they inhibit the hexose monophosphate pathway.

In a set of experiments, PMN were incubated with 1 μg/ml of anti-LDLR in PBS-gel for 30 min at 4°C before adding DCFH-DA and LDL. As a control, PMN were incubated either with an irrelevant IgG2b or without antibody for the same period of time.

Determination of PKC activity

The measurement of PKC activity was performed as described by the manufacturer (GIBCO) using the protocol described by Thomas *et al.* [27] and modified by Yasuda *et al.* [28] which includes a pseudosubstrate inhibitor PKC (19–36). Membrane and cytosol fractions were separated by ultracentrifugation at 100 000 ***g*** for 60 min at 4°C; the membrane fraction was then solubilized again with 1% (w/v) of Nonidet P-40 and recentrifuged at 100 000 ***g*** for 60 min at 4°C. The enzyme from both fractions was partially purified through DEAE52 columns following the kit's instructions. The specificity of the assay was determined by subtracting the radioactivity obtained in the presence of the pseudosubstrate inhibitor from the total radioactivity of the assay. The results are expressed as percentage of control values.

In order to evaluate the effects of activation of PKC by LDL, 2×10^{-5} M of H7 (1-(5-isoquenoline sulfonyl)-2-methylpiperazine dihydrochoride) was added 1 h before the addition of LDL, and a set of experiments without adding LDL was used as control.

RESULTS

A typical LDLR expression, assessed by flow cytometry, is shown in Fig. 1 in fresh cells. The expression of the receptor ($90 \pm 6\%$, $n = 8$) in PMN was significantly different to that observed for monocytes ($28 \pm 4\%$, $P < 0{\cdot}05$) and lymphocytes ($16 \pm 5\%$, $P < 0{\cdot}05$). In parallel to the studies performed with LDLR expression, the internalization of the LDL-DiI was assessed. A characteristic example of LDL-DiI internalization, appraised by flow cytometry, is pictured in Fig. 2. The specific uptake of LDL-DiI by different cell populations is represented in Fig. 3a; it depicts a saturable system with a maximum uptake at 60 μg/ml. The maximum internalization of LDL-DiI by PMN cells was three-fold higher than the uptake observed with monocytes or lymphocytes. The Kd of LDL-DiI uptake for the different cell populations was calculated using the Lineweaver–Burk equation (Fig. 3b). The calculated Kd (or apparent Kd) for PMN (15×10^{-9} M) was seven-fold lower than the observed Kd for monocytes ($1{\cdot}1 \times 10^{-7}$ M) and 21-fold lower than the observed Kd for lymphocytes ($3{\cdot}2 \times 10^{-7}$ M). Incubation of mononuclear cells with medium RPMI–0·5% BSA (fatty acid-free) for 48 h reduced the Kd for LDL from $3{\cdot}2 \times 10^{-7}$ M to $7{\cdot}53 \times 10^{-9}$ M (results not shown).

In order to ascertain the binding of LDL to purified PMN, LDL-^{125}I was used as ligand and the results are illustrated in Fig. 4a. The figure represents a saturable system with a maximum binding at 60 μg/ml of LDL-^{125}I as it was observed for LDL-DiI. The Scatchard analysis of these binding experiments is illustrated in Fig. 4b. The calculated number of binding sites is 25 000 and the Kd is $9{\cdot}6 \times 10^{-9}$ M. The estimated Kd obtained

