Free Rad. Res., Vol. 24, No. 2, pp. 107–114
Reprints available directly from the publisher
Photocopying permitted by license only

Respiratory Burst is Decreased by Human Hyperlipemic Serum in Rat Peritoneal Macrophages

MANUEL CONDE, M. DOLORES CHIARA, M. GRACIA MARQUEZ, JOSEFA ANDRADE, CONSUELO SANTA MARIA*, FRANCISCO BEDOYA and FRANCISCO SOBRINO**

*Laboratorio de Sistemas Inmunológicos, Dpto. Bioquímica Médica y Biología Molecular, Facultad de Medicina, Universidad de Sevilla; *Dpto. de Bioquímica, Bromatología y Toxicología, Facultad de Farmacia, Universidad de Sevilla*

Accepted by Professor H. Sies

(Received April 5th, 1995; in final form, June 12th, 1995)

The effect of hyperlipemic human serum on superoxide anion (O_2^-) production by rat peritoneal macrophages was investigated. Phorbol myristate acetate (PMA)-stimulated O_2^- production was inhibited when cells were preincubated with hyperlipemic human serum. This inhibition was specifically carried out by a lipid fraction and was dependent on both cholesterol and triglyceride serum levels. This inhibitory effect was not exerted by a direct effect on NADPH-oxidase activity, nor by a putative superoxide dismutase activity present in the serum. With human neutrophils, we observed a decreased mobility of the cytosolic factor p47-phox to the membrane during the activation process, caused by hyperlipemic serum. We did not find any effect of hyperlipemic serum on NO_2^- production by cultured rat macrophages. These results suggest that a pathological increase of circulating plasma lipids may be associated with an impaired inflamatory capacity of macrophages.

INTRODUCTION

The role of macrophages on the pathogenesis of atherosclerosis has been recognized.[1] There is a recruitment of monocytes in the subendothelial space in the early events of the atherosclerotic process. Macrophages in the arterial wall are derived from blood monocytes and were shown to possess both the low density lipoprotein (LDL) receptor and a scavenger receptor, which takes up modified forms of LDL, primarily oxidized-LDL.[2] Macrophages first play a protective role by their ability to remove LDL from their environment, but when cholesterol is supplied in excess, these cells can be converted into foam cells.[3]

An index of macrophage inflamatory activity is the capacity to produce superoxide anion (O_2^-). O_2^- is produced in response to a variety of membrane stimulants which activate the respiratory burst by the membrane-bound NADPH-oxidase. This complex has at least four components: two subunits of the membrane bound cytochrome b, p91-phox and p22-phox, and two cytosolic components, p47-phox and p67-phox, which

**Correspondence to: F. Sobrino. Dpto. Bioquímica Médica y Biología Molecular, Facultad de Medicina. Avda. Sánchez Pizjuán, 4.-41009-Sevilla, Spain.

TABLE 1 Effect of hyperlipemic serum on NADPH-oxidase activity in rat peritoneal macrophages

			nmol O_2^-/min × mg protein	
[Triglycerides]	[Cholesterol]	[NADPH]...	50 μM	200 μM
–	–		5.07 ± 0.42	11.46 ± 0.84
0.74 g/l	1.95 g/l		5.64 ± 1.22	11.35 ± 0.21
12.8 g/l	2.06 g/l		5.58 ± 1.90	12.11 ± 0.69
0.8 g/l	3.49 g/l		6.20 ± 2.18	12.14 ± 1.29

Rat peritoneal macrophages were incubated with 100 nM PMA in the presence of 2% human serum (v/v) with the cholesterol and triglyceride concentrations as indicated. After 15 min, the cells were treated as described in "Materials and Methods". The NADPH-oxidase was measured in the postnuclear supernatant. Data are means ± S.E.M. of three experiments performed in triplicate.

translocate to the membrane during activation of the cell.[4,5,6]

Production of O_2^- has been demonstrated to be one of the main functional responses by which macrophages kill invading microbes or tumor cells.[7] Superoxide generated by the macrophages oxidizes the native LDL and transforms it into modified LDL,[8] which is able to bind to macrophage-specific receptors. With regard to LDL oxidation by macrophages, no consensus on the mechanism exists: lipoxygenase activity has been reported to be involved in LDL oxidation by murine peritoneal macrophages[9] but this is controversial.[10] Moreover, Wilkins and Leake[11] described that NADPH-oxidase was not essential for the modification of LDL by macrophages.

