

Methodology article

## A simple method to assess the oxidative susceptibility of low density lipoproteins

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### Abstract

**Background:** Oxidative modification of low density lipoproteins (LDL) is recognized as one of the major processes involved in atherogenesis. The in vitro standardized measurement of LDL oxidative susceptibility could thus be of clinical significance. The aim of the present study was to establish a method which would allow the evaluation of oxidative susceptibility of LDL in the general clinical laboratory.

**Results:** LDL was isolated from human plasma by selective precipitation with amphipathic polymers. The ability of LDL to form peroxides was assessed by measuring thiobarbituric acid reactive substances (TBARS) after incubation with  $\text{Cu}^{2+}$  and  $\text{H}_2\text{O}_2$ . Reaction kinetics showed a three-phase pattern (latency, propagation and decomposition phases) which allowed us to select 150 min as the time point to stop the incubation by cooling and EDTA addition. The mixture  $\text{Cu}^{2+}/\text{H}_2\text{O}_2$  yielded more lipoperoxides than each one on its own at the same time end-point. Induced peroxidation was measured in normal subjects and in type 2 diabetic patients. In the control group, results were  $21.7 \pm 1.5$  nmol MDA/mg LDL protein, while in the diabetic group results were significantly increased ( $39.0 \pm 3.0$  nmol MDA/mg LDL protein;  $p < 0.001$ ).

**Conclusion:** a simple and useful method is presented for the routine determination of LDL susceptibility to peroxidation in a clinical laboratory.

### Background

Atherosclerosis is a pathology that affects many people and may cause their death or disability due to myocardial infarction or strokes. Although the clinical manifestations of the disease have been established, the underlying mechanism of atherogenesis is still unclear. Recent theory points toward the oxidative modification of LDL (LDL-Ox) as one of the major involved processes [1]. Nevertheless, hardly any of the biological effects of LDL-Ox have been tested in vivo.

Taking into account the potential clinical importance of the oxidative modification of LDL, many studies have been carried out to quantify their in vitro susceptibility to oxidation. This measurement is thought to correlate with the LDL oxidative susceptibility within the arterial wall [2].

Plasmatic LDLs may be isolated by different methods, which include sequential and density-gradient ultracentrifugation, chromatography, electrophoresis and selec-

tive precipitation [3]. Lipid peroxidation is a very complex process that involves the chain reaction of free radicals with polyunsaturated fatty acids. These reactions lead to rearrangements of double bonds in conjugated dienes, hydroperoxide generation, lipid breakdown into lower molecular weight fragments, as well as chemical modifications in the apo B protein [4,5,6,7,8]. The extent of lipid peroxidation can be estimated by measurement of thiobarbituric reactive substances (TBARS). This method, although nonspecific, is of value in purified systems. TBARS determination mainly measures malondialdehyde (MDA) derived from the hydroperoxidation of unsaturated fatty acids with three or more double bonds.

Many studies have been carried out to establish the role of Fe<sup>3+</sup>, Fe<sup>2+</sup> and Cu<sup>2+</sup> in the oxidation of LDL [1,8,9]. In biological systems, the reduction of oxygen yields hydrogen peroxide and superoxide radical. The reaction between these two species generates a hydroxyl radical, which is the reactive oxygen species with the shortest half life and highest reactivity. This reaction, which is kinetically slow, can be accelerated by catalytic amounts of iron or copper salts [10].

In the present study we present a simple method which would allow the high-throughput routine evaluation of the oxidative susceptibility of LDLs in the simultaneous presence of Cu<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub> in the general clinical laboratory. LDLs were isolated by selective precipitation and their oxidative susceptibility was evaluated through the quantitation of TBARS.

