HDL antioxidant effects as assessed using a nonexchangeable probe to monitor particle-specific peroxidative stress in LDL-HDL mixtures[1]

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Abstract High density lipoproteins (HDL) have been reported to inhibit oxidation of low density lipoproteins (LDL) based in part on observations that oxidative changes occur more slowly in LDL-HDL mixtures than in LDL alone. In the current studies, we developed an approach to discern particle-specific oxidation kinetics within mixed particle systems using the oxidation-labile fluorescent probe parinaric acid cholesteryl ester (PnCE) and applied this to the study of HDL inhibition effects. PnCE was introduced into acceptor lipoproteins by cholesteryl ester transfer protein (CETP)-mediated transfer from donor microemulsions. Incubation of PnCE-containing LDL and HDL with non-probe-containing HDL and LDL, respectively, followed by measurement of reisolated fractions, indicated that PnCE does not transfer appreciably between lipoprotein fractions. Oxidative loss of lipoprotein-associated PnCE occurred essentially in tandem with changes in conjugated dienes, suggesting that PnCE loss reflects the course of peroxidation of endogenous lipoprotein lipids. Using PnCE to separately monitor LDL- and HDL-specific oxidation within LDL-HDL mixtures, we obtained direct evidence that HDL inhibits both Cu^{2+}- and Fe^{3+}-induced peroxidation of LDL-associated lipids. Notably, in the presence of Cu^{2+}, loss of HDL-associated PnCE fluorescence also was inhibited in LDL-HDL co-incubations, suggesting that LDL exert an antioxidant effect under these conditions as well. Thus, results obtained using this new methodology are consistent with previously reported antioxidant effects of HDL, but indicate that the behavior of individual lipoprotein particles may be more complicated than can be predicted from the collective behavior of the lipoprotein mixture.—**Tribble, D. L., B. M. Chu, E. L. Gong, F. van Venrooij, and A. V. Nichols.** HDL antioxidant effects as assessed using a nonexchangeable probe to monitor particle-specific peroxidative stress in LDL-HDL mixtures. *J. Lipid Res.* 1995. **36:** 2580-2589.

Supplementary key words parinaric acid • cholesteryl ester • lipid peroxidation • conjugated diene formation • Cu^{2+}-induced peroxidation • Fe^{3+}-induced peroxidation

In view of evidence that oxidation increases the atherogenicity of low density lipoproteins (LDL), considerable effort has been directed toward identifying factors that determine LDL oxidative behavior (1, 2). Results have shown that the oxidative susceptibility of LDL in vitro, and presumably in vivo, is influenced by extrinsic or environmental factors, which determine the oxidative pressure to which particles are exposed, and intrinsic lipoprotein properties, which determine their tendency to resist or succumb to that pressure (2). Among the extrinsic factors of possible physiological relevance are high density lipoproteins (HDL), which have been reported to exhibit antioxidant effects based on observations that oxidative changes occur more slowly in LDL-HDL mixtures than in LDL alone (3–7). The degree to which HDL actually inhibit oxidative modifications within LDL has been difficult to discern, however, because oxidative changes occurring within LDL and HDL particles cannot be distinguished without their subsequent reisolation, which is complicated by alterations in lipoprotein chemical and physical properties upon oxidation.

We recently described use of the oxidation-labile fluorescent probes parinaric acid (PnA) and parinaric acid methyl ester (PnME) to monitor peroxidative stress after their incorporation into LDL particles (8, 9). These probes were found to be useful for characterizing the structural dynamics of LDL oxidation due to their selective localization within the surface (PnA) and outer core (PnME) particle domains (10, 11). Despite the greater depth of penetration of PnME, like PnA, this probe readily exchanges between lipoproteins and rapidly

Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein; PnCE, parinaric acid cholesteryl ester; CETP, cholesteryl ester transfer protein; FPLC, fast protein liquid chromatography.

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equilibrates among particles within a mixture. More recently, we have used the cholesteryl ester of parinaric acid (PnCE) as a probe of core-specific oxidation (D. L. Tribble, B. M. Chu, E. L. Gong, and A. V. Nichols, unpublished observations). In the course of these studies, we observed that when PnCE is incorporated into lipoproteins from donor microemulsions using cholesteryl ester transfer protein (CETP), it does not readily exchange between particles (as described herein). We exploited the nonexchangeability of PnCE to develop a new method for monitoring particle-specific oxidation kinetics in mixed lipoprotein systems.

In the current report, we describe applications of PnCE to the study of HDL inhibition of LDL oxidation, with particular emphasis on deciphering the extent of peroxidative stress experienced within either LDL or HDL when these fractions are co-incubated in oxidizing environments. Our results indicate that measures of bulk lipid peroxidation (e.g., conjugated dienes), and possibly other measures that do not distinguish particle-specific oxidative changes, are not adequate for describing LDL-HDL interactive effects.

