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OXIDIZED LOW-DENSITY LIPOPROTEIN STIMULATES NITRIC OXIDE RELEASE BY RABBIT AORTIC ENDOTHELIAL CELLS

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Incubation of rabbit aortic endothelial cells (RAEC) with oxidized low-density lipoprotein (LDL) for 16 h resulted in stimulation of calcium uptake and increased release of nitric oxide (NO) by these cells. Accompanying inhibition of protein tyrosine phosphatase (PTP) activity in these cells was also observed. Conversely, native LDL was unable to produce any of those effects. These observations suggest that oxidized LDL could modulate two major signalling processes in endothelial cells: tyrosine dephosphorylation and NO synthesis. Such modulation may be of importance in the early phase of the atherogenic process.

Nitric oxide (NO) a free radical gas, has been characterized as an important signal transducing molecule in the vascular system. NO released by endothelial cells regulates the vascular tone, inhibits platelet aggregation and smooth muscle cells contraction (1). NO is synthesized from the guanidino nitrogen of L-arginine by dioxygenases known as NO synthases. There are two basic isoforms of NO synthases, the constitutive form which is calcium/calmodulim dependent and is predominant in endothelial cells, and an inducible form which is calcium insensitive and is present in smooth muscle cells and macrophages (1). It was reported that activation of endothelial cells by agents that promote increase in the free cytosolic calcium

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Abbreviations: Epidermal growth factor, EGF; low-density lipoprotein, LDL; malondialdehyde, MDA; nitric oxide, NO; phosphate-buffered saline, PBS; protein tyrosine phosphatases, PTP; rabbit aortic endothelial cells, RAEC; thiobarbituric acid reactive substances, TBARS.

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concentration, mediates the release of NO by these cells (2). Besides calcium-dependent activation, NO synthesis could be up regulated by tyrosine phosphorylation. Recently, Marczin et al. (3) reported that NO synthesis in rat aortic smooth muscle cells could be stimulated by tyrosine phosphorylation. Protein tyrosine kinases and their counterparts, protein tyrosine phosphatases (PTP) are of major importance in cell signalling processes (4,5) and their activities can be modulated by intracellular calcium levels and oxidative stress inducing agents (6-12).

Low density lipoprotein (LDL) is essential for cholesterol delivering to perypheral cells and its oxidized form is frequently associated to the atherogenic process (13). Recently, it was reported that oxidized LDL provokes an elevation of the cytosolic calcium in bovine aortic endothelial cells (14). Furthermore, endothelial cells from hypercholesterolemic and atherosclerotic rabbit aortas released substantially higher amounts of NO than normal aortas (15). Therefore, in the present report we tested the hypothesis that oxidized LDL, which could function as a calcium ionophore in endothelial cells (14), is capable of stimulation of NO release by rabbit aortic endothelial cells (RAEC) in culture. The effects of oxidized LDL on PTP activities in these cells were also examined. The relationships between the two events are discussed.

MATERIALS AND METHODS

Cell cultures - The rabbit aortic endothelial cell line (RAEC) (16) was kindly provided by Dr. Vincenzo Buonassisi (Escola Paulista de Medicina, S.Paulo, Brazil) and was grown in Ham's Nutrient mixture F12 (F12 medium) supplemented with 10% heat inactivated fetal bovine serum in 100 mm tissue culture Petri dishes. Cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

LDL isolation - LDL was isolated from human blood plasma from healthy donors in the presence of EDTA (1 mg/ml), benzamidine (2 mM), aprotinin (1μg/ml) and phenylmethylsulfonyl fluoride (1 mM) by sequential ultracentrifugation (17). The LDL fraction was isolated between densities of 1.019-1.063 g/ml. Total protein content of the LDL preparation was determined according to the Lowry procedure (18). The purity of the LDL preparation was estimated by gradient 2.5-15% SDS polyacrylamide gel electrophoresis.

LDL oxidation - Native LDL (2 mg/ml) was oxidized by incubation with a solution of 10 μM $CuSO_4$ in phosphate buffered saline (PBS) in a Dubnoff metabolic waterbath shaker during 17 hs at 37 °C. After this period, copper oxidized LDL was extensively dyalised against PBS containing the chelating resin Chelex, followed by sterilization in 0.45 μm filters. The extent of LDL oxidation was evaluated using the thiobarbituric acid reactive substances (TBARS) assay (19) and expressed in malondialdehyde (MDA) equivalents per miligram of protein.

