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Measurement of oxidation in plasma Lp(a) in CAPD patients using a novel ELISA

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Measurement of oxidation in plasma Lp(a) in CAPD patients using a novel ELISA.

Background. LGE_2 is produced by the cyclooxygenase- or free radical-mediated modification of arachidonate and is formed during the oxidation of low density lipoprotein (LDL) with subsequent adduction to lysine residues in apo B. We have developed a sensitive enzyme-linked sandwich immunosorbent assay (ELISA) for detection and measurement of LGE_2-protein adducts as an estimate of oxidation of plasma LDL and Lp(a).

Methods. The assay employs rabbit polyclonal antibodies directed against LGE_2-protein adducts that form pyrroles, and alkaline phosphatase-conjugated polyclonal antibodies specific for apo B or apo (a). It demonstrates a high degree of specificity, sensitivity and validity.

Results. Epitopes characteristic for LGE_2-pyrroles were quantified in patients with end-stage renal disease (ESRD) that had undergone continuous ambulatory peritoneal dialysis (CAPD) and in a gender- and age-matched control population. In addition to finding that both LDL and Lp(a) levels were elevated in CAPD patients, we also found that plasma Lp(a) but not LDL was more oxidized in CAPD patients when compared to corresponding lipoproteins from healthy subjects. Using density gradient ultracentrifugation of plasma samples, we found that modified Lp(a) floats at the same density as total Lp(a).

Conclusions. The results of this study demonstrate that oxidation of plasma Lp(a) is a characteristic of ESRD patients undergoing CAPD. This ELISA may be useful for further investigations on oxidation of lipoproteins in the circulation of specific patient populations.

One of the characteristics of end-stage renal disease (ESRD) is the presence of oxidative stress [1–3]. Mortality in this patient population is primarily due to coronary and/or peripheral artery diseases as a result of atherosclerosis [4]. Oxidation of plasma low density lipoproteins (LDL), claimed to be present in such patients [5], has been linked to the atherosclerotic process by the ability of such oxidized (ox-) LDL to induce a number of events in model systems that are considered to be atherogenic [6]. Increased plasma levels of ox-LDL would be expected to accelerate the further oxidation of LDL in the interstitial space of tissues such as the arterial intima following insudation from the circulation. Evidence for the presence of ox-LDL in plasma came from measurements in the circulation of hydroperoxides [7], thiobarbituric acid reactive substances (TBARS), conjugated dienes, affinity of lipoproteins for specific ion exchange matrices [8], autoantibodies [9, 10], and epitopes specific for oxidized lipid-protein adducts [11–14]. However, several of these measurements are nonspecific, while others require initial isolation of LDL. Some studies reported an increased susceptibility to *in vitro*-induced oxidation of LDL isolated from plasma of ESRD patients [9, 15, 16], while others failed to find such an increase [17, 18].

In contrast to LDL, little attention has been paid to the possible oxidation of plasma Lp(a) in ESRD patients, including those on continuous ambulatory peritoneal dialysis (CAPD), even though Lp(a) is believed to be as susceptible to oxidation under *in vitro* conditions as is LDL [19–23]. Oxidation of LDL or Lp(a) is commonly characterized by the formation of aldehydes that react with their respective apoprotein moieties [6, 24]. Two immunoassays have been reported that directly measure oxidatively-modified lipoproteins in plasma samples. One employed antibodies to epitopes specific for MDA-modified proteins [11–13], and the other to epitopes in specific fractions of oxidized phosphatidylcholine-modified proteins [14], but which required first isolating the lipoprotein [11–14]. Given that MDA modification of proteins requires free radical-induced scission of lipid hydroperoxides [24], an event occurring in more extensively-oxidized lipoproteins [24], we sought a modification of LDL that represented an earlier stage in the oxidation of lipoproteins, one that would more likely be still present in the circulation.

Phospholipid endoperoxide formation represents an early event during oxidation of LDL. It is the result of

Key words: LGE_2, protein adducts, lipoproteins, oxidative stress, coronary artery disease, atherosclerosis, phospholipid endoperoxide.

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Table 1. Characteristics of CAPD patients and age matched controls

	Controls ($N = 15$)	CAPD ($N = 27$)
Age	48 ± 10	52 ± 15
Sex (m/f)	6/9	14/13
Race (w/b/others)	8/7	14/11(1 Spanish/1 Indian)
Diabetes	0	48%
Presence of cardiovascular disease	0	41%
Creatinine clearance	—	76.8 ± 39.4
KT/V	—	2.2 ± 0.6
Creatinine *mg/ml*	—	9.8 ± 3.5
Duration of PD procedure	0	30 ± 21 months
LDL concentration *mg/dl*	92.2 ± 19.1	112.7 ± 31.6[a]
Lp(a) concentration *mg/dl*	16.1 ± 13.6	30.9 ± 23.4[a]