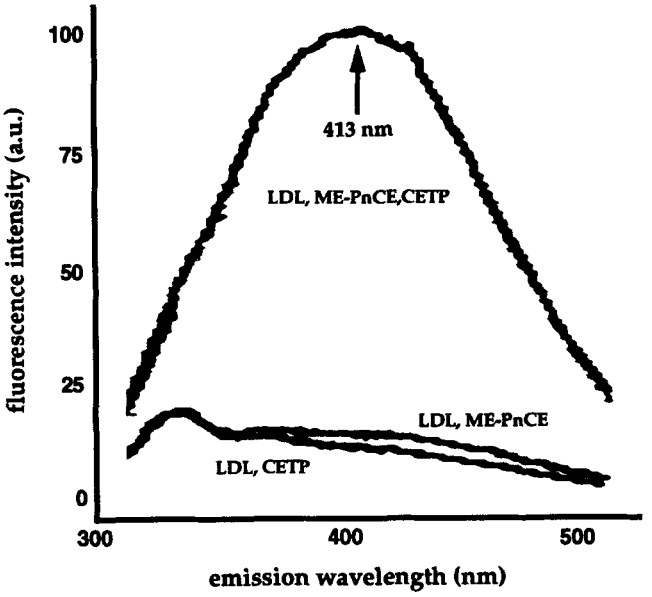

Fig. 1. Fluorescence characteristics of LDL and LDL-associated PnCE. Fluorescence emission spectra (from 300 to 550 nm) are shown for LDL incubated with PnCE-containing microemulsions (ME-PnCE) in the presence and absence of CETP, and with CETP in the absence of ME-PnCE for 24 h. The excitation wavelength was set at 324 nm.

METHODS

Chemicals

The cholesteryl ester of PnA (9,11,13,15-*cis-trans-trans-cis*-o ctadecatetraenoic acid) was synthesized upon special request by Molecular Probes (Eugene, OR). Recombinant cholesteryl ester transfer protein (CETP) was generously supplied as a gift to A. V. Nichols by Genentech, Inc. (South San Francisco, CA). TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Aldrich Chemical Co. (Milwaukee, WI). EDTA, $CuCl_2$, and $FeCl_3$ were from Sigma Chemical Co. All reagents, buffer components, and HPLC solvents were of the highest grade commercially available.

Isolation and characterization of plasma lipoproteins

Blood was obtained from healthy normolipidemic adult volunteers not using vitamin supplements or taking hormones or drugs known to alter plasma lipids or lipoproteins. Samples were collected into vacutainers containing 1 mg/ml EDTA. Cells were removed by centrifugation at 2000 *g* for 30 min at 4°C, and 10 μM TROLOX was immediately added to plasma. LDL (d 1.019–1.063 g/ml) and HDL (d 1.063–1.21 g/ml) were isolated from plasma by sequential ultracentrifugation using standard methods (12). In some cases, apoA-I-containing particles also were isolated by immunoaffinity chromatography as previously described (13), and results were compared with those obtained using centrifugally isolated HDL. EDTA was included during all stages of lipoprotein isolation to prevent artifactual oxidation.

Lipoprotein preparations were routinely characterized according to composition of protein and the major lipid constituents, and the effects of PnCE incorporation on these constituents were examined for a subset of samples. Protein concentrations were determined using the Lowry method modified to include sodium dodecyl sulfate (14). Phospholipid phosphorus was analyzed according to the method of Bartlett (15) and was expressed as phosphatidylcholine equivalents. Triglycerides and cholesterol (total, free and esterified) were measured using standard enzymatic methods on a System 3500 Gilford Computer Directed Analyzer.

Fatty acid composition was determined for a subset of samples before and after probe incorporation. Fatty acids were extracted into hexane and were derivatized to the corresponding trimethylsilyl esters according to published procedures (16). Analyses were performed using a Hewlett-Packard 5890 Gas Chromatograph coupled to a Hewlett-Packard Mass Selective Detector using previously described conditions (16).

Incorporation of PnCE into LDL and HDL

Lipoproteins were concentrated to 3.5–5.0 mg protein per ml using Centriprep-10 concentrators (Amicon Corp., Beverly, MD). Microemulsions (egg yolk phosphatidylcholine/triolein/cholesteryl oleate) containing

TABLE 1. Lipid and protein mass composition of LDL and HDL before and after PnCE incorporation

	TG	FC	CE	PL	Pro
			% mass		
LDL	4.5 ± 1.7	8.4 ± 1.6	42.6 ± 3.3	22.8 ± 1.7	21.8 ± 2.2
LDL*	4.3 ± 2.0	9.3 ± 1.4	42.2 ± 1.0	22.4 ± 1.9	21.7 ± 2.7
P value	0.71	0.10	0.81	0.58	0.98
HDL	3.1 ± 0.9	5.1 ± 3.7	13.8 ± 3.8	29.1 ± 4.6	48.9 ± 7.1
HDL*	3.8 ± 1.4	6.0 ± 4.0	16.4 ± 4.4	25.8 ± 1.6	48.1 ± 3.7
P value	0.32	0.72	0.30	0.12	0.81

Mean values (± SD) are shown for 15 LDL and 6 HDL preparations and are expressed as percent total lipoprotein mass, with the sum of these constituents taken as 100%. *P* values for differences before and after PnCE incorporation were determined using two-group *t*-tests. Abbreviations: TG, triglycerides; FC, free cholesterol; CE, cholesteryl esters; PL, phospholipids; Pro, protein; LDL*, LDL containing PnCE; HDL*, HDL containing PnCE.

