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Reactive oxygen species generation in peripheral blood monocytes and oxidized LDL are increased in hyperlipidemic patients

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Abstract

Objectives: Experimental and *in vitro* evidences have established that reactive oxygen species (ROS) generated by vascular wall cells play a key role in atherogenesis. Here, we evaluated the rate of ROS generation by resting peripheral monocytes in naive hyperlipidemic subjects.

Design and methods: Primary hypercholesterolemic, combined hyperlipidemic, and normolipidemic individuals were studied. ROS generation and the mitochondrial electrical transmembrane potential were estimated by flow cytometry. Plasma oxidized (ox) LDL levels and lipid profile were measured by ELISA and enzymatic colorimetric methods.

Results: Both hyperlipidemic groups presented significantly higher rates of monocyte ROS generation and elevated plasma levels of ox-LDL. Combined hyperlipidemic subjects presented increased levels of small dense LDL and insulin. Significant positive correlations between monocyte ROS generation and ox-LDL concentrations were found in pooled data.

Conclusions: These data provide evidence that ROS production by circulating monocytes from hyperlipidemic subjects may contribute to the systemic oxidative stress and possibly to atherogenesis.

Keywords: Reactive oxygen species; Monocytes; Hyperlipidemia; Oxidized low density lipoprotein; Atherosclerosis

Introduction

Atherosclerosis is characterized by low density lipoprotein (LDL) deposition in the arterial wall. It is initiated by the recruitment of circulating monocytes into the vascular intima with their subsequent transformation in foam cells. Foam cell formation results from monocyte derived macrophage scavenging of modified lipoproteins that have undergone oxidative modification in the vascular wall [1,2]. The scavenger receptor (SR) and CD36 mediated oxidized LDL uptake by macrophages lead to the initial formation of the well-defined fatty streaks in the arterial intima. Such lesions then progress to more complex ones and are prone to rupture precipitating clinical events such as heart attack and stroke [2].

Clinical studies have shown that traditional risk factors for atherosclerosis predispose to endothelial dysfunction [3,4]. One of the most consistent proposed mechanisms for endothelial dysfunction involves the establishment of a local oxidative stress [4]. Reactive oxygen species (ROS) can be generated by aerobic cells during reduction of molecular oxygen by enzymatic reactions (NADH/NADPH oxidase, xanthine oxidase, lipoxygenase, and cyclooxygenase systems), mitochondrial electron transport chain and autoxidation of diverse substances [5]. Mitochondria represent a major intracellular source of ROS [6–8] and may mediate cellular oxidative stress leading to cell death [9].

Cellular ROS and their non-physiological derivatives may oxidize LDL *in vitro* and *in vivo* [1,10]. Oxidation process

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increases the atherogenic potential of LDL [1] by: 1) favoring the recruitment of circulating monocytes into the intimal space, 2) inhibiting the ability of resident macrophages to leave the intima, 3) enhancing the rate of lipoprotein uptake leading to foam cell formation, and 4) being cytotoxic and leading to endothelial dysfunction [2].

Oxidized (ox) LDL contains various toxic lipids such as lipid peroxides, oxysterols and aldehydes that can induce modifications in protein structure, and further increase cell ROS generation in a vicious cycle manner [3]. Defective catabolism of LDL and other lipoproteins in hypercholesterolemic and hypertriglyceridemic states results in a prolonged half-life of these particles in the circulation, increasing the possibility of these lipoproteins to be exposed to ROS generated by circulating and vascular cells. Although it is well established that LDL may undergo oxidation and that oxidized LDL is present in the blood stream [11] and in the arterial lesions [12], the issues of how and when LDL become oxidized *in vivo* are still not well clarified [1].

Previous studies have shown elevated extracellular release of ROS by mononuclear cells from hypertriglyceridemic [13] and combined hyperlipidemic [14] patients. However, these data were obtained after activating these cells with phorbol esters or opsonized zymosan. In this work, we investigated whether circulating monocytes from untreated hyperlipidemic individuals, freshly isolated and under resting (non-stimulated) condition, present elevated intracellular ROS generation. In addition, we determined mitochondrial electrical transmembrane potential of these cells, plasma levels of oxidized LDL and other biochemical parameters and checked whether they correlated with intracellular ROS production.