Treatment of intact cells - Cells were starved for 24 hs in F12 medium supplemented with 0.5% fetal bovine serum, before each experiment. They were then incubated in fresh medium supplemented with 1 mg/ml bovine serum albumin and 1 mM $CaCl_2$, in

the presence or absence of sterilized native LDL or oxidized LDL at three different protein concentrations (50, 100 and 200 μg/ml).

Measurement of $^{45}Ca^{2+}$ uptake - Uptake of $^{45}Ca^{2+}$ by RAEC in the absence (basal uptake) or presence of native and oxidized LDL was determined in confluent monolayers grown in 12 multiwell plates, essentially as described by Orlov et al (20). Briefly, uptake was initiated by simultaneous addition of $^{45}CaCl_2$ (5 μCi/ml) and lipoproteins (50 - 200 μg/ml) to the cultures maintained in F12 medium supplemented with 1 mg/ml bovine serum albumin and 1 mM $CaCl_2$. A control without adddition of lipoproteins was also performed. The uptake was determined by standard liquid scintillation counting techniques after 16 hs incubation.

Assay for nitric oxide - NO generated by RAEC incubated in the presence and absence of native or oxidized LDL, was estimated by determining the concentrations of nitrite in the incubation medium using the Greiss reagent as described by Stuehr and Nathan (21).

Assay of PTP activities - PTP activities were determined in RAEC lysates as the epidermal growth factor (EGF) receptor tyrosine phosphatase activity (11). Briefly, after incubations cells were lysed in lysis buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride). After 30 min incubation on ice, lysates were clarified by centrifugation for 10 min at 4°C at 12,000 x g in a microcentrifuge. The supernatants were collected, had their protein content determined (22) and served as the source of PTP activity. PTP activity was assayed by mixing 100 μg protein from lysates with a suspension containing immunoprecipitated [^{32}P]-labeled EGF receptors prepared as described by Honneger et al. (23). Reactions were carried out for 30 min at 37°C with agitation and terminated by aspiration of the supernatants. The radiolabeled immunoprecipitates were collected and mixed with 50 μl of 2 fold concentrated Laemmli's (24) sample buffer and resolved on 7.5% SDS PAGE. Afer drying, the gels were exposed to autoradiographic films for 2-4 hs at room temperature. PTP activity was estimated as a decrease of intensity of the corresponding band to the EGF receptor in the autoradiographies. Band intensities in the autoradiographies were subjected to densitometric analysis using a LKB Ultroscan Laser Enhanced Densitometer.

Statistical analysis - Statistical analysis was performed using Student's t-test for unpaired data.

RESULTS

Exposure of native LDL (2 mg/ml) to an aerated solution of $CuSO_4$ (10 μM) in PBS for 17 hr resulted in extensive oxidation of the lipoprotein as measured by the TBARS assay (13.38 ± 8.21 nmols MDA/mg of protein; n = 8). Native LDL presented only negligible oxidation levels (0.16 ± 0.07 nmols MDA/mg of protein; n = 6).

The ability of oxidized LDL to promote calcium influx in endothelial cells was checked. In agreement with previous observations (14), incubation of RAEC cultures in F12 medium containing (5μCi/ml) $^{45}Ca^{2+}$ and 50-200 μg/ml of oxidized LDL during 16 hs, stimulated the isotope uptake by the cells (Fig. 1). Stimulated uptake was

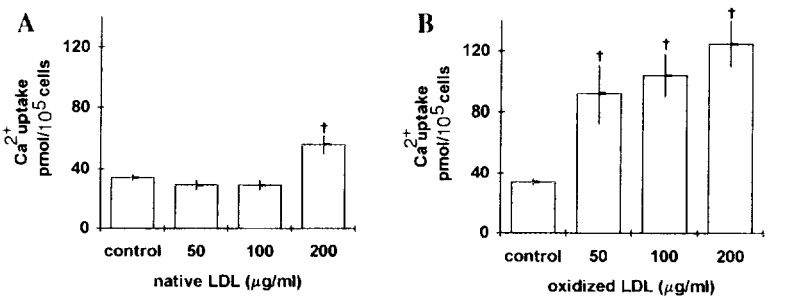

Figure 1. $^{45}Ca^{2+}$ uptake in RAEC cultures induced by the native and oxidized forms of LDL. RAEC were serum-starved during 24 hs before incubation with native (A) and oxidized LDL (B) for additional 16 hs. After this period, $^{45}Ca^{2+}$ uptake was determined as described in Materials and Methods. Results are the means ± S.E.M. of triplicates of two independent experiments. Values are significantly higher ($p < 0.05$) than uptake in the absence of the lipoproteins if indicated by +.