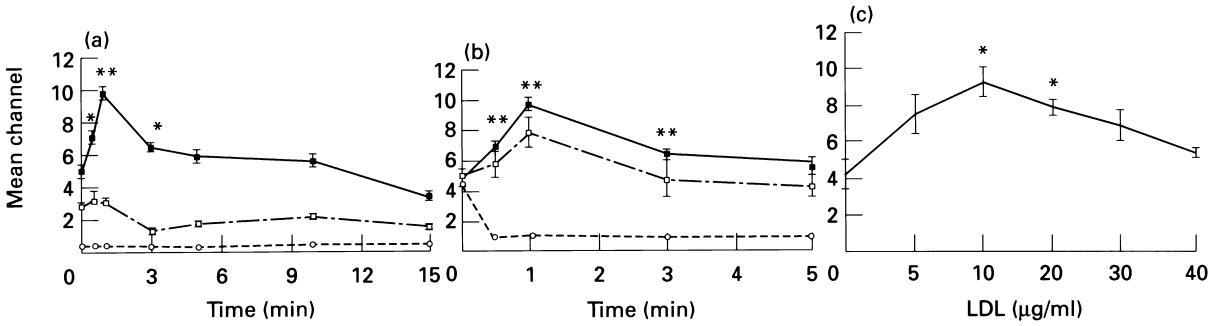

Fig. 5. Low density lipoprotein **(**LDL) effect on the oxidative burst in PMN. Cells were incubated with 2′,7′ dichloro-fluorescein diacetate (DCFH-DA) as described in Materials and Methods, and the formation of DCF was monitored using flow cytometry. The figures represent the mean channel fluorescence intensity (fluorescence emitted in logarithmic units) recorded, which is dependent upon DCF formation. (a) Effect of LDL on DCF production by the different leucocyte cell populations. Significant differences were observed when comparing the values obtained at different time intervals with the 0 time point for PMN (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$). Significant differences were observed at different time intervals when PMN were compared with monocytes ($P < 0{\cdot}05$) and lymphocytes ($P < 0{\cdot}001$). ■, PMN; ○, lymphocytes; □, monocytes. (b) Time kinetics for LDL induction of DCF formation in the presence and absence of anti-LDLR. PMN were incubated with an irrelevant IgG2b or with anti-LDLR before the assay. The results represent the mean and s.e.m. of five different experiments. Significant differences were observed when comparing the cells incubated in the absence of antibody or in the presence of an irrelevant IgG2b (at 1 min) with the cells pre-treated with anti-LDLR before stimulation with LDL (**$P < 0{\cdot}01$, $n = 5$). No significant differences were observed between cells treated or untreated with the irrelevant mouse IgG2b and stimulated with LDL. ■, LDL; ○, LDL + anti-LDLR; □, LDL + IgG2b. (c) Effect of different concentrations of LDL on the formation of DCF (1 min incubation). The results represent the mean and s.d. of five different experiments. The increment in DCF formation with 10 and 20 μg/ml of LDL is significant with respect to the control ($P < 0{\cdot}05$).

with the Scatchard plot ($9{\cdot}6 \times 10^{-9}$ M) is similar to the estimated Kd obtained with the Lineweaver–Burk plot (15×10^{-9} M).

Figure 5 illustrates the effect of LDL on the oxidative burst of different leucocytes. In Fig. 5a, the effect of LDL on PMN, lymphocyte and monocyte oxidative burst is illustrated. LDL induced a two-fold transient increase (peak at 1 min) in the mean channel fluorescence intensity in PMN, without affecting monocytes and lymphocytes. This transient increase was blocked by 1 μg/ml of anti-LDLR, but not by an irrelevant IgG2b as observed in Fig 5b. The specificity of the assay is represented in Table 1. Mannose and maleimide block basal and PMA-induced oxidative burst. The respiratory burst induced by LDL is concentration-dependent and has a maximum peak at 10 μg/ml, as observed in Fig. 5c. Concentrations of LDL > 40 μg/ml did not induce oxidative burst, instead LDL decreased cell viability, assessed by trypan blue exclusion and flow cytometry (results not shown).

In order to evaluate the effect of LDL and PMA on the oxidative burst, four sets of experiments with time kinetics were

Table 1. Effect of mannose and maleimide on DCF fluorescence (mean channel fluorescence intensity in logarithmic units)

Inhibitor	Control	Percent control	PMA	Percent control
None	5·12 ± 1·18		52·10 ± 15·1	
5 mM mannose	4·30 ± 1·15	84·0	33·5 ± 10·4	64·3
10 mM mannose	2·44 ± 1·08	47·0	27·3 ± 10·6	52·4
20 mM mannose	2·36 ± 1·10	46·0	29·2 ± 6·2	56·0
0·5 mM maleimide	0·53 ± 0·27	10·4	0·50 ± 0·38	1·0
1 mM maleimide	0·63 ± 0·35	12·3	1·10 ± 0·70	2·0
2 mM maleimide	0·45 ± 0·4	8·8	0·82 ± 0·76	1·5

The specificity of the formation of peroxides was assessed using two different inhibitors of the hexose monophosphate pathway. Purified PMN were incubated with the concentrations of inhibitors before the assay as described in Materials and Methods, the cells were then either non-stimulated or stimulated with phorbol myristate acetate (PMA). The numbers correspond to the mean ± s.d. of the mean channel fluorescence intensity expressed in logarithmic units for four different donors. The percentages represent the remaining DCF production in the presence of inhibitors compared with the untreated control.

