Lipolysis-induced iron release from diferric transferrin: possible role of lipoprotein lipase in LDL oxidation

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Abstract **Conditions leading to oxidation of LDL in vivo are still unknown. While the occurrence of oxidized lipoproteins and catalytic free iron in advanced atherosclerotic lesions has been demonstrated, the origin of both is unclear. In vivo, iron metabolism is tightly regulated by iron-binding proteins that ensure that virtually no free iron exists. We examined whether physiological events such as lipolysis might reduce pH, facilitate iron release from transferrin (Tf), and promote low density lipoprotein (LDL) oxidation. Lipolysis is brought about by lipoprotein lipase (LpL), a triglyceride hydrolase present on endothelial cell surfaces and in atherosclerotic lesions. LpL hydrolysis of Intralipid lowered pH from 7.40 to 7.00 in 10% human serum and from 7.40 to 6.88 in phosphate-buffered saline. Similar decreases in pH were also observed when very low density lipoproteins were hydrolyzed by LpL. Lipolysis was accompanied by a 2-fold increase in the release of ^{59}Fe from Tf. Tf binding to subendothelial matrix (SEM), a site of key events in atherosclerosis, increased 2-fold as the pH decreased from 7.40 to 6.00. More free iron also bound to SEM as the pH decreased below 7.40. We next tested whether a reduction in pH promotes LDL oxidation. More oxidation products were found in LDL incubated at low pH for 24 h in 10% human serum. Malonaldehyde contents (nmol/mg protein), measured as TBARS, were 7.11 ± 0.34 at pH 7.40, 7.65 ± 0.49 at pH 7.00, 9.00 ± 1.18 at pH 6.50, and 11.54 ± 0.63 at pH 6.00. Based on these results, we hypothesize that lipolysis-induced acidic conditions enhance iron release from Tf and increase formation of oxidized LDL.**—Balagopalakrishna, C., L. Paka, S. Pillarisetti, and I. J. Goldberg. **Lipolysis-induced iron release from diferric transferrin: possible role of lipoprotein lipase in LDL oxidation.** ***J. Lipid Res.*** **1999.** 40: **1347–1356.**

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The conditions that lead to oxidation of circulating low density lipoproteins (LDL) are unknown (1–4). It is believed that the presence of abundant antioxidants in plasma prevents LDL oxidation by free radicals and catalytic free metal ions such as iron and copper (5–11). Even trace amounts of free iron are barely detectable in plasma due to the presence of iron-binding proteins such as ferritin and transferrin (Tf) (10, 11). In contrast, lipid hydroperoxides and catalytic free iron are found in the gruel taken from atherosclerotic lesions (12–17). How this iron is generated is unknown. In addition, higher concentrations of the iron binding proteins, Tf and ferritin, are found in atherosclerotic lesions than in normal arteries (17–21). Therefore, conditions must exist that allow release of free iron from these and other proteins, and/or prevent the released iron from re-associating with Tf or ferritin.

Intracellularly, iron is released from Tf in lysosomal compartments at low pH (22–24). Iron release from Tf due to pH changes may also occur extracellularly, but this has not been investigated. We hypothesized that physiological events such as lipolysis on the arterial wall would lower pH, cause iron release, and promote oxidative modification of lipoproteins. Such a process could generate minimally modified (MM) LDL (25). We found that triglyceride lipolysis by lipoprotein lipase (LpL), a triglyceride hydrolase found on the endothelial cell surface and in atherosclerotic lesions, generated free fatty acids (FFA) and lowered pH. This lipolysis-induced acidity, in turn, triggered iron release from Tf. We further showed that MM-LDL was formed when acidic conditions prevailed in serum. Thus, iron release can occur extracellularly during lipolysis. This suggests that free iron generation can promote lipoprotein oxidation under conditions that may occur during the postprandial period.

MATERIALS AND METHODS

Chemicals and reagents

Human apo-Tf (siderophilin, iron-poor), bovine serum albumin (BSA), nitrilotriacetic acid (NTA), malonaldehyde bis

Abbreviations: LpL, lipoprotein lipase; FFA, free fatty acids; Tf, transferrin; SEM, subendothelial matrix; MDA, malonaldehyde; TBARS, thiobarbituric acid reactive substances; PPi, tetrasodium pyrophosphate; NTA, nitrilotriacetic acid.

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(dimethylacetal), pyrophosphate (tetrasodium) (PPi), Bis-Tris buffer, butylated hydroxytoluene (BHT), serum total iron binding capacity (TIBC) assay, and linoleic acid were obtained from Sigma Chemical Co. (St. Louis, MO). $Na^{125}I$ (100 mCi/ml) and ^{3}H-labeled triolein (glycerol tri- 9-10 [^{3}H]oleate) were obtained from Amersham Corp. (Arlington Heights, IL). Lactoperoxidase/glucose oxidase immobilized on agarose beads (Enzymobeads) was obtained from Bio-Rad Laboratories (Hercules, CA). $^{59}FeCl_3$ in 0.5 M HCl (~14 μCi/ug) was obtained from DuPont NEN Research Products (Boston, MA). Prepacked PD-10 Sepharose columns were obtained from Pharmacia Inc. (Clayton, NC). LpL was purified from fresh unpasteurized bovine milk (26); protein concentration was determined by the method of Lowry et al. (27); and enzymatic activity was assayed using the method of Nilsson-Ehle and Schotz (28). The purified LpL was stored at −70°C. Intralipid (20%) (20 g/dl soybean oil and 1.38 g/dl eggyolk phospholipids, Pharmacia Inc.) was used for most lipolysis experiments.

Subendothelial matrix (SEM)

Bovine aortic endothelial cells were isolated and cultured as described (29). The cells (5–12 passages) were grown in 24-well culture dishes in Dulbecco's modified Eagle's medium (Life Technologies) containing 10% fetal bovine serum. SEM was prepared as detailed elsewhere (30).

pH measurements

Bovine LpL (10 μg/ml) was incubated at room temperature (or at 37°C where indicated) with Intralipid (10 μg/ml) in 5 ml of PBS (or 0.1 × PBS, where indicated) containing 3% BSA (and 10% human serum, where indicated) and the changes in pH were monitored as a function of time. pH measurements were performed using a Corning pH meter Model 220. The pH was adjusted to 7.40 at the start and lipolysis was initiated by the addition of LpL. The mixture was constantly agitated on a shaker to ensure Intralipid mixing.