**Table 1: Recovery of 1.44 nmol MDA/tube added to duplicated resuspended LDL samples obtained from three independent plasma samples.**

Sample	nmol MDA / tube in the original sample	Observed increment (nmol MDA/ tube)	% Recovery mean ± SD
A	0.63	1.15	85 ± 7
	0.69	1.30	
B	1.07	1.24	78 ± 12
	1.32	1.00	
C	0.47	1.43	94 ± 8
	0.47	1.27	

## Results

### Optimization of oxidative susceptibility assay

As expressed in Materials and Methods, different volumes of solubilizing solution were used to resuspend LDL precipitates. Relatively low volumes (0.4 ml) gave lower intra-assay coefficients of variation (4.8 %) than relatively high volumes of 1.0 ml (CV = 10.8 %). These results correspond to the analysis of 22 samples determined in duplicate. In order to assess the recovery of standard, a fixed amount of 1.44 nmol MDA / tube was added to aliquots of previously assayed duplicated resuspended LDL samples from three different plasmas. As can be seen in Table 1 the MDA recovery varied between 78.1 and 93.8 % of the true value.

In experiments aimed at adjusting the number of precipitate washes needed, LDL precipitate was washed once or twice with precipitating solution, or not washed at all, prior to solubilizing. The protein concentration and cholesterol content of the resulting resuspended LDL samples was then determined in duplicate. The first wash diminished the protein content of the resuspended LDL sample by 20 %, whereas the second wash further decreased protein content by 3 %. On the other hand, the cholesterol content of resuspended LDL samples did not vary as a consequence of successive washes (99 ± 3 and 98 ± 2 % of non washed precipitate, for 1 or 2 washes respectively). The samples were also submitted to agarose electrophoresis, and bands revealed with Coomassie brilliant blue, in order to evaluate the possible presence of contaminating plasma proteins. The unwashed precipitate showed a clearly visible band corresponding to albumin, as well as another band of greater intensity with the electrophoretic mobility of LDL. One and two washes with precipitating reagent greatly diminished - but did not completely eliminate - the albumin band, without provoking any changes in the intensity of the LDL band (data not shown). As the washing procedure eliminates non-apoB co-precipitating plasma proteins without cholesterol losses, a single wash was selected as the standard procedure.

**Table 2: Effect of Triton X-100 on the LDL oxidative susceptibility assay.**

Solubilizing reagent	MDA nmol / mg LDL protein mean ± SD (n = 3)
NaCl	20.3 ± 0.6
NaCl + Triton X-100	21.0 ± 0.7

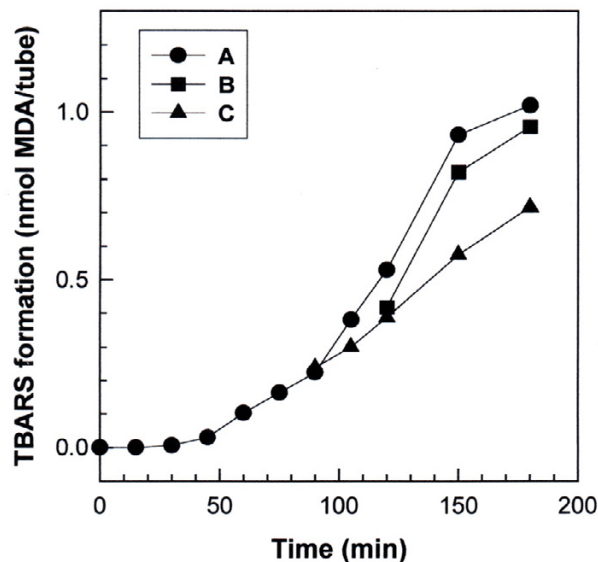
No significant differences between the two procedures were observed.

In other experiments, the effect of the presence of Triton X-100 in the solubilizing solution was evaluated by assaying the oxidative susceptibility in three independent precipitations of the same sample. Two solubilizing reagents were investigated, 50 g/l NaCl and 0.1 % Triton X-100 in 50 g/l NaCl. Table 2 shows that the same results for LDL oxidative susceptibility were obtained with the two procedures. However, since resuspending the precipitate with Triton X-100 was found to be less time-consuming, it was chosen as the standard method.