Materials and methods

This study was approved by the Medical Ethics Committee of the Medical Sciences Faculty of the University of Campinas. Written consent was obtained from the patients or their relatives.

Fourteen primary hypercholesterolemic (HC) patients, aged 37±12 y (29% male and 71% female), 15 combined hyperlipidemic patients (CH) aged 42±12 y (60% male and 40% female), with high serum cholesterol (>200 mg/dL) and triglycerides (>150 mg/dL) levels, according to National Cholesterol Education Program (NCEP) [15] and 18 normolipidemic individuals (NL, controls) aged 32±6 y (33% male and 67% female), all non-smokers and naive for lipid-lowering treatment, were enrolled. Patients with secondary causes of hyperlipoproteinemia or who had suffered myocardial infarction, coronary artery bypass grafting, percutaneous transluminal coronary angioplasty, stroke, transient ischemic attack and peripheral arterial disease were excluded from the study. Fasting (12 h) serum cholesterol (Chol), HDL-cholesterol (HDL-chol), triglycerides (TG) and glucose were measured by enzymatic colorimetric methods in an automated system (Roche, Germany). The LDL-cholesterol (LDL-chol) and VLDL-cholesterol (VLDL-chol) were calculated by the Friedewald's formula (only if TG levels <400 mg/dL). The apolipoproteins A1 (Apo A1), B100 (Apo B) and lipoprotein "a" [Lp (a)] were measured by nephelometric methods (Dade-Behring, USA). Free fatty acids (FFA) and free cholesterol (FC) were determined by enzymatic colorimetric methods (Wako Chemicals GmbH, Japan), ox-LDL by enzyme immunoassay (Mercodia Oxidized LDL ELISA, Sweden) and insulin by chemiluminescence assay (DPC MEDLAB, USA).

LDL particle size was estimated by the ratios LDL-chol/apo B-100 and TG/HDL-chol [16,17]. The homeostasis model assessment (HOMA), that estimates insulin resistance, was calculated using the formula: fasting glucose level (mmol/L)× fasting insulin level (μU/mL)/22.5 [17].

Red and white blood cell counts were made in automated system (SYSMEX SE or SYSMEX XE).

For evaluation of intracellular ROS generation and mitochondrial membrane potential ($\Delta\psi_m$) the mononuclear cells of NL, HC and CH groups were isolated by Ficoll-Hypaque gradient adjusted to 1.076 g/mL [18], and used immediately after the blood collection. Cellular viability was checked by Trypan blue staining and cells were used when the viability was >98%. The samples were analyzed in flow cytometer (FACSCalibur — Becton & Dickinson) equipped with an argon laser and CellQuest software. Ten thousand events were collected for each sample.

Mononuclear cells (10^6 cells/mL) were pre-incubated in RPMI 1640 medium supplemented with 1% bovine fetal serum (FBS) and glutamine 200 mM (Cutilab, Campinas, Brazil) at 37 °C in a humidified CO_2 incubator (5% CO_2) for 30 min. Then, cells were incubated with 5 μM dihydroethidium (DHE, Molecular Probes Inc., Eugene, OR, USA) for 70 min. The probe DHE, a membrane permeable cation, is converted to ethidium when oxidized by ROS (particularly superoxide). The ethidium fluorescence was measured in the FL2 channel (FACSCalibur — Becton & Dickinson) [19].

Mitochondrial electrical transmembrane potential was measured in intact mononuclear cells. The monocyte population was identified by their light-scattering characteristics, enclosed in electronic gates, and analyzed for the intensity of the fluorescent probe signal. Monocytes (10^6 cells/mL) were incubated with 0.2 nM 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$, Molecular Probes Inc., Eugene, OR, USA] with or without 1 μg/mL oligomycin (Sigma, St. Louis, MO, USA) in RPMI 1640 medium supplemented with 1% FBS and 200 mM glutamine at 37 °C in a humidified CO_2 incubator (5% CO_2), for 20 min. One half of the above cell preparation (200 μL) was incubated with 50 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP, Sigma, St. Louis, MO, USA), an uncoupling agent that dissipates the mitochondrial electrical membrane potential [20], for further 10 min. After both incubation periods, cells were stained with anti-human CD14 labeled with fluorescein isothiocyanate (FITC) or an isotype-matched control (BD Pharmingen, San Diego, USA) for an additional 10 min. incubation period. The samples were analyzed in a FACSCalibur flow cytometer. $DioC_6(3)$, at non-saturating concentration, binds preferentially to mitochondria, since the magnitude of mitochondrial electrical membrane potential is much higher (−180 mV) than the plasma membrane potential

Table 1
Plasma lipoproteins, biochemical and anthropometric profiles of normolipidemic (NL), hypercholesterolemic (HC) and combined hyperlipidemic (CH) individuals.