linearly dependent on the concentrations of the oxidized lipoprotein present in the medium (Fig. 1B). Conversely, under our experimental conditions, native LDL at concentrations of 50 and 100 μg/ml did not promote $^{45}Ca^{2+}$ uptake by RAEC. However, at a concentration of 200 μg/ml, native LDL significantly ($p < 0.05$) promoted $^{45}Ca^{2+}$ uptake by RAEC in comparison with non-treated cells (Fig. 1A). Yet, the enhancement (56.4 pmol $Ca^{2+}/10^5$ cells) was 61% lower than the stimulation evoked by oxidized LDL at 50 μg/ml (92.3 pmol $Ca^{2+}/10^5$ cells). Oxidized LDL is cytotoxic to the endothelium (13). However, as judged by the trypan blue uptake after 16 hr incubation, oxidized LDL at 50-200 μg/ml had no adverse effect on cell survival (>95% viable cells).

It is known that agonists which elevate the intracellular concentrations of Ca^{2+} in endothelial cells can activate their constitutively express NO synthases resulting in enhancement of NO release by these cells (2). Therefore, we decided to investigate if LDL could stimulate NO release by RAEC when these cells were treated with the native and oxidized forms of the lipoprotein. Incubation of confluent cultures of RAEC during 16 hs with increasing concentrations (50-200 μg/ml) of oxidized LDL stimulated the accumulation of nitrite in the incubation medium (Fig. 2B) Otherwise, native LDL at the same concentrations was unable to significantly induce nitrite accumulation above the basal levels (Fig. 2A).

Taken in account that phosphorylation/dephosphorylation processes are of major consequences in cellular metabolism (4,5) and both can be modulated by oxidants and calcium ionophores (6-12), we investigated the effects of the native and oxidized forms of LDL on PTP activities in RAEC. RAEC associated PTP activities, determined as the EGF receptor tyrosine phosphatase activity were strongly inhibited when cells were incubated in the presence of 100 and 200 μg/ml of oxidized LDL (Fig.3B, lanes 4 and 3, respectively). However, at 50 μg/ml oxidized LDL only gave a

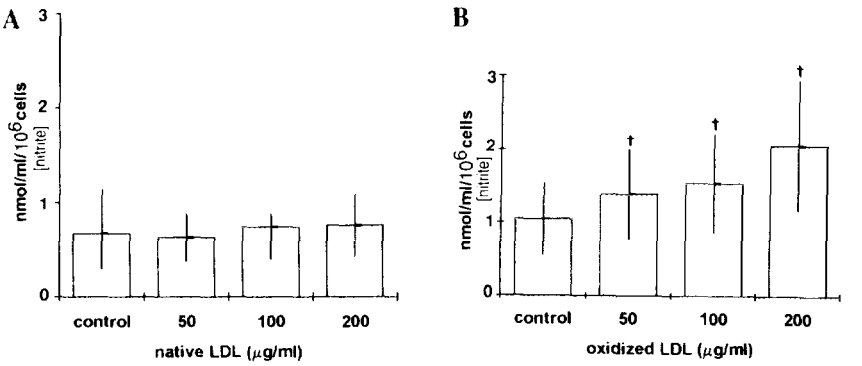

Figure 2. Effects of native and oxidized LDL on NO release by RAEC. RAEC were serum-starved during 24 hs before incubation with native (A) and oxidized LDL (B) for additional 16 hs. After this period NO release was determined as accumulated nitrite in the supernatants of the cell cultures as described in Material and Methods. Results are the means ± S.E.M. of 8 (native LDL) or 12 (oxidized LDL) independent experiments. Values are significantly higher ($p < 0.05$) than basal NO release if indicated by +.

slight inhibition of PTP activity (Fig. 3B, lane 5). By contrast, PTP activities in cells treated with native LDL (50, 100 and 200 μg/ml) remained practically without alterations (Fig. 3A, lanes 5, 4 and 3). In parallel experiments, sodium orthovanadate