~ 15 mol % PnCE were prepared according to the method of Bisgaier et al. (17), and were concentrated 5-fold. PnCE was incorporated into acceptor lipoproteins by incubating the concentrated lipoprotein preparations (1.0 ml) with the concentrated donor microemulsions (1 ml) in the presence of CETP (25 μg/mg protein). Incubations were carried out at 37°C in 50 mM Tris buffer containing 2 mM EDTA for 4, 8, or 24 h. The latter condition was found to be optimal for promoting uptake of adequate probe to perform oxidation experiments (as indicated by fluorescence intensity) while avoiding over-incorporation leading to fluorescence quenching and elimination of the linear (stoichiometric) fluorescence loss upon oxidation. Probe-containing lipoproteins were separated from CETP and donor microemulsions by fast protein liquid chromatography (FPLC) using two Superose 6 columns in series.

Measurement of lipoprotein oxidation

Lipoproteins were dialyzed against 0.9% NaCl, pH 7.4, for 48 h to remove NaBr, EDTA, and TROLOX prior to oxidation. The dialysis solution was initially de-oxygenated by bubbling with nitrogen. LDL and HDL were incubated, separately, in 0.9% NaCl at 0.1 and 0.2 mg protein/ml, respectively. Co-incubations of LDL and HDL were performed with a total protein concentration of 0.3 mg per ml (0.1 plus 0.2 mg protein/ml, respectively). Oxidation experiments were carried out at 37°C using either $CuCl_2$ or $FeCl_3$ (5 μM) as initiating agents. Oxidation of lipoprotein-associated PnCE was monitored by following fluorescence loss using a Shimadzu RF-5000 Spectrofluorophotometer equipped with a thermostatically controlled cuvette and a magnetic stirring device. Excitation and emission wavelengths were set at 324 nm (slit width 1.5 nm) and 413 nm (slit width 20 nm), respectively. In separate incubations, conjugated diene formation was determined as an index of LDL lipid peroxidation by monitoring the increase in absorbance at 234 nm (18). Measurements were performed in a Shimadzu UV2101-PC scanning spectrophotometer equipped with a thermostatically controlled 6-position automatic sample changer. Initial absorbance was set at zero and was recorded every 2 min for up to 16 h.

Statistical analyses

Statistical analyses were performed using the Statview II statistical program. The significance of differences in lipoprotein composition, conjugated diene lag times, and PnCE oxidation parameters were determined using two-group *t*-tests. All significance levels are based on two-tailed tests.

RESULTS

Characteristics of PnCE-containing lipoproteins

LDL incubated with PnCE-containing microemulsions in the presence of CETP showed a marked increase in fluorescence that was characteristic of lipid-associated parinaroyl derivatives (max. em.: 413 nm) (see **Fig. 1**). Fluorescence intensity at 413 nm was ~10-fold higher than in LDL incubated with microemulsions in the absence of CETP or LDL incubated with CETP but without microemulsions. The extent of PnCE incorporation (as indicated by relative fluorescence intensity at 413 nm) was similar for all LDL preparations tested. Probe incorporation into HDL particles also was dependent on the presence of CETP. The extent of incorporation was ~30% lower than in LDL under similar conditions (data not shown).

In an effort to optimize conditions for probe incorporation, we compared probe intensity in three LDL samples after incubation with microemulsions and CETP

for 4, 8, and 24 h. Fluorescence intensities at 4 and 8 h were 65 ± 5% and 76 ± 10%, respectively, of that observed after 24 h incubation. Based on fluorescence intensity in relation to that obtained with PnA, which can be titrated (8, 19), we estimate that these procedures led to the incorporation of approximately 2–4 molecules of PnCE per LDL particle. In subsequent oxidation experiments, we used an 18–24 h incubation period to achieve optimal probe incorporation.

Compositional analyses of LDL and HDL before and after PnCE incorporation are shown in **Table 1.** The PnCE incorporation procedures did not appear to appreciably affect lipid and protein mass distributions in either LDL or HDL. LDL fatty acid composition also remained unchanged upon probe incorporation (**Table 2**), whereas a slight (13%) albeit insignificant increase was observed for 18:1 in HDL, likely due to the exchange of endogenous HDL lipids for microemulsion cholesteryl oleate.

As indicated in **Fig. 2** for a representative LDL sample exposed to 5 μM Cu^{2+}, oxidative loss of PnCE-associated fluorescence (ex.: 324 nm; em.: 413 nm) occurred essentially in tandem with changes in conjugated diene formation. These findings (observed in all LDL and HDL preparations tested) suggest that the kinetics of PnCE oxidation reflect the kinetics of peroxidation of endogenous lipids. As with conjugated dienes, PnCE loss appeared to be bi-phasic in some samples, although notably, the lag phase rate was greater for PnCE than for conjugated dienes. Incorporation of PnCE had variable effects on the oxidative behavior of acceptor lipoproteins as indicated by changes in conjugated dienes. In general, there were no differences in the course of conjugated diene formation before and after PnCE incorporation, although in few samples, a slight (~ 15%) decrease in conjugated diene lag times was observed.

The exchangeability of PnCE between LDL and HDL was investigated by co-incubating PnCE-containing LDL (LDL*) or HDL (HDL*) with non-PnCE-containing HDL or LDL, respectively, for 17 h followed by reisolation of the individual lipoprotein fractions by FPLC. Even at lipoprotein concentrations 10- to 20-fold greater than those used in oxidation experiments, the extent of exchange of the probe was small (< 15%), as indicated by comparing lipoprotein fluorescence at 413 nm (ex: 324 nm) before and after co-incubation. Redistribution of PnCE-associated fluorescence from HDL to LDL appeared to be slightly greater than that from LDL to HDL, but was considered to be minimal and unlikely to tangibly affect the utility of these probes for determination of particle-specific oxidation.