Data are presented as mean ± SD.
Comparison of Lp(a) levels was performed on log transformed data.
[a] Significance $p < 0.05$ vs. control group

free-radical-induced oxidation of phospholipid-associated arachidonate in LDL [25]. The structurally-similar prostaglandin endoperoxide (PGH_2 derived from arachidonate) rearranges to generate prostaglandins and secoprostanoic acid levulinaldehyde derivatives, defined as LGE_2 [26]. These aldehydes can then react with lysyl residues on proteins to form adducts that subsequently partition to form pyrroles [27, 28]. We had previously shown by immunochemical procedures that such formation of LGE_2-derived protein-bound pyrroles from arachidonate occurs as a very early event during oxidation of LDL [29], as contrasted to MDA modification. Moreover, in preliminary studies we found that the degree of modification of proteins and lipoproteins in plasma by LGE_2 is far less than the amount of adduction required to induce uptake by macrophages in culture [30]. In this present study we have developed a sandwich ELISA employing polyclonal antibodies to LGE_2-protein pyrroles and polyclonal antibodies to apo B and apo (a) to quantify the degree of oxidation in plasma LDL and Lp(a) of CAPD patients and age-matched normal individuals.

METHODS

Materials

All chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless specified otherwise.

Patient and control populations

Plasma was obtained from patients undergoing CAPD treatment at the Cleveland Clinic Foundation. All patients were on CAPD treatment for an average of 30 months and received 8 to 12 liter of Dianeal® dialysate per day (Baxter Travenol, Deerfield, IL, USA). Baseline characteristics of the 27 CAPD patients are summarized in Table 1. Patients typically received medications for hypertension (primary calcium channel blockers) and were treated with erythropoetin, phosphorus binders, plus iron and vitamin D supplements. The study protocol was approved by the Institutional Review Board, and all patients gave their written informed consent prior to participation. Control plasma was obtained from a group of randomly selected volunteers within the Cleveland Clinic Foundation and was comprised of 15 subjects with no history of renal disease who were matched with the CAPD patients for gender and race.

Blood samples

Whole blood was collected by venipuncture into vacutainer tubes (Becton Dickinson & Co., Rutherford, NJ, USA) containing EDTA (0.15% final concentration) and placed in an ice bucket. Plasma was then separated by centrifugation at 3000 rpm. Butylated hydroxytoluene (BHT), leupeptin, pepstatin and aprotinin were added immediately to plasma to final concentrations of 40 μM, 35 μM, 5 μM and 18 μg/ml, respectively. Samples were either used immediately for assaying or quench-frozen in liquid nitrogen and stored at −70°C.

Lipoprotein isolation and density gradient ultracentrifugation

LDL was isolated from fresh human plasma by sequential ultracentrifugation at a $1.019 < d < 1.063$ g/ml density fraction [31]. The LDL was dialyzed against phosphate buffered saline (PBS), pH 7.4 containing 1 mg/ml EDTA and 40 μM BHT, filter-sterilized and used for subsequent modification. Lp(a) was isolated from a male donor with an Lp(a) plasma level of 30 mg/dl in the density range of 1.050 to 1.110 g/ml, and further purified on Sephacryl S 400-HR as reported previously [32]. Lp(a) purity was assessed by agarose gel electrophoresis and by 4 to 12% gradient SDS-PAGE. The procedure for density gradient ultracentrifugation was performed according to Redgrave, Roberts and West [33] with minor changes. Solutions of NaBr with decreasing density were layered sequentially from the bottom to the top into Beckman 14 × 89 mm Ultra-Clear™ tubes (0.5 ml d = 1.478 g/ml; 2 ml of plasma at d = 1.21 g/ml, 3 ml d = 1.1 g/ml, 3 ml d = 1.05 g/ml and 2 ml d = 1.006 g/ml). The tubes were centrifuged in a Beckman SW-41 rotor at 39, 000 rpm for 44 hours. After centrifugation, 22 fractions were collected sequentially from the bottom of the tubes. The densities of the fractions were measured by refractometry on parallel tubes.

Low density lipoprotein and Lp(a) modification

LGE_2 ([R]-acetyl-9[R]-formyl-12[S]-hydroxy-5[Z],10[E]-heptadeca-dienoic acid) and ^{3}H-labeled LGE_2 were prepared as described previously [34, 35]. 0.5 ml of LDL or Lp(a) (0.5 mg/ml) in PBS/EDTA were combined with 0.5 ml ^{3}H LGE_2 (0.2 mg/ml) in PBS and incubated at 37°C for 12 hours. The mixture was then dialyzed against PBS/EDTA, centrifuged for 10 minutes at 10,000 g, and sterilized by filtration. The amount of protein was determined by the bicinchoninic acid assay [36], and the amount of LGE_2

bound was assessed by quantifying the radioactivity associated with lipoprotein.

Antibodies and conjugates

Goat anti-human plasma LDL was purchased from Bethyl Laboratories (Montgomery, TX, USA). Affinity-purified goat anti-LDL IgG was obtained using LDL-Sepharose prepared by coupling of LDL to CNBr-activated Sepharose®-4B (Pharmacia, Stockholm, Sweden) as described in detail elsewhere [37]. Goat anti-human plasma Lp(a) was purchased from International Enzymes, Inc. (Fallbrook, CA, USA) and demonstrated no crossreactivity with human plasminogen or apo B. Alkaline phosphatase conjugates of anti-LDL and anti-Lp(a) were prepared as described previously [37]. Preparation of rabbit antibodies to keyhole limpet hemocyanin (KLH)-LGE_2 was described by us earlier [38]. The IgG fraction was purified from the serum using protein G-agarose [ImmunoPure® (G) Immobilized Protein G; Pierce, Rockford, IL, USA] according to the manufacturer's protocol. The concentration of isolated IgG was determined by absorbance at 280 nm. Specificity of antibodies to LGE_2 was assessed previously in competitive inhibition studies [38]. No crossreactivity of this antibody was shown with LDL, MDA-modified LDL, or HNE-modified LDL.