In another experiment, pH changes as a function of linoleic acid concentration were measured by adding indicated concentrations of linoleic acid to PBS containing 3% BSA (460 μM) ± 10 serum. The solutions were stirred well and the pH of the medium was measured immediately to avoid fatty acid oxidation and decomposition.

Measurement of FFA released during lipolysis

The method of Nilsson-Ehle and Schotz (28) was used for this purpose. Briefly, 200 μC_i of [^{3}H]triolein (60 μl) containing 1 g triolein was mixed with 0.05 g of l-α phosphatidylcholine and dried under a nitrogen purge for 10 min. Glycerol (12.5 ml) was added and the emulsion was sonicated, vortexed vigorously, and incubated at 37°C for 30 min (solution A). Tris-HCl (0.3 M) containing 3% BSA was prepared and the pH was adjusted to 8.6 (solution B). Serum from a normal patient was heat-inactivated by incubating at 55°C for 1 h (solution C). An emulsion was prepared containing 1 part of solution A, 4 parts of solution B, and 1 part of solution C. Four ml of the emulsion was mixed with 4 ml of PBS/3% BSA, pH 7.40 (or with PBS/3% BSA/10% normal human serum, pH 7.40). LpL (40 μg, 5 μg/ml final concentration) was added and the reaction was allowed to proceed at 37°C. At various time points, aliquots (200 μl) were taken from the above lipolytic reaction mixture and mixed with 3.5 ml of a mixture of 1.25 L of $CHCl_3$, 1.41 L of CH_3OH, 1 L of n-heptane plus 100 μl of oleic acid, followed by the addition of 1 ml of borate solution (pH 10.0). The solution was vortexed and centrifuged (2300 rpm) for 30 min at room temperature and released fatty acids in 1 ml of the supernatant were determined in a liquid scintillation counter. The controls did not have LpL added.

Labeling and purification of ^{59}Fe-Tf

Labeled and unlabeled diferric Tf were prepared from apo-Tf (22). Briefly, apo-Tf (6 mg) was dissolved in 0.5 ml Tris chloride (0.25 M, pH 8.0) containing 0.1 mM $NaHCO_3$ and combined with NTA (100 mM, 0.5 ml) containing 100 μl of $^{59}FeCl_3$ solution, followed by incubation at room temperature for 1 h. For the unlabeled protein, the apo-Tf solution was mixed with 20 μl of 100 mM NTA/12.5 mM $FeCl_3$. The excess free iron was removed by passing through a PD-10 column that had been previously equilibrated with 0.15 M NaCl/0.02 M Tris chloride, pH 7.40. The amount of iron loading in Tf was estimated from the A_{465}/A_{280} nm; it was ~0.046, consistent with full saturation (31). The specific activity of the labeled protein used for the experiments was in the range 8–10 cpm/ng.

Iron release during lipolysis

The kinetics of iron release from ^{59}Fe-Tf (17 μM) was studied in 500 μl of PBS/3% BSA, Intralipid (0.5 mg), and LpL (5 μg) at room temperature. PPi (10 mM in PBS/3% BSA) was added to chelate the newly released iron. Released and bound ^{59}Fe were determined as detailed by Bali, Zak, and Aisen (32) using excess unlabeled Tf (0.2 mM). The controls did not have LpL in the medium.

In a different experiment, the above procedure was repeated to compare the ^{59}Fe release kinetics in 0.1 M HEPES/3% BSA (pH 7.40) using 1 mM PPi.

Iodination of Tf

Iodination of Tf with $Na^{125}I$ was carried out as reported previously (22). Briefly, Tf (100 μg) in 0.15 M NaCl/0.02 M Tris chloride was combined with 50 μl of Enzymobeads suspension. $Na^{125}I$ (1 mCi) followed by 50 μl of β-d-glucose were added to this mixture. After a 30 min incubation at room temperature, the samples were chromatographed using a PD-10 column equilibrated with 0.15 M NaCl/0.02 M Tris chloride, pH 7.4. The specific activity of the recovered ^{125}I-labeled Tf was 1280 cpm/ng.

Binding of Tf and the release and retention of its iron on SEM

A. ^{125}I-labeled Tf binding to SEM. To study binding of Tf to SEM as a function of pH, ^{125}I-labeled Tf (5 μg/well, 80 μl) and 0.2 M Bis-Tris buffer/3% BSA (420 μl, pH 7.40, 7.00, 6.50, and 6.00) were added to 24-well plates containing SEM and incubated for 2 h at 37°C. After incubation, the wells were washed thrice with 4°C, 0.2 M Bis-Tris buffer/3% BSA (at pH 7.40, 7.00, 6.50, and 6.00). Matrix-associated radioactivity was released by incubation with 0.5 N NaOH (500 μl) for 30 min at 37°C.

B. Release of ^{59}Fe from Tf on SEM. ^{59}Fe-Tf (22 μM, 175 μg, 100 μl) was incubated with SEM in Bis-Tris buffer/3% BSA (400 μl) at different pHs for 2 h at 37°C. Free ^{59}Fe released from Tf was then determined as described by Bali et al. (32).

C. Binding of ^{59}Fe to SEM. To SEM-containing wells maintained at different pHs with 475 μl of 0.2 M Bis-Tris buffer/3% BSA, 240 ng of ^{59}Fe metal ion in the form of $FeCl_3$ (25 μl) was added. This concentration corresponded to the concentration of ^{59}Fe in ^{59}Fe-Tf used in previous SEM experiments. Albumin-coated wells produced by overnight incubation of 24-well plates with PBS/3% albumin solution served as controls. Incubation and elution of bound radioactivity were done under the same conditions as described above for labeled Tf.