The kinetics of  $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ -induced LDL peroxidation was monitored by measuring the TBARS levels in aliquots of three resuspended LDL samples incubated from 15 to 180 min with  $\text{Cu}^{2+}/\text{H}_2\text{O}_2$  (Figure 1). An initial lag phase could be observed with no increments in the absorbance, followed by another with a maximum slope (propagation phase). A final phase was evident with lower absorbance increments (decomposition phase). Figure 1 represents three examples of various LDL preparations. In most cases, the propagation phase reached a maximum at about 150 min. Thus, this incubation period was selected for the LDL oxidation reaction. In further experiments, EDTA was validated as an effective inhibitor of the basal and induced oxidation reaction. After an incubation of 150 min, the results for samples submitted to oxidation in the presence of EDTA (30 nmol/tube) monitored for TBARS formation, did not show significant differences when compared with the blanks (with  $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ , without sample). Thus, EDTA at this concentration was subsequently used to effectively stop the oxidative reaction induced by  $\text{Cu}^{2+}/\text{H}_2\text{O}_2$ .

The basal LDL oxidation (as defined in Materials and Methods) was extremely low and was arbitrary assigned 100 % value (Figure 2). In absence of EDTA and oxidating agents, the samples showed an inherent oxidability, with TBARS values of approximately twice that of basal LDL oxidation. We next analyzed the effect of  $\text{Cu}^{2+}$  and/or  $\text{H}_2\text{O}_2$  as inducers of LDL oxidation reaction. When added separately,  $\text{Cu}^{2+}$  and  $\text{H}_2\text{O}_2$  increased TBARS formation by approximately 5- fold. However; the simultaneous addition of  $\text{H}_2\text{O}_2$  and  $\text{Cu}^{2+}$  induced a synergistic increase in TBARS levels (approximately 13- fold). Doubling doses of  $\text{Cu}^{2+}$  or  $\text{H}_2\text{O}_2$  did not further increase the oxidation levels of LDL.

The influence of LDL protein content on the TBARS reaction, was evaluated by increasing the volume of resuspended LDL samples, under constant TBARS reagent volume and incubation period (Figure 3). The reaction was linear up to an LDL protein content of approximately 300  $\mu\text{g}/\text{tube}$ .



**Figure 1**  
**Kinetics of in vitro LDL peroxidation assessed by TBARS formation.** An aliquot of 100  $\mu\text{l}$  plasma from three different patients (A, B, C) was assayed. All sample precipitates were redissolved in 0.4 ml of solubilizing solution, incubated with  $\text{Cu}^{2+}/\text{H}_2\text{O}_2$  for different periods of time, and 100  $\mu\text{l}$  of oxidized LDL was employed for TBARS assay. Results are the average of duplicate determinations.

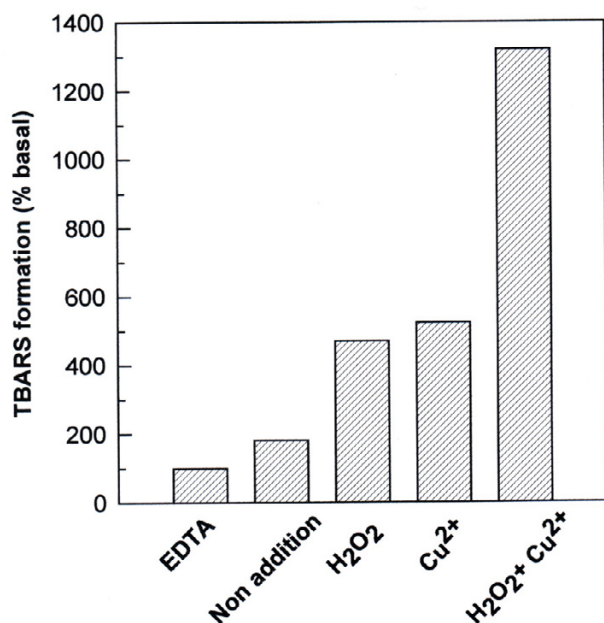
In order to characterize the LDL isolated by selective precipitation, and to investigate the possible damage of the inner structure of LDL caused by this method, we performed an agarose electrophoresis of the resuspended LDL sample in parallel with the LDL isolated by ultracentrifugation and the corresponding whole plasma sample. As can be seen in Figure 4, LDL fraction isolated by both methods showed the same electrophoretic mobility and no contamination by other lipoprotein fractions.