In the present study we have studied the effect of hyperlipemic human serum on superoxide anion production by rat peritoneal macrophages. An inhibition on O_2^- production has been observed when the cells were preincubated with hyperlipemic serum for 30 min before PMA addition. The inhibition was produced by a lipid fraction, and both cholesterol and triglyceride-rich serum had the same effect. No change in NADPH-oxidase activity was found.

MATERIALS AND METHODS

Materials

Chemicals were of analytical grade and were obtained from Merck, Darmstadt, Germany. Biochemical reagents and enzymes were from Boehringer Mannheim, Germany, or from Sigma Chemical Co., St Louis, MO, U.S.A. Human serum was obtained from University Hospital 'Virgen Macarena', Seville. Anti-p47-phox antibody was kindly provided by Prof. O.T. Jones (Dept. of Biochemistry, Bristol University, England).

Macrophage Preparation and Culture

Macrophages were obtained from peritoneal lavage of rats as previously described.[12] Peritoneal cells were suspended in Krebs-Ringer bicarbonate buffer (KRB) containing 10 mM glucose and 2% bovine serum albumin (w/v) and incubated in plastic Petri dishes at 37 °C under air/CO_2 (19:1) for 3 h. The dishes were then vigorously rinsed with cold KRB buffer and the adherent cells were detached using a rubber policeman, washed twice and suspended in KRB buffer without albumin but containing 10 mM glucose. Cell viability was estimated by Trypan blue exclusion. In some experiments macrophages were resuspended in culture medium (RPMI 1640, Hepes 20 mM, 2 mM glutamine, 10% FCS and antibiotics), plated (5×10^5 cells/300 μl per well) in flat bottom 96-well culture plates and incubated at 37 °C for 2 h. Non adherent cells were removed by washing three times with RPMI 1640 and macrophage monolayers were then cultured in culture medium. The components added to the culture medium are indicated in Table 2. After 72h of culture

TABLE 2 Effect of hyperlipemic serum on nitrite production by rat peritoneal macrophages

Serum added [Cholesterol; Triglycerides] (g/l)	Nitrite production nmol/well
Control (None)	32.7 ± 3.14
Normal serum	
1 [1.73; 0.76]	12.1 ± 1.21
2 [2.01; 0.99]	13.9 ± 1.69
Hyperlipemic serum	
1 [4.16; 4.94]	3.38 ± 0.56
2 [3.32; 0.98]	6.44 ± 1.04
3 [2.90; 1.09]	9.22 ± 0.98
4 [4.32; 7.47]	7.99 ± 1.09
5 [1.99; 3.60]	10.9 ± 1.53
6 [1.81; 2.89]	6.82 ± 1.83

Rats were injected intraperitoneal with E. Coli (previously killed by heat in an autoclave at 120 °C during 20 min). After 5 days, macrophages were isolated and cultured with serum. Final serum concentration in the culture media was 2% (v/v). After 72 h, aliquots of culture media were removed to measure accumulated nitrite. Data are means ± S.E.M. of three experiments performed in triplicate.

supernatants were removed to measure accumulated nitrite.

Assay of Superoxide Anion Release

O_2^- was measured by following the superoxide dismutase-inhibitable reduction of cytochrome c at 550 nm as described.[13] Macrophages (10^6 cells/ml) were incubated at 37 °C with 10 mM glucose, 80 μM cytochrome c and stimulated with 100 nM PMA. The reduction of cytochrome c was recorded continuously using a Hewlett Packard spectrophotometer. The O_2^- released was calculated by using a molar absorption coefficient of 21.1 mM^{-1} cm^{-1}.

