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References

Comparison of Three Methods for Measuring LDL Resistance against Copper-Ir Ourzed Oxidation, Peter G. Scheffer,1* Stephan J.L. Bakker,2 Erik E. Musch, Corrie Popp-Snijders,1,2 Robert J. Heine,1 and Tom Teerlink1 (Departments of 1 Clinical Chemistry and 2 Endocrinology, Research Institute for Endocrinology, Reproduction and Metabolism, Academic Hospital Vrije Universiteit, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands; * author for correspondence: fax 31-20-4443895, e-mail p.scheffer@azvunl)

Oxidation of LDL in the subendothelial space of the arterial wall initiates a series of events leading to enhanced uptake of LDL by scavenger receptors on macrophages and subsequent foam cell formation. Therefore, LDL oxidation is supposed to play a role in the development of atherosclerosis (1, 2).

The most uniformly accepted procedure for measuring the resistance of LDL to in vitro oxidation is determination of the lag time (LT) for conjugated diene (CD) formation, initiated by catalytic amounts of transition metal ions (3). Another method is based on the oxidation of paraaminic acid (PA), a fluorescent and oxidation-sensi-
tive polyunsaturated fatty acid (FA) probe, incorporated into LDL (4). A third method monitors fluorescence development (FD) during LDL oxidation (5), caused by reaction of protein amino groups with aldehydes generated during decomposition of peroxidized FAs (6).

The three methods described above measure different stages of the oxidation process. Oxidation of the PA probe represents one of the earliest stages of oxidation. The formation of CDs represents an intermediate stage of the oxidation process, and finally FD represents a late stage of the oxidation process.

The methods for measurement of LDL oxidizability were evaluated with respect to reproducibility and potential for automation. Their relationship with LDL constituents, as determinants of LDL oxidation, was also studied.

Subjects were 98 normolipidemic type 2 diabetic patients with good glycemic control (mean hemoglobin A1c, 6.2% ± 1.0%). LDL was isolated by ultracentrifugation between densities 1.019 and 1.063 kg/L, as described elsewhere (7). PA was purchased from Molecular Probes. Other reagents used were obtained from Merck or Sigma.

Total cholesterol, free cholesterol, triglycerides, and phospholipids were analyzed using enzymatic test kits (Boehringer Mannheim). The cholesterol ester content of LDL was calculated as total minus free cholesterol. LDL lipid constituents were standardized for LDL protein quantified by a modified Lowry procedure (8), with bovine serum albumin as the calibrator.

The mean diameter of the LDL particles was measured by the high performance gel-filtration chromatography method we described recently (7). Lipid peroxides, analyzed as malondialdehyde equivalents, were measured by HPLC with fluorescence detection after sample pretreatment and derivatization with thiobarbituric acid, according to the method of Tsai et al. (9). The FA composition of LDL was assessed by capillary gas chromatography, as described previously (10, 11). α-Tocopherol and coenzyme Q10 were simultaneously determined by reversed-phase HPLC.

LDL oxidation was performed at 37 °C. The molar ratio of copper ions to LDL was equal in all oxidation experiments. Before oxidation, salts and EDTA were removed by desalting on a 5-mL dextran column (Pierce). The three LDL oxidation methods used are described below.

LT was measured by monitoring CD formation (3) at 3-min intervals on a Hitachi U2000 spectrophotometer at 234 nm. The calculated LT was independent of the amount of LDL between 0.025 and 0.100 g/L LDL-protein. We routinely used a final concentration of 0.04 g/L LDL-protein. The final copper concentration was 15 μmol/L.

The oxidation of PA after its incorporation into LDL (4, 12) was monitored on a Cobas Bio centrifugal analyzer (Roche). The fluorescent PA probe (0.9 μmol/L) was incorporated into LDL (0.07 g/L LDL-protein/L) by incubation for 10 min at 37 °C. During automated analysis, LDL preparations were further diluted to 0.02 g/L LDL-protein and 0.26 μmol/L PA with phosphate-buffered saline.

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The fluorescence decay (emission wavelength, 450 ± 45 nm; excitation wavelength, 324 nm) was monitored every 2 min for 1 h.

Measurement of the increase in autofluorescence intensity during LDL oxidation was also adapted to the Cobas Bio analyzer. During a 5-h period, the fluorescence was measured every 10 min at 450 ± 45 nm with excitation at 360 nm. The final LDL-protein and copper concentrations were equal to the CD method.

Throughout the present study, results are expressed as the mean ± SD. Relationships between LTs were evaluated by linear regression analysis. Spearman correlation coefficients were calculated to evaluate relationships between variables. Because multiple comparisons were made, significance was set at \( P < 0.01 \).

The mean CD-LT was 49.0 ± 6.6 min. Corresponding values for PA-LT and FD-LT were 20.4 ± 4.3 and 66.7 ± 8.0 min, respectively. As illustrated in Fig. 1A, PA-LT values were highly correlated to those obtained by the CD method \( [y = (0.49 ± 0.04)x - (3.53 ± 2.16); r = 0.78; S_{xy} = 2.85 \text{ min}] \). FD-LTs were also highly correlated to CD-LTs \( [y = (1.02 ± 0.07)x + (16.67 ± 3.22); r = 0.86; S_{xy} = 4.25 \text{ min}] \), as shown in Fig. 1B.