Variables	NL (n=18)	HC (n=14)	CH (n=15)	p value
Cholesterol (mg/dL)	168.1±28.3[a]	244.1±30.5[b]	274.3±84.8[b]	<0.0001
HDL-cholesterol (mg/dL)	57.8±13.0	62.9±13.8	50.7±14.6	0.5819
LDL-cholesterol (mg/dL)	110.1±28.2[a]	169.4±30.5[b]	156.0±45.6[b]	<0.0001
VLDL-cholesterol (mg/dL)	14.7±5.6[a]	23.7±4.8[b]	52.9±11.1[c]	<0.0001
TG (mg/dL)	73.5±28.9[a]	118.9±24.0[b]	445.5±400.2[c]	<0.0001
Apolipoprotein A1 (mg/dL)	152.2±20.6	167.9±23.2	154.7±33.8	0.0768
Apolipoprotein B (mg/dL)	83.0±20.4[a]	126.6±17.3[b]	137.8±24.8[b]	<0.0001
Lipoprotein "a" (mg/dL)	13.4±16.4	43.8±55.8	34.7±31.5	0.2392
Free fatty acids (mEq/L)	0.47±0.2[a]	0.5±0.2[a]	1.4±0.6[b]	<0.0001
Free cholesterol (mg/dL)	33.0±7.3[a]	45.6±7.7[b]	73.1±53.6[b]	<0.0001
Cholesteryl ester (mg/dL)	132.9±24.0[a]	194.0±18.0[b]	219.1±70.0[b]	<0.0001
LDL-chol/Apo B	1.3±0.2[a]	1.3±0.1[a]	1.1±0.2[b]	0.0325
TG/HDL-chol	1.4±0.8[a]	2.0±0.8[b]	9.8±9.5[c]	<0.0001
Glucose (mg/dL)	80.9±8.8	83.6±5.6	91.7±15.4	0.3553
Insulin (μUI/mL)	6.6±2.8[a]	8.5±4.0[a]	17.5±9.4[b]	0.0002
HOMA	1.2±0.5[a]	1.8±0.9[b]	3.4±2.0[b]	0.0003
Weight (kg)	62.8±12.2[a]	64.4±13.2[b]	77.6±15.9[c]	<0.001
BMI (kg/m^2)	22.5±2.4[a]	23.8±3.1[b]	27.2±4.5[c]	<0.001
WC (cm)	80.8±10.6[a]	86.4±10.8[b]	99.2±11.0[c]	<0.001

Values are expressed as mean±standard deviation. HOMA: Homeostasis Model Assessment; WC: waist circumference; BMI: body mass index. Statistical analyses by ANCOVA (adjusted for age, sex and BMI) for biochemical analyses and ANOVA for anthropometric measurement, both with Tukey–Kramer post test. [a,b,c]Different letters indicate significant differences between groups.

(−60 mV). The results were normalized using F/F_{CCCP} ratio where F is the fluorescence intensity of $DioC_6(3)$ (maximum fluorescence) and F_{CCCP} is the fluorescence intensity in the presence of CCCP (minimum fluorescence).

Comparative analyses of sex, age and body mass index (BMI) were performed using the tests Chi-Square and ANOVA, respectively. Other results from the three groups were compared by the Analysis of Covariance (ANCOVA) in ranked variables, adjusting for age, sex and BMI. The Tukey–Kramer post-hoc test was used for multiple comparisons and Mann–Whitney for two group comparisons. The correlations were calculated by partial Spearman's correlation coefficient [21]. The differences were considered significant when $p<0.05$.