Application of PnCE methodology to the study of HDL inhibition of Cu^{2+}-induced LDL oxidation

Figure 3 shows the course of conjugated diene formation for LDL, HDL, and LDL plus HDL following exposure to 5 μM Cu^{2+}. In this example, LDL appeared to be the most susceptible and HDL the least susceptible to Cu^{2+}-induced oxidation, while LDL plus HDL exhibited an intermediate response. When lipoproteins from three different subjects were compared, incubations of HDL and of LDL plus HDL were observed to exhibit significantly slower oxidation responses than LDL alone ($P < 0.05$). Mean lag time values (±SD) were 71± 29, 102± 31, and 138± 51 min, respectively. These results are consistent with previous observations of a delay in the course of oxidative changes in LDL-HDL mixtures relative to LDL alone (3–7), which have been interpreted to indicate an antioxidant effect of HDL.

Figure 4 shows changes in fluorescence intensity (ex.: 324 nm; em.: 413 nm) in LDL and HDL preparations both in the absence and presence of 5 μM Cu^{2+}. Data are included for PnCE-containing (*) and non-PnCE-containing samples to illustrate changes in both probe-associated fluorescence and in the fluorescence of intrinsic lipoprotein components upon oxidation. No loss of fluorescence was observed in either LDL or HDL in the

TABLE 2. Fatty acyl composition of LDL and HDL before and after (*) PnCE incorporation

	16:0	18:0	18:1	18:2	20:4	22:6
			%			
LDL	21.4 ± 1.5	6.8 ± 0.8	20.8 ± 2.0	35.1 ± 3.3	13.3 ± 1.0	2.7 ± 0.4
LDL*	21.2 ± 1.5	8.3 ± 3.2	20.0 ± 2.4	34.8 ± 3.3	13.1 ± 0.3	2.5 ± 0.5
P value	0.88	0.46	0.69	0.92	0.80	0.74
HDL	22.3 ± 2.9	10.5 ± 1.5	17.8 ± 1.0	31.9 ± 2.2	14.0 ± 3.1	3.4 ± 0.8
HDL*	22.9 ± 0.8	10.0 ± 3.1	20.2 ± 1.5	32.2 ± 3.0	12.0 ± 4.0	2.9 ± 1.3
P value	0.78	0.80	0.09	0.89	0.52	0.60

Mean values (± SD) are shown for 3 LDL and 3 HDL preparations and are expressed as percent, with the sums of these constituents taken as 100%. *P* values for differences before and after PnCE incorporation were determined using two-group *t*-tests.

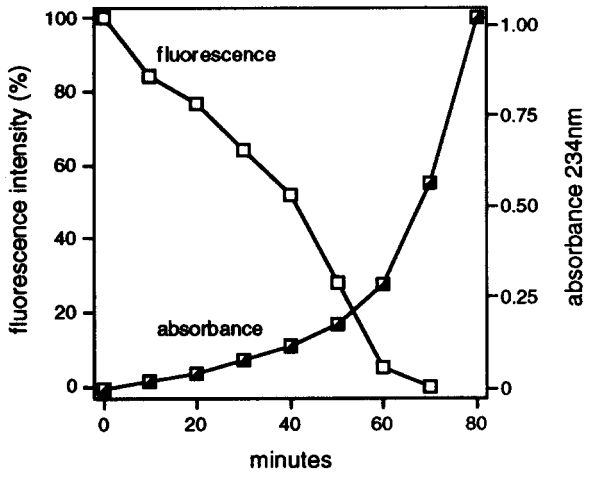

Fig. 2. Course of PnCE fluorescence loss and conjugated diene formation in PnCE-containing LDL exposed to Cu^{2+}. PnCE was incorporated into LDL by incubating with donor microemulsions in the presence of CETP for 24 h. Oxidation was initiated by addition of 5 μM $CuCl_2$ and fluorescence loss (ex: 324 nm; em: 413 nm) and conjugated diene formation (increase in absorbance at 234 nm) were continuously monitored, in separate incubations, for up to 3 h.

absence of Cu^{2+}. Addition of Cu^{2+} produced a rapid loss of fluorescence in both LDL* and HDL* that leveled off at values similar to those in the corresponding unlabeled LDL and HDL fractions. Afterwards, a slow increase in fluorescence intensity was observed in both probe-containing and non-probe-containing samples. This likely reflected the formation of fluorescent protein adducts (max. em.: 430 nm) which we (20) and others (21) have observed to occur subsequent to bulk lipid peroxidation in Cu^{2+}-exposed LDL.

As shown in **Fig. 5** (left panel), when LDL* were combined with HDL, fluorescence loss was slower than in LDL* alone. As probe oxidative behavior did not exhibit a clear bi-phasic response in all samples, relative oxidative probe depletion was evaluated on the basis of time (min) to 50% fluorescence loss (t_{50}). Based on this index, addition of HDL slowed the rate of loss of LDL* fluorescence 1.5-fold (t_{50}: 22.4 ± 13.2 for LDL* and 34.1 ± 9.8 for LDL* plus HDL, $P < 0.05$, n = 6). As PnCE is not exchangeable, these results indicate that HDL inhibit peroxidative stress within the LDL particle.