NADPH-oxidase Activity

NADPH-oxidase activity was quantified as O_2^- production by measuring the superoxide dismutase inhibition of the reduction of cytochrome c at 25 °C as described.[14] Macrophages were incubated with 100 nM PMA in the absence (control) or in the presence of 2% human serum (v/v) for 5 min. The cells were harvested and homogenized with a Potter-Elvehjem homogenizer in 10 mM Tris, 0.35 M sucrose, pH 7.2. The homogenate was centrifuged at 1000 × g to eliminate debris and unbroken cells. Enzyme activity was assayed in the supernatant (postnuclear supernatant). Reaction mixture (1 ml) contained 10 mM phosphate buffer, pH 7.2, 100 mM NaCl, 1 mM $MgCL_2$, 80 μM cytochrome c and the postnuclear supernatant. The reaction was started by addition of 200 μM (maximal activity) or 50 μM (submaximal activity) of NADPH (final concentration). The absorbance change at 550 nm was recorded.

Preparation of Human Serum Lipid Extracts

Lipid extracts were obtained from human serum as follow: 4 ml human serum were mixed with 150 ml chloroform:methanol (2:1). This mixture was shaken for 10 min and then filtered. 20% $CaCl_2$ (w/v) was added to the filtered volume and it was shaken for 5 min. After two phases were formed, the lower phase, which contained the lipids, was collected and evaporated. The lipid extract was dissolved in 1 ml ethanol and added to the cells. Control cells had the same amount of solvent.

Neutrophils Preparation, Electrophoresis and Immunoblotting

Human neutrophils were prepared from venous blood of healthy donors by standard methods.[15] Cells (10^7 cells/ml) were resuspended in Krebs Ringer bicarbonate buffer containing 10 mM Hepes pH 7.4, 0.5 mM $CaCl_2$ and 5 mM glucose. After incubation as indicated in Figure 4, cells were centrifuged 30 seconds in a microfuge and resuspended in 10 mM Tris-HCl pH 7.4, 1 mM PMFS and 100 U/ml aprotinin and were disrupted by sonication (3 × 20 seconds). Fractionation was carried out as described in.[16] First, sonicated cells were centrifuged for 10 min at 10,000 × g and postnuclear supernatants were then recentrifuged for 1 h at 100,000 × g. Similar aliquots (80 μg of protein) of cytosolic fractions were

electrophoresed on a 10% SDS/PAGE gel as.[17] Proteins were transferred to nitrocellulose membranes in semi-dry conditions with a Bio-Rad Trans Blot cell apparatus. The blots were incubated with purified rabbit anti-p47-phox antibody (dilution 1:1000). After washing, blots were newly incubated with horseradish peroxidase labelled sheep anti-rabbit IgG antibody. Detection of bound antibody was performed by an enhanced chemiluminescence method, using luminol and other enhancers (manuscript in preparation).

Other Methods

Protein concentration was determined by the modified Lowry method[18] with bovine albumin as standard.

O_2^- production by phenazine methosulphate/NADH was followed by cytochrome c reduction at 20 °C.[19] The reaction mixture contained 20 mM pyrophosphate, pH 8.3, 5 μM cytochrome c and 100 μM NADH. The reaction was triggered by 10 μM phenazine methosulphate and followed at 550 nm. Superoxide dismutase activity in serum was assayed according to the method described by Beyer.[20] Venous blood was sampled an overnight fast and serum was obtained by low-speed centrifugation. The cholesterol and triglycerides were analyzed enzymatically on a Technicon RA-1000 autoanalyzer.

The accumulation of NO_2^- in culture supernatant was measured with the Griess reagent.[21] NO_2^- concentration was calculated from a $NaNO_2$ standard curve.

RESULTS

Peritoneal rat macrophages were preincubated in the presence of 2% hyperlipemic human serum (v/v) for 15 min before stimulation with PMA. Figure 1 illustrates results from different experiments in which PMA-stimulated O_2^- production was measured. In the presence of hyperlipemic serum macrophages displayed a reduced capacity