LDL isolation

Human VLDL (d < 1.006 g/ml) and LDL (d 1.019–1.063 g/ml) was isolated from EDTA-containing plasma by sequential ultracentrifugation (33). LDL was dialyzed against 2 changes of EDTA-free PBS at 4°C and used immediately. Total protein con-

centration was determined by the method of Lowry et al. (27) with BSA as a standard.

LDL oxidation

LDL samples (1.5 mg) were incubated in triplicate in 2 ml of 0.2 m Bis-Tris/0.15 m NaCl/10% human serum solution at pH 7.40, 7.00, 6.50, and 6.00 for 24 h. Oxidation was terminated with BHT (20 μm). The samples were subjected to ultracentrifugation (45,000 rpm) at 10°C in 1.12 density solution for 4 h to isolate LDL and oxidation was measured.

Malonaldehyde (MDA) content of the oxidized lipoprotein samples was determined using a fluorescence method by slightly modifying a reported assay (34) to attain greater sensitivity in the range 0.1–5 nmol. One hundred twenty-five μl of 5% H_3PO_4 was added with mixing to 10 μl of oxidized LDL samples (as above). After precipitation, 1 ml of 0.6% TBA was added and the final reaction volume was increased to 3 ml with water. The mixture was incubated at 95°C for 45 min, cooled, and subsequently centrifuged at 4000 *g* for 20 min. The emission intensity of the supernatant was determined at 553 nm using an excitation wavelength of 515 nm. Malonaldehyde bis (dimethylacetal) was used as the standard. To assess the formation of conjugated dienes (35), LDL (0.1 mg) was incubated in 2 ml of 0.2 m Bis-Tris, 0.15 m NaCl, with 10% human serum at pH 7.40 and 7.00. Diene formation was monitored spectrophotometrically by following the absorbance changes at 234 nm.

Estimation of free iron in serum under acidic conditions

Free iron release from human serum under acidic conditions was measured by complexation with Ferrozoin using a serum total iron binding capacity assay (TIBC, Sigma). Human serum was acidified with 1 N HCl, added to iron buffer solution, and incubated for 30 min. Absorbance (at 560 nm) was determined.

RESULTS

Lipolysis of triglycerides decreases pH

We hypothesized that lipolysis along the arterial surface would reduce pH. pH changes during a lipolytic reaction using Intralipid (1–10 μg/ml) in PBS containing 3% BSA at 37°C were monitored. pH dropped during lipolysis from 7.40 to 7.07 in the first hour (**Fig. 1A**, solid triangles), and to 6.90 over a period of 20 h (data not shown). With lipolysis in 0.1 × PBS, the pH dropped to 6.91 in 10 min and gradually dropped to 6.47 over a period of 3 h (diamond). The controls that did not have either LpL (closed circle) or Intralipid (open circle) maintained a constant pH at 7.40 with little variation for 6 h. pH also dropped when the lipolysis was carried out in PBS/3% BSA containing 10% human serum, reaching 7.10 in 10 min (solid squares). Unlike in PBS (solid triangle), measurements performed in the presence of serum (solid squares) had a small subsequent increase in pH during the incubations. The pH of the serum control lacking LpL, shown with the asterisk, increased from pH 7.4 to 7.53 during the first 2 h of incubation. After reaching a pH of 7.06 at 45 min, the pH of the Intralipid plus LpL mixture in serum (solid squares) also increased approximately 0.1 pH unit. This may be due to the equilibration between the atmospheric and serum CO_2. Nonetheless, the data show that lipolysis reduces the pH of the solutions.

To test whether the decrease in pH correlated with the generation of FFA during lipolysis, lipolysis was performed using radiolabeled triolein and liberated FFA were measured. As shown in Fig. 1B, FFA release during lipolysis paralleled the observed pH changes above in Fig. 1A. The rise in the amount of FFA during the first hour (in PBS, solid triangles; and in serum, solid squares) coincided with the drop in pH seen in Fig. 1A during the same time period. Thus, the present data suggest that FFA released during lipolysis alter pH.

To prove that the FFA were, in fact, responsible for the pH lowering, linoleic acid, which is 55% of the acylester in Intralipid, was added at various concentrations to PBS/3% BSA (Fig. 1C) or to PBS/3% BSA/10% human serum. pH dropped with greater concentrations of the added linoleic acid.

Finally, we next tested whether such pH changes would also occur with lipolysis of VLDL, a physiological substrate of LpL. VLDL was incubated with LpL and pH was monitored over time (Fig 1D). pH decreased from 7.4 to 7 in 15 min and to 6.85 in 1 h.

Free iron release from Tf increases during lipolysis

Iron release from Tf in lysosomal compartments is accelerated at low pHs (22), but evidence that Tf loses its iron extracellularly is lacking. For this reason, we next tested whether reduction of pH in the range found with lipolysis was sufficient to alter iron–Tf interaction. The kinetics of iron release from Tf were studied during lipolysis in PBS/3% BSA, containing Intralipid (1 mg/ml), LpL (5 μg), and PPi (10 mm). Iron release was monitored by tracing ^{59}Fe released from Tf as a function of time. More iron was released during lipolysis than under control conditions (**Fig. 2A**). The steep increase in free iron release during the first hour coincided with the steep drop in pH observed during the same time (Fig. 1A). Fig. 2B shows the corresponding decrease in the protein-associated iron that parallels the free iron release shown in Fig. 2A. Some iron was also released in controls that did not have LpL, presumably due to the chelating effects of PPi (32). However, the amount of dissociation without lipolysis was clearly less than that found with LpL. Thus, lipolytic events can trigger iron release from Tf even in the narrow ranges of pH (7.40 to 7.00).