Results

Biochemical and anthropometric data are reported in Table 1. Both hyperlipidemic groups of patients presented elevated total cholesterol, LDL-cholesterol, VLDL-cholesterol, triglycerides, apolipoprotein B, and insulin resistance index (HOMA) when compared to normolipidemic subjects. As expected, VLDL-cholesterol and triglycerides were higher in combined

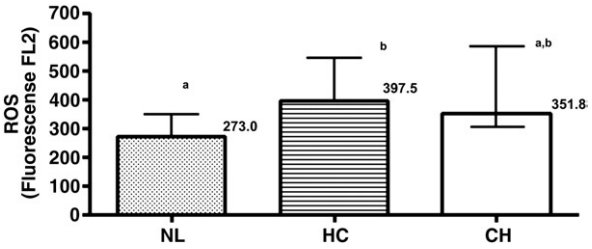

Fig. 1. ROS generation in monocytes of normolipidemic (NL, n=17), hypercholesterolemic (HC, n=12) and combined hyperlipidemic (CH, n=12) groups measured by flow cytometry (FACS). Monocytes were loaded with DHE (5 μM) for 70 min. The average of 2-hydroxy-ethidium fluorescence intensity (arbitrary unit — a.u.) was detected in FL2 channel. Data presented as median and interquartile range. [a,b]Different letters indicate significant differences (p=0.0491 for NL vs HC by ANCOVA; $p<0.05$ for NL vs HC and NL vs CH by Mann–Whitney test).

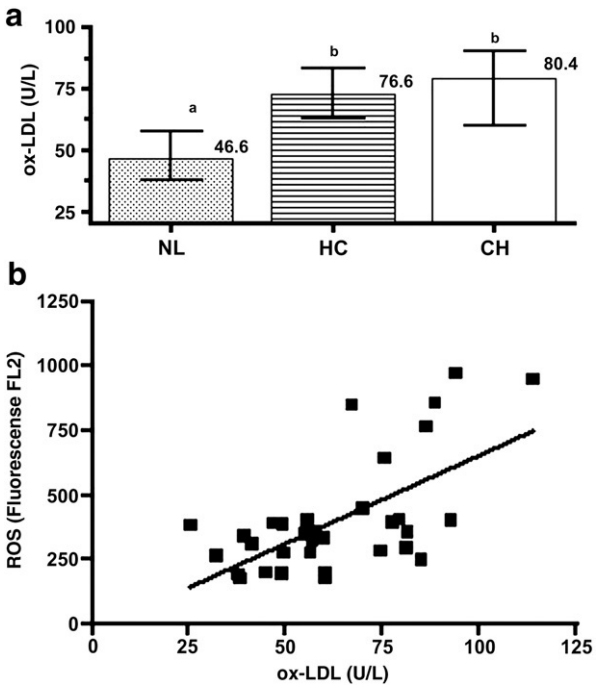

Fig. 2. Oxidized LDL serum level (a) and its correlation with monocyte ROS generation (b) Panel a: Oxidized LDL serum levels in normolipidemic (NL), hypercholesterolemic (HC) and combined hyperlipidemic (CH) individuals (expressed as median and interquartile range). ANCOVA (adjusted for age, sex and BMI) with Tukey–Kramer post test (p=0.0003). [a,b]Different letters indicate significant differences. Panel b: Correlation between monocyte ROS generation and serum ox-LDL (n=41). Partial Spearman's correlation coefficient (adjusted for age, sex and BMI): r=0.35 and p=0.0385.

hyperlipidemia than in hypercholesterolemia. Combined hyperlipidemic patients presented additionally increased insulin and free fatty acids levels.

The presence of small dense LDL particles was estimated by the LDL-chol/apoB-100 and TG/HDL-chol ratios (Table 1). A lower LDL-chol/ApoB-100 ratio in combined hyperlipidemic individuals than in normolipidemic and hypercholesterolemic groups indicates the presence of these small dense atherogenic LDL particles in the formers. A higher TG/HDL-chol ratio in combined hyperlipidemic and hypercholesterolemic groups confirmed the presence of small dense LDL particles in both hyperlipidemic groups.