Measurement of LDL and Lp(a) concentrations

Lp(a) concentrations in plasma samples were measured according to Fless, Snyder and Scanu [39] and expressed as Lp(a) mass per dl of plasma. In this method goat anti-human apo (a) polyclonal antibodies were used to capture Lp(a) in samples diluted 1/100 to 1/400 in carbonate buffer. Affinity-purified goat anti-human LDL (apo B) conjugated to alkaline phosphatase was used to detect the bound Lp(a). This method detects Lp(a) isoforms with equal sensitivity and does not detect plasminogen [39]. This same method was used for measurement of LDL concentrations with the following modifications: the coating antibody was goat anti-LDL, plasma samples were diluted 1500-fold, and standard LDL concentrations ranged from 0.25 to 2 μg/ml. Absorbance was read with a Bio-Rad Model 3550 microplate reader at 405 nm using 595 nm as the reference wavelength. The sigmoidal dose-response curve was transformed using the logit function as described by Tijssen [40] and analyzed by least square regression.

Detection of immunoreactive LGE_2-LDL and LGE_2-Lp(a) by ELISA

Microtiter plates (Corning, Cambridge, MA, USA) were coated with 200 μl of anti LGE_2 IgG (20 μg/ml) in PBS overnight at 4°C, followed by blocking with 300 μl of PBS containing 3% bovine serum albumin (BSA), for two hours at 37°C. Following washing of the plates with PBS, a 200 μl test sample, prepared by diluting plasma 750- to 1500-fold in PBS containing 3% BSA, was added to plates and incubated for 90 minutes at 37°C. A 200 μl sample of LGE_2-modified LDL or LGE_2-modified Lp(a), in a concentration range of 5 ng to 2 μg, was also added and served as standards. Plates were subsequently washed three times with PBS containing 0.1% Tween 20 and two times with PBS. Two hundred microliters of goat anti-LDL or goat anti-Lp(a) conjugated to alkaline phosphatase (diluted 1/500 in PBS/BSA) were then added, and plates incubated at 37°C for 90 minutes. After washing the plates with PBS, 200 μl of p-nitrophenyl phosphate in Tris buffer (SIGMA FAST™ pNPP tablets; Sigma) was added to each well. The plates were then placed in a dark chamber, and the enzyme reaction was allowed to proceed for 30 to 60 minutes at 37°C. Absorbance was read as reported above. The sigmoidal dose-response curve was transformed using the logit function and analyzed by least square regression.

Other methods

Proteins were measured by the bicinchoninic acid assay (Pierce) using bovine serum albumin as a standard [36]. Oxidized LDL was prepared by incubation of LDL (0.3 mg/ml) with 5 μM copper sulfate at 37°C for 18 hours and subsequent dialysis against PBS/EDTA.

Statistical analysis

A log transformation of Lp(a) concentration data was used to approach normal distribution. Differences were assessed by an unpaired Student's *t*-test.

RESULTS

ELISA for quantification of oxidized plasma LDL or Lp(a)

As was shown previously by us [38], antibodies generated against KLH that had been modified with LGE_2 specifically recognize pyrrole epitopes on LGE_2-protein adducts [38]. No detectable crossreactivity of LGE_2-KLH antibodies was found with MDA-LDL or HNE-LDL [38]. During preliminary studies we found that the sensitivity of our ELISA was insufficient to measure oxidized LDL and Lp(a) in plasma when we used an initial immunoprecipitation of LDL or Lp(a) by anti-LDL or anti-Lp(a) respectively, and subsequent detection of LGE_2-protein adducts by a phosphatase-conjugated LGE_2-KLH antibody. However, as shown previously [14], the sensitivity of the assay can be significantly increased by first absorbing modified lipoproteins with specific antibodies recognizing chemically-modified macromolecules, and then using phosphatase-conjugated antibodies to native lipoproteins to detect and quantify the amount of bound lipoprotein. We used this second approach and found that LGE_2-modified LDL and LGE_2-modified Lp(a) bind to wells coated with anti-LGE_2-KLH in a dose-dependent fashion up to 0.5 μg/ml of LDL or Lp(a) (Fig. 1 A, B). When nonimmune rabbit IgG was used to precoat wells instead of anti-LGE_2-KLH, only negligible

