

# Atherogenic Lipoproteins Support Assembly of the Prothrombinase Complex and Thrombin Generation: Modulation by Oxidation and Vitamin E

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The importance of lipoproteins in the etiology of atherosclerosis is well established. Evidence is now accumulating to implicate thrombin in the pathogenesis of atherosclerosis. We have investigated whether atherogenic lipoproteins can support thrombin generation. In the absence of platelets or endothelial cells, both very low-density lipoprotein (VLDL) and oxidized low-density lipoprotein (LDL) support assembly of the prothrombinase complex and generation of thrombin. Thrombin generation (per  $\mu\text{g}$  of apolipoprotein) supported by VLDL was 19.4-fold greater than that supported by high-density lipoprotein (HDL),  $P < .00001$ , and 11.7-fold greater than that supported by LDL,  $P < .00001$ . Oxidation of LDL increased lipoprotein-supported thrombin generation 12-fold compared to unmodified LDL,  $P < .0001$ . We have

**T**HERE IS INCREASING evidence to support the hypothesis that thrombin generation may influence the development of atherosclerosis.<sup>1-7</sup> Thrombin generation is dependent on assembly of the prothrombinase complex on a phospholipid surface.<sup>8,9</sup> Whether platelets provide the only important phospholipid surface for prothrombinase assembly is uncertain. Thrombin can also be generated in vitro in the presence of phospholipid bilayers and micelles; and it is therefore possible that cell-free phospholipid may be able to support assembly of the prothrombinase complex in vivo. Various cell-free phospholipids appear capable of binding the individual components of the prothrombinase complex. Liposome vesicles containing phosphatidyl choline were able to support thrombin generation in the presence of factor Va.<sup>10</sup> Very low-density lipoprotein (VLDL) has also been shown to bind prothrombin<sup>11,12</sup> and it has been suggested that intact VLDL and VLDL phospholipid extracts were able to accelerate prothrombin conversion, although contamination of prothrombin by factor V may have contributed to this process.<sup>13</sup>

It is well established that hyperlipidemia is a risk factor for coronary heart disease (CHD) but the relationship between increased plasma lipoprotein concentrations and CHD is poorly defined. Given the role of phospholipids in thrombin generation we hypothesized that lipoprotein-supported thrombin generation provides a link between increased concentrations of atherogenic

shown that the phenomenon of lipoprotein-supported thrombin generation is mediated predominantly by specific phospholipids and is enhanced by oxidation of these phospholipids. The addition of vitamin E ( $\alpha$ -tocopherol) markedly reduced the increase in thrombin generation observed after oxidation of LDL ( $822 \pm 57$  v  $138 \pm 47$  nmol/L;  $P < .0001$ ). These effects suggest that lipoproteins are important in the production of thrombin and that vitamin E may confer protection from the detrimental effects of lipoprotein oxidation by limiting thrombin formation. These results suggest that atherogenic lipoproteins are linked to the development of atherosclerosis in part by their capacity to support thrombin generation.

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lipoproteins and CHD. There is indirect evidence supporting this notion because subjects with hyperlipidemia have increased concentrations of markers of thrombin generation<sup>14,15</sup> and treatment of hyperlipidemia with lipid-lowering drugs reduced levels of these markers<sup>14-16</sup> without necessarily affecting platelet function.<sup>17</sup> Thus, there is in vitro, pathological, and clinical evidence suggesting that cell-free plasma lipids and lipoproteins may be capable of supporting assembly of the prothrombinase complex and thrombin production. We have tested the ability of lipoprotein fractions to support thrombin generation and examined whether modification of lipoproteins by oxidation, nonenzymatic glycosylation, or vitamin E alters this function.