Lipolysis products mediate iron release largely through the pH effect

We next tested whether the iron release from Tf during lipolysis was due to the pH drop or chelation of the iron by the fatty acid anions by performing the lipolysis reaction in the presence of a stronger buffer (0.1 m HEPES, pH 7.4). A lower concentration of PPi (1 mm) was used to decrease spontaneous iron dissociation. As shown in **Fig. 3A**, compared to the experiment performed in HEPES, much more iron was released in PBS. The amount of protein-bound iron was reduced by 60% in 3 h while the reduction in HEPES was less than 5%. This occurred in spite of the fact that the amount of FFA generated in the two buffers was not significantly different (Fig. 3B). A control

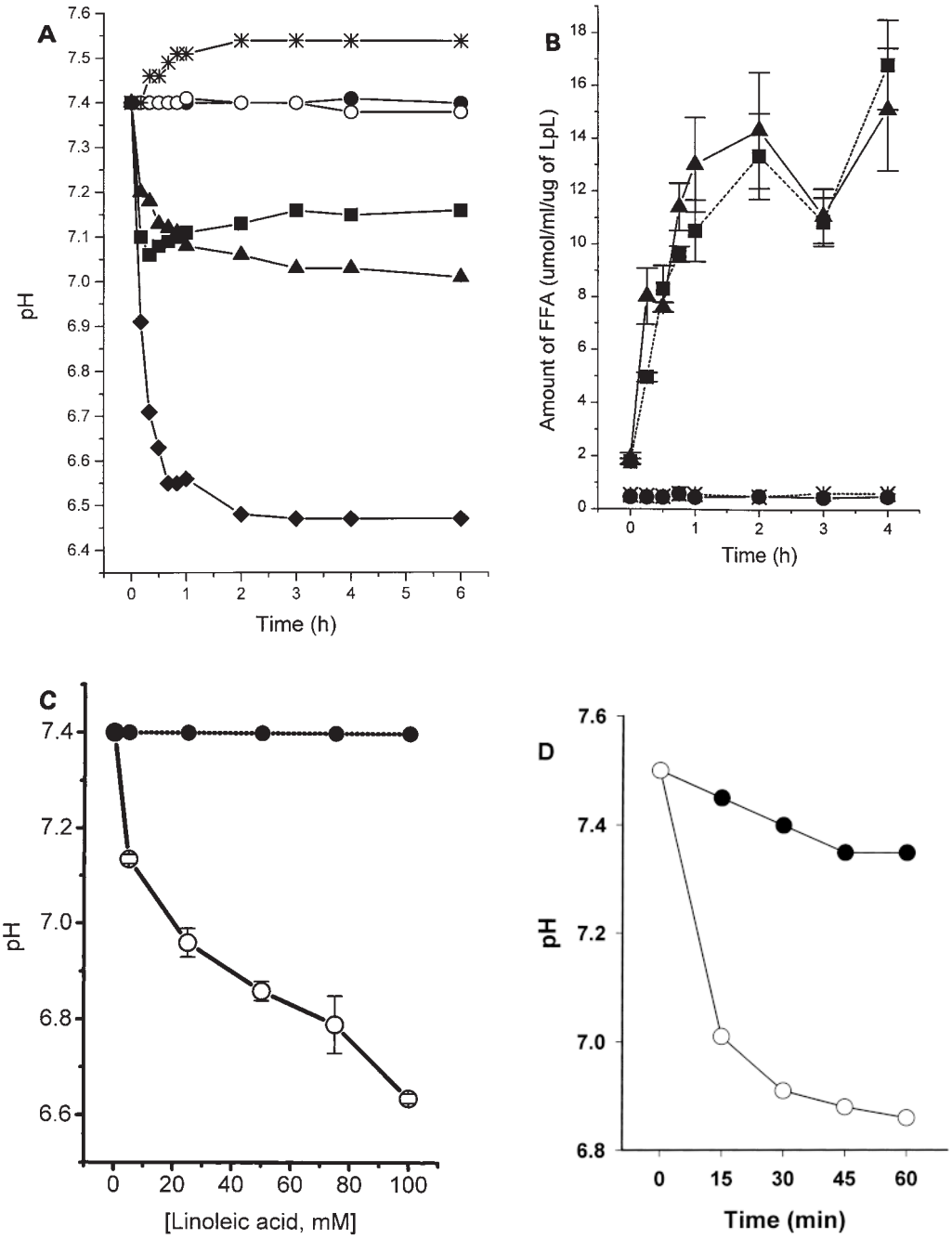

Fig. 1. A: pH drops during lipolysis. In 5 ml of the reaction mixture, Intralipid suspension (20%, 1 mg/ml) was incubated with bovine LpL (10 μg/ml) in PBS/3% BSA (▲), or in 0.1 × PBS/3% BSA (◆) or in PBS/3% BSA/10% human serum (■) at room temperature. pH changes were followed as a function of time. The controls did not have LpL, but were with (○) and without Intralipid (●) in PBS/3% BSA. A third control used for pH changes in 10% human serum contained Intralipid alone (*). pH dropped steadily only in the medium containing LpL. B: Measurement of free fatty acid (FFA) release during lipolysis. FFA released during lipolysis were measured by the method of Nilsson-Ehle and Schotz (26). An emulsion was prepared containing ^{3}H-labeled triolein (200 μCi). Four ml of the emulsion was mixed with 4 ml of PBS/3% BSA, pH 7.40 (▲) (or with PBS/3% BSA/10% normal human serum, pH 7.40 [■]) containing LpL (40 μg, final concentration 5 μg/ml) and incubated at 37°C. At various time points, aliquots (200 μl) were taken from the lipolytic reaction mixture and the liberated FFA were determined. The controls for PBS (●) and for 10% human serum (*) did not have LpL in the medium. C: Linoleic acid increases acidity. To demonstrate that FFA are responsible for the pH lowering effect seen during lipolysis, linoleic acid was added at various concentrations (1–20 mm) to PBS/3% BSA, 7.40. pH decreased as the concentration of the acid increases in the medium (○). The control that did not have linoleic acid maintained a constant pH (●) D: Lipolysis of VLDL decreases pH. In 2.5 ml of the reaction mixture, VLDL (1 mg/ml) was incubated with bovine LpL (10 μg/ml) in 0.1 × PBS/3% BSA (○) or at 37°C. pH changes were followed as a function of time. The controls (●) had VLDL but no LpL. pH dropped (from 7.4 to 7) in 15 min only in reaction containing LpL.

