A Microassay to Assess the Oxidative Resistance of Low-Density Lipoproteins, László Ilhélyi,1 József Balla,1 László Muszbek,1 György Kakuk,1 John Belcher,2 Harry S. Jacob,3 Gregory M. Vercelotti,3 and György Balla2* (Departments of 1 Medicine, 2 Pediatrics, and 3 Clinical Chemistry, Medical University of Debrecen, Debrecen H-4012, Hungary; and 4 Department of Medicine, University of Minnesota, Minneapolis, MN 55455; * address correspondence to this author at: Department of Pediatrics, Medical University of Debrecen, Nagyerdei krt. 98. Pf. 19., Debrecen H-4012, Hungary; fax 36-52-413 653, e-mail balla@ibel.dote.hu)

Oxidative modification of LDL is implicated in the pathogenesis of atherosclerosis (1, 2). Susceptibility of LDL to oxidative modification is suggested to be an independent risk factor for coronary atherosclerosis, and recent epidemiological studies revealed protective effects of antioxidants on development and progression of atherosclerosis (3–5).

We have previously demonstrated that hemin readily intercalates into LDL particles and rapidly oxidizes LDL in vitro (6). Hemin-catalyzed oxidation of LDL can be accelerated by activated inflammatory cells, small amounts of hydrogen peroxide, or preformed lipid hydroperoxides within the LDL. That such hemin-induced oxidative modification of LDL may be involved in atherogenesis is supported by the finding that hemin-sensitive genes in endothelium (7–8) are up-regulated in atherosclerotic lesions (9).

The aim of the present study was to establish a clinical laboratory microassay, based on the time kinetics of hemin-catalyzed lipid peroxidation of LDL, for assessing LDL resistance to oxidative modification, and to determine the optimal conditions and reproducibility of the assay.

Plasma LDL was isolated from 1 g/L Na2EDTA-anticoagulated venous blood after a 2000g centrifugation for 20 min at 4 °C, and the density of plasma was adjusted to 1210 g/L with KBr. After a two-layer gradient was made in a 5.1-mL Quick-Seal polyallomer tube (Beckman Instruments) by layering normal saline containing 100 mg/L of Na2EDTA on 1.5 mL of density-adjusted plasma, a single spin gradient ultracentrifugation at 228 000g at 4 °C for 90 min (VTi 65.2 rotor, Beckman Instruments) was performed to isolate LDL. The LDL fraction proved pure in agarose gel electrophoresis, running homogeneously with β-lipoprotein. The LDL protein concentration, which is proportional to LDL molarity, was determined by the bicinchoninic acid (BCA) protein assay (Pierce). During preparative procedures and storage, the samples were kept at 4 °C in room air and protected from shaking and light.

Hemin-catalyzed lipid peroxidation of LDL was monitored spectrophotometrically at 405 nm in a reaction mixture containing LDL (200 mg/L protein), hemin (4 μmol/L), hydrogen peroxide (75 μmol/L), and HEPES buffer (10 mmol/L, pH 7.4), in a final volume of 200 μL, in triplicate. In the hemin-hydrogen peroxide-mediated LDL modification system, hemin degradation was shown to occur inversely with conjugated diene formation (6, 10); thus hemin degradation may function as a probe of lipid peroxidation process. The reaction was monitored in an Automated Microplate Reader Model EL340 (Bio-Tek Instruments) in a 96-well flat bottom tissue culture plate at 37 °C. To run the reader and to analyze serial measurements taken every minute for 4 h, we applied KC3 software (Bio-Tek Instruments). The oxidative resistance of LDL was characterized by ΔT at maximum velocity (Vmax) in seconds, the time period until the maximal velocity of hemin disappearance in the propagation phase. The Vmax of hemin degradation, as defined by the maximum change in absorbance of hemin at 405 nm, was calculated using absorbance values detected every minute, with four absorbance values used to calculate the slope. Data are given as means ± SD. The linearity of the relationship between ΔT at Vmax and lag time was assessed by use of correlation coefficients.

Fig. 1A demonstrates the kinetics of lipid peroxidation of an LDL sample catalyzed by hemin. The lipid peroxidation process and hemin degradation were monitored spectrophotometrically at 405 nm, using a kinetic microplate reader. The maximal velocity of the propagation phase was −6.4848 milliabsorbance units/min, and ΔT at Vmax was 4590 s. Because ΔT at Vmax has a strong linear relationship (r2 = 0.957) with the lag time, the length of initiation phase, the ΔT at Vmax also characterizes the oxidative resistance of LDL (Fig. 1B). The lag time was 84% of ΔT at Vmax.