Next, we evaluated ROS production in monocytes from normolipidemic and hyperlipidemic groups utilizing a cell permeable probe (dihydroethidium) that becomes fluorescent when oxidized by ROS. ROS generation by monocytes either from hypercholesterolemic or from combined hyperlipidemic patients was approximately 40% higher than from normolipidemic subjects (Fig. 1).

Serum levels of ox-LDL were significantly higher in hypercholesterolemic and combined hyperlipidemic patients ($p=0.0003$) when compared to normolipidemic individuals (Fig. 2a). When data from normolipidemic and both hyperlipidemic groups were pooled and adjusted for age, sex and body mass index, a significant positive correlation between monocyte ROS generation and serum concentration of ox-LDL was found (Fig. 2b, $r=0.35$, $p=0.0385$). Also, a significant positive correlation between TG/HDL-chol ratio and ox-LDL levels ($r=0.56$, $p=0.0002$) was found for pooled data. No significant correlations were found between HOMA index, FFA and HDL-chol levels with monocyte ROS generation or oxidized LDL levels.

Mitochondrial electrical transmembrane potential was measured as the $DiOC_6(3)$ fluorescence intensity ratio in the absence and presence of the mitochondria uncoupler CCCP. The signal obtained by depolarizing the cells with CCCP is a necessary and reliable control for measuring specific mitochondrial membrane potential. Mitochondrial electrical transmembrane potential in monocytes (Fig. 3) did not differ between groups and the inhibition of ATP-synthase by oligomycin increased the fluorescence ratio in all groups in a similar manner, indicating preserved oxidative phosphorylation in their cells.

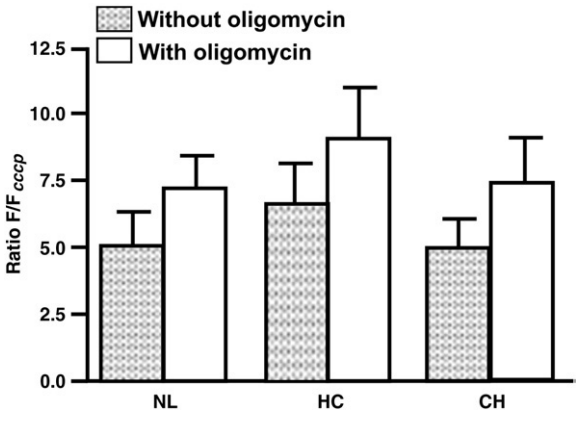

Fig. 3. Mitochondrial electrical transmembrane potential ($\Delta\psi_m$) evaluation measured by flow cytometry in monocytes from normolipidemic (NL), hypercholesterolemic (HC) and combined hyperlipidemic (CH) groups. The ratio of $DiOC_6(3)$ fluorescence in the absence and in the presence of 50 μM CCCP was measured to estimate changes in $\Delta\psi_m$. Experiments performed without (gray bars) or with 1 μM oligomycin (white bars). ANCOVA (adjusted for age, sex and BMI) with Tukey–Kramer post test. Data expressed as mean ± standard error.

The hematologic profiles of normolipidemic, hypercholesterolemic and combined hyperlipidemic patients were similar (data not shown) and no statistical correlations were found between white cell count and monocyte ROS generation or oxidized LDL levels.

Discussion

Plasma low density lipoprotein is a major target for oxidation in the vascular compartment, particularly in the intimal environment. The ox-LDL exhibits pro-inflammatory properties and is thought to lead to plaque formation and progression [22,23]. Ehara et al. [24] found that plasma ox-LDL levels were higher in patients with coronary syndromes than in control subjects and these concentrations were directly related to the severity of disease.

Several studies have shown that LDL becomes oxidized when incubated with vascular wall cells *in vitro* [1,10]. On the other hand, other studies have shown elevated extracellular release of ROS by activated mononuclear cells from hypertriglyceridemic [13] and combined hyperlipidemic [14] subjects. Here, we demonstrated that resting monocyte ROS generation was significantly higher in naive hypercholesterolemic and combined hyperlipidemic patients. Araujo et al. [14] observed a greater ROS production by monocytes from combined hyperlipidemic, but not from hypercholesterolemic patients. The reasons for these discrepant results may be related to the methodology and patients' metabolic state. Those authors used a luminol-derived chemiluminescence assay, which has been reported to be elicited in response to an array of intracellular and extracellular generated ROS. Indeed they showed that exogenous superoxide dismutase and catalase inhibited 70 to 90% the luminol signal, thus demonstrating almost exclusively extracellular production of ROS. The DHE assay used here solely detects intracellular ROS, mainly superoxide radical [25]. Thus, the luminol reagent may not be as sensitive as DHE to detect intracellular generation of ROS in hypercholesterolemic monocytes.