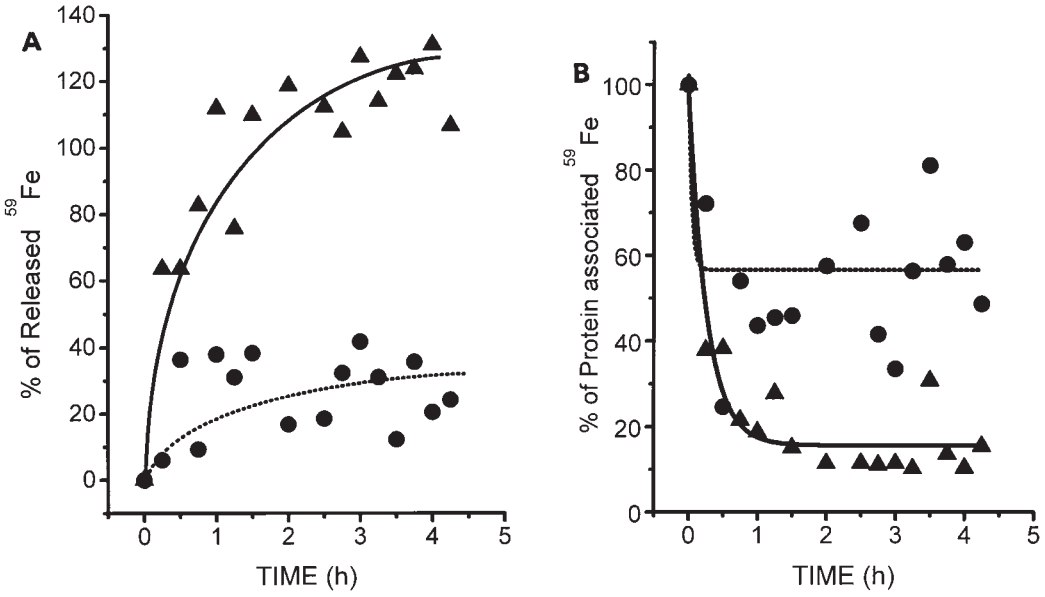

Fig. 2. A: Lipolysis induces greater iron release from ^{59}Fe-transferrin (Tf). Release of ^{59}Fe from Tf was measured during a lipolysis reaction (▲) under conditions noted in Fig. 1A. The reaction was performed in 500 μl of a reaction mixture containing PBS/3% BSA, ^{59}Fe Tf (17 μm), Intralipid (0.5 mg), and LpL (5 μg). To achieve a measurable amount of iron release, PPi (10 mm in PBS/3% BSA) was added to prevent the dissociated iron from rebinding to Tf. Released ^{59}Fe was determined as detailed by Bali et al. (30) using excess unlabeled Tf (0.2 mm). The controls (●) did not have LpL in the solution. In the figure, 100% corresponds to 2.3 μg. B: Decrease of protein Tf-associated ^{59}Fe concentration during lipolysis. In the same experiment, the Tf in the solution was precipitated with 40% polyethylene glycol to determine the iron content of the protein. Precipitate (% of protein-associated ^{59}Fe) was measured at identical time points as in 2A in control (●) or with LpL (▲).

experiment showed that the pH did not change from 7.40 in HEPES during lipolysis. These data suggest that the change in pH, and not the amount of generated FFA, was responsible for the iron dissociation from Tf.

Lower pH enhances Tf binding to SEM

SEM is the site of major atherosclerosis events. Although more Tf is present in atherosclerotic vessels (18–21), the factors responsible for this are not known. We initially studied the binding of ^{125}I-labeled Tf (5 μg/well, a non-saturating concentration, data not shown) to SEM over the range of pH seen during lipolysis. As shown in **Fig. 4**, binding increased with the lowering of pH. Lowering pH to 7.00 increased binding 20%; at pH 6.00 binding increased 65%. These data suggest that acidic conditions facilitate Tf association with SEM.

We also performed experiments using ^{59}Fe-labeled Tf and, as expected, incubation of ^{59}Tf with SEM led to more iron release under acidic conditions. The percent free iron released at each pH was 100% at pH 7.40, 341.6% at pH 7.00, 633% at pH 6.4, and 802.4% at pH 6.00. Therefore, Tf interaction with SEM was greater at lower pH. At lower pH, however, the Tf contained less iron, i.e., there was more apo and monoferric and less diferric Tf.

Acidic conditions increase free iron association with SEM

We explored whether the iron released from Tf under acidic conditions could be a source of intimal iron by dissociating from Tf and then binding to SEM. To test this, protein-free iron association with the matrix was studied using $^{59}FeCl_3$ (240 ng of Fe^{3+}/well) under the same conditions as above. Albumin-coated wells were used as controls. As shown in **Fig. 5**, more iron bound to the matrix than to albumin at all the pHs studied. While the binding with albumin remained largely unaffected as the pH was lowered, free iron interaction with SEM increased as the pH was lowered. Thus, under similar conditions in vivo, free iron originating from Tf may be retained in the intima and cause LDL oxidation.

Acidic conditions promote LDL oxidation in serum

It is generally believed that LDL oxidation is a non-plasmic event due to the rich antioxidant nature of plasma (9–11, 36–39). While this may be true at physiological pH, we hypothesized that iron released from serum proteins such as Tf under acidic conditions promotes LDL oxidation. To assess this possibility, EDTA-free LDL (3 mg/ml) was incubated in 0.2 m Bis-Tris/0.15 m NaCl/10% human serum, maintained at 4 different pHs (7.40, 7.00, 6.50, and 6.00) at 37°C. MDA content of LDL was determined at 24 h. As shown in **Fig. 6**, MDA was greater in LDL that had been incubated under acidic conditions. The TBARS values at each pH were, in nmol/mg protein, 7.11 ± 0.34 at pH 7.4, 7.65 ± 0.49 at pH 7.0, 9.00 ± 1.18 at pH 6.5, and 11.54 ± 0.63 at pH 6.0. Therefore, our results demonstrate that LDL oxidation can be initiated even in the presence of human serum under lipolysis-induced acidic conditions. Because reduced pH might have a number of effects, it is possible that release of metals from other metal-binding proteins could contribute to this process. A second mea-