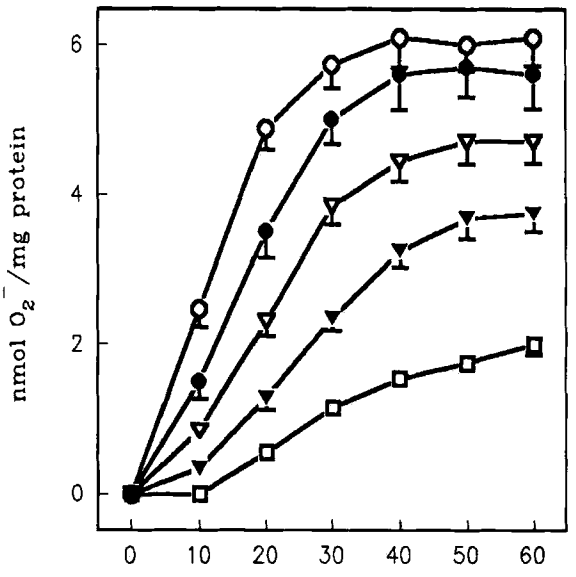

FIGURE 1 Effect of hyperlipemic human serum on O_2^- production by peritoneal macrophages. Macrophages were preincubated in the absence (○) or in the presence of human serum with several cholesterol and triglyceride concentrations: 1.86 g/l cholesterol and 0.9 g/l triglycerides (●), 2.55 g/l cholesterol and 1.1 g/l triglycerides (▽), 3.1 g/l cholesterol and 1 g/l triglycerides (▼) and 2.1 g/l cholesterol and 12.8 g/l triglycerides (□). After 15 min at 37 °C the respiratory burst was triggered with 100 nM PMA and the O_2^- production was recorded at different times. Data shown are means ± S.E.M. of five experiments performed in triplicate.

to produced O_2^- in response to 100 nM PMA. The inhibition ranged from 22% to 67% compared with control cells. It was also observed that O_2^- production was weakly decreased when normolipemic human serum was used. However, this inhibition did not exceed 14% regarding control cells (preincubated without serum).

In order to determine what lipidic component (e.g. cholesterol or triglycerides) were responsible for the inhibitory effect on O_2^- production, macrophages were preincubated with serum containing different levels of either cholesterol or triglycerides. Figure 2A shows that serum with increasing concentrations of cholesterol (but normal triglyceride concentration) inhibited, in dose-dependent manner, the O_2^- produced at 60 min of PMA addition. A similar inhibitory effect on respiratory burst was found when serum with high triglyceride levels (but normal cholesterol concentration)

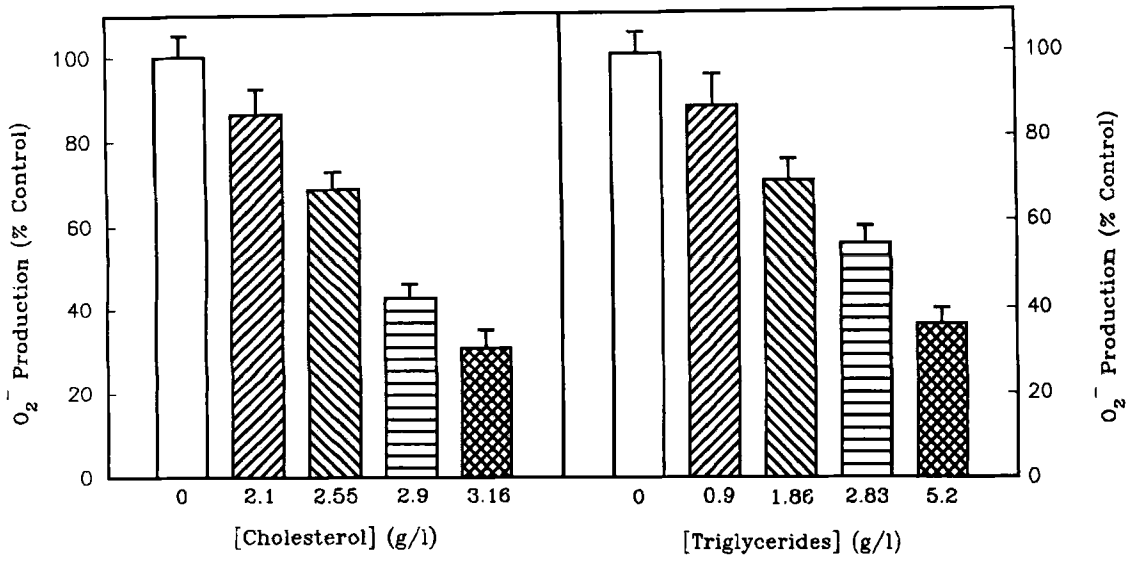

FIGURE 2 Dose effect of cholesterol and triglycerides on O_2^- production by rat peritoneal macrophages. The cells were incubated in the absence (control) or in the presence of 2% human serum with increasing concentrations of cholesterol but triglycerides concentrations lower than 1 g/l (A), or with 2% human serum with increasing concentrations of triglycerides but cholesterol concentrations lower than 2 g/l (B). The respiratory burst was stimulated, after 15 min of the preincubation with serum, with 100 nM PMA and the O_2^- released was estimated at 60 min. The results are means ± S.E.M. of three experiments performed in triplicate.