#### **LDL oxidation susceptibility in control and diabetic samples**

In order to evaluate the method's usefulness in separating a control population from another with increased risk for cardiovascular disease, a group of 30 normal subjects and 12 type 2 diabetic patients were submitted to this assay. The oxidative susceptibility of LDL was significantly greater in the diabetic group than in the control population ( $39.0 \pm 3.0$  vs.  $21.7 \pm 1.5$  nmol MDA / mg LDL protein;  $p < 0.001$ ).

#### **Discussion**

The oxidative modification of LDL appears to be involved in the development of various degenerative dis-



**Figure 2**

**Basal and Cu<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub> -induced LDL oxidation.** Samples were incubated with EDTA (basal LDL oxidation), without additions, or in the presence of Cu<sup>2+</sup> (100 μM) and/or H<sub>2</sub>O<sub>2</sub> (300 ml/l). Results are expressed as % basal TBARS values.

eases such as atherosclerosis, carcinogenesis, aging and diabetes mellitus [11,12]. Standard reference methods to prepare LDL from plasma employ ultracentrifugation. However, the selective precipitation methods, which are more accessible than ultracentrifugation, are widely used in the clinical laboratory for the measurement of the cholesterol content in different lipoprotein fractions. In particular, selective precipitation of LDL may be approached in different ways: by addition of heparin at an exactly controlled pH of 5.12 in the absence of divalent cations; or with polyvinylsulphate in the presence of EDTA and polyethylene glycol methyl ether; with amphipathic polymers in imidazole buffer at pH 6.10 (bioMerieux). An excellent statistical correlation is obtained when these methods are compared with reference ultracentrifugation methods, providing samples with triglyceride concentration above 8 mmol/l and those from patients with hyperlipoproteinemia Type III are excluded [13]. In particular, the precipitating reagent used in the present work (bioMerieux), shows a good correlation coefficient ( $r = 0.96$ ) when compared with ultracentrifugation methods [3]. Its selectivity and the preservation of the immunological properties as well as the lipid composition of the native original LDLs have also been demonstrated [3,9,14,15]. In our present study, we were unable to find differences in agarose electro-

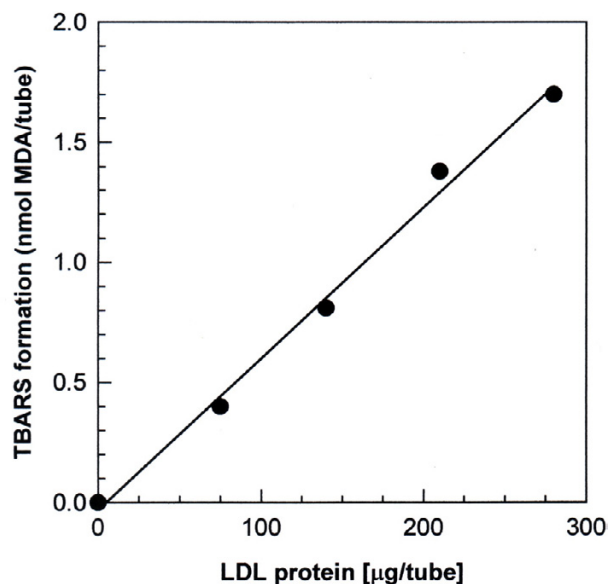
phoretic mobility between LDL fractions obtained by this method of selective precipitation and ultracentrifugation. In addition, no contaminating lipoprotein fractions were observed by this electrophoretic method. In our standard procedure we washed the LDL precipitate once prior to solubilizing. Thus, it was necessary to establish whether there were changes in LDL cholesterol content, which could invalidate the original method's correlation with ultracentrifugation. However, we were unable to find cholesterol losses as a consequence of one or two washes with precipitating reagent.