## MATERIALS AND METHODS

*Preparation of lipoprotein subclasses.* Lipoprotein preparation was undertaken as described previously.<sup>18</sup> Initially, experiments were undertaken in the presence of protease inhibitors, antibiotics, and an antioxidant to determine whether lipoprotein-supported thrombin generation was affected by these reagents. Omission of protease inhibitors and antioxidant did not alter lipoprotein-supported thrombin generation and therefore subsequent experiments were undertaken immediately after preparation of lipoproteins without addition of any of these reagents. Preparation of each lipoprotein class was undertaken by collection of venous blood from 12 healthy volunteers into EDTA (1.5 mg/mL of blood). Plasma was separated by centrifugation at  $1,750 \times g$  for 20 minutes and the density adjusted to 1.215 g/mL by adding KBr. After ultracentrifugation at  $235,000 \times g$  for 36 hours at  $4^\circ\text{C}$ , the lipoprotein fraction was removed. Purified lipoprotein fractions were obtained by separation through a Sepharose CL-6B gel filtration column (Sigma, St. Louis, MO), in phosphate-buffered saline (PBS), pH 7.2. Analysis of lipoprotein fractions collected from the gel filtration column by agarose gel electrophoresis (1% agarose, 0.4% bovine serum albumin (BSA), 50 mol/L sodium barbital, 1.9 mol/L EDTA, pH 8.6, at 220 V, 150 mA for 30 minutes) was undertaken to ensure that each fraction used in the thrombin generation assay contained only a single class of lipoprotein. Agarose gels were fixed by incubation with 5% trichloroacetic acid and dried at  $90^\circ\text{C}$  for 15 minutes. Lipoproteins were visualized by incubation with Sudan Black in 60% ethanol. Using the above technique, pure fractions of chylomicrons, VLDL, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) were obtained. The protein content of each lipoprotein fraction was measured by a modified Lowry method.<sup>19</sup> Cholesterol and triglyceride concentrations were measured by a standard enzymatic method and apo B concentrations measured by enzyme-linked immunosorbent assay (ELISA) with a

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polyclonal antibody apo B and apo B standard from The Binding Site (Birmingham, UK).<sup>20</sup>

**Quantitation of lipoprotein-supported thrombin generation.** A chromogenic assay to quantify thrombin generation by lipoproteins and platelets was developed based on established work.<sup>21,22</sup> Prothrombin, factor V, and factor Xa purified from human plasma were obtained from Diagnostica, Stago (Asnières, France). Each factor was reconstituted in 0.05% human albumin in phosphate-buffered saline (PBS), pH 7.2. Lipoprotein [5  $\mu$ L of 100  $\mu$ g/mL (apolipoprotein)] was added to 1  $\mu$ mol/L prothrombin, 1.5 nmol/L factor V, and 1 nmol/L factor Xa (final concentrations). After incubation for 15 minutes at 37°C, 2.5 mmol/L CaCl<sub>2</sub> in 6.25 mmol/L Tris HCl, 12.5 mmol/L NaCl, 0.0625% fatty acid-free BSA, pH 7.5 was added. After a further incubation for 25 minutes at 37°C, 10  $\mu$ L of this solution was added to tubes containing 465  $\mu$ L of buffer (50 mmol/L Tris HCl, 20 mmol/L EDTA, 0.1 mol/L NaCl, 0.5% fatty acid-free BSA at pH 7.5) and 25  $\mu$ L of 4 mmol/L S2238, a thrombin-specific chromogenic substrate (Kabi Diagnostica, Kabi Vitrum, Stockholm, Sweden). After 4 minutes the reaction was stopped by the addition of 300  $\mu$ L of 1 mol/L citric acid. Three hundred  $\mu$ L of the final reaction mixture was transferred to flat-bottomed 96-well microtitre plates (Nunc Maxisorp, Roskilde, Denmark) and the absorbance determined at 405 nm in an Anthos HTII, Anthos Labtec HT2 version 1.05 microtitre plate reader. The ability of each lipoprotein class to support thrombin generation was quantified separately for individual subjects and the results (mean  $\pm$  standard error of the mean [SEM]) were expressed as nmol/L thrombin, calculated from a standard curve generated with  $\alpha$  thrombin. The thrombin generation assay was also used to measure thrombin generation from unactivated platelets. Venous blood was collected into EDTA (1.5 mg/mL of blood) and centrifuged at 800 $\times$ g for 8 minutes at room temperature. Platelets were prepared by gel filtration (Sephacose CL-2B [Sigma] in PBS, pH 7.2) of platelet-rich plasma after the platelet count was measured with a Coulter STKS counter (Coulter, Hiale, FL). Thrombin generation of 1 $\times$ 10<sup>6</sup> platelets was quantified immediately as described for lipoproteins.