We analyzed how the concentrations of hemin and hydrogen peroxide influence ΔT at Vmax. Between 3.0 and 5.0 μmol/L hemin, ΔT at Vmax had minimum values; therefore, 4.0 μmol/L of hemin was optimal for assays generating the characteristic, shortest ΔT at Vmax. Increases in the concentration of hydrogen peroxide decreased ΔT at Vmax. From a technical point of view, i.e., length of kinetic spectrophotometric measurement, 75 μmol/L was the optimal hydrogen peroxide concentration. The presence or absence of Na2EDTA in the reaction mixture does not affect the reaction kinetic curve of LDL lipid peroxidation catalyzed by hemin; therefore, dialysis of samples is not required for running the assay.

We tested the effect of storage of isolated LDL on the values of ΔT at Vmax. Five LDL samples were stored in 150 mmol/L NaCl solution containing 100 mg/L of Na2EDTA at 4 °C in room air, and ΔT at Vmax was determined on 5 consecutive days. The decreases in ΔT at Vmax were 2.6% ± 0.9% and 9.4% ± 5.7% at 24 and 48 h after LDL isolation, respectively. There were more substantial
changes at later time points, even in the presence of Na₂EDTA. We emphasize that the intraassay CVs were 10–20% for these LDL samples. The decrease in ΔΤ at Vₘₐₓ during storage of LDL samples is the consequence of spontaneous LDL lipid peroxidation and not the failure of our method. The spontaneous lipid peroxidation of LDL was reflected in endogenous lipid hydroperoxide formation (not shown). It is advisable to process the samples within 24 h after blood drawing. Because isolation of LDL from plasma (n = 10) stored at −70 °C for 4 weeks also decreased ΔΤ at Vₘₐₓ by 8.7% ± 12.4%, we advise not to use frozen plasma for the assay.

The intraassay CVs of ΔΤ at Vₘₐₓ were 1.6–2.7% (n = 20, Table 1). When the ΔΤ at Vₘₐₓ values of 54 LDL samples from healthy subjects were analyzed, a broad spectrum of LDL resistance to oxidative stress was observed (Table 1). The extreme values were 1275 s and 8495 s.

Because α-tocopherol increases LDL resistance to oxidation, we assessed the effect of oral vitamin E supplementation on ΔΤ at Vₘₐₓ in 11 healthy volunteers taking 800 IU of dl-α-tocopherol acetate per day for 2 weeks. The ΔΤ at Vₘₐₓ increased 1.2- to 2.8-fold (mean, 1.8-fold) and the α-tocopherol content of LDL increased 1.5- to 4.1-fold (mean, 2.4-fold). Significant correlation between the increase in ΔΤ at Vₘₐₓ and the increase in LDL α-tocopherol content was found during supplementation (r = 0.609).

This novel assay is suitable for testing large numbers of LDL samples on an automated microplate reader. The advantages of our method over existing measurements (2, 11) are the ability to follow the kinetics of LDL lipid peroxidation at a visible wavelength and the use of Na₂EDTA during isolation and analysis of LDL. Because the readily and exactly measurable ΔΤ at Vₘₐₓ has a strong linear relationship with the lag time, the ΔΤ at Vₘₐₓ also characterizes the oxidative resistance of LDL.

This work was supported in part by US-Hungarian joint fund 349/93-B, ETT 116,136/96, OTKA T 21.023, and MEC-1/96. We thank Alice G. Dobolyi for technical assistance.

References

Table 1. Intraassay precision of ΔΤ at Vₘₐₓ of LDL samples (n = 20) and ΔΤ at Vₘₐₓ of LDL samples (n = 54) from healthy subjects.

<table>
<thead>
<tr>
<th>Intraassay precision</th>
<th>Healthy subjects</th>
</tr>
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<tbody>
<tr>
<td>Mean ΔΤ at Vₘₐₓ, s</td>
<td>SD, s</td>
</tr>
<tr>
<td>2336</td>
<td>63</td>
</tr>
<tr>
<td>3777</td>
<td>69</td>
</tr>
<tr>
<td>6664</td>
<td>105</td>
</tr>
<tr>
<td>Maximum</td>
<td>8495</td>
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</table>

ΔΤ at Vₘₐₓ increased 1.2- to 2.8-fold (mean, 1.8-fold) and the α-tocopherol content of LDL increased 1.5- to 4.1-fold (mean, 2.4-fold). Significant correlation between the increase in ΔΤ at Vₘₐₓ and the increase in LDL α-tocopherol content was found during supplementation (r = 0.609).
Changes in the Concentrations of Plasma Selenium and Selenoproteins after Minor Elective Surgery: Further Evidence for a Negative Acute Phase Response? Colin Nichol,1 Jacqueline Herdman,2 Naveed Sattar,3 Patrick J. O’Drayer,4 Denis St. J. O’Reilly,5 and Gordon Fell1* (1 Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow G11X, UK; 2 Department of Pathological Biochemistry, Glasgow Royal Infirmary University NHS Trust, Glasgow G4 OSF, UK; and 3 Department of Surgery, Western Glasgow Hospitals University NHS Trust, Glasgow G16NT, UK; * address correspondence to this author at: Trace Element Unit, Macewen Building, Royal Infirmary University and NHS Trust Hospitals, Glasgow G4 0SF, UK; fax 0044 141-555-1703, e-mail gpya11@udcf.gla.ac.uk)