Arshad et al. [16] developed a simple method to assess whole plasma susceptibility to peroxidation by Cu<sup>2+</sup>/H<sub>2</sub>O<sub>2</sub> incubation. They used thiobarbituric acid reactivity to evaluate lipid peroxidation, a method which is not entirely specific. However, it proved to be easy to perform and accessible for the analysis of many samples. In the present work, we measured LDL-associated TBARS after induction of lipid peroxidation with a mixture of Cu<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub>.

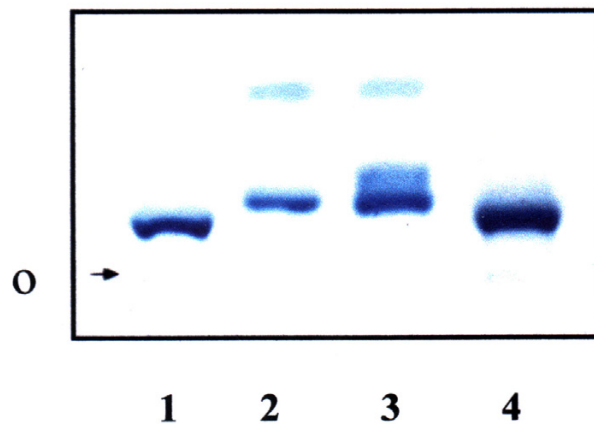
Several methodological aspects of our procedure were subsequently addressed, in order to achieve its optimization. a) The intra-assay precision was found to depend on the volume of solubilizing solution employed. In our standard working conditions, the CV was 4.8 %, which is lower than the precision limit established for the determination of selectively precipitated lipoprotein cholesterol (CV < 5%) [3], and so can be considered acceptable. b) The observed percentage of recovery for exogenously added MDA (Table 1) was comparable to that of the TBARS reaction (82-100 %) [10]. These results suggest that the added MDA was still TBA reactive and did not generate any interfering substances, since the observed increment in MDA content did not significantly differ from that of the true value. c) It is important to ensure that the precipitate is not contaminated with non-LDL serum proteins, since results are expressed per LDL protein content. This contribution to variability was eliminated by washing the LDL precipitate. d) When the composition of the solubilizing solution was evaluated, precipitate redissolution effectively occurred in 50 g/l NaCl. However, the addition of Triton X-100 was chosen because it shortened the period of LDL redissolution. e) Lipid peroxidation kinetics have been extensively studied [8,10]. It is known that LDL oxidation in the presence of Cu<sup>2+</sup> shows three phases: latency, propagation and decomposition. This has been established by determination of hydroperoxides, TBARS or other aldehydes, fluorescent products and conjugated dienes. It has been shown that during the latency and propagation phases, as well as during the early stages of the decomposition phase, the time-courses of diene, TBARS and lipid hydroperoxide formation, are practically coincident [8]. In-

