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Lipoproteins induce tyrosine phosphorylation in a murine macrophage cell line

Henry Rivera, Danuta Radzioch, Isaac Blanca, Nicolás E. Bianco and Juan B. De Sanctis

Instituto de Inmunología, Facultad de Medicina, Universidad Central de Venezuela, Caracas, Venezuela

Requests for reprints to Dr Juan B. De Sanctis, Instituto de Inmunología, Universidad Central de Venezuela, Aerocav #1216, PO Box Miami, FL, PO Box #02-5304, Miami, FL, 33102-5304, USA. Tel: (58) (2) 605-3429; Fax (58) (2) 693-2734; E-mail: inmuno@cantv.net

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Abstract: Lipoproteins, chylomicrons (CM), very low density lipoprotein (VLDL), low density lipoprotein (LDL), high density lipoprotein (HDL) and acetyl modified LDL (AcLDL) are able to stimulate macrophages upon binding to specific receptors. Using flow cytometry and a specific monoclonal antibody against phosphotyrosine, we assessed the effect of lipoproteins on total intracellular expression of immunoreactive phosphotyrosine. Tyrosine phosphorylation increased when lipoproteins were added to a macrophage cell line, which was previously cultured in RPMI–0.5% fatty acid free bovine serum albumin. The optimal effect occurred at 15 min for all the lipoproteins used. The concentrations of lipoprotein were 10 µg of protein/ml of CM, and 20 µg of protein/ml for the other lipoproteins. The effect of lipoproteins on phosphotyrosine positiveness was as follows: AcLDL > HDL > CM > VLDL > LDL. Thus lipoproteins may activate macrophages through tyrosine phosphorylation and this signal transduction pathway seems to be enhanced when these cells are activated with AcLDL.

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Introduction: Lipoproteins, chylomicrons (CM), very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) have always been considered important for cell and tissue physiology [1]. However, deregulation of the metabolic control of lipoprotein may cause vascular pathologies such as atherosclerosis [2,3].

Macrophages have been studied extensively in atherosclerosis. Most of the research has been directed towards scavenger receptor expression and foam cell formation [1–3]. Only a few reports have dealt with the signal transduction generated by different lipoprotein receptors.

We have studied the generation of phosphotyrosine upon lipoprotein stimulation in order to ascertain the possible activation of these cells upon lipoprotein binding.

Materials and methods: Lipoproteins were separated as described previously [4]. The levels of oxidized lipoproteins, assessed by the thiobarbituric acid assay (TBARS), were undetectable [4]. LDL was acetylated as described by Basu *et al.* [5]. All the lipoproteins were washed, dialysed and filter-sterilized before the assay.

Immunofluorescent quantification of tyrosine phosphorylation of cellular proteins was performed as described previously by Far *et al.* [6]. The assay was done in a synchronized culture of ANA-1, a murine macrophage cell line, [7] incubated with RPMI – 0.1% BSA fatty acid free. The cells were removed from the plates using a rubber policeman and subjected to different stimuli.

The cells were fixed with a 1% paraformaldehyde, permeabilized with saponin, incubated with 0.01 M phosphate buffer solution (PBS) – 0.1% bovine serum albumin (BSA) 0.1% Tween-20 to block non-specific binding and finally incubated with anti-phosphotyrosine-FITC (Sigma Chemical Company, St. Louis, MO, USA). As controls, the cells were treated with pervanadate (positive) and 1 µM staurosporine (negative) as described previously [6].

Specificity was assessed by adding phosphotyrosine or incubating pervanadate-stimulated cells with unlabelled anti-phosphotyrosine before incubation with the labelled antibody. Finally, the fluorescence intensity was quantified in an EPICS ELITE flow cytometer (Coulter Corporation, Miami, Florida, USA) previously calibrated with DNA check beads.

ANOVA test and Student's *t*-test were used to analyse the results. The results were considered significant when the *P* values were < 0.05.