Within-run CVs, determined on an LDL pool, were 2.0% (\( n = 6 \)), 2.5% (\( n = 12 \)), and 1.5% (\( n = 12 \)) for the CD, PA, and FD methods, respectively. Between-run reproducibility was determined for the entire procedure, including isolation of LDL. Between-run CVs were 7.9% (\( n = 17 \)), 11% (\( n = 15 \)), and 3.6% (\( n = 9 \)) for the CD-LT, PA-LT and FD-LT determinations, respectively.

The free cholesterol, cholesterol ester, phospholipid, and triglyceride content of LDL were not associated with any of the LTs (data not shown). Correlations between LTs and lipid peroxides, LDL antioxidants, FAs, and particle diameter are presented in Table 1. CD-LT, PA-LT, and FD-LT were positively associated with the LDL \( \alpha \)-tocopherol content; however, only the association with PA-LT reached statistical significance \( (r = 0.27; P = 0.008) \). Positive and significant associations between coenzyme Q10 and both PA-LT and FD-LT \( (r = 0.34, P < 0.001; \text{ and } r = 0.29, P = 0.004, \text{ respectively}) \) were also found. Negative correlations between the arachidonic acid concentrations and both CD-LT and FD-LT were observed \( (r = -0.40, P < 0.001; \text{ and } r = -0.47, P < 0.001, \text{ respectively}) \). An inverse significant association was also found between CD-LT and lipid peroxides \( (r = -0.27; P = 0.008) \).

The aim of this study was to compare three methods to measure the LT at different stages of in vitro oxidation of LDL. In the CD technique, additional indices of LDL oxidizability can be derived, such as total amount of dienes and their rate of formation \( (13) \). This additional information is not obtained with the PA and FD methods. However, because an inverse relationship has been found between LT and severity and progression of coronary atherosclerosis \( (14–17) \), the LT is considered the most distinctive index of LDL oxidizability.

The PA and FD assays offer practical advantages in

### Table 1. Correlation coefficients between LTs* and lipid peroxides, LDL size, antioxidants, and total amount of FAs.\(^{b}\)

<table>
<thead>
<tr>
<th>LTs</th>
<th>Lipid peroxides</th>
<th>LDL particle size</th>
<th>( \alpha )-Tocopherol</th>
<th>Coenzyme Q10</th>
<th>Saturated FAs</th>
<th>Monounsaturated FAs</th>
<th>Polyunsaturated FAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD-LT</td>
<td>(-0.27^{c})</td>
<td>(-0.16)</td>
<td>(-0.12)</td>
<td>(-0.04)</td>
<td>(-0.13)</td>
<td>(-0.14)</td>
<td>(-0.23)</td>
</tr>
<tr>
<td>PA-LT</td>
<td>(-0.20)</td>
<td>(-0.12)</td>
<td>(-0.11)</td>
<td>(-0.24)</td>
<td>(-0.25)</td>
<td>(-0.24)</td>
<td>(-0.25)</td>
</tr>
<tr>
<td>FD-LT</td>
<td>(-0.20)</td>
<td>(-0.20)</td>
<td>(-0.20)</td>
<td>(0.29^{c})</td>
<td>(0.15)</td>
<td>(0.10)</td>
<td>(0.09)</td>
</tr>
</tbody>
</table>

* The LTs were measured by following the production of CDs (CD-LT), by monitoring the fluorescence intensity after incorporation of PA into LDL (PA-LT), and by monitoring the autofluorescence development of LDL (FD-LT).

\(^{b}\) \( n = 98 \).

\(^{c}\) \( P < 0.01 \).

\(^{d}\) \( P < 0.001 \).
terms of simplicity in comparison with the conventional CD method because we have adapted both methods to a Cobas Bio analyzer. We obtained the shortest LTs with the PA technique, confirming that the PA probe, because of its surface localization, monitors an early stage of the oxidation process. The FD method is the most reproducible for determining LTs.

It has been shown that small, dense LDL subfractions are more readily oxidized than large, buoyant LDL subfractions (18, 19). When we measured the mean LDL particle diameter by high performance gel-filtration chromatography (7), a very precise and reproducible technique, we found no significant association with LTs. This does not contradict the above mentioned studies, but it does demonstrate that a relationship between size and LT of LDL oxidation is observed only in subfractions isolated from the same subject and not in a cross-sectional study design.