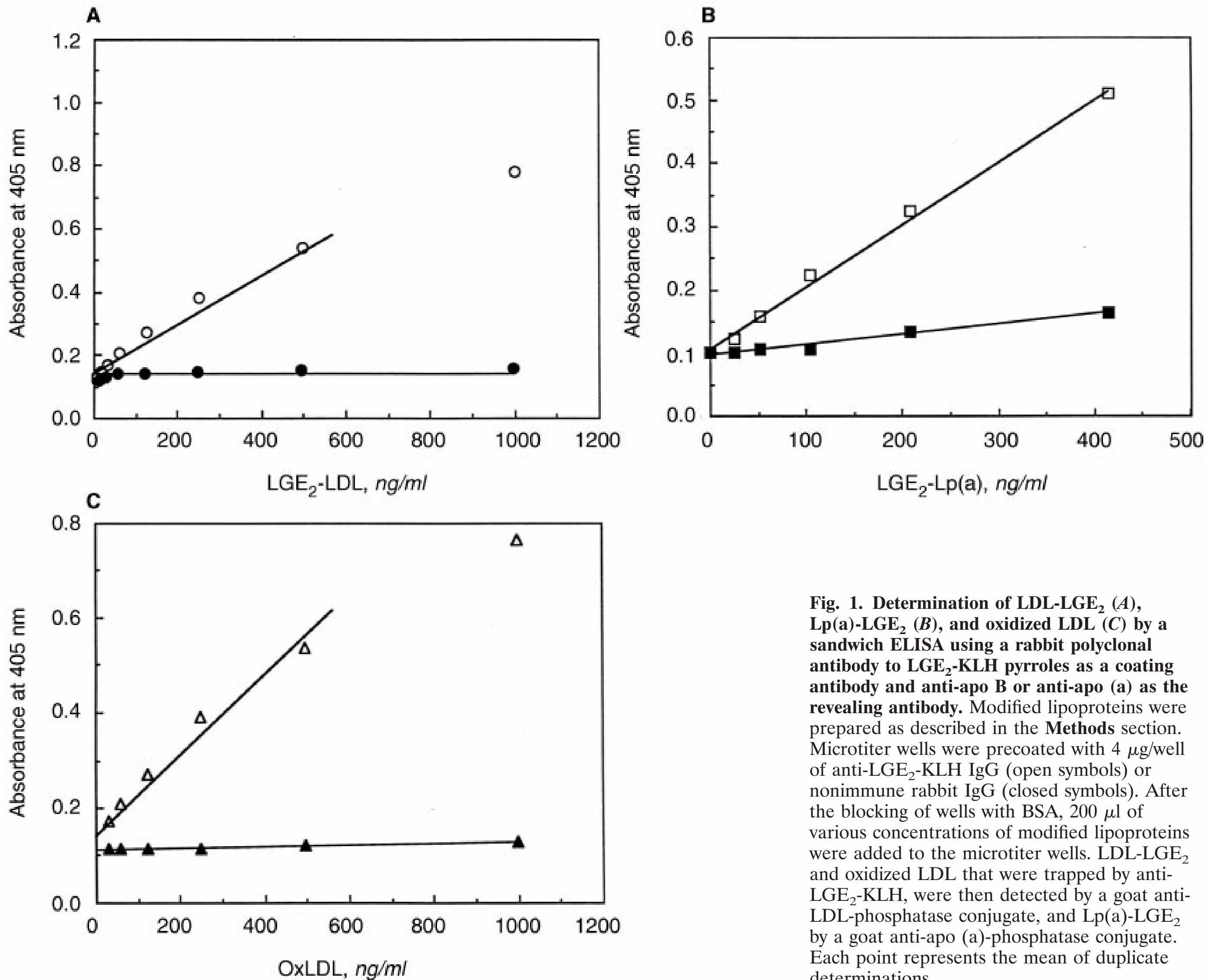

Fig. 1. Determination of LDL-LGE_2 (*A*), Lp(a)-LGE_2 (*B*), and oxidized LDL (*C*) by a sandwich ELISA using a rabbit polyclonal antibody to LGE_2-KLH pyrroles as a coating antibody and anti-apo B or anti-apo (a) as the revealing antibody. Modified lipoproteins were prepared as described in the **Methods** section. Microtiter wells were precoated with 4 μg/well of anti-LGE_2-KLH IgG (open symbols) or nonimmune rabbit IgG (closed symbols). After the blocking of wells with BSA, 200 μl of various concentrations of modified lipoproteins were added to the microtiter wells. LDL-LGE_2 and oxidized LDL that were trapped by anti-LGE_2-KLH, were then detected by a goat anti-LDL-phosphatase conjugate, and Lp(a)-LGE_2 by a goat anti-apo (a)-phosphatase conjugate. Each point represents the mean of duplicate determinations.

lipoprotein binding was observed. As such, the assay system measures the number of lipoprotein particles that contain at least one LGE_2-protein adduct. Excess amounts of LGE_2-modified HSA (100-fold) inhibited LGE_2-modified lipoprotein binding to anti-LGE_2-KLH by 90%. Cu^{++}-oxidized LDL demonstrated a degree of binding to anti-LGE_2-KLH which was similar to that of LGE_2-modified LDL (Fig. 1C), suggesting that LGE_2-protein pyrroles were formed in LDL by oxidation, as was shown previously [29]. The intra- and inter-assay coefficient of variation for the determination of LGE_2-LDL and LGE_2-Lp(a) was $<5\%$ and 10%, respectively. The high sensitivity of the assay was achieved by virtue of the fact that each modified lipoprotein particle that was attached to wells precoated with anti-LGE_2-KLH, can bind a large number of anti-apo B or anti-apo (a) molecules. In this ELISA the conjugated antibodies against apo B or apo (a) are unable to detect other proteins that could bind to anti-LGE_2-KLH. Thus, our assay will detect all apo B or apo (a) modified by LGE_2 including lipoprotein-free fragments of apo B or apo (a).

