Measurement 64 (2015) 105–111

Contents lists available at ScienceDirect

Measurement

journal homepage: www.elsevier.com/locate/measurement

Detecting LDL-oxidation process using Langmuir monolayer technique

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ARTICLE INFO

Article history:
Received 24 March 2014
Received in revised form 11 September 2014
Accepted 23 December 2014
Available online 2 January 2015

Keywords:
Adsorption
Free radical
LDL oxidation
Bovine serum albumin

ABSTRACT

We introduced a novel method to detect the low density lipoprotein (LDL) oxidation process by transition metals using the Langmuir monolayer technique. This process has been studied because of its potential relevance to pathological processes like atherosclerosis. In particular, we investigated the influence of copper ions on the oxidation susceptibility of the LDL and the ability of bovine serum albumin (BSA) to inhibit this oxidation. Langmuir monolayer and Wilhelmy techniques have been successfully used to achieve these aims. In additions, our results agree qualitatively with the other conventional methods which detect LDL-oxidation. Our results indicate that the oxidation of LDL depends strongly on the ratio between Cu^{2+} and BSA. Moreover, the BSA–Cu^{2+} binding affinity may play an important role in the oxidation process of the LDL particles. Our study showed that BSA is an effective radical trap as evidenced by its ability to prevent LDL oxidation induced by Cu^{2+}. These simple *in vitro* experiments of the Langmuir monolayer technique may serve as an elementary tool in clinical tests or pharmaceutical applications.

1. Introduction

The LDL is a quasi-spherical particle which composes two major compartments, a hydrophobic core, comprised primarily of cholesteryl esters and triglycerides, surrounded by a surface monolayer of phospholipids (i.e., phosphatidylethanolamine, phosphatidylinositol, sphingomyeline, phosphatidylcholine and Lyso-PC) with unesterified cholesterol [1–3]. Also one copy of the apolipoprotein B100 (apoB) is presented per LDL particle, and is believed to wrap around the outer surface [4], with sites of hydrophobicity immersed in the lipid phase [5]. On the other hand it is accepted that LDL plays a critical role in human diseases such as atherosclerosis [6–12]. This complex chronical disease is firstly recognizable by the so-called 'fatty streak' [5], an aggregation of lipid-rich macrophages and T lymphocytes within the innermost layer of the artery wall, the intima. As the process continues, fatty steaks precede the development of intermediate phase, which is composed of layers of macrophages and smooth muscle cells [13–18] and, in turn, develop into more advanced, complex accumulation of polyunsaturated fatty acids (PUFA) [19]. At the final stage of oxidation process PUFA decomposes into aldehydes and other water soluble compounds. These highly potent substances react with the protein moiety of the LDL-apoB, thus modifying it to an extent that it is no longer recognized by the LDL receptor. This mechanism, unlike the uptake of the non-oxidized LDL receptor pathways, is not well regulated and therefore, results in the formation of foam cells and finally cell death.

Determination of the susceptibility of LDL to oxidation *in vivo* is not available due to obvious clinical difficulties. Adopting various promoters to initiate the oxidation, most of the effort has been devoted in developing *in vitro* models for the assessment of the susceptibility of LDL to oxidation.

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http://dx.doi.org/10.1016/j.measurement.2014.12.039
0263-2241/

Because of the similarity observed in the changes induced in LDL morphology and biochemistry by these two procedures, several experimental works have been devoted to the evaluation of the correlation between lipid related pathologies and the susceptibility of isolated LDL to oxidation *in vitro*. In most of the mentioned studies, oxidation requires or is facilitated by the presence of redox active transition metal ions such as copper. According to these experiments of monitoring the kinetics of LDL-induced oxidation, the most common used model is Cu^{2+}-induced oxidation. This strategy, therefore, was carried out extensively in most of the experiments due to its rapidity and diversity. In normal conditions, the kinetics of this reaction is characterized by a 'lag-phase' of relatively slow oxidation, which precedes the auto-oxidation of the most oxidizable lipids. During this phase vitamin E is produced, and lipid oxidation is limited. After all the vitamin E becomes oxidized, auto-oxidation occurs, yielding rapid production of conjugated dienic hydroperoxides, which finally decomposes into aldehydes and other products. Many Studies have shown that the amount of copper bounded to LDL increases during the incubation time, where more than 80% of the copper ions bound to the LDL particles in the protein phase of LDL, suggesting that most of the copper ions form complexes with the ligand-binding sites of the apoB [19–31].