Recently, Kontush et al. (20) found no significant correlations between CD-LT and LDL composition. In agreement with this study, we found no significant correlations between LTs and the concentrations of cholesterol esters, free cholesterol, phospholipids, and triglycerides in LDL. We did find significant positive associations between coenzyme Q10 and both PA-LT and FD-LT (Table 1), although the concentration of this antioxidant in LDL is very low (<1 mol/mol). In this and several other studies (13, 21, 22), CD-LT and FD-LT were not related to α-tocopherol, the most abundant antioxidant in LDL. In general, significant relationships between α-tocopherol content and LT have been observed only after supplementation with pharmacological doses (22–24). In our study, however, the α-tocopherol content of LDL was significantly correlated to PA-LT. These results suggest that monitoring PA oxidation in LDL is a useful technique for analyzing early oxidation processes taking place in the surface monolayer of the LDL particle.

Polyunsaturated FAs are the main substrates for lipid peroxidation. We observed no correlation between linoleic acid and any of the LTs. In contrast, arachidonic acid was inversely related to both CD-LT and FD-LT, showing that the presence of this FA increases the oxidative susceptibility of LDL. We found a much weaker correlation between PA-LT and arachidonic acid, presumably because the PA probe is itself highly unsaturated and thus extremely prone to oxidation.

Peroxidation of lipids in isolated LDL requires the presence of traces of preformed peroxides (25). The lipid peroxide concentrations were indeed inversely related to LTs; however, only the association with CD-LT reached statistical significance.

In summary, LTs as measured by the three methods are highly correlated, but the magnitude of correlations found with LDL constituents are method dependent. LTs measured by the reproducible FD assay can be an alternative for the conventional CD method. The PA technique seems to be particularly suitable for studying an early stage of the oxidation process and relationships between LDL oxidation and antioxidants.

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References
Simple Sampling Method and Routine Gas Chromatography-Mass Spectrometry Analysis in 13C Breath Tests, Ágnes Keszler,* László Kótaí, and Klára Szentmihályi (Institute of Chemistry, Chemical Research Center, Hungarian Academy of Sciences, P.O. Box 17, H-1525 Budapest, Hungary; * author for correspondence: fax 36-1-325-7554, e-mail agi@cric.chemres.hu)

The 13C-labeled CO2 breath tests are convenient methods for various uses. They can be used to measure gastric emptying rate of solids (1), detect fat malabsorption (2), and evaluate pancreatic lipase activity (3), exocrine pancreatic insufficiency (4), glucose adsorption and utilization (5), liver function control (6), and lactase deficiency (7) with appropriate 13C isotope-labeled compounds. In recent years, the 13C-urea breath test has been used to detect Helicobacter pylori (8). With an appropriate analytical background and the facilities required for the detection or quantification of labeled CO2, the 13CO2 breath test could be widely applied for diagnostic purposes in the medical practice.

Although several infrared or mass spectrometric methods (9) are available, the transport of large volumes of gaseous samples to analytical centers as well as storage before measurement involves great problems. Therefore, we have developed a simple method that allows storage of these samples in minimal liquid volume. Although CO2 is absorbed in water or in alkaline solutions (10), rapid and quantitative absorption can hardly be achieved in these solutions. As has been described (11, 12), some organic amine-containing nonaqueous solutions are more suitable for CO2 absorption. These solutions contain the carbamate anions formed and the protonated ammonium cations of the starting amine (11, 12). Therefore, the use of 2-phenylethylamine, ethanolamine, or 3-methoxypropylamine (12) in pure form or dissolved in alcohol-type solvents is preferable. Because the absorption of CO2 is most efficient in 3-methoxypropylamine with a high gas bubbling rate (12), we used the ethanolic solution of this amine in our experiments.

The reaction can be described as follows:

\[
\text{MeO-CH}_2\text{CH}_2\text{CH}_2\text{-NH}_2 + \text{CO}_2 = \text{MeO-CH}_2\text{CH}_2\text{CH}_2\text{-NH-C(=O)-O}^- + \text{H}_3\text{N-CH}_2\text{CH}_2\text{CH}_2\text{-OMe}
\]

The salt formed is stable in ethanolic solution, and the volume of the sample container is small; it is easy to handle and greatly facilitates storage and transportation. The samples are acidified at the site of measurement when the free carbamic acid formed, which is unstable in acidic solution, immediately decomposes to amine and CO2. The amine formed is fixed in salt form in the acidic solution so that equilibrium is shifted completely toward the liberation of CO2. The gas sample is removed from the sealed sampling vessel via a septum and is measured directly by GC-MS. The retention time of the CO2 gas is short (1.4 min); thus, the measurement time is short (10 min). The relative amount of 13CO2 can be readily distinguished from mass spectra obtained from the extracted ion chromatogram (M = 44 or 45, 12CO2 or 13CO2). The chromatographic peak-area ratio of the 44- or 45-amu ions depends on the 13CO2 content of the air sample. We studied the correlation between the 13C content of the gas and the 12CO2/13CO2 peak-area ratios (Table 1). This peak-area ratio continuously decreased from the nonlabeled sample to the undiluted, labeled sample.

This method is quick and inexpensive, and the presence or absence of 13CO2 can be readily observed. Although the analytical method applied typically is qualitative, by calibration of the mass detector it may be extended to quantitative measurements. The relationship between 13CO2 content and the peak-area ratio of the 44/45 peak.