**Inhibition by annexin V.** Lipoproteins (5  $\mu$ L of 100  $\mu$ g/mL) were incubated with 5  $\mu$ mol/L annexin V at 37°C for 30 minutes. The thrombin generation assay was then undertaken as described above. Control experiments were undertaken by adding equal volumes of PBS instead of annexin V. The small volume of PBS used did not produce a calcium precipitate when added to the solution of coagulation factors and calcium chloride.

**Quantitation of phospholipid concentration and identification of lipoprotein phospholipids.** Phospholipid concentrations were measured using a commercial kit (Randox Laboratories Ltd, Crumlin, Co Antrim, UK). Phospholipids were analyzed by high performance thin-layer chromatography (HPTLC). Two hundred  $\mu$ g by total weight of each lipoprotein class was dried down under nitrogen. Lipoproteins were resuspended in 30  $\mu$ L chloroform and methanol (2:1) and analyzed by HPTLC (silica gel, 60 precoated plates; MERCK, Darmstadt, Germany); in methyl acetate:isopropanol:chloroform:methanol:0.25% KCl (25:25:25:10:9) polar solvent. Commercial phospholipid standards were obtained from Sigma. Lipids were stained with a solution containing 3% cupric acetate and 8% phosphoric acid. Phospholipid bands were photographed using an Eagle Eye II Still Video System (Stratagene, La Jolla, CA). The image was exported in a TIFF file and phospholipid bands were quantified using NIH Image 1.55 software for Macintosh<sup>23</sup>(Apple Computer, Cupertino, CA).

**Quantitation of phospholipid-supported thrombin generation.** To test each component of lipoproteins for thrombin generating capacity, the thrombin generation assay was modified and optimized for use in 96-well microtitre plates. Lipid or apolipoprotein was dried onto microtitre wells (PolySorp [lipid], MaxiSorp [protein] Nunc), blocked in 1% fatty acid-free BSA in PBS for 1 hour, and washed in PBS. The assay was performed in the microtitre plate with the same ratio of prothrombin, factor V, and factor Xa as described above. The factors were incubated for 15 minutes at 37°C before adding CaCl<sub>2</sub>. The plate

was then incubated for a further 60 minutes before subsampling into the substrate solution. After 8 minutes incubation the reaction was terminated and assessed as described above. The results are expressed as nmol/L thrombin, calculated from a standard curve generated with  $\alpha$  thrombin with the above conditions. In addition, the inhibitory action of annexin V on phospholipids was examined with 10  $\mu$ mol/L annexin V.

**Oxidation, glycosylation, and vitamin E treatment of lipoproteins and phospholipids.** Lipoproteins were modified by oxidation and nonenzymatic glycosylation. Oxidation of lipoprotein fractions was undertaken by incubation with 2.5  $\mu$ mol/L CuSO<sub>4</sub> (final concentration; Sigma) at 37°C for 17 to 20 hours.<sup>24</sup> Lipoprotein oxidation was checked by comparison of diene conjugates before and after incubation with CuSO<sub>4</sub><sup>25</sup> and by agarose gel electrophoresis. Oxidation was stopped by addition of 0.1 mol/L EDTA and refrigeration at 4°C. Lipoprotein fractions were also modified by nonenzymatic glycosylation with 5 mmol/L, 20 mmol/L, 50 mmol/L, and 100 mmol/L glucose by incubation for 3 days at 37°C.<sup>26</sup> After modification, the lipoprotein fractions were washed with PBS by centrifugation with Centricon 30 concentrators (Amicon, Beverly, MA) and were resuspended at 100  $\mu$ g/mL protein concentration. The effect of vitamin E on thrombin generation produced after copper oxidation of LDL was examined. LDL from 9 of the subjects was mixed and incubated with 850 IU vitamin E/mL [(+)- $\alpha$ -tocopherol, mixed isomers; Sigma] for 30 minutes. The samples were then copper oxidized as described above. Vitamin E and the copper-containing solution were removed by washing in centricons with several volumes of PBS.