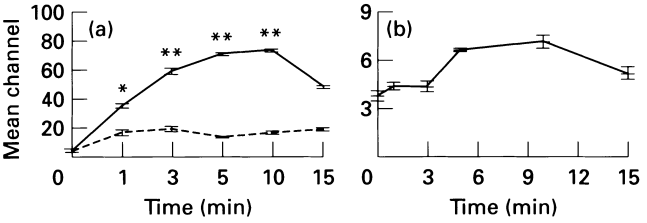

Fig. 6. Effect of phorbol myristate acetate (PMA) and low density lipoprotein **(**LDL) and both agents on the oxidative burst of PMN. (a) Effect of LDL on the oxidative burst induced by PMA. The cells were incubated with PMA, as described in Materials and Methods, either in the absence (—) or presence of LDL (---). The decrease in DCF production is significant when comparing PMA stimulation alone with PMA plus 10 μg/ml LDL (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$). When ethanol was added to the mixture of LDL plus PMA, the supernatant induced a similar amount of DCF production as observed when the cells were stimulated with PMA. (b) Effect of PMA on the oxidative burst induced by LDL. The cells were incubated with LDL, as described in Materials and Methods, and then stimulated with PMA. The induction of the formation of DCF is lower than the observed value of PMA alone shown in (a) ($P < 0{\cdot}001$ for the time interval after 30 s). The scale is different from that of (a).

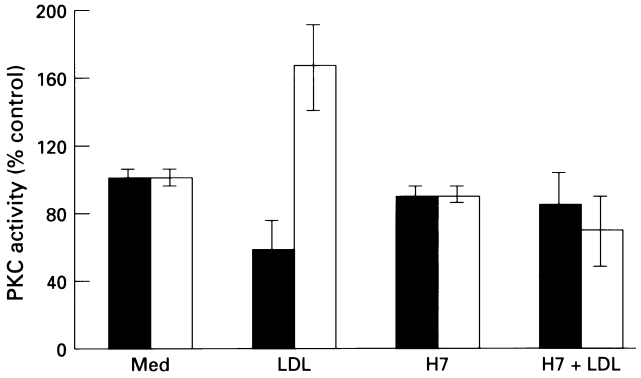

Fig. 7. Effect of low density lipoprotein (LDL) on the translocation of protein kinase-C (PKC) in PMN. Purified cells were incubated with 10 μg/ml of LDL, H7 or H7 plus LDL as described in Materials and Methods, and the activity of PKC was assessed on both fractions. The figure represents the percentage of enzyme activity using 100% as control for non-stimulated cells. The results represent the mean and s.d. of five different experiments. ■, Cytosol; □, membrane.

performed: (i) cells were incubated with 150×10^{-9} M PMA alone; (ii) cells were incubated with 150×10^{-9} M PMA and 10 μg/ml LDL simultaneously; (iii) cells were incubated with PMA 5 min before LDL kinetics; and (iv) cells were treated with 10 μg/ml LDL for 0·5, 1, 3, 5, 10 and 15 min and then stimulated with PMA for 5 min. The results of this experiment are represented in Fig. 6. Figure 6a illustrates the amount of DCF produced by PMN incubated with 150×10^{-9} M of PMA (PMA) or 150×10^{-9} M PMA along with LDL (PMA + LDL). The effect of LDL was similar if the lipoprotein was added 5 min after PMA stimulation (results not shown). The addition of LDL to PMA-stimulated cells markedly decreased DCF formation. In Fig. 6b, PMA induced only a slight increase in the oxidative burst of cells previously treated with LDL. This increment was significantly less when it was compared with the values obtained with PMA alone ($P < 0{\cdot}001$) and PMA plus LDL ($P < 0{\cdot}05$). In addition, ethanol precipitation of LDL, in the mixture PMA–LDL, prevented the inhibitory effect of LDL and consequently DCF formation was similar to the observed values in Fig. 6a.

After 1 min of stimulation, the two-fold increase in the oxidative burst observed with LDL alone (Fig. 5a) was significantly lower ($P < 0{\cdot}05$) compared with the eight-fold increase observed using 150 ng/ml of PMA (Fig. 6a).

PKC activity in the cytosol and membrane of treated cells was assessed and the results are shown in Fig. 7. LDL induced an increment in particulate membrane fraction of PKC compared with the control by two-fold; similarly, there was a 60% reduction of the PKC observed in the cytosol. The effect of LDL was blocked by H7. H7 alone did not affect PKC distribution compared with controls.

DISCUSSION

Normal LDL receptor function is involved in the anabolism and catabolism of LDL and other lipoproteins. On the other hand, a delayed clearance of LDL from the plasma due to unimpaired receptor function renders this molecule more susceptible to modifications which have been demonstrated to be involved in the transformation of macrophages to foam cells [1–3]. Recently, several reports [3–6,8] have shown that free radicals produced by stimulated PMN and macrophages (N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), PMA, or zymosan) avidly modified LDL. This oxLDL is overlooked by normal LDLR interacting only with scavenger receptors [3,11]. OxLDL altered lymphocyte and macrophage proliferative and cytotoxic responses [3–8], suggesting that this is one of the key physiopathological events in atheroma formation.