Generation of extracellular and intracellular ROS can be mediated by a membrane associated, multi-component protein enzyme complex named NADPH oxidase [26]. This enzyme complex is inactive in unstimulated polymorphonuclear and mononuclear cells and is only assembled upon cell activation [27–29]. Since we worked with resting monocytes, membrane NADPH oxidase is likely not involved in the ROS production measured in the present work, however this hypothesis could be reinforced by measurements of NADPH oxidase activation.

Mitochondria are considered one of the most important cellular source of ROS production [2,30,31]. Since the probe DHE accumulates in high concentrations preferentially in the

mitochondrial matrix [32], although not exclusive in these organelles, we suggested that the increased ROS production in hypercholesterolemic monocytes may be mediated by mitochondria. Previous experiments in our laboratories using MitoSOX, a mitochondrial specific probe [33] and DHE in parallel, in cells from two hypercholesterolemic subjects showed similar values for ROS production as follows: 427 vs 489 and 216 vs 267 fluorescence intensity units, for MitoSOX and DHE, respectively . Further experiments with the same model and groups should be performed to definitely confirm this mechanistic hypothesis.

Interestingly in hypercholesterolemic LDL receptor knockout mice previous experimental studies in our laboratories [34,35] showed higher ROS production by isolated mitochondria from several tissues such as liver, heart, brain and spleen intact mononuclear cells. The hypercholesterolemic mice mitochondria did not sustain matrix NADPH in the reduced state. This nucleotide is the main source of reducing equivalents to reconstitute the enzymatic mitochondrial antioxidant defense glutathione/thioredoxin reductases and peroxidases [9]. The authors suggested that this mitochondrially mediated redox imbalance could explain LDL oxidation observed in familiar hypercholesterolemia [36]. On the other hand, *in vitro* studies have shown that oxidized LDL can increase mitochondrial ROS generation [3,37]. Asmis and Begley showed that oxidized LDL promotes peroxide, but not superoxide, mediated mitochondrial dysfunction in human macrophages [38]. Here, the DHE fluorescence data indicates elevation of superoxide production [39] in the monocytes from hypercholesterolemic subjects. Together, these evidences suggest the existence of a vicious cycle in hypercholesterolemia, i.e., intracellular ROS may increase LDL oxidation, and ox-LDL in its turn stimulates ROS generation. These concerted actions lead to cell death and may be an important part of the pathogenesis of atherosclerosis in hypercholesterolemic individuals.

Since mitochondria are also targets for cell ROS, we measured their electrical transmembrane potential which did not differ among groups. Increased fluorescence ratio after inhibition of ATP-synthase by olygomicin indicates preserved oxidative phosphorylation in all groups and suggests that the elevated ox-LDL levels in the sera of hyperlipidemic patients did not affect this mitochondrial function in resting monocytes. Previous *in vitro* studies with endothelial cell of human umbilical vein and monocyte U937 derived macrophage [40,41] described mitochondrial transmembrane electric potential depolarization induced by addition of ox-LDL to the culture medium. These results may reflect cell specific differential responses or excessive concentration of exogenous ox-LDL.

Small dense LDL, which is independently associated with the incidence and extent of coronary artery disease [42], is more susceptible to oxidation than the native LDL [43]. In the present study, we found increased levels of indicators of small dense LDL particles and increased levels of ox-LDL in both hyperlipidemic groups of patients when compared to normolipidemic individuals. Both, small dense LDL and ox-LDL were positively correlated with ROS production by circulating monocytes in grouped data analysis.

In summary, we found that, in addition to the expected atherogenic lipoprotein profile, resting monocytes from untreated hyperlipidemic individuals exhibit an elevation of intracellular ROS production rate, likely derived from mitochondria. Monocyte ROS production rates positively correlated with the plasma levels of oxidized LDL of both hyperlipidemic groups. These data provide evidence that ROS production by circulating monocytes from both hyperlipidemic groups may contribute to the systemic oxidative stress and possibly to atherogenesis in these subjects.