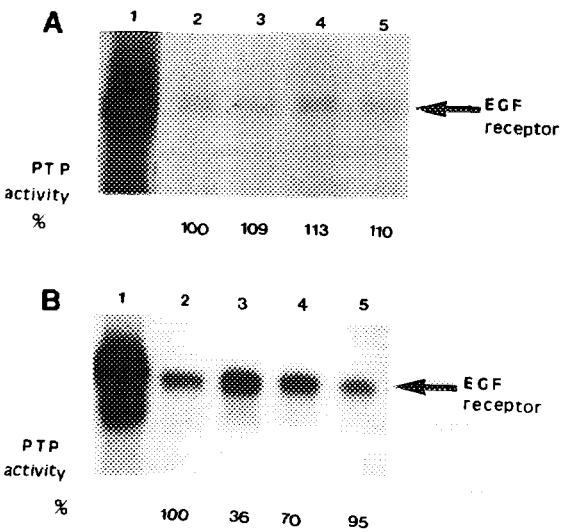

Figure 3. Modulation of PTP activity in RAEC by the native and oxidized forms of LDL. Serum-starved RAEC were incubated for 16 hs with increasing concentrations of native LDL (A) (50, 100 and 200 μg/ml in lanes 5, 4 and 3, respectively) or with increasing concentrations of oxidized LDL (B) (50, 100 and 200 μg/ml in lanes 5, 4 and 3, respectively). Control cells were maintained in the absence of lipoproteins for the incubation period (lane 2, A and B). Reaction was terminated and homogenates were prepared to measure PTP activity on ^{32}P-labeled EGF receptors as a substrate (see Materials and Methods). A representative experiment out of three is shown. The corresponding area for the EGF receptor band was determined by laser densitometry analysis. PTP activity is determined as a percentage of this area.

(1 mM) promoted NO release by RAEC with concomitant inhibition of PTP activities (data not shown).

DISCUSSION

The present report describes our findings on the oxidized LDL dependent-stimulation of NO release by RAEC. In endothelial cells, NO is synthesized from the oxidation of the guanidino nitrogen of L-arginine by a constitutively expressed NO synthase (1). The enzyme can reversibly bind calmodulin in a calcium-dependent fashion, and consequently can be activated by substances which elevate intracellular calcium levels (2). Furthermore, Negre-Salvayre et al. (14) had previously shown that oxidized LDL produced a delayed and sustained rise in the free cytosolic calcium concentration of the bovine aortic endothelial cell line, GM 7372A, after cells were incubated with the lipoprotein for16 hs. Accordingly, in our experimental model, we observed that oxidized LDL stimulated NO release by RAEC as well as calcium uptake by these cells. We also observed an stimulation of calcium uptake by the highest concentration of the native form of LDL used in our study. However, this elevation appeared not to be sufficient to promote NO release above basal levels. It has been reported that both, deficient generation of NO and enhanced production of this free radical can be injurious to the vascular system (25-28). Impairment on endothelium-dependent vasorelaxation in coronary atherosclerosis, was suggested to be associated with a reduced capacity of the endothelium to synthesize NO (29 and references therein). However, mounting experimental evidence in the literature (15,30,31) converge to the idea that such impairment may instead occur as a result of an enhanced inactivation of NO leading to a reduction in its vasoactive properties. In fact, Minor et al. (15) showed that the production of nitrogen oxides is augmented in hypercholesterolemic and atherosclerotic vessels. Our findings on the oxidized LDL-dependent stimulation of NO release by RAEC are in agreement with these observations and put forward the discussion on the role of oxidized LDL as an initiator of such process.

Oxidized LDL-dependent inhibition of RAEC associated PTP activity was observed concomitantly with nitrite accumulation. This inhibition could be imputed to : (a) lipid hydroperoxides present in the oxidized LDL particle (7); (b) NO (10) released by the cells under the effects of oxidized LDL; (c) elevated intracellular calcium levels (11,12) promoted by the oxidized form of the lipoprotein. Additionaly, vanadate, a well described inhibitor of PTP activities (32), also promoted an enhancement of NO release by RAEC. Taken together, our findings and the observations in the literature, suggest that oxidant modulation of the tyrosine dephosphorylation signalling pathway may be linked to the modulation of NO synthase activity in endothelial cells. Such modulation carried out by the oxidized form of LDL may be of importance in the early steps of the atherogenic process.