deed, the corresponding maxima coincide temporally. However, each individual's LDL shows its own particular kinetics so that sample to sample variations could represent a problem when - as in the present study - a single measurement of only one parameter is taken after a long incubation time. This does not allow us to conclusively establish whether the sample is at the end of its propagation phase, or has already begun its decomposition phase. In our preliminary studies of TBARS time-course, we found a lag phase followed by a maximum slope which ended at 150 min, the time point adopted for our standard procedure. A slower increment in absorbance was observed from this point on, a fact that may have been due to the decomposition of accumulated products. f) In the absence of oxidation inhibitors, LDL oxidation may continue throughout the TBA reaction period, thus contributing to the method's variability. This was effectively prevented by the addition of EDTA prior to the TBA reaction, which acts as an inhibitor of LDL oxidation by  $\text{Cu}^{2+}$  sequestration. g) Our experiments show that the combination of  $\text{Cu}^{2+}$  and  $\text{H}_2\text{O}_2$  is more effective for the induction of LDL oxidation, than each agent its own. The observed results suggest a synergistic mechanism of action between both reagents. Previous studies have addressed the  $\text{Cu}^{2+}$ -induced in vitro oxidation of plasma LDL [17]. These authors found a value of  $21 \pm 3$  nmol MDA / mg LDL protein, obtained from four normal subjects, for LDL isolated by ultracentrifugation. This is practically coincident with the results which we obtained with our control healthy population ( $21.7 \pm 1.5$  nmol MDA / mg LDL protein), as would be expected from the reported correlation between LDL obtained by ultracentrifugation and by the LDL-precipitating method of bioMerieux. Recently, Guerci et al.[14] studied the LDL oxidation susceptibility of normolipidemic diabetic and non-diabetic patients. These authors found a significant increase in type 2 diabetic patients vs. healthy subjects, particularly in the group of type 2 diabetic females, in which LDL oxidation susceptibility was highest. In coincidence with these reported results, LDL oxidative susceptibility of our type 2 diabetic patients was significantly greater ( $39.0 \pm 3.0$  nmol MDA / mg LDL protein) than the control group.

The LDL precipitation method which we have used in this study is based on interaction with glycosaminoglycans (GAG). However, both lipid composition and the content of sialic acid can modulate the interaction with GAG. In this context, particles such as small dense LDL can interact with GAG with high affinity. In addition, the precipitation procedure may increase the susceptibility for oxidation by copper since copper penetrates the LDL particle more easily after precipitation. In consequence, we cannot discard the possibility that our results may reflect a preselection of LDL with higher susceptibility for oxidation.



**Figure 3**  
**Influence of the LDL protein content on TBARS reaction linearity.** Increasing doses of a LDL sample were submitted to constant oxidative conditions. Results are expressed as mean of duplicate determinations.  $y = 4.35 \cdot 10^{-3} \times - 6.57 \cdot 10^{-3}$ ,  $r^2 = 0.994$ ;  $p < 0.001$ .



**Figure 4**  
**Agarose electrophoresis of whole plasma and LDL fractions.** Bands correspond to: 1, LDL fraction obtained by selective precipitation; 2, whole plasma of a normolipidemic patient; 3, whole plasma of a hyperlipidemic patient; 4, LDL fraction obtained by ultracentrifugation. Samples were stained with Sudan black.

## Conclusion

A simple method for the *in vitro* measurement of LDL oxidation susceptibility has been optimized, and applied to a group of healthy subjects and type 2 diabetic patients. This straightforward approach could facilitate the comparison of results obtained from an increased number of general clinical laboratories, and thus allow us to move a step further towards the standardization of a procedure of potential clinical importance.

## Materials and methods

### Materials

LDL Cholesterol kit (cat. Number 61532) was provided by bioMerieux (Marcy l'Etoile France). Hydragel Lipo+Lp(a) kit for agarose electrophoresis was obtained from Sebia. Thiobarbituric acid was obtained from Merck. 1,1',3,3'-tetra-methoxy-propane or malondialdehyde (MDA) was used as standard and purchased from SIGMA Co. St. Louis, MO, USA. All chemicals were of analytical grade and used without further purification.

### Sample collection

Twelve type 2 diabetic patients of both sexes (37 - 65 years old) were studied. The degree of metabolic control was assessed by the measurement of fasting plasma glucose (mean  $7.2 \pm 1.1$  mmol/l), fasting plasma HbA<sub>1c</sub> (mean  $6.8 \pm 1.3$  %; NV 4.8-6.0 %) and they were normolipidemic. A series of 30 control non-diabetic subjects of both sexes (age range, 35-60 year old) was processed in parallel. All controls were normolipidemic according to the Alfedian criteria [18], and none of the subjects were taking any drug known to influence lipid or lipoprotein metabolism. Blood samples were obtained on heparin (5 U/ml) by venipuncture from subjects with 12 hours fasting. Plasma was separated rapidly and processed immediately. Alternatively, the samples were stored at 4 °C for 24 hours or at -20 °C for not more than 2 days.