Results: The analysis of phosphotyrosine revealed a difference in the intensity of fluorescence that was dependent on the concentration of the lipoprotein used. Figure 1 illustrates the effect of lipoprotein on phosphotyrosine positiveness in three sets of experiments. The cells stimulated with LDL

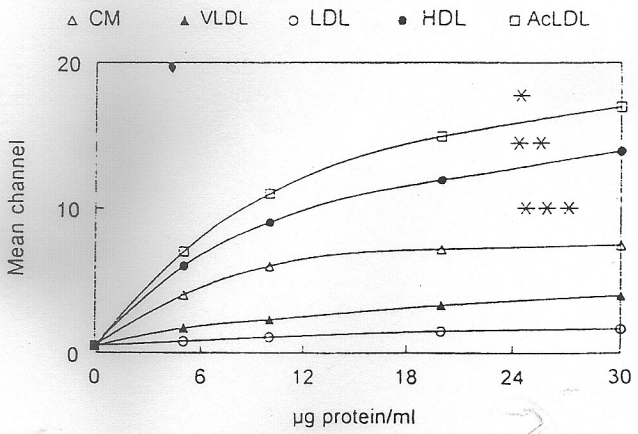

*Figure 1. Effect of lipoproteins on tyrosine phosphorylation. The mean channel fluorescence intensity (logarithmic units) represents the amount of immunoreactive phosphotyrosine detected with anti-phosphotyrosine-FITC and assessed by flow cytometry. *As compared with LDL, P < 0.001 (ANOVA test); **as compared with LDL, P < 0.001 (ANOVA test); ***as compared with LDL, P < 0.005, P < 0.05 (ANOVA test).*