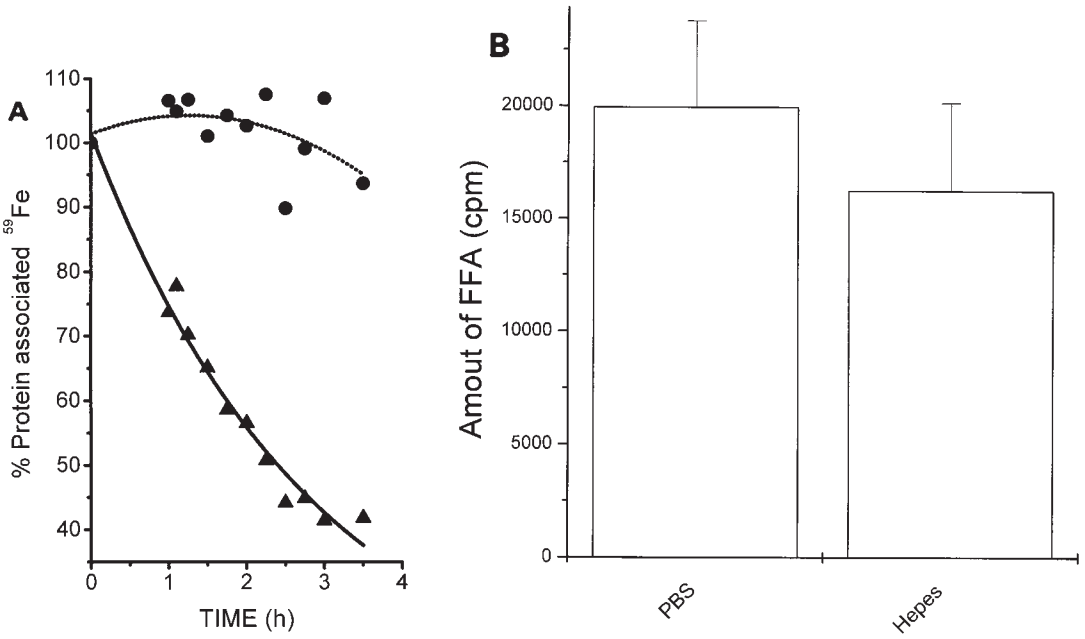

Fig. 3. A: Lipolysis products-mediated iron release is largely through pH effect. Under conditions similar to those for Fig. 2A, the ^{59}Fe release kinetics and the kinetics of the resultant decrease in ^{59}Fe-associated protein concentration in 0.1 m HEPES/3% BSA (●) was compared with that in PBS/3% BSA (▲) using PPi (1 mm). The pH at the start of lipolysis was 7.40. The small decrease seen in HEPES results from the well-known effect of PPi. In contrast, a 2-fold decrease is seen in PBS. In the figure 100% corresponds to 3.6 μg. B: Amount of FFA released in PBS and HEPES were not significantly different. Lipolysis was performed in PBS/3% BSA and in 0.1 m HEPES/3% BSA using ^{3}H-labeled triolein (200 μCi) as a substrate and released FFA were determined as detailed under Fig. 1B. The amount of released FFA determined were not significantly different ($P = 0.15$) in the two buffers.

sure of oxidation, conjugated diene production, was also used. Although lower pH appeared to increase diene formation, conjugated dienes were 1.35 ± 0.34 nmol/mg protein at pH 7.40 and 19.66 ± 0.68 at pH 7.0. These measurements were obtained after subtracting a very high background due to serum and are, therefore, less reliable.

To further confirm that free iron was released from Tf under acidic conditions, we measured the free iron content of human serum under different pH conditions. As shown in **Fig. 7** a small but significant increase in the free iron content of serum occurred with greater acidity 136 ± 2.5 μg/dl at pH 7.4 to 155 ± 6.3 at pH 6.

The hypothesis that released free iron causes oxidation suggests that iron complexed to Tf is less likely to promote oxidation. To test this, LDL oxidation by free and Tf-bound iron was compared (**Fig. 8**). The amount of conjugated dienes formed over a period of 10 h was greater when iron was in free form at pH 7.40. Thus, free iron is a superior oxidizing agent.

DISCUSSION

While the in vivo site of LDL oxidation is unknown, it is generally believed that LDL oxidation does not take place in serum because of the antioxidant properties of plasma. Serum is believed to suppress LDL oxidation under normal conditions (9–11, 36–39) through the protective actions of antioxidant enzymes and molecules (superoxide dismutase, catalase, ascorbic acid, alpha tocopherol, etc.), metal binding proteins (apo forms of ferritin, transferrin, and lactoferrin, albumin, etc.), and reducing agents (reductases, glutathiones, etc.). Our results suggest that in 10% serum these antioxidant capabilities are altered during acidic conditions.

We first studied a physiological process that could alter pH. In our studies, lipolysis caused pH to drop by 0.5 units in PBS and by 0.4 units in the presence of serum (Fig. 1A). This drop in pH coincided with the increase in released FFA (Fig. 1B). The major increase in released FFA was seen in the first 30 min. This pH drop was sufficient to trigger iron release from Tf (Figs. 2A and 2B). Iron dissociates from Tf within very acidic intracellular compartments (22–25); we now show that mild acidification will also lead to the release of free iron (Figs. 2A and 2B). Thus, other acidifying conditions such as ketoacidosis and hypoxia may lead to the release of catalytic iron.