When HDL* were combined with LDL, fluorescence loss was reduced relative to HDL* alone (Fig. 5, right panel). Addition of LDL, in turn, slowed the rate of loss of HDL* fluorescence 1.8-fold (t_{50}: 29.2 ± 8.0 for HDL* and 53.2 ± 20.2 for HDL* plus LDL, n = 3). Thus, it would appear that LDL and HDL act reciprocally to inhibit peroxidative changes within the other under these conditions.

Inhibition of LDL-associated PnCE loss by HDL isolated by density ultracentrifugation versus apoA-I immunoaffinity chromatography

ApoA-I-containing particles (HDL_{AI}) isolated by immunoaffinity chromatography have been shown to be more effective in inhibiting LDL oxidation than HDL fractions isolated by ultracentrifugation (HDL_d) (5). We compared these two particle subpopulations with respect to their ability to inhibit oxidative loss of LDL-associated PnCE. The degree of inhibition conferred by HDL_{AI} was ~ 15% greater than that conferred by HDL_d, but this difference did not reach significance for four subjects. Mean t_{50} values (± SD) for LDL-associated PnCE fluorescence were 22.5 ± 15 min in LDL alone, 39.6 ± 19.4 min in LDL plus HDL_{AI}, and 33.8 ± 12.1 min in LDL plus HDL_d.

Application of PnCE methodology to the study of HDL inhibition of LDL oxidation by Fe^{3+}

Figure 6 shows the course of Fe^{3+}-induced conjugated diene formation in LDL, HDL, and co-incubations of LDL and HDL. The kinetics of conjugated diene formation differed from those of Cu^{2+}-exposed lipoproteins, with the response being hyperbolic rather than sigmoidal in character. This hyperbolic response was observed in all preparations tested, but the magnitude of the initial increase differed. In some preparations, a subsequent propagation phase also was observed, but was markedly delayed relative to Cu^{2+}-exposed samples. Based on the magnitude of the initial response, LDL plus HDL appeared to be the most susceptible and HDL

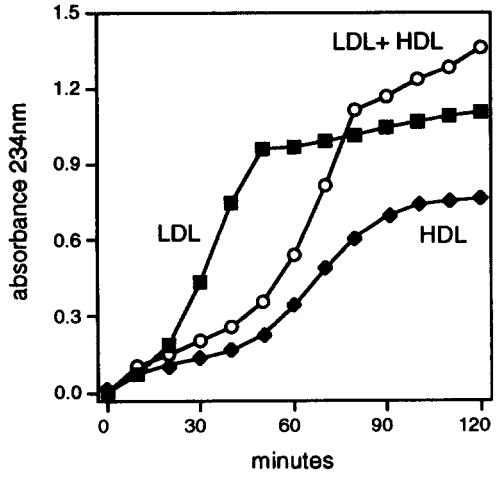

Fig. 3. Cu^{2+}-induced formation of conjugated dienes in LDL and HDL alone and when co-incubated. Conjugated diene formation, as indicated by the increase in absorbance at 234 nm, was monitored in incubations of LDL and HDL exposed to 5 μM Cu^{2+}. Results are shown for LDL (0.1 mg protein/ml), HDL (0.2 mg protein/ml), and a mixture of LDL plus HDL (0.1 and 0.2 mg protein/ml, respectively).

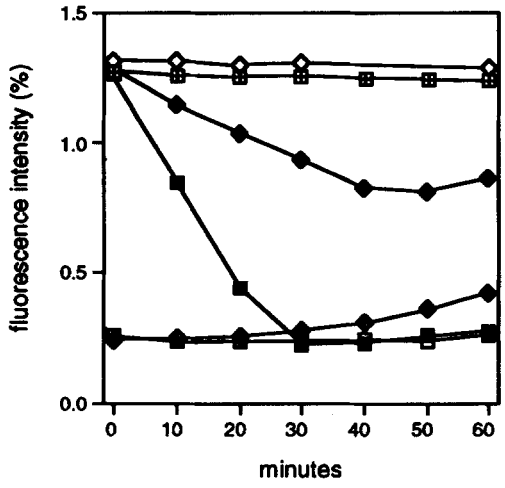

Fig. 4. Cu^{2+}-induced changes in the intensity of lipoprotein-associated fluorescence at 413 nm. Fluorescence (ex.: 324 nm; em: 413 nm) was monitored in PnCE-containing (*) and non-PnCE-containing LDL and HDL, either in the absence or presence of 5 μM Cu^{2+}. Results are shown for a representative set of LDL and HDL fractions; LDL* (⊞); HDL* (◇); LDL*, Cu^{2+} (■); HDL*, Cu^{2+} (◆); LDL, Cu^{2+} (□); HDL, Cu^{2+} (◈).

appeared to be the least susceptible. When lipoproteins from six subjects were compared, incubations of LDL plus HDL were observed to exhibit a significantly greater response (at $P < 0.05$) than either LDL or HDL alone. Due to the hyperbolic nature of the response, relative responses were evaluated on the basis of absolute absorbance (234 nm) at 300 min. Mean values (± SD) were 0.46 ± 0.16, 0.38 ± 0.18, and 0.74 ± 0.26 min for LDL, HDL, and LDL plus HDL, respectively. Thus, in marked contrast to results obtained with Cu^{2+}, Fe^{3+}-induced formation of conjugated dienes appeared to be enhanced in co-incubations of LDL plus HDL relative to either of these preparations alone.