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References

[1] Chisolm GM, Steinberg D. The oxidative modification hypothesis of atherogenesis: an overview. Free Radic Biol Med 2000;28:1815–2186.
[2] Stocker R, Keaney Jr JF. Role of oxidative modifications in atherosclerosis. Physiol Rev 2004;84:1381–478.
[3] Napoli C. Oxidation of LDL, atherogenesis, and apoptosis. Ann NY Acad Sci 2003;1010:698–709.
[4] Duvall WL. Endothelial dysfunction and antioxidants. Mt Sinai J Med 2005;72:71–80.
[5] Halliwell B, Gutteridge JMC. The importance of free radicals and catalytic metal ions in human diseases. Mol Aspects Med 1985;8:89–193.
[6] Boveris A. Mitochondrial production of superoxide radical and hydrogen peroxide. Adv Exp Med Biol 1977;78:67–82.
[7] St-Pierre J, Buckingham JA, Roebuck SJ, Brand MD. Topology of superoxide production from different sites in the mitochondrial electron transport chain. J Biol Chem 2002;277:44784–90.
[8] Starkov AA, Fiskum G, Chinopoulos C, et al. Mitochondrial alpha-ketoglutarate dehydrogenase complex generates reactive oxygen species. J Neurosci 2004;24:7779–88.
[9] Kowaltowski AJ, Castilho RF, Vercesi AE. Mitochondrial permeability transition and oxidative stress. FEBS Lett 2001;495:12–5.
[10] Steinberg D, Parthasaraty S, Crew TE, Khoo JC, Witztum JL. Beyond cholesterol: modification of low-density lipoprotein that increase its atherogenicity. N Engl J Med 1989;320:915–24.
[11] Itabe H, Ueda M. Measurement of plasma oxidized low-density lipoprotein and its clinical implications. J Atheroscler Thromb 2007;14:1–11.
[12] Palinski W, Ylä-Herttuala S, Rosenfeld ME, et al. Antisera and monoclonal antibodies specific for epitopes generated during oxidative modification of low density lipoprotein. Arteriosclerosis 1990;10:325–35.
[13] Prónai L, Hiramatsu K, Saigusa Y, Nakazawa H. Low superoxide scavenging activity associated with enhanced superoxide generation by monocytes from male hypertriglyceridemia with and without diabetes. Atherosclerosis 1991;90:39–47.
[14] Araujo FB, Barbosa DS, Hsin CY, Maranhão RC, Abdalla DSP. Evaluation of oxidative stress in patients with hyperlipidemia. Atherosclerosis 1995; 117:61–71.
[15] National Cholesterol Education Program. Executive summary of the third report the National Cholesterol Education Program. Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Adults Treatment III). JAMA 2001;285:2486–97.