More Tf and free iron are found in human atherosclerotic plaques than in normal aortic intimal tissue (18–21). The factors responsible for this are unknown. Our results show that when acidic conditions prevail, SEM retains increasing amounts of Tf (Fig. 4A). Moreover, a 3-fold increase in the release of iron from Tf for a 0.40 unit drop in pH was also observed even in the absence of an external chelator (32). Our data also suggest that some of the released iron associated with matrix. Presumably, the anion-rich matrix served as a chelator and provided sites of attachment for the cationic iron. Not surprisingly, even

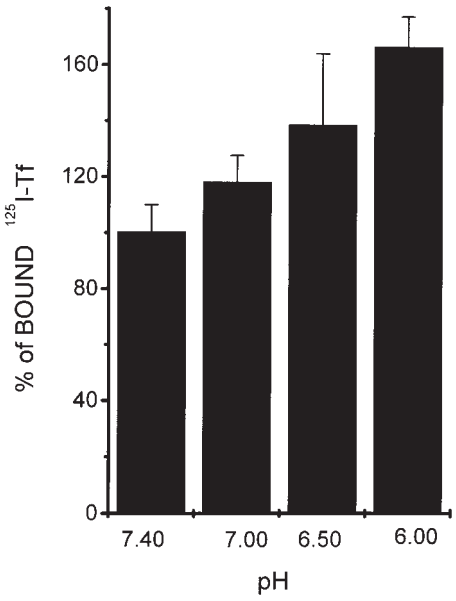

Fig. 4. ^{125}I-labeled Tf binding to SEM increases with acidity. To SEM prepared in 24-well plates, ^{125}I-labeled Tf (5 μg/well, 80 μl) was added along with 0.2 m Bis-Tris buffer/3% BSA (420 μl, pH 7.40, 7.00, 6.50, and 6.00) in triplicate and incubated for 2 h at 37°C. The wells were then washed with ice-cold 0.2 m Bis-Tris buffer/3% BSA (pH 7.40, 7.00, 6.50, and 6.00) and matrix-associated radioactivity was released by incubation with 0.5 N NaOH (500 μl) for 30 min at 37°C. This represents the Tf fraction bound to SEM. Values represent mean ± SD, n = 3. In the figure, 100% corresponds to 100.4 ng of the protein.

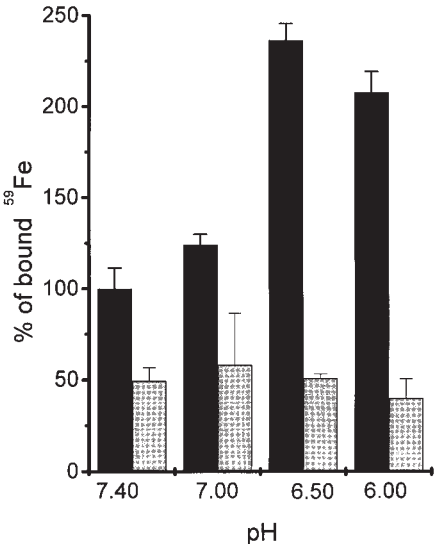

Fig. 5. More free iron is associated with SEM under acidic conditions. To SEM in 24-well plates maintained at different pH (7.40, 7.00, 6.50, and 6.00) with 475 μl of 0.2 m Bis-Tris/3% BSA, 240 ng of ^{59}Fe (3^+) as $FeCl_3$ (25 μl) was added. This concentration corresponds to the concentration of ^{59}Fe in ^{59}Fe-Tf used in the previous SEM experiments, shown in Fig. 4. Albumin-coated wells, obtained with overnight incubation of 24-well plates with PBS/3% BSA, served as controls (light bars). Incubation and elution of bound radioactivity were done as described under Fig. 4A. The dark bars correspond to ^{59}Fe ions bound to SEM. More iron associated with the matrix at lower pH studied. Values represent mean ± SD, n = 3. In the figure, 100% corresponds to 0.246 ng of ^{59}Fe.

more iron bound to these sites under acidic conditions (Fig. 5). Our results confirm that iron is a stronger oxidant when "off" Tf than while it is "on" Tf (10, 39, 40). We have not yet studied whether matrix-bound iron is also a pro-oxidant.

Several mechanisms have been proposed for LDL oxidation; these may require a long residence time of LDL in the vessel wall (41, 42). In pre- and early lesions, the amount of LDL oxidation is thought to be relatively small and MM-LDL typically contain only 2–5 nmoles of MDA per mg of the protein. Nevertheless, such MM-LDL are an important factor in several atherosclerosis-initiating events (43, 44) including induction of granulocyte and macrophage stimulating factors (45, 46), expression of tissue factor (47), inhibition of PDGF expression (48), and stimulation of Gi and inhibition of Gs protein functions (49). Our TBARS results indicate that such a degree of oxidation can be attained even in the presence of human serum under acidic conditions (Fig. 6); after subtraction of the baseline data a 4 nmol/mg protein increase occurred. Using cysteine, Lamb and Leake (50) have shown that under acidic conditions Tf promotes LDL oxidation. Cysteine (0.18 mm) was included in their reactions, probably to ensure that the iron released from Tf under acidic conditions was reduced to Fenton's reagent (Fe^{2+}), a powerful pro-oxidant (51, 52). In the current study, increased LDL oxidation occurred in serum containing medium in the absence of added pro-oxidants such as cysteine. Therefore, acidification, within physiologic limits, allows slow but measurable increases in LDL oxidation in the presence of serum.

Our studies suggest that a normal event such as lipolysis on the arterial wall can lead to appreciable iron release from Tf and identify a possible, and hitherto unexplored, pathological event that may occur during postprandial lipolysis. Other non-iron-mediated pathways may be involved in acidification-mediated increases in LDL oxidation. Ceruloplasmin-derived copper also oxidizes LDL (52). There are no data on whether LDL oxidation by known oxidative enzymes, such as myeloperoxidase or lipoxygenase, is increased during acidosis. It should be noted that Tf is a protein that is usually considered to be an antioxidant because it scavenges free iron. We propose that Tf can become a pro-oxidant when it transports plasma iron to SEM and releases it within lesions.