was used (Figure 2B). The lipid fraction from hyper- or normolipemic serum was extracted as described in the Materials and Methods section. This lipid fraction was used to determine whether a serum lipid component or a non-lipemic factor was the responsible for the inhibitory effect observed on respiratory burst. Figure 3 illustrates that the lipid fraction exerted an inhibitory effect on PMA-stimulated O_2^- production, similar to those obtained when complete hyperlipemic serum was used. These data suggest that the serum lipid component is likely the responsible for the inhibitory effect.

The cellular viability was not altered by human serum (tested by exclusion of Trypan blue test). Alternatively, the inhibitory effect might be the consequence of the scavenger effect on anion superoxide of an unidentified serum component or by the presence of high superoxide dismutase activity in the serum. Both possibilities were analyzed. The results shown that serum assayed did not interfere with the O_2^- generated by the phenazine methosulphate/NADH system, and direct measurements of superoxide dismutase failed to reveal detectable activity in any of the serum samples used (data not shown). Thus, both possibilities seem unlikely.

A direct interaction of the serum lipid fraction with the O_2^- producing enzyme (NADPH-oxidase) could be suggested. To test for this possibility, the NADPH-oxidase activity was assayed in crude supernatants from PMA-stimulated macrophages in the presence or absence of serum. Table 1 illustrates that NADPH-activity was not affected by the preincubation with 2% hyperlipemic serum, indicating that the serum lipid component did not induce an irreversible modification of NADPH-oxidase. The inhibitory effect of hyperlipemic serum is more probably due to an alteration of the activation mechanism of the NADPH-oxidase rather than a direct effect on the enzyme. To investigate this possibility, we have studied by immunoblotting whether hyperlipemic serum alters the mobility of p47-phox in human neutrophils. Figure 4 illustrates that in resting cells most of p47-phox remains in the

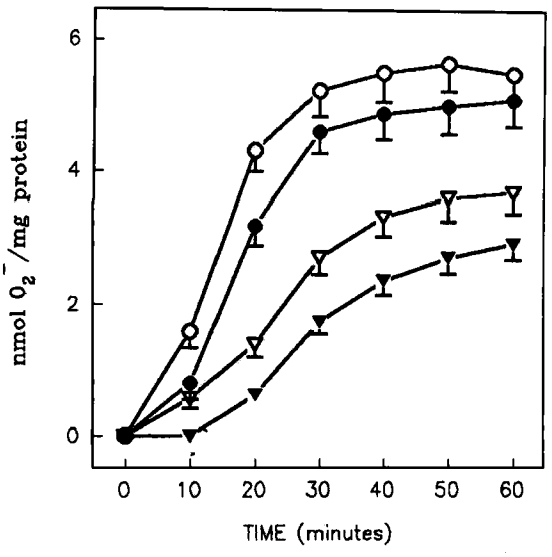

FIGURE 3 O_2^- production by rat peritoneal macrophages in the presence of human serum lipid extracts. The cells were incubated in the absence (○) or in the presence of 2% lipid extracts (v/v) obtained from human serum with several cholesterol and triglyceride concentrations: 1.86 g/l cholesterol and 0.9 g/l triglycerides (●), 3.1 g/l cholesterol and 1 g/l triglycerides (▽) and 2.1 g/l cholesterol and 12.8 g/l triglycerides (▼). After 15 min at 37 °C the respiratory burst was triggered with 100 nM PMA and the O_2^- production was recorded at different times. The values are means ± S.E.M. of three experiments performed in triplicate.