Changes in fluorescence intensity (ex.: 324 nm; em.: 413 nm) were monitored in PnCE-containing and non-PnCE-containing samples in the absence or presence of 5 μM Fe^{3+} (**Fig. 7**). Fe^{3+} produced a slow loss of fluorescence in LDL* that corresponded with the course of conjugated diene formation (as shown in Fig. 6). Little to no loss of fluorescence was observed for HDL* in the absence or presence of Fe^{3+}. A minor increase in fluorescence was observed during the same period for non-PnCE-containing LDL and HDL preparations.

As shown in **Fig. 8** (left panel), in apparent contrast to the increased accumulation of conjugated dienes in co-incubations of LDL and HDL, addition of HDL markedly inhibited Fe^{3+}-induced fluorescence loss in LDL*. The significance of differences in PnCE loss between LDL* and LDL* plus HDL was evaluated by comparing percent fluorescence remaining at 300 min for samples from three subjects. In LDL* alone, 34 ± 30% of the fluorescence was still present at 300 min, whereas in LDL* plus HDL, 63 ± 7% remained at 300 min ($P < 0.05$). No alterations in the response of HDL* to Fe^{3+} were observed when co-incubated with LDL (Fig. 8, right panel). Mean percent fluorescence remaining at 300 min (± SD) for three subjects was 68 ± 29% for HDL* alone and 69 ± 25% for HDL* plus LDL.

DISCUSSION

We developed an approach for monitoring particle-specific oxidation kinetics within mixed lipoprotein sys-

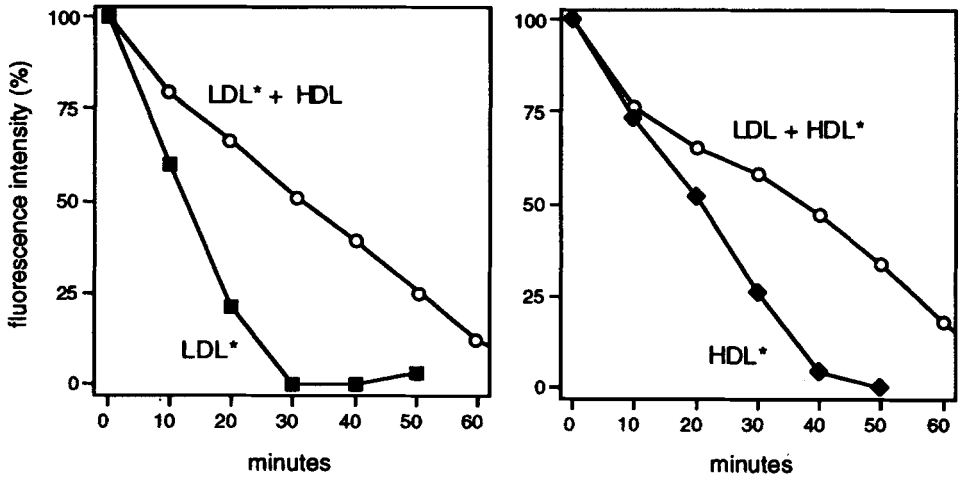

Fig. 5. Cu^{2+}-induced PnCE-associated fluorescence loss in co-incubations of LDL and HDL. PnCE was incorporated into LDL (LDL*) and HDL (HDL*) by CETP-mediated exchange from donor microemulsions. Oxidation was initiated by 5 μM Cu^{2+}. Loss of PnCE-associated fluorescence was monitored at 413 nm (ex: 324 nm). Left panel: LDL* in the absence or presence of non-probe-containing HDL. Right panel: HDL* in the absence or presence of LDL.

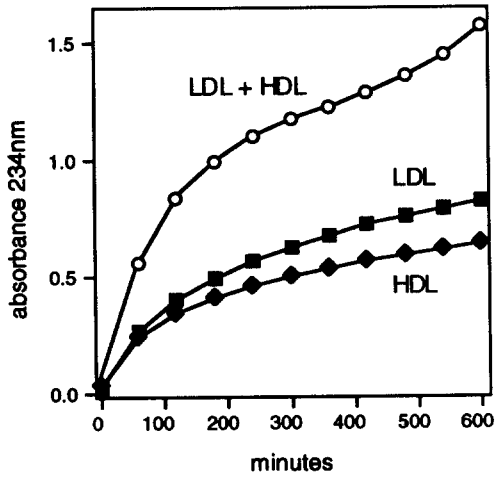

Fig. 6. Fe^{3+}-induced formation of conjugated dienes in LDL and HDL alone and when co-incubated. Conjugated diene formation, as indicated by the increase in absorbance at 234 nm, was monitored in incubations of LDL and HDL exposed to 5 μM Fe^{3+}. Results are shown for LDL (0.1 mg protein/ml), HDL (0.2 mg protein/ml), and a mixture of LDL plus HDL (0.1 and 0.2 mg protein/ml, respectively).

tems using the cholesteryl ester derivative of PnA, an oxidation-labile fluorescent reporter molecule that can be used to directly monitor peroxidative stress following incorporation into lipid environments (8, 9, 19,22–25). PnCE was incorporated into both LDL and HDL by CETP-mediated transfer from donor microemulsions. When incorporated in this manner, PnCE was found to be virtually non-exchangeable, a property crucial to its use as a probe of particle-specific behavior. The extent of exchange was not analyzed under oxidizing conditions, which conceivably could alter lipid exchange properties. It can be argued, however, that because PnA is more oxidatively labile than most endogenous fatty acyl components, PnCE-associated fluorescence is unlikely to still be present under conditions that alter the particle enough to facilitate lipid exchange.