Contrary to previous reports, Aviram & Rosenblat [5] observed that macrophage-mediated oxidation of extracellular LDL required an initial binding of the lipoprotein to its receptor, since the blockage of LDLR with specific antibodies inhibited LDL oxidation. It is concluded that LDL–LDLR interaction is crucial for cell activation and oxidative burst induction. A similar mechanism of cell activation could take place in PMN.

In this study, LDL receptor expression and function in PMN were fully assessed by its recognition with the specific MoAb and the internalization of the ligand LDL-DiI or the binding of LDL-^{125}I to purified PMN. The expression of the receptor was studied in fresh leucocytes and freshly purified PMN close to physiological conditions. In freshly isolated mononuclear leucocytes (lymphocytes and monocytes), LDL receptors (assessed with LDL-DiI) showed a higher Kd ($1{\cdot}1 \times 10^{-7}$ M for monocytes and $3{\cdot}2 \times 10^{-7}$ M for lymphocytes) than the calculated Kd value for PMN (15×10^{-9} M). Scatchard and Lineweaver–Burk analysis, performed with a different tracer, showed similar Kd values ($9{\cdot}6 \times 10^{-9}$ M and 15×10^{-9} M, respectively) for PMN. These results suggest that LDLR expression and avidity are higher in PMN compared with mononuclear cells. However, long-term incubation (48 h) of mononuclear cells in the absence of lipoprotein decreased the Kd for LDL from $3{\cdot}2 \times 10^{-7}$ M to $7{\cdot}73 \times 10^{-9}$ M (results not shown). This calculated Kd is similar to that observed in the literature ($7{\cdot}3 \times 10^{-9}$ M) [1,15].

LDL was able to specifically (blocked by anti-LDLR) induce an increase in PMN oxidative burst which was significant, but transient. Bonneau *et al.* [12] reported that 5 min preincubation of PMN with LDL induced superoxide generation over the following 15 min. The difference in the kinetic activation of PMN may be due to the method used for free radical quantification. In the experimental tests performed in this study we were unable to observe, after 5 min of stimulation with LDL, an increase in the oxidative burst. Flow cytometry identification of peroxides by DCF production permitted us to monitor specifically the fluorescence emitted by viable cells that maintain granularity and antigen expression, while the ferrocytochrome assay may not distinguish ferrocytochrome c reduction by viable cells when they are incubated for long periods of time.

LDL induction of DCF production was maximal at 10 μg of LDL and slowly decreased as the concentration of LDL increased. This effect may be due to a decrease in cell viability observed when unstimulated or PMA-primed cells were incubated for long periods of time with increasing concentrations of LDL. These results contrast with those of Bonneau *et al.* [12], in which LDL induction of superoxide generation did not reach a maximum.

The observed effects of LDL on PMA-activated PMN are similar to those reported by Bonneau *et al.* [12]. These authors [12] suggested that LDL alters PMA affinity to PKC, since LDL does not interact with PMA and LDL is unable to modify opsonized zymosan activation of PMN. However, LDL was internalized by PMN by its specific receptor, and this internalization decreased PKC-induced oxidative burst (monitored by

DCF formation). In neutrophils, contrary to macrophages, NADPH oxidase has been located preferentially in the phagolysosome [29] and cytoskeleton rearrangements upon phagolysosome formation diminished PKC induction of peroxide formation [30]. Furthermore, LDL has been shown to scavenge peroxides produced by the cells, which readily modified the molecule [3,11]. It can be proposed that LDL reduction of DCF accumulation induced by PMA stimulation may be a consequence of either phagolysosome formation or peroxide scavenging by LDL.

LDL induction of PKC translocation to the membrane seems to be the mechanism responsible for the increment in oxidative burst. This effect is specific, since the addition of H7 before LDL stimulation blocked PKC translocation to the membrane. These results are in agreement with those reported by Li & Catchcart [4]. These authors [4] have shown that PKC activity is required for lipid oxidation of LDL in activated human macrophages.

The functional expression of LDLR in PMN implies the involvement of these cells in the physiological oxidation of LDL in normal human plasma and in the generation of vascular damage as observed in cardiac reperfusion injury [11]. A tentative hypothesis can be proposed in relation to the amounts of oxidized LDL produced. In normal physiological conditions, low amounts of oxLDL are produced, and it may be cleared up by scavenger receptors, yet if a massive activation of peripheral PMN by different agents takes place, these cells will originate an enormous increment in oxLDL which could not be cleared out from the plasma and may precede the formation of an atheroma.

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