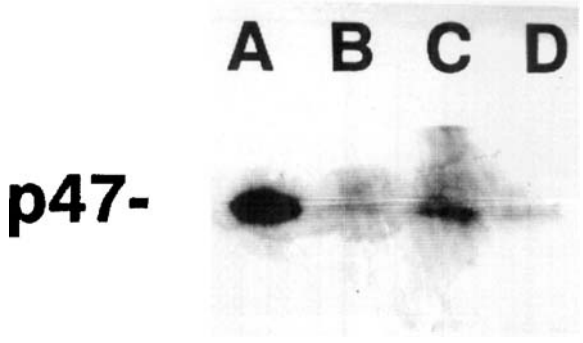

FIGURE 4 Effect of hyperlipemic serum on disappearance of p47-phox cytosolic factor from cytosol. Human neutrophils (10^7 cells) were preincubated without additions (lane A and B), with 2% hyperlipemic serum (cholesterol 4.16 g/l, triglycerides 4.96 g/l) (lane C) or with 2% normal serum (cholesterol 1.73 g/l, triglycerides 0.73 g/l) (lane D). After 15 min at 37 °C 100 nM PMA was added (lanes B,C and D) and the cells were incubated for 5 min at 37 °C. Following these treatments cytosolic fraction was prepared, electrophoresed and immonoblotted with a polyclonal antibody anti-p47-phox.

cytosolic fraction (lane A). The activation of NADPH-oxidase with PMA is associated with the disappearance of p47-phox from the cytosolic fraction after PMA addition (lane B). The simultaneous presence of PMA with hyperlipemic serum (lane C) caused that a relative amount of p47-phox remained in the cytosol, which implicated a lower activity of NADPH-oxidase. If normal serum was added with PMA (lane D) no modification was detected compared with PMA alone (lane B).

We have also performed experiments to analyze the effect of hyperlipemic serums on nitric oxide production by macrophages maintained in culture for 48 h. Nitric oxide has been identified as a potent and pleotropic mediator with biological roles in inflammatory responses, blood vessel reactivity and neurotransmission.[22,23] Table 2 shows that addition of normolipemic serum (control serum) to culture media produced strong inhibition on NO_2^- production (about 60%) by rat macrophages. When hyperlipemic serums were added the same degree of inhibition was observed. Thus, no relationship between cholesterol or triglycerides levels of serum and inhibition of NO_2^- production by macrophages maintained in cultured could be established.

DISCUSSION

The superoxide anion production by rat peritoneal macrophages when cells are incubated with hyperlipemic serum was studied. The results show that human hyperlipemic serum produces an inhibition of the PMA-stimulated respiratory burst in rat peritoneal macrophages, which depended on cholesterol and trigliceride serum levels and was specifically exerted by a serum lipid fraction. The activity of NADPH-oxidase, measured in crude extracts, was not affected by previous exposure to hyperlipemic serum.

Perhaps oxidized-LDL might be the factor responsible for the respiratory burst inhibition. However, we have found a similar inhibitory

effect using serum with high triglyceride levels and normal cholesterol concentration and serum with increasing cholesterol concentration and normal triglyceride levels.

Another possibility is that exposition to hyperlipemic serum might alter macrophage lipid composition and alter membrane fluidity. The respiratory burst is membrane-dependent, so changes in membrane lipid composition and fluidity may affect the anion superoxide production. Indeed, changes in macrophage membrane fluidity due to variation in membrane lipid composition was associated with a decrease in respiratory burst.[24] Mahoney *et al.*[25] showed that other membrane-associated functions such as receptor-mediated phagocytosis and pinocytosis were dependent on lipid environment.

A negative modulatory effect of fatty acids or derived products on macrophage functions has been described. Engels *et al.*[26] observed that a linoleic acid metabolite inhibited O_2^- production when added exogenously. Other lipids such as sphinganine and sphingosine are potent inhibitors of the neutrophil respiratory burst and PKC activity.[27] Also dietary n-3 fatty acid supplementation reduces O_2^- production[28] and synthesis of interleukin-1 and tumor necrosis factor by macrophages.[29] In contrast, an enhanced O_2^- production in neutrophils[30] and increase NADPH-oxidase activity in cell free systems[14] by polyunsatured fatty acid has been described. However, Badwey *et al.*[31] and Curnutte *et al.*[32] observed that the polyunsatured fatty acid-stimulated O_2^- production was inhibited by albumin.