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REFERENCES

1. Nathan, C. (1992) FASEB J. 6, 3051-3064.
2. Bossaler, c., Habibi, G.B., Yamamoto, H., Williams, C., Wells, S. and Henry, P.D. (1987) J. Clin. Invest. 79, 170-174.
3. Marczin, N., Papapetropoulos, A. and Catravas, J.D. (1993) Am. J. Physiol. 265, H1014-H1018.
4. Schlessinger, J. and Ullrich, A. (1990) Cell 61, 203-212.
5. Dixon, J. E. and Walton, K. M. (1993) Ann. Rev. Biochem. 62, 101-120.
6. Chan, T. M., Chen, E. , Tatoyan, A., Shargill, N. S., Pleta, M. and Hochstein, P. (1986) Biochem. Biophys. Res. Commun. 139, 439-445.
7. Heffetz, D. and Zick, Y. (1989) J. Biol. Chem. 264, 10126-10132.
8. Monteiro, H. P., Ivaschenko, Y., Fischer, R. and Stern, A. (1991) FEBS Lett. 295, 146-148.
9. Monteiro, H. P. , Ivaschenko, Y., Fischer, R. and Stern, A. (1993) Intl. J. Biochem. 25, 1859-1864.
10. Monteiro, H.P., Peranovich, T.M.S., Fries, D.M., Stern, A. and Silva, A.M. (1994) In Frontiers in Reactive Oxygen Species in Biology and Medicine (K. Asada, and T. Yoshikawa, eds.) pp 215-218, Elsevier Science, Amsterdam.
11. Ostergaard, H. L. and Trowbridge, I. S. (1991) Science 253, 1423-1425.
12. Errasfa, M. and Stern, A. (1993) Eur. J. Pharmacol. 247, 73-80.
13. Ross, R. (1993) Nature 362, 801-809.
14. Negre-Salvayre, A., Fitoussi, G., Reaud, V., Pieraggi, M. T., Thiers, J. C. and Salvayre, R. (1992) FEBS Lett. 299, 60-65.
15. Minor, R. L., Myers, P. R., Guerra Jr., R., Bates, J. N. and Harrison, D. G. (1990) J. Clin. Invest. 86, 2109-2116.
16. Buonassisi, V. and Venter, J.C. (1976) Proc. Natl. Acad. Sci USA 73, 1612-1616.
17. Havel, R.J., Eder, H.A. and Bragdon, J.H. (1955) J. Clin. Invest. 34, 1345-1353.
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
19. Buege, J.A. and Aust, S.D. (1978) Meths Enzymol. 52, 302-310.
20. Orlov, S. , Resink, T. J., Bernhardt, J., Ferracin, F. and Buhler, F. R. (1993) Hypertension 21, 195-203.
21. Stuehr, D.J. and Nathan, C.F. (1989) J. Exp. Med. 169, 1543-1545.
22. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
23. Honegger, A. M., Dull, T. J., Felder, S., Van Obbberghen, E., Bellot, F., Szapary, D., Schmidt, A., Ullrich, A. and Schlessinger, J. (1987) Cell 51, 199-209.
24. Laemmli, U.K. (1970) Nature 227, 680-685.
25. Cox, D.A., Vita, J.A., Treasure, C.B., Fish, R.D., Alexander, R.W., Ganz, P. and Selwyn, A.P. (1989) Circulation 80, 458-465.
26. Panza, J.A., Quyyumi, A.A., Brush, J.E. Jr and Epstein, S.E. (1990) N.Engl. J. Med. 323, 22-27.
27. Palmer, R.M.J., Bridge, L., Foxwell, N.A. and Moncada, S. (1992) Br. J. Pharmacol. 105, 11-12.
28. Westenberger, U., Thanner, S., Ruf, H.F., Gersonde, K., Sutter, G. and Trentz, O. (1990) Free Rad. Res. Commun. 11, 167-178.
29. Flavahan, N.A. (1992) Circulation 85, 1927-1938.
30. Ohara, Y., Peterson, T.E. and Harrison, D.G. (1993) J. Clin. Invest. 91, 2546-2551.
31. White, C.R., Brock, T.A., Chang, L.Y., Crapo, J., Briscoe, P., Ku, D., Bradley, W.A., Gianturco, S.H., Gore, J., Freeman, B.A. and Tarpey, M.M. (1994) Proc. Natl. Acad. Sci. USA 91, 1044-1048.
32. Swarup, G., Cohen, S. and Garbers, D.L. (1982) Biochem. Biophys. Res. Commun. 107, 1104-1109.