These results imply an additional pro-atherogenic role for LpL as an indirect generator of free iron. LpL produces remnant particles on the arterial wall, and this has led to speculations about the role of the enzyme in the atherogenic process (53). Several human clinical studies have shown a positive correlation between postprandial lipemia and atherosclerosis (54–56). Remnant particles are more likely to enter the vessel wall (53). Other possibilities are that the postprandial particles alter other lipoproteins, e.g., HDL and LDL (54), or that the protracted lipolytic process occurring along the artery wall alters the

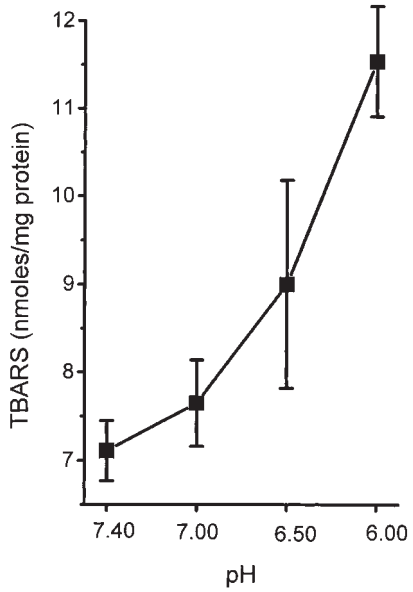

Fig. 6. LDL oxidation increases even in serum under acid conditions. LDL (1.5 mg) was incubated for 24 h in 2 ml of 0.2 m Bis-Tris/0.15 m NaCl/10% human serum at pH 7.40, 7.00, 6.50, and 6.00. The oxidation was terminated with BHT (20 μm). The solutions were then subjected to ultracentrifugation (45,000 rpm) at 10°C in 1.12 density solution for 4 h to separate VLDL and LDL fractions from the rest of the serum proteins and the extent of oxidation was measured by determination of TBARS. Values represent mean ± SD, n = 3.

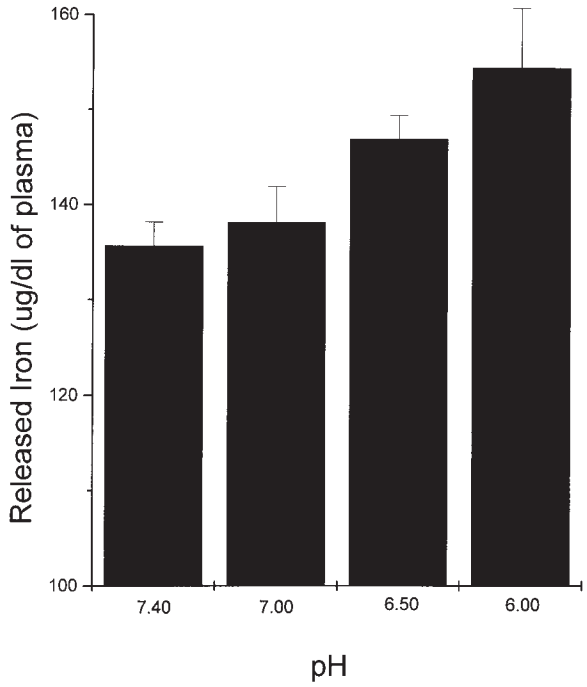

Fig. 7. Increased iron content of serum under acidic conditions. The pH of the human serum was adjusted with HCl (1 N) to pH 7.40, 7.00, 6.50, and 6.00 and then added to iron buffer solution (Sigma TIBC assay) and incubated for 30 min. The amount of free iron was determined from the intensity of absorbance at 560 nm. Values represent mean ± SD, n = 3.

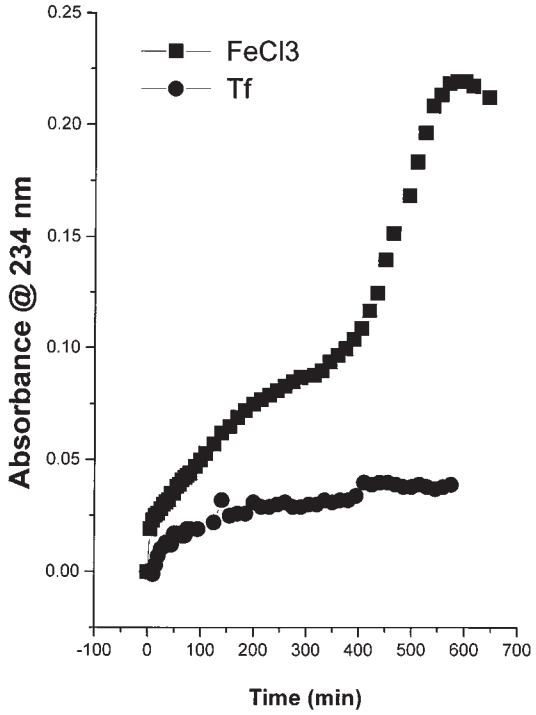

Fig. 8. LDL oxidation by free iron and iron bound to Tf under physiological pH conditions. The formation of conjugated dienes on LDL (0.1 mg) incubated in 1.5 ml of 0.2 m Bis-Tris/0.15 m NaCl solution at pH 7.40 containing $FeCl_3$ (5 μm) or Tf (16 μm) was monitored by following the absorbance changes at 234 nm. At these concentrations, both $FeCl_3$ and Tf have same concentration of iron. Absorbance was corrected for protein background absorption.

permeability barrier of the endothelial cell layer (53, 57). Our data suggest yet another atherogenic mechanism, namely, iron release in local areas of lipolysis that, in turn, increases LDL modification (58).

The present studies provide a model for LDL oxidation when acidic conditions prevail in vivo. We hypothesize that along the endothelial surface, LpL engages a large triglyceride carrier, generates FFA, and causes transient reductions in pH. As reported elsewhere (59, 60), the interaction of LpL with model lipoproteins is fairly tight and such a cooperative binding to several LpL molecules would hold the chylomicron firmly at the lipolysis sites. These interactions may also produce a protected compartment that excludes plasma perfusion. Such a protected local domain created by a chylomicron along an endothelial surface would be analogous that those made by platelet plugs at the site of vascular injury, and neutrophils and macrophages on ligand-coated surfaces (61–64). Prior to tissue uptake, newly formed FFA may (albeit transiently) alter local pH in the protected domains close to or beneath the endothelial cell layer.

In conclusion, we show that LDL oxidation occurs even in the presence of serum under acidic conditions induced by lipolysis. Elevated plasma levels of FFA (2–5 mm) are quite frequently observed during postprandial feeding sessions (59). These levels are greater than those required to alter pH in our experiments. We also show that this re-

duction of pH will alter iron–Tf interaction, and will create a pro-oxidant environment. We hypothesize that Tf and its iron are the origin of increased iron deposits in the intimal tissue in atherosclerotic lesions. Under slightly acidic, yet physiologic conditions, both plasma and arterial wall iron may initiate atherosclerotic events.

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