In aging, where there is an increase in plasma cholesterol levels in human as well as in rats,[33] a decrease in O_2^- production by rat peritoneal macrophages stimulated with PMA has been described.[34] NADPH-oxidase activity does not change in macrophages after the incubations with hyperlipemic serum. This suggests that the inhibitory effect is more probably due to a dysfunction in the NADPH-oxidase activation mechanism than a direct effect on the enzyme. Similar results has been described during aging.[34] In agreement with this possibility we observed a partial inhibition of translocation to membrane of cytosolic factor p47-phox during PMA-dependent activation of respiratory burst in the presence of hyperlipemic serum. It has been shown that the translocation of p47-phox appears to depend on the presence of membrane-bound cytochrome b_{554}, which cytoplasmic domain play a critical role in interaction with p47-phox.[5] From present data (Figure 4) could be suggested that the putative alterations of membrane exposed to hyperlipemic serum are produced by a decreased translocation of p47-phox to membrane.

Acknowledgments

Financial support for this research was provided by the Fondo de Investigaciones Sanitarias (FISS) (Grants 94/1484 and 94/1489) and by the Consejería de Salud/Junta de Andalucía (RS 46), Spain.

References

1. M.J. Mitchinson and R.Y. Ball (1987) Macrophages and atherogenesis. *Lancet*, **2**, 146–149.
2. J.L. Goldstein, Y.K. Ho, S.K. Basu and M.S. Brown (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proceedings of National Academy of Science USA*, **76**, 333–337.
3. N.M. Agel, R.Y. Ball, H. Waldmann and M.J. Mitchinson (1984) Monocytic origin of foam cells in human atherosclerotic plaques. *Atherosclerosis*, **53**, 265–271.
4. R.A. Clark, B.D. Volpp, K.G. Leidal and W.M. Nauseef (1990) Two cytosolic components of the human neutrophil respiratory burst oxidase translocate to the plasma membrane during cell activation. *Journal of Clinical Investigation*, **85**, 714–721.
5. F. Morel, J. Doussiere and P.V. Vignais (1991) The superoxide-generating oxidase of phagocytic cells. Physiological, molecular and pathological aspects. *European Journal of Biochemistry*, **201**, 523–546.
6. A.W. Segal and A. Abo (1993) The biochemical basis of the NADPH-oxidase of phagocytes. *Trends in Biochemical Science*, **18**, 43–47.
7. B. Halliwell and J.M.C. Gutteridge (1985). Free radicals as useful species. In *Free Radicals in Biology and Medicine*. Clarendon Press, Oxford, pp. 246–278.
8. S. Parthasarathy, D.J. Printz, D. Boyd, L. Joy and D. Steinberg (1986) Macrophage oxidation of low density lipoprotein generates a modified form recognized by the scavenger receptor. *Atherosclerosis*, **6**, 505–510.
9. S.M. Rankin, S. Parthasarathy and D. Steinberg (1991) Evidence for a dominant role of lipoxygenase(s) in the oxidation of LDL by mouse peritoneal macrophages. *Journal of Lipid Research*, **32**, 449–456.
10. C.P. Sparrow and J. Olszwski (1992) Cellular oxidative