Oxidation of LDL (oxLDL) can be inhibited by both water soluble and lipid-soluble anti-oxidations. In addition, plasma proteins such as bovine serum albumin (BSA) or Cu, Zn-superoxide dismutase from bovine erythrocytes may also inhibit free-radical-induced oxLDL [27,28]. Recent studies proposed that BSA has a protective effect and that this effect is not limited to the 'metal sequestering potential' of albumin but also relates to its capacity as 'a radical trapping protein' and/or to its interaction with LDL [27,28]. Chromatography methods showed that the amount of Cu^{2+} recovered with the protein-containing fractions indicated that BSA bounds greater than 90% of Cu^{2+}. Since Albumin may be present in the intima of arteries, especially due to permeability of the arterial wall by lipidic oxidation products, the effect of albumin on LDL oxidation carries in its nature a pathophysiological significance [21]. Along this direction, we intended to use Langmuir monolayer technique at the air/water interface to determine the oxidation of LDL by Cu^{+2} and the ability of the BSA in inhibiting the oxLDL particles when adsorbs into the air/water interface. These simple *in vitro* experiments may serve as an elementary tool for clinical tests or pharmaceutical applications.

2. Materials and methods

2.1. Materials

Low Density Lipoprotein (LDL), bovine serum albumin (BSA) and Copper (II) chloride hydrate were purchased from Sigma Chemical. Butylated hydroxytoluene (BHT), disodium ethylenediamine-tetraacetic acid (EDTA), phenylmethysulphonyl fluoride (PMSF) and Tris-buffered saline (TBS) (0.14 M NaCl and 5 mM Tris–HCl, pH 7.4) were also purchased from Sigma Chemical. All the chemicals were of analytical grade and used without further purification.

2.2. Lipoprotein solutions preparation

After adjustment of the LDL concentration at 1.5 mg/mL (expressed as total LDL concentration), lipoprotein preparations were dialyzed against 100 volumes using phosphate-buffered saline (PBS) in the absence of any antioxidants. The composition of PBS was: 10 mM sodium phosphate, containing 150 mM sodium chloride, pH = 7.4. The dialyzed LDL was stored in dark at 4 °C and used within 1 week after preparation for peroxidation experiments.

2.3. In vitro LDL peroxidation

Aliquots of LDL were subject to one of the following conditions, either (a) Autoxidation: 0.2 or 0.8 mg/ml of LDL in TBS (1 or 5 ml final volume), placed in loosely capped glass tubes, were exposed to air, in dark, at 37 °C for time period more than 96 h to achieve full oxidation [32], or (b) Copper-induced oxidation: 0.2 or 0.8 mg/ml LDL protein in 10 mM TBP (pH = 7.4), was added to solutions of 1–5 μM $CuSo_4$, and the samples were treated as in (a) for time period more than 24 h to achieve full oxidation [33].

In each condition, oxidation was arrested by adding 0.01% EDTA, 0.25 μM BHT, and 0.2 μM PMSF to the samples, and placing the tubes in dark, at 4 °C. For the subsequent experiments, the buffers used were supplemented with the above antioxidants and proteolytic inhibitors.

The evaluation of the oxidative process leading to oxLDL formation was essentially the same during autoxidation or copper-induced oxidation. In fact, for the same incubation time the number of oxLDL particles recognized by autoxidation was lower than upon copper-induced oxidation. Also, the observed similarity in the changes in oxLDL morphology and biochemistry by these two procedures, led us to carry out most experiments with copper-oxidized LDL in which the rapidity and intensity of the modifications were the highest.

2.4. Effect of albumin on lipoprotein LDL peroxidation and inhibition

These experiments were conduced in conditions which support the oxidative modification of lipoproteins by copper-induced oxidation, in the absence or presence of serum albumin. The latter was added to the incubation medium to final concentrations ranging from 0.5 to 10 μM. After the arrest of oxidation and measurement of pressure isotherms, for further analysis of lipoproteins were re-stored in conditions similar to those used for the preparation of LDL particles.

The effect of albumin was evaluated using concentration dependency in which increasing amounts of albumin (ranging from 1 to 5 μM) were added. Also, to determine whether albumin can interfere with an already initiated oxidative process, in separate experiments, albumin was