[16] Hirano T, Ito Y, Yoshino G. Measurement of small dense low-density lipoprotein particles. J Atheroscler Thromb 2005;12:67–72.
[17] Maruyama C, Imamura K, Teramoto T. Assessment of LDL particle size by triglyceride/HDL-cholesterol ratio in non-diabetic, healthy subjects without prominent hyperlipidemia. J Atheroscler Thromb 2003;10:186–91.
[18] Boyum A. Isolation of lymphocytes, granulocytes and macrophages. Scand J Immunol 1976;5:9–15.
[19] Becker LB, Hoek TLV, Shao ZH, Li CQ, Schumacker PT. Generation of superoxide in cardiomyocytes during ischemia before reperfusion. Am J Physiol 1999;277:H2240–6.
[20] Rottenberg H, Wu S. Quantitative assay by flow cytometry of the mitochondrial membrane potential in intact cells. Biochim Biophys Acta 1998;1404:393–404.
[21] Montgomery DC. Design and analysis of experiments. 1st ed. John Wiley & Sons: New York; 1991. p. 95–133.
[22] Jessup W, Kritharides L, Stocker R. Lipid oxidation in atherogenesis: an overview. Biochem Soc Trans 2004;32:134–8.
[23] Robbesyn F, Garcia V, Auge N, et al. A. HDL counterbalance the proinflammatory effect of oxidized LDL by inhibiting intracellular reactive oxygen species rise, proteasome activation, and subsequent NF-kB activation in smooth muscle cells. FASEB J 2003;17:743–5.
[24] Ehara S, Ueda M, Naruko T, et al. Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. Circulation 2001;103:1955–60.
[25] Walrand S, Valeix S, Rodriguez C, Ligot P, Chassagne J, Vasson MP. Flow cytometry study of polymorphonuclear neutrophil oxidative burst: a comparison of three fluorescent probes. Clin Chim Acta 2003;331:103–10.
[26] Babior BM. NADPH oxidase: an update. Blood 1999;93:1464–76.
[27] Hampton MB, Kettle AJ, Winterbourn CC. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood 1998;92:3007–17.
[28] Aljada A, Ghanim H, Dandona P. Translocation of p47phox and activation of NADPH oxidase in mononuclear cells. Methods Mol Biol 2002;196:99–103.
[29] Bey EA, Xu B, Bhattacharjee A, et al. Protein kinase C delta is required for p47phox phosphorylation and translocation in activated human monocytes. J Immunol 2004;173:5730–8.
[30] Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. Production of reactive oxygen species by mitochondria: central role of complex III. J Biol Chem 2003;278:36027–31.
[31] Cadenas E. Mitochondrial free radical production and cell signaling. Mol Aspect Med 2004;25:17–26.
[32] Nicholls G, Fergunson SJ. Mitochondria in the cell. In: Nicholls G, Fergunson SJ, editors. Bioenergetics 3. Second edition. London: Academic Press Elsevier; 2002. p. 267.
[33] Payne CM, Weber C, Crowley-Skillicorn C, et al. Deoxycholate induces mitochondrial oxidative stress and activates NF-kappaB through multiple mechanisms in HCT-116 colon epithelial cells. Carcinogenesis 2007;28:215–22.
[34] Oliveira HCF, Cosso RG, Alberici LC, et al. Oxidative stress in atherosclerosis-prone mouse is due to low antioxidant capacity of mitochondria. FASEB J 2005;19:278–80.
[35] Paim BA, Velho JA, Castilho RF, Oliveira HC, Vercesi AE. Oxidative stress in hypercholesterolemic LDL (low-density lipoprotein) receptor knockout mice is associated with low content of mitochondrial NADP-linked substrates and is partially reversed by citrate replacement. Free Radic Biol Med 2008;44:444–51.
[36] Vercesi AE, Castilho RF, Kowaltowiski AJ, Oliveira HC. Mitochondrial energy metabolism and redox state in dyslipidemias. IUBMB Life 2007;59:263–8.
[37] Zmijewski JW, Moellering DR, Goffe CL, Landar A, Ramachandran A, Darley-Usmar VM. Oxidized low density lipoprotein induces mitochondrially associated reactive oxygen/nitrogen species formation in endothelial cells. Am J Physiol Heart Circ Physiol 2005;289:H852–61.
[38] Asmis R, Begley JG. Oxidized LDL promotes peroxide-mediated mitochondrial dysfunction and cell death in human macrophages: a caspase-3-independent pathway. Circ Res 2003;92:e20–9.
[39] Bindokas VP, Jordán J, Lee CC, Miller RJ. Superoxide production in rat hippocampal neurons: selective imaging with hydroethidine. J Neurosci 1996;16:1324–36.
[40] Ou HC, Chou FP, Sheen HM, Lin TM, Yang CH, Sheu WHH. Resveratrol, a polyphenolic compound in red wine, protects against oxidized LDL-induced cytotoxicity in endothelial cells. Clin Chim Acta 2006;364:196–204.
[41] Deng T, Xu K, Zhang L, Zheng X. Dynamic determination of Ox-LDL-induced oxidative/nitrosative stress in single macrophage by using fluorescent probes. Cell Biol Int 2008;32:1425–32.
[42] Kwon SW, Yoon SJ, Kang TS, et al. Significance of small dense low-density lipoprotein as a risk factor for coronary artery disease and acute coronary syndrome. Yonsei Med J 2006;47:404–5.
[43] Homma Y. Predictors of atherosclerosis. J Atheroscler Thromb 2004;11:265–70.