modification of low density lipoprotein does not require lipoxygenases. *Proceedings of National Academy of Science*, **89**, 128–131.
11. G.M. Wilkins and D.S. Leake (1994) The effect of inhibitors of free radical generating-enzymes on low-density lipoprotein oxidation by macrophages. *Biochimica et Biophysica Acta*, **1211**, 69–78.
12. D.E. Mosier (1984) Separation of macrophages on plastic and glass surfaces. *Methods in Enzymology*, **108**, 294–297.
13. B.M. Babior, R.S. Kipnes and J.T. Curnutte (1973) Biological defence mechanism: the production of superoxide a potential bactericidal agent. *Journal of Clinical Investigation*, **52**, 741–744.
14. R.A. Heyneman and R.E. Vercauteren (1984) Activation of a NADPH-oxidase from horse polymorphonuclear leukocytes in a cell-free system. *Journal of Leukocyte Biology*, **36**, 751–759.
15. A. Böyum (1968) Isolation of mononuclear cells and granulocytes from blood. *Scandinavian Journal of Clinical Laboratory Investigation*, **97**, 77–89.
16. I. Aviram and S. Sharabani (1989) Kinetics of cell-free activation of neutrophil NADPH-oxidase. Effects of neomycin and guanine nucleotides. *Biochemical Journal*, **261**, 471–482.
17. U.K. Laemmli (1970) Cleavage of structural proteins during the assembly of bacteriophage T4. *Nature*, **227**, 680–685.
18. O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall (1951) Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.
19. M. Nishikimi, N.A. Rao and K. Yagi (1972) The occurrence of superoxide anion in the reaction of reduced phenazine methosulphate and molecular oxygen. *Biochemical and Biophysical Research Communications*, **46**, 849–854.
20. W.F. Beyer Jr. and I. Fridovich (1987) Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Analytical Biochemistry*, **161**, 559–566.
21. L.C. Green, D.A. Wagner, J. Glogowsky, P.L. Skipper, J.S. Wishnok, and S.R. Tannenbaum (1982) Analysis of nitrate, nitrite and [^{15}N]nitrate in biological fluids. *Analytical Biochemistry*, **126**, 131–138.
22. H.H.W. Schmidt and U. Walter (1994) NO at work. *Cell*, **78**, 919–925.
23. D.S. Bredt and S.H. Snyder (1994) Nitric oxide: A physiologic messenger molecule. *Annual Review in Biochemistry*, **63**, 175–195.
24. E. Alvarez, V. Ruiz-Gutierrez, C. Santa-María and A. Machado (1993) Age-dependent modification of lipid composition and lipid structural order parameter of rat peritoneal macrophages. *Mechanism Ageing Development*, **71**, 1–12.
25. E.M. Mahoney, A.L. Hamill, W.A. Scott and Z.A. Cohn (1977) Response of endocytocis to altered fatty acyl composition of macrophage phospholipids. *Proceedings of National Academy of Science USA*, **74**, 4895–4899.
26. F. Engels, P.A.J. Henricks, H. Van der Vliet and F.P. Nijkamp (1989) Modulatory activity of 9-hydroxy- and 9-hydroperoxy-octadecadienoic and towards origin species from guinea-pig pulmonary macrophages. *European Journal of Biochemistry*, **184**, 197–203.
27. E. Wilson, M.C. Olcott, R.M. Bell, A.H. Merril Jr. and J.D. Lambeth (1986) Inhibition of the oxidative burst in human neutrophils by sphingoid long-chain bases. *Journal of Biological Chemistry*, **261**, 12616–12623.
28. M. Fisher, P.H. Levine, B.H. Weiner, M.H. Johnson, E.M. Doyle, P.A. Ellis and J.J. Hoogasian (1990) Dietary n-3 fatty acid supplementation reduces superoxide production and chemiluminescence in a monocyte-enriched preparation of leukocytes. *American Journal of Clinical Nutrition*, **51**, 804–808.
29. S. Endres, R. Ghorbani, V.E. Relley, K. Georgilis, G. Lonnemann, J.W.M. Van der Meer, J.G. Cannon, T.S. Rogers, M.S. Klempner, P.C. Weber, E.J. Schaefer, S.M. Wolff and C.A. Dinarello (1989) The effect of dietary supplementation with n-3 polyunsatured fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. *New England Journal of Medicine*, **320**, 265–271.
30. J.A. Badwey, J.T. Curnutte and M.L. Karnovsky (1981) Cis-polyunsatured fatty acid induce high levels of superoxide production by human neutrophils. *Journal of Biological Chemistry*, **256**, 12640–12643.
31. J.A. Badwey, J.T. Curnutte, J.M. Robinson, C.B. Berde, M.J. Karnovsky and M.L. Karnovsky (1984) Effect of free fatty acids on release of superoxide and on change of shape by human neutrophils. *Journal of Biological Chemistry*, **259**, 7870–7877.
32. J.T. Curnutte, R. Kuver and P.J. Scott (1987) Activation of neutrophil NADPH-oxidase in a cell-free system. Partial purification of components and characterization of the activation process. *Journal of Biological Chemistry*, **262**, 5563–5569.
33. A.G. Lacko and J.L. Davis (1979) Age-related changes in rat and primate plasma cholesterol metabolism. *Journal American Geriatric Society*, **37**, 212–217.
34. E. Alvarez, C. Santa-María and A. Machado (1993) Respiratory burst reaction changes with age in rat peritoneal macrophages. *Biochimica et Biophysica Acta*, **1179**, 247–252.