Since we were measuring the amount of bound apolipoprotein immunoreactivity after trapping modified lipoproteins by anti-LGE_2-KLH, we needed to determine whether anti-apo B and anti-apo (a) recognized the modified lipoproteins to the same degree as native lipoproteins. At a level of modification used in this study, apo B recognition by anti-apo B was not changed appreciably, nor was apo (a) recognition by anti-apo (a) (data not shown).

Oxidation of LDL and Lp(a) in CAPD and in a control population

Since in preliminary experiments we found that quench-freezing of plasma samples in liquid nitrogen did not induce significant changes in measurement of lipoproteins

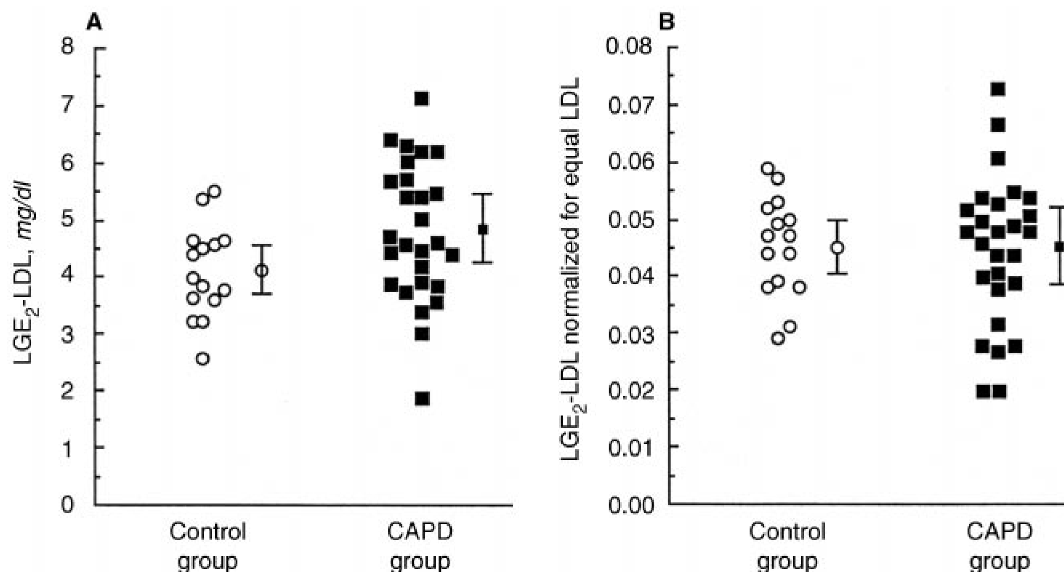

Fig. 2. Concentrations of LGE_2-modified LDL in the plasma of patients with ESRD undergoing CAPD and in a control group. Plasma samples from 27 patients (■) and from 15 age-matched control individuals (○) were obtained and subjected to measurements described in the **Methods** section. The anti-apo B-phosphatase conjugate was used for the detection of lipoproteins bound to microtiter wells. The average concentration of LGE_2-LDL was 4.09 ± 0.81 mg/dl in plasma of controls, and 4.86 ± 1.22 mg/dl in those of patients (*A*). The difference was statistically significant as assessed by an unpaired Student's *t*-test. Normalization of LGE_2-LDL concentrations was performed by dividing each value by its corresponding LDL concentration (*B*). Mean values were 0.045 ± 0.013 for patients and 0.045 ± 0.008 for controls. In A, $P < 0.05$; in B, $P = 0.92$.

or LGE_2-lipoprotein immunoreactivity, we performed all subsequent measurements on such quench-frozen samples. The plasma from 27 CAPD patients and 15 control subjects was assayed to determine the levels of LDL and Lp(a) and the levels of oxidized LDL and Lp(a), indicated by the amounts of LGE_2-LDL and LGE_2-Lp(a) levels, respectively. As seen in Table 1, the mean levels of both LDL and Lp(a) were statistically significantly elevated when compared to corresponding levels in the control population. The level of plasma LGE_2-LDL ranged from 2.5 to 5.5 mg/dl, and from 1.9 to 7.1 mg/dl in the control group and in the CAPD group, respectively (Fig. 2A). The level of LGE_2-LDL was found to be 19% higher in the CAPD group than in the control group ($P < 0.05$). However, the level of LGE_2-LDL when normalized for equal concentrations of plasma LDL was about the same in the CAPD patients as in the control group (Fig. 2B), since the mean plasma concentration of LDL was higher in patients than in controls (Table 1).