Thrombin generation was also determined after copper-induced oxidation of the phospholipids. The phospholipids were dried onto the microtitre wells, then incubated for 17 to 20 hours with 2.5  $\mu$ mol/L CuSO<sub>4</sub>. The thrombin generation assay was then undertaken as described above.

**Statistics.** Lipoprotein and phospholipid-supported thrombin generation was normally distributed. Presented data of means  $\pm$ SEM and comparison of means was undertaken by unpaired Student's *t*-test.

## RESULTS

### Subject Characteristics

Twelve healthy Caucasian subjects (6 men and 6 women) were recruited, mean ( $\pm$ SD) age of subjects was 30.3 ( $\pm$ 5.4) years and mean body mass index [BMI] ( $\pm$ SD) was 22.0 ( $\pm$ 2.4) kg/m<sup>2</sup>. All subjects gave informed consent and underwent a fasting full lipid profile. Mean ( $\pm$ SD) values were total cholesterol 5.1 ( $\pm$ 0.9) mmol/L, triglyceride 1.0 ( $\pm$ 0.4) mmol/L, HDLc 1.5 ( $\pm$ 0.3) mmol/L and LDL 3.1 ( $\pm$ 0.9) mmol/L. On a different day, 60mL of blood were drawn from an antecubital vein at a standardized time, 2 hours after a light meal, to ensure that adequate amounts of chylomicrons and VLDL could be obtained. Plasma was immediately prepared by centrifugation, and chylomicrons, VLDL, LDL, and HDL separated by ultracentrifugation followed by gel filtration. Analysis of lipoprotein fractions collected from the gel filtration column by agarose gel electrophoresis was undertaken to ensure that each fraction used in the thrombin generation assay contained only a single class of lipoprotein. Fractions were also examined for platelet microparticle contamination with an ELISA for GPIIIa and no evidence of microparticle contamination was detectable in any of the fractions. Lipoproteins from the same subjects were isolated from a second plasma sample 6 months later and retested for their capacity to support thrombin generation. The results were consistent on both occasions.

### Optimization of Thrombin Generation Assay

The thrombin generating properties of chylomicrons, VLDL, LDL, and HDL lipoproteins were investigated. Purified components of the prothrombinase complex were used to quantify the ability of lipoproteins to support thrombin generation. Omission of any of these components abolished thrombin generation. The assay was designed so that thrombin generation was limited only by the components of the lipoprotein rather than any other constituent of the assay. The factors were added in excess, as was the substrate. In addition, prolonged incubation periods showed that the activity of the factors was not diminished during the assay. The assay was also optimized to allow near maximal thrombin generation for VLDL, whereas allowing the optical density to be read off the linear section of the thrombin standard curve. Thrombin generation was linear with time during incubation of factors with lipoproteins and calcium, and during substrate development and proportional to the amount of lipoprotein or phospholipid in the assay. The thrombin generation assay was standardized using human  $\alpha$  thrombin that had been active-site titrated. The standard curve was linear from 25 nmol/L to 1000 nmol/L thrombin generation. The limit of detection of the assay was 25 nmol/L. The interassay coefficient of variance (cv) was 14%,  $n = 10$  and the intra-assay cv was 14%,  $n = 10$ . The assay for phospholipids was optimized separately. The difference between drying the phospholipids in nitrogen gas or air was found to be negligible so all subsequent experiments were carried out in air. The specificity of prothrombinase complex formation on phospholipid components was studied. It has been shown previously that up to 59 annexin V molecules are able to bind to a single phospholipid molecule,<sup>27-29</sup> however it was anticipated that not all phospholipid bound to microtitre wells would be available for binding to annexin V, and therefore experiments were undertaken with 10  $\mu$ mol/L annexin V. We have shown that the chromogenic substrate is specific for thrombin by using the specific thrombin inhibitor hirudin, derived from *Hirudo medicinalis*, the medicinal leech. Hirudin is the most potent and specific inhibitor of thrombin known and incubation with hirudin reduced absorbances to those of the detection limit of the assay. Thus, a specific and sensitive method for thrombin generation quantitation was developed.