The acute phase plasma protein response is part of the complex series of physiological, hematological, and biochemical events that constitute the inflammatory response after tissue injury or infection. The magnitude and duration of the response are related to the nature and severity of the injury and the presence of sepsis (1). We have previously reported alterations in plasma iron, transferrin, zinc, albumin, copper, and ceruloplasmin concentrations after major surgery to a marked rise in plasma C-reactive protein (CRP) concentration (2). In both acute (3, 4) and chronic (5) illnesses, the plasma concentration of selenium also decreases in proportion to the magnitude of the inflammatory response. There is concern about the decline in dietary intake of selenium in some areas of the world (6), because the antioxidant activities of several selenoproteins may be important in preventing free radical damage (7). If plasma selenium concentrations decrease during an inflammatory response, independently of dietary intake, then this would have important implications for the interpretation of the plasma selenium values reported in a wide range of illnesses. In this study, total plasma selenium concentration and changes in plasma selenoproteins after minor elective surgery (inguinal hernia repair) were determined and related to the accompanying alterations in plasma CRP.

Ten male patients (mean age, 51 years; range, 18–90 years) requiring inguinal hernia repair were recruited to the study. All patients were healthy before surgery, and none were taking any relevant medication. Samples of head hair and toe nails (80–100 mg) were obtained 24 h before surgery as a measure of long-term selenium nutritional status, along with venous blood collected into plain (10 mL) and lithium heparin tubes (20 mL). Blood samples were taken on the mornings of day 1 and day 6 after surgery. None of the patients received intravenous fluids or blood products postsurgery, and all had uncomplicated clinical courses. Routine ward diet was resumed the day after surgery. The study was approved by the local Ethics Committee, and all subjects gave their informed consent. Plasma and serum were separated as soon as possible and stored in plastic tubes at −20 °C.

For selenium determination, a Perkin–Elmer 1100B atomic absorption spectrometer, equipped with a PE HGA 700 programmer, a PE AS 70 autosampler, and an Epson FX800 printer, was used to obtain integrated absorption signals. A selenium electrodeless discharge lamp (5 W) was used, and atomic absorption measured at the 196.0 nm selenium line, with palladium as a matrix modifier (8). This method has a within-batch imprecision of 2.3%. The limit of detection is 0.05 μmol/L. Hair and nail samples were prepared by microwave digestion using concentrated nitric acid and hydrogen peroxide; the selenium content was then determined by electrothermal atomic absorption spectrometry (9). The selenoproteins in human plasma were separated by column affinity chromatography (10). Blue Sepharose CL-6B (Pharmacia) was used to bind both selenoprotein-P (Se-P) and albumin, thus releasing the glutathione peroxidase (GSHPx) fraction. Heparin Sepharose CL-6B (Pharmacia) was used to separate Se-P from albumin, and was then eluted from the column. The selenium content of the separated fractions was then determined by electrothermal atomic absorption spectrophotometry. The plasma GSHPx protein concentration was measured by a commercial enzyme-linked immunosorbent immunoassay kit, using polyclonal antibodies that were specific for human plasma GSHPx (pi-GPx-EIA kit from Bioxtech S.A.). The detection limit was 2.5 mg/L, and the within-batch imprecision was 5.1% at 5.4 mg/L. Plasma and red cell GSHPx activity was measured by a rate reaction method using t-butyl peroxide as a substrate (11). The plasma GSHPx assay had a within-batch imprecision of 3.0%, and red cell GSHPx activity measurement had a between-batch imprecision of 7.3%. Serum albumin (colorimetric), transferrin and ceruloplasmin (immunoturbidimetric) iron (colorimetric), zinc and copper (inductively coupled plasma atomic emission spectrometry), and CRP (immunoturbidimetric) were measured as described previously (5).

Results are presented as the mean and observed range. Comparisons between different days after surgery were made by paired t-test (Minitab statistical software).

Selenium concentrations found in hair and nail samples taken from the patients were 0.64 ± 0.05 mg/kg and 0.35 ± 0.11 mg/kg, respectively. These results were similar to those found in the local population (0.52 ± 0.11 mg/kg and 0.44 ± 0.10 mg/kg, n = 25) (9). The minor surgery of inguinal hernia repair caused a modest rise in plasma CRP concentration (mean concentration increased to 20.7 mg/L; P < 0.01; Table 1). This is less than the increases in CRP concentration found in more major surgical procedures (12). Nevertheless, this increase in plasma CRP was associated with significant alterations in the plasma concentrations of trace metals and carrier proteins in a manner similar to that seen after major surgical procedures (2). The plasma concentrations of iron, transferrin, zinc, and albumin decreased as CRP