The levels of LGE_2-modified Lp(a) varied from undetectable to 11.9 mg/dl in the control group and from undetectable to 27.8 mg/dl in the CAPD group (Fig. 3A). The mean level of LGE_2-Lp(a) was found to be 2.4 times higher in the CAPD patient group than in the control group (Fig. 3A, $P < 0.05$). Since this difference may merely reflect the difference in Lp(a) concentrations, as was found for LDL, we again compared the LGE_2-Lp(a) level in the two groups after normalizing for their respective Lp(a) concentrations. We found that such normalization of data slightly decreased the difference between the CAPD group and the control group, but normalized LGE_2-Lp(a) levels were still significantly higher in the CAPD group than in the control group (1.6 times, $P < 0.05$, Fig. 3B). Thus, not only is the total amount of LGE_2-Lp(a) increased in CAPD patients, but the percent of Lp(a) particles that are associated with at least one LGE_2-protein epitope is also increased.

Since many of the CAPD patients had diabetes and/or CVD, which themselves could be causative factors for the oxidation of Lp(a), we separated the CAPD group into those with or without documented CVD, as well as those with or without diabetes. Although no significant differences in Lp(a) levels were found in the subgroups with and without diabetes, we did find that Lp(a) levels were higher in the CVD subgroup relative to the subgroup without CVD, but the degree of significance was borderline (Table 2). When subsequently examining the level of oxidation of Lp(a) in such subgroups, we found that there was no statistically significant difference in LGE_2-Lp(a) level between the CAPD subgroups with or without CVD, nor those with- and without diabetes (Table 2). This result suggests that the oxidation of Lp(a) was probably not the result of CVD nor diabetes. The level of oxidation of Lp(a) was still higher in the different CAPD subgroups relative to the control group (Table 2), although not all reached the $P < 0.05$ level of significance. When KT/V values, creatinine clearance levels, and time of dialysis for each CAPD patient were each correlated with corresponding LGE_2-Lp(a) values, no associations could be found (not shown). Thus, it is unlikely that the dialysis procedure was responsible for the oxidation of Lp(a). This would leave renal

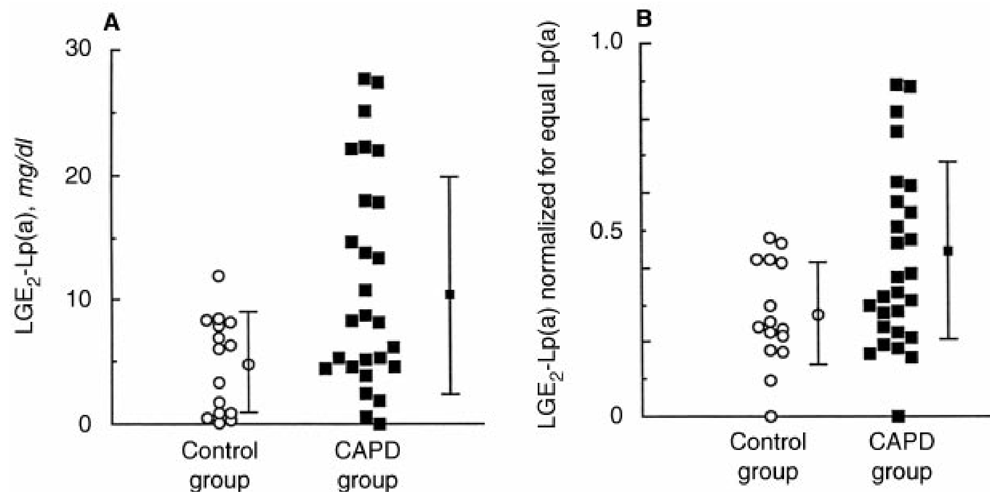

Fig. 3. Concentrations of LGE_2-modified Lp(a) in the plasma of patients with ESRD undergoing CAPD and in controls. An anti-apo (a)-phosphatase conjugate was used to reveal bound lipoproteins (*A*). The average concentration of LGE_2-Lp(a) was 11.28 ± 8.74 mg/dl and 4.77 ± 3.89 mg/dl in patient and control plasma, respectively ($P < 0.01$). A log transformation of data was used to calculate statistical differences. Normalization of LGE_2-Lp(a) concentrations was performed by dividing each value by its corresponding Lp(a) concentration (*B*). Mean values were 0.443 ± 0.238 for patients and 0.275 ± 0.141 for controls ($P < 0.05$).

Table 2. Level of Lp(a) and Lp(a)-LGE_2 in specific subgroups of CAPD patients and in the control population

		CAPD patients			
	Controls ($N = 15$)	with diabetes ($N = 13$)	without diabetes ($N = 14$)	with CVD ($N = 11$)	without CVD ($N = 16$)
Lp(a) *mg/dl*	16.1 ± 13.6	27.9 ± 21.3	33.5 ± 25.0[a]	36.7 ± 24.5[b]	26.8 ± 21.9
Lp(a)-LGE_2	4.8 ± 3.9	9.8 ± 8.4[a]	12.7 ± 9.0[b]	10.2 ± 7.1[a]	12.6 ± 9.8[b]

Data are presented as mean ± SD. Comparison of Lp(a) levels was performed on log-transformed data.
[a] $P < 0.05$ vs. control group
[b] $P < 0.01$ vs. control group

failure as the most likely factor responsible for this oxidation.