### Method of LDL isolation

LDL was selectively precipitated from 100 µl of plasma by addition of bioMerieux precipitating reagent of LDL-Cholesterol kit and vortex-mixed [14]. The mixture was incubated for 30 min at 2-8 °C, and centrifuged for 5 min. The supernatant was discarded and the precipitate was washed with precipitating reagent. The washed precipitate was redissolved in different volumes of solubilizing solution (0.01% Triton X100 in 50 g/l NaCl) at 37 °C, and vortex-mixed (resuspended LDL sample) [15]. Bradford's method [19] was used to determine the total protein content of the resuspended LDL sample, using bovine serum albumin as a standard. For selected experiments, the LDL fraction was obtained by density gradient ultracentrifugation as has been previously described [13].

### Characterization of LDL isolated by selective precipitation

Representative samples were subjected to ultracentrifugation and selective precipitation (0, 1 or 2 washes) in order to isolate the LDL fraction. Subsequently, LDL fractions obtained by both methods, as well as the whole plasma, were electrophoresed in agarose according to the manufacturer's instructions. Briefly, electrophoresis was performed at a constant voltage of 130 V and initial intensity of 25 mA, for 80 minutes. The gel was dried and bands were revealed with either Sudan Black or Coomassie brilliant blue.

### LDL-cholesterol determination

LDL was obtained by selective precipitation of representative samples, and the resulting precipitates were washed once, twice, or not at all with the precipitating reagent, prior to resuspending with the solubilizing solution. Cholesterol content of the resulting resuspended LDL samples was determined by a commercial enzymatic kit (Colestat, Wiener Laboratories Argentina).

### Basal and induced LDL oxidation

Basal LDL oxidation was determined by incubating an aliquot of 100 µl resuspended LDL sample, containing 50-90 µg protein, with 30 µl of 1 mM EDTA and 45 µl of distilled water. The corresponding blank was determined substituting the resuspended LDL sample by solubilizing solution.

In other experiments, resuspended LDL sample was mixed with 50 µl of 100 µM Cu<sup>2+</sup> (freshly prepared in phosphate buffer saline solution, PBS, pH 7.4) and 25 µl of H<sub>2</sub>O<sub>2</sub> solution (300 ml/l H<sub>2</sub>O<sub>2</sub> in PBS, stock solution corresponds to 10 volume commercial H<sub>2</sub>O<sub>2</sub>). Blank was performed with solubilizing solution instead of resuspended LDL sample. In all cases, sample and blank were incubated at 37 °C for different periods of time with occasional stirring. At the end of the incubation period, the lipid peroxidation was stopped by cooling and addition of 30 µl of 1 mM EDTA.

### TBARS determination

Lipid peroxidation of LDL was assessed by TBARS formation [20]. Briefly, samples were incubated with 0.5 ml of 20% acetic acid, pH 3.5 and 0.5 ml of 0.78% aqueous solution of thiobarbituric acid. After heating at 95 °C for 45 minutes, the samples were centrifuged at 4000 r.p.m. for 5 minutes. The red pigment in the supernatant fractions was estimated by absorbance at 532 nm. A calibration curve was prepared with an MDA standard. Results were expressed as nmol MDA /mg LDL protein. All samples gave results which were within the linear portion of the MDA standard curve. A recovery assay was also performed by adding a defined amount of MDA before incubating with the oxidant mixture.

### Statistical analysis

Results were expressed as mean  $\pm$  SD and mean  $\pm$  SEM. Statistical analysis was performed by Student's t test; a p value  $< 0.05$  was considered statistically significant. Linear regression analysis was used for testing correlations between variables.

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