### Thrombin Generating Capacity of Lipoproteins and Effect of Annexin V Binding

Thrombin generation [per  $\mu$ g of (apolipo)protein] supported by VLDL was 19.4-fold greater than that supported by HDL,  $P < .00001$  (and 11.7-fold greater than that supported by LDL,  $P < .00001$ ). Levels of thrombin generation supported by LDL and HDL were not significantly different from each other. Annexin V reduced lipoprotein-supported thrombin generation and platelet-specific thrombin generation by similar amounts (Table 1) (45.7%,  $P = .002$  and 47.4%,  $P = .015$ , respectively). Annexin V is a member of a family of  $\text{Ca}^{2+}$ -dependent phospholipid-binding proteins. The exact prerequisites for binding are not fully established but binding is known to be strongly dependent on the presence of an sn-3 phosphate group and hydrophobic interaction with the sn-2 acyl chain of the phospholipid.<sup>30</sup> Therefore, the reduction in lipoprotein-supported thrombin

**Table 1. Effects of Annexin V and Modification by Oxidation and Glycosylation on Lipoprotein-supported Thrombin Generation**

Lipoprotein/ Platelets	Unmodified	Annexin V	Oxidation	Glycosylation
Thrombin (nmol/L) (mean $\pm$ SEM)				
Chylomicrons	292 $\pm$ 60*		224 $\pm$ 115	
VLDL	503 $\pm$ 58*	273 $\pm$ 73†	542 $\pm$ 91	499 $\pm$ 95
LDL	43 $\pm$ 12		526 $\pm$ 87†	94 $\pm$ 40
HDL	26 $\pm$ 11		77 $\pm$ 46	
Platelets	338 $\pm$ 50	178 $\pm$ 18§		

Thrombin generation supported by unmodified lipoproteins, lipoproteins modified by oxidation and nonenzymatic glycosylation, and lipoproteins incubated with annexin V. Experiments were undertaken with lipoproteins from 12 subjects (mean  $\pm$  SEM) containing 1  $\mu$ g of total (apolipo)protein or  $1 \times 10^6$  platelets in the absence and presence of annexin V. Lipoproteins were oxidized by incubation with 2.5  $\mu$ mol/L cupric sulfate. Of the lipoproteins, only VLDL was incubated with annexin V (5  $\mu$ mol/L). Results for nonenzymatic glycosylation of lipoproteins were obtained after incubation with 20 mmol/L glucose (nonenzymatic glycosylation was only undertaken with VLDL and LDL). Specific comparisons between mean thrombin concentrations were undertaken by Student's *t*-test.

\* $P < .00001$  for chylomicrons or VLDL versus HDL.

† $P < .0001$  for oxidized LDL versus unmodified LDL.

‡ $P = .002$  for VLDL with annexin V versus unmodified VLDL.

§ $P = .015$  for platelets with annexin V compared to platelets alone.

generation caused by annexin V strongly suggested that phospholipids are an important mediator of this phenomenon.

Because lipoproteins differ in both type and content of lipid and protein between classes, we explored whether observed differences in lipoprotein-supported thrombin generation could be explained by standardizing expression of thrombin generation to a different denominator [rather than per  $\mu$ g of total (apolipo)protein]. Because our results strongly suggested that phospholipids were an important mediator of this phenomenon we investigated whether variation in total amount of phospholipids within lipoproteins could account for the differences in lipoprotein-supported thrombin generation between lipoprotein classes. The concentration of phospholipid in each lipoprotein class was measured. Differences in amount of phospholipid did not explain the marked differences in lipoprotein-supported thrombin generation observed between lipoprotein classes. In a lipoprotein solution containing 100  $\mu$ g/mL (apolipo)protein, mean ( $\pm$ SD) concentrations of phospholipid were 117 ( $\pm$ 33)mg/dl for HDL, 98 ( $\pm$ 32)mg/dl for LDL, 152 ( $\pm$ 62)mg/dl for VLDL, 307 ( $\pm$ 131)mg/dl for chylomicrons. By using mg of phospholipid rather than  $\mu$ g of (apolipo)protein as the denominator in calculating thrombin generation for each lipoprotein class, VLDL-supported thrombin generation remained markedly different from both LDL- and HDL-supported thrombin generation. There was a 21-fold increase in thrombin generation supported by VLDL compared to HDL ( $P = 0.006$ ) and an 18-fold increase in thrombin generation supported by VLDL compared to LDL ( $P = .004$ ). In contrast to VLDL, the difference between chylomicron and HDL-supported thrombin generation became less marked and was reduced to fivefold greater thrombin generation supported by chylomicrons ( $P = .034$ ). As a further test of whether the concentration of phospholipid determined the level of lipoprotein-supported