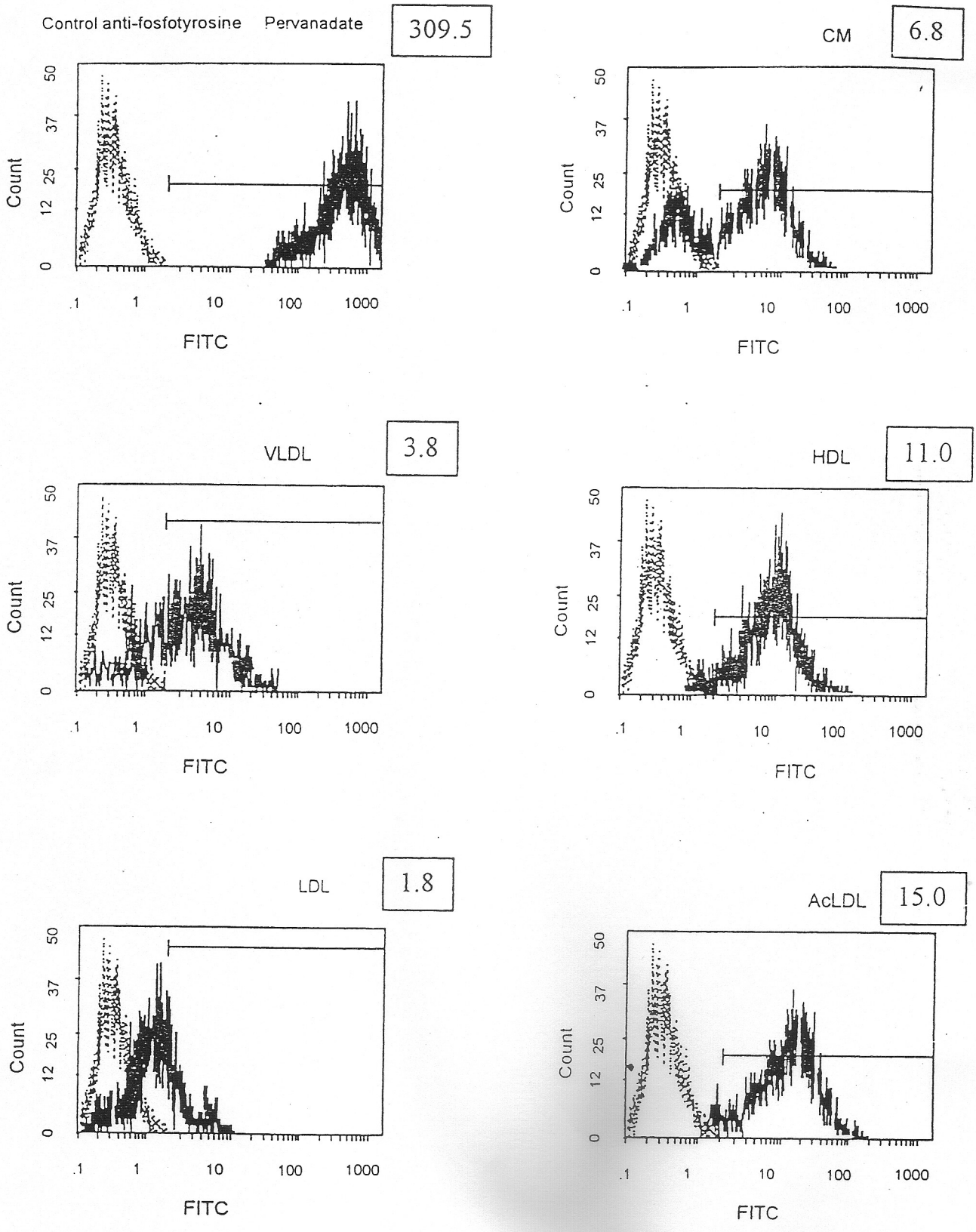

Figure 2: Flow cytometry histograms of stimulated cells. In this typical flow cytometry experiment, the dotted line refers to control (non-stimulated cells) and the dark lines represent the lipoprotein stimulated cells. The number on the top right of the histograms represents the mean channel fluorescence intensity in logarithmic units.

had the lowest levels of phosphotyrosine, which were significantly lower than those of cells stimulated with HDL or AcLDL ($P < 0.001$) and those of CM stimulated cells ($P < 0.05$). Similarly, the cells stimulated with VLDL expressed low levels of phosphotyrosine as compared to HDL or AcLDL ($P < 0.005$) and CM ($P < 0.01$) stimulated cells.

Figure 2 represents a typical assay for phosphotyrosine determination, at optimal concentrations of lipoprotein, using flow cytometry. The mean channel fluorescence intensity

reported in logarithmic units was assessed in the total cell population. The dotted line represent the basal phosphotyrosine expression. The dark lines represents the phosphotyrosine from stimulated cells.

In concordance to the figure, the amount of phosphotyrosine recorded upon LDL stimulation was the lowest, contrasting to the higher levels of phosphotyrosine observed in HDL and AcLDL. Two peaks were always observed in the CM-stimulated cells.

Discussion: Lipoprotein receptors are important for lipid homeostasis in cell metabolism, but may also be important in the generation of foam cells present in atheromas [2,3]. The signal transduction generated upon lipoprotein binding to its receptor may be important in macrophage physiological response.

The effects of the different types of lipoprotein on tyrosine phosphorylation are clear. High levels of phosphotyrosine were observed upon AcLDL and HDL stimulation, contrasting with the low levels seen upon LDL stimulation.

Previous studies [8,9] have shown that LDL activation induces phosphorylation of the mitogen-activated protein kinase (MAP) in the U937 cell line and smooth muscle cells. As a consequence of this activation, the proliferative response of these cells increased upon LDL activation. The effect of LDL was inhibited by HDL.

Similarly, Lara *et al.* [10] have shown that LDL increased the translocation of PKC to the particulate membrane fraction of polymorphonuclear cells, with a corresponding increase in peroxide production. Miki *et al* [11] suggested a direct activation of the *lyn* tyrosine kinase upon AcLDL binding to the scavenger receptor in the THP-1 cell line, indicating a direct involvement of this kinase with the receptor.

These reports leads us to postulate that lipoprotein-induced signal transduction is an important event in cell activation. These primed cells, in the presence of proinflammatory cytokines, may magnify the original inflammatory response, leading to the formation of atheroma [2,3].

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