It was shown previously that some apo (a) in Lp(a) in human plasma can be detected in a fragmented state, and therefore can be found in non-lipoprotein fractions [41]. This was particularly true for ESRD patients on dialysis [42]. If Lp(a) oxidation induces free-radical scission of apo (a), fragments should also be oxidatively modified and, therefore, be detected by our ELISA. Since our method of measuring of LGE_2-Lp(a) does not discriminate between free apo (a) and apo (a) within an Lp(a) particle, we attempted to separate Lp(a) and fragments of apo (a) by density gradient ultracentrifugation with subsequent detection of LGE_2-apo (a) immunoreactivity in the different density fractions. If oxidatively modified apo (a) fragments were present in plasma samples of CAPD patients tested, they should be detected in a non-lipoprotein fraction (d > 1.21 g/ml) by our method. Fresh plasma samples from four CAPD patients were therefore individually subjected to density gradient ultracentrifugation and the density distribution of Lp(a) and LGE_2-Lp(a) determined. The hydrated density of Lp(a) peaks varied from 1.08 to 1.1 g/ml in different samples. Lp(a) immunoreactivity was not found at a non-lipoprotein density in three of the four samples (Fig. 4A). However, in one sample a small additional peak was found at a density higher than that of intact Lp(a) (Fig. 4B). Since our revealing antibody is specific for apo (a), this peak probably represents apo (a) not associated with an intact Lp(a) particle. In this sample, as in others, there was little if any change in the hydrated density distribution of Lp(a) particles with LGE_2-protein pyrroles when compared to that of total Lp(a). Furthermore, no apo (a) with LGE_2-protein epitopes was found in a density > 1.21 g/ml where fragments of apo (a) would appear. These results suggest that the immunoreactive LGE_2-Lp(a) being measured in our ELISA do not represent fragments of Lp(a), but rather intact Lp(a) particles.

DISCUSSION

The major result of this study is that Lp(a), but not LDL, is oxidized in plasma of CAPD patients. That such oxidation has occurred is not unexpected, since oxidative stress has been shown to be a characteristic of ESRD [1–3]. It should be noted that any oxidation of lipoproteins in the circulation would be at a level below that required for it to be a ligand for scavenger receptors on tissue macrophages.

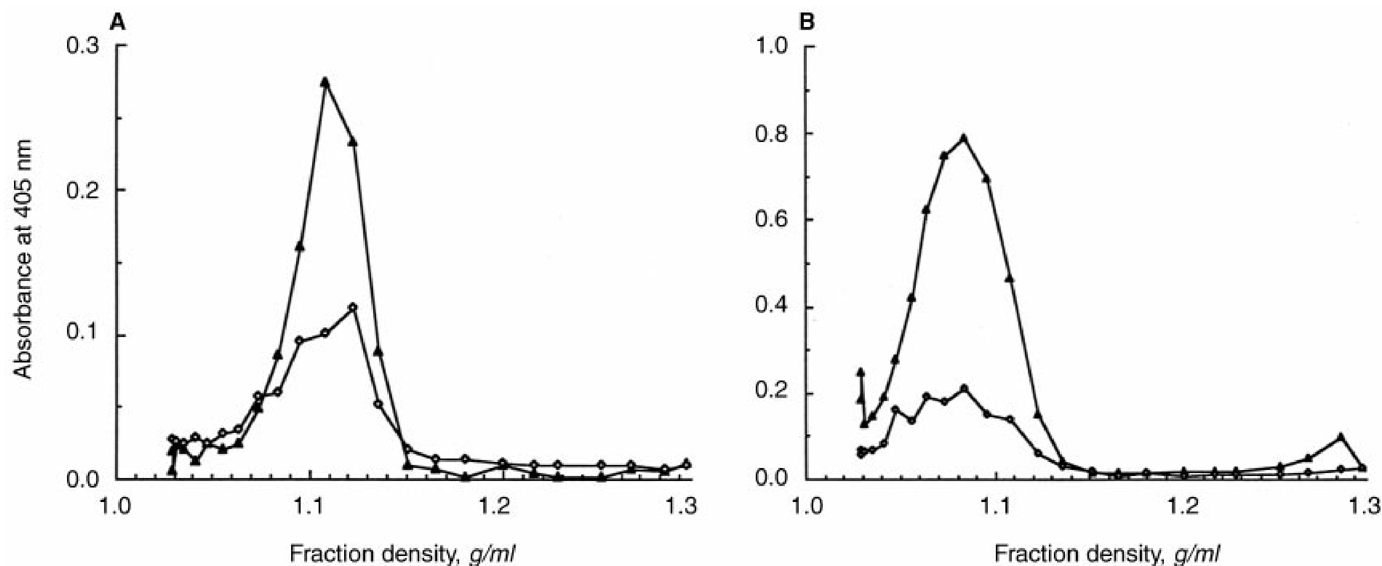

Fig. 4. Density gradient profiles of Lp(a) and LGE_2-Lp(a) in plasma samples from patients with ESRD undergoing CAPD. Two milliliters of fresh plasmas from four individual patients was subjected to density gradient ultracentrifugation as described in the **Methods** section. The fractions were collected from the bottom of the tube, and their densities were measured by refractometry. Apo (a) immunoreactivity was measured by first coating the plates with anti-apo (a) and then revealing the bound apo (a) with the conjugated anti-apo (a). LGE_2-apo (a) immunoreactivity was measured by first coating wells with anti-LGE_2-KLH and then revealing with anti-apo (a). It is presented as absorbance at 405 nm. Two of the four samples studied are presented. Each point represents the mean of triplicate determinations. Symbols are: (▲) apo (a); (◇) LGE2-apo (a).