Examination of Cu^{2+}-induced fluorescence loss of PnCE in relation to conjugated diene formation suggests that this probe reflects the peroxidation of endogenous lipoprotein lipids, particularly the accelerated phase of lipid peroxidation, although changes in PnCE are slightly faster than those of the bulk lipids. This is likely due to the parinaroyl conjugated polyene system, which renders PnCE more susceptible than most endogenous lipoprotein fatty acyl components. This probe is thus expected to be a particularly sensitive indicator of peroxidative fatty acyl degradation. Notably, despite its greater susceptibility, PnCE does not appear to increase lipoprotein oxidative susceptibility (when incorporated in amounts ranging from 2 to 4 molecules per lipoprotein particle).

In the current studies, we used PnCE to examine the interactive effects of LDL and HDL in oxidizing environments. Based on the course of conjugated diene formation, oxidative changes were slower in mixtures of LDL and HDL than in LDL alone. Comparisons of the oxidative fluorescence loss of PnCE-associated with either LDL or HDL in the presence or absence of non-probe-containing HDL or LDL, respectively, showed that addition of HDL reduces Cu^{2+}-induced peroxidative stress within the LDL particle, consistent with a direct antioxidant effect of HDL. In addition, and contrary to expectations, we observed simultaneous inhibition by LDL of peroxidative stress within the HDL particle. Based on these results, it would appear that LDL acts reciprocally as an antioxidant for HDL in the presence of Cu^{2+}. These findings indicate that the kinetic response of individual lipoproteins to oxidation by Cu^{2+} is not always accurately predicted by the course of conjugated diene formation, nor probably, by other non-particle-specific measures of oxidation, in lipoprotein mixtures.

In contrast to results obtained with Cu^{2+}, conjugated diene accumulation induced by Fe^{3+} was enhanced in co-incubations of LDL and HDL relative to either of these lipoprotein fractions alone. In fact, based on this parameter, the response in lipoprotein mixtures appeared to be additive. Surprisingly, however, results obtained by monitoring loss of lipoprotein-associated PnCE fluorescence indicated that Fe^{3+}-induced peroxidation within the LDL particle is inhibited by HDL, and that this occurs without any apparent increase in peroxidative stress to HDL. Moreover, HDL-associated PnCE showed marked resistance to oxidation by Fe^{3+} both in the absence and presence of LDL. The apparent dispar-

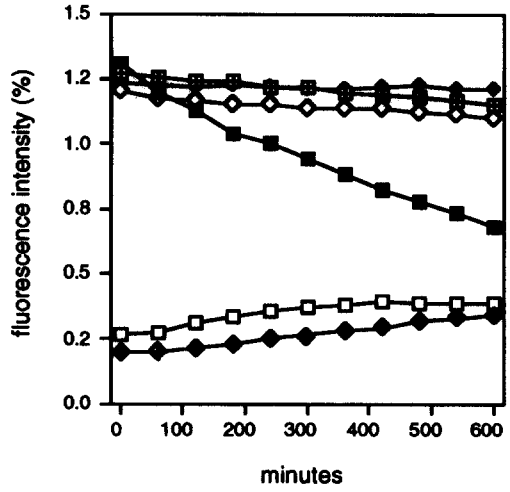

Fig. 7. Fe^{3+}-induced changes in intensity of lipoprotein-associated fluorescence at 413 nm. Fluorescence (ex.: 324 nm; em: 413 nm) was monitored in PnCE-containing (*) and non-PnCE-containing LDL and HDL, either in the absence or presence of 5 μM Fe^{3+}. Results are shown for a representative set of LDL and HDL fractions; LDL* (⊞); HDL* (◇); LDL*, Fe^{3+} (■); HDL*, Fe^{3+} (◆); LDL, Fe^{3+} (□); HDL, Fe^{3+} (⊕).