Indeed, if oxidation of Lp(a) were to increase to such threshold levels, such particles would be rapidly removed from the circulation by Kupffer cells after passage through the liver [43]. We had shown previously that when LDL was modified directly with LGE_2, recognition of such modified LDL by macrophages in culture occurred only when about 30 or more molecules of LGE_2 formed pyrroles with lysine residues on one molecule of apo B in LDL [30]. Thus, any measurement of oxidation of lipoproteins in this study represents only an initial stage of oxidation.

Our results showing no oxidation of LDL in CAPD patients are in agreement with those of Westhuyzen, Saltissi and Healy [17], who found no evidence for the oxidation of plasma LDL in ESRD patients. However, in those studies hemodialysis rather than CAPD patients were investigated. Our results on the oxidation of Lp(a) appear to be consisitent with those of Kotani et al [12]. Using an antibody to MDA-protein adducts, this group demonstrated that the greatest modification of apo B-containing lipoproteins in plasma was found at a hydrated density in which Lp(a) is normally found. However, these authors interpreted their results to mean that the modified apo B in their study represented small dense LDL [12] and not Lp(a).

It is unclear as to why Lp(a), but not LDL, was more oxidized in the plasma of CAPD patients in this study. One explanation is that Lp(a) itself is more prone to oxidation than LDL, although the data in the literature on such a comparison give conflicting conclusions [19, 21, 22, 44]. Another possibility is that Lp(a) is more oxidized in ESRD patients due to a lower plasma clearance of Lp(a) than of LDL [45]. By increasing the residence time of Lp(a) in the circulation, these particles would be expected to be more prone to oxidation. Since Lp(a) is capable of binding to endothelial cells and platelets to a greater degree than LDL [46], it is also possible that such binding can facilitate Lp(a) oxidation *in vivo*, thereby resulting in preferential oxidation of Lp(a). The increase in oxidation of Lp(a) found in our ESRD patients could have been due to the dialysis procedure, or to the presence of such risk factors as CVD or diabetes. However, since we did not find an association between the oxidation of Lp(a) and any of these factors, it is unlikely that they induced this oxidation. It seems more likely that the increased oxidation of Lp(a) is the result of renal failure, consistent with the oxidative stress that characterizes this disease process [1–3].

Oxidized Lp(a) possesses several atherogenic properties that it shares with oxidized LDL, which could be partially responsible for the association between elevated Lp(a) levels and CVD in several patient populations including patients with ESRD undergoing hemodialysis [47] or CAPD [48]. One of them is an induction of the cell adhesion molecule Mac-1 on the monocyte surface [44], responsible for enhanced binding of monocytes to endothelial cells. This is followed by entry of these monocytes into the arterial intima and their development into tissue macrophages [49]. Finally, recognition of more extensively-oxidized Lp(a) by the scavenger receptor on tissue macrophages will occur, leading to lipid loading of these cells [21]. Both ox-LDL and ox-Lp(a) were shown to stimulate the

release of renin from juxtaglomelar cells [50]. However, ox-Lp(a) also possesses properties not shared by ox-LDL. It exhibits increased binding to the plasminogen receptor, thereby potentially competing with plasminogen for this site, and ultimately inhibiting fibrinolysis [51]. Also, ox-Lp(a) is capable of increasing the production of plasminogen activator inhibitor-1 by the endothelial cells [46]. Finally, ox-Lp(a) was shown to be more potent than ox-LDL in inhibiting endothelium-dependent vasodilation [52].

In conclusion, this study describes a sandwich ELISA for measuring oxidation of LDL and Lp(a) in plasma. We have used it to provide evidence that Lp(a), but not LDL, is more oxidized in patients with ESRD undergoing CAPD when compared to corresponding lipoproteins from healthy subjects. Additional studies are still needed to evaluate the significance of a relationship between ESRD and lipoprotein oxidation, as well as a possible role of additional clinical factors in this relationship.

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APPENDIX

Abbreviations used in this article are: apo B, apolipoprotein B; apo (a), apolipoprotein (a); BHT, butylated hydroxytoluene; CAPD, continuous ambulatory peritoneal dialysis; ELISA, enzyme-linked sandwich immunosorbent assay; ESRD, end-stage renal disease; KLH, keyhole limpet hemocyanin; LDL, low density lipoprotein; LGE_2, [R]-acetyl-9[R]-formyl-12[S]-hydroxy-5[Z],10[E]-heptadeca-dienoic acid; Lp(a), lipoprotein(a); PBS, phosphate buffered saline; TBARS, thiobarbituric acid reactive substance.

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