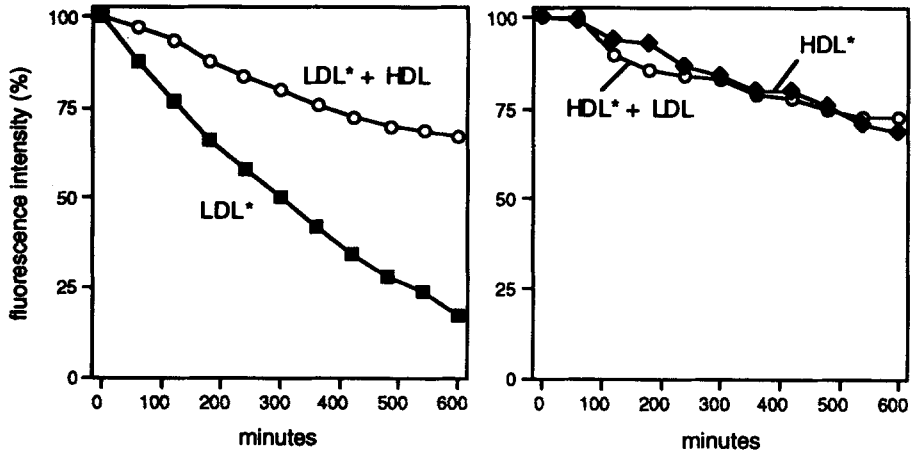

Fig. 8. Fe^{3+}-induced PnCE fluorescence loss in co-incubations of LDL and HDL. PnCE was incorporated into LDL (LDL*) and HDL (HDL*) by CETP-mediated exchange from donor microemulsions. Oxidation was initiated by 5 μM Fe^{3+}. Loss of PnCE-associated fluorescence was monitored at 413 nm (ex: 324 nm). Left panel: LDL* in the absence or presence of HDL. Right panel: HDL* in the absence or presence of LDL.

ity between results obtained with conjugated diene accumulation and PnCE fluorescence loss may be explained by a basic difference in these two assays. Namely, that the extent of conjugated diene accumulation is determined both by lipid peroxide formation and decomposition, whereas PnCE loss is unaffected by decomposition reactions. Thus, oxidizing conditions favoring formation, but not decomposition, would be expected to show greater accumulation of conjugated dienes (234 nm-absorbing species) relative to the extent of fluorescence loss than oxidizing conditions favoring decomposition reactions. Another relevant factor may be that, in Fe^{3+}-induced oxidation, conjugated diene formation reflects peroxidation of surface phospholipids whereas PnCE provides an index of peroxidative stress within the lipoprotein core. In separate studies, we have examined surface-to-core injury transfer efficiency in Cu^{2+}- and Fe^{3+}-exposed LDL using PnA and PnCE as probes of surface and core oxidation, respectively (D. L. Tribble, B. M. Chu, P. M. Thiel, and E. L. Gong, unpublished observations). Results show that surface-to-core injury transfer is impaired in Fe^{3+}-exposed LDL and thus that core lipids are relatively resistant to oxidation by this agent. Moreover, movement of injury from surface to core corresponds temporally with movement into the propagation phase of conjugated diene formation.

Possible mechanisms for the antioxidant effects of HDL in LDL-HDL mixtures have been suggested to include metal ion-sequestration by HDL-associated proteins (5), HDL-associated paraoxonase activity (6), HDL-associated platelet activating acetylhydrolase activity (26), HDL-associated hydroperoxide reducing activity (27), and redistribution of oxidized lipids from LDL to HDL (28, 29). It was not possible to determine which, if any, of these factors were operating in the current studies. Notably, in the Cu^{2+} oxidizing system, the degree of inhibition by HDL appeared to increase with time, at least in the initial phases, whereas in the Fe^{3+} oxidizing system, the degree of inhibition appeared to be similar throughout. The latter is more consistent with metal binding activity (i.e., a non-competitive type of inhibition) than the former.

Kunitake et al. (5) found that HDL protective effects in the presence of Cu^{2+} and Fe^{3+} were more pronounced in column-isolated HDL. They attributed this to the loss of ceruloplasmin and ferritin, and associated metal-binding activity, in centrifugally isolated HDL. In the current studies, we compared the effects of particles obtained by apoA-I immunoaffinity chromatography versus centrifugation on Cu^{2+}-induced oxidation of LDL-associated PnCE. In general, we did not find column isolation to be critical to the antioxidant potential of HDL, suggesting that particle-related factors altered by centrifugation but not immunoaffinity isolation are not essential. It is possible that a greater difference between these particle subpopulations may occur in the presence of Fe^{3+}. This was not examined in the current studies due to limitations in sample material.

The unexpected antioxidant effects exhibited by LDL in the presence of Cu^{2+} may reflect specific properties of these particles. Alternatively, such behavior may indicate that total lipoprotein mass is more important than lipoprotein identity in modulating lipoprotein oxidation in the presence of Cu^{2+}. Because of the greater lipoprotein mass in co-incubates, the ratio of oxidant to substrate was diluted relative to LDL and HDL alone, and this may have reduced oxidant effectiveness. Were this the basis of the LDL antioxidant effects, however, the reciprocity of the response should differ at lower

total protein concentrations or different ratios of LDL to HDL. In separate experiments, we did not observe qualitative differences in lipoprotein response when total lipoprotein mass was reduced (data not shown). Thus, it appears possible that a direct antioxidant effect of LDL on HDL oxidation may occur when these lipoproteins are co-incubated in the presence of Cu^{2+}.

In conclusion, we developed a method for monitoring particle-specific oxidation kinetics in mixed lipoprotein systems and applied this to the study of HDL inhibition of LDL oxidation. We observed that HDL directly inhibits peroxidative stress within the LDL particle in the presence of both Cu^{2+} and Fe^{3+}. In the latter case, this inhibition occurs without marked peroxidative stress to the HDL particle. A reciprocal antioxidant effect of LDL also was observed in the presence of Cu^{2+}. While mechanisms underlying these behaviors cannot be determined from the present studies, the results do indicate that the oxidative behavior of individual lipoproteins is not accurately predicted from the overall response of lipoprotein mixtures and thus that such measures should not be strictly relied upon for examining interactive effects of lipoproteins under oxidizing conditions.

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