thrombin generation, the amount of HDL in the thrombin generation assay was increased, by concentrating HDL threefold and sevenfold. No significant increase in thrombin generation was found.

#### Phospholipid Composition of Lipoproteins

Next, we analyzed the phospholipid components of each lipoprotein by HPTLC to determine if the presence of different phospholipids between lipoprotein classes might be responsible for the marked difference in lipoprotein-supported thrombin generation between classes. Similar percentages of each type of phospholipid were observed across lipoprotein classes. Typical values of individual phospholipids expressed as a percentage of total phospholipid extracted from the lipid core of a lipoprotein were: phosphatidyl choline, 46%; sphingomyelin, 12%; phosphatidyl serine, 5%; phosphatidyl ethanolamine, 12%; phosphatidyl inositol, 11%; and lysophosphatidyl choline, 14%. Sphingomyelin varied most in percentage content ( $\pm 5\%$ ) between lipoprotein classes.

#### Thrombin Generating Capacity of VLDL and LDL Lipoproteins Calculated With apo B100 Concentration as Denominator

Because neither the total amount nor the type of phospholipid could explain the marked differences in lipoprotein-supported thrombin generation between VLDL and HDL (or LDL), we excluded the possibility that the observed differences with VLDL were attributable to different numbers of VLDL and LDL particles in the thrombin generation assay. Therefore, we determined lipoprotein-supported thrombin generation results per ng of apo B100, for VLDL and LDL, because this represented an indirect measure of the amount of thrombin generated per lipoprotein particle (VLDL and LDL contain a single apo B100 molecule per particle). VLDL-supported thrombin generation was 32-fold greater than LDL-supported thrombin generation per ng of apo B100 ( $P < .001$ ). These data provided further support for the suggestion that the differences between lipoprotein classes were not an artifact resulting from the choice of denominator for expression of thrombin generation results.

#### Thrombin Generation Supported by Phospholipids

The magnitude of the difference in lipoprotein-supported thrombin generation between lipoprotein classes suggested that our findings had not occurred by chance. Confounding by amount and type of phospholipid or lipoprotein particle number was also excluded and therefore we investigated the mechanism of lipoprotein-supported thrombin generation by analyzing individual components of lipoproteins for thrombin generating capacity. Of the phospholipids present in each lipoprotein class, phosphatidyl inositol, phosphatidyl ethanolamine, and phosphatidyl serine provided the greatest support for assembly of the prothrombinase complex and thrombin generation and this phenomenon could be inhibited partially by annexin V (Table 2). Although sphingomyelin, lysophosphatidyl choline, and phosphatidyl choline were present in highest concentration in each lipoprotein class, thrombin generation supported by these phospholipids was at levels close to the limit of detection of the assay. Thrombin generation supported by sphingomyelin (685

**Table 2. Effects of Annexin V and Oxidation on Phospholipid-supported Thrombin Generation**

Lipid	Unmodified	Annexin V	Oxidation
Thrombin (nmol/L) (mean $\pm$ SEM)			
Phosphatidyl inositol (125 $\mu$ mol/L)	741 $\pm$ 130	259 $\pm$ 85†	
Phosphatidyl inositol (72 $\mu$ mol/L)	204 $\pm$ 86		382 $\pm$ 131†
Phosphatidyl ethanolamine (677 $\mu$ mol/L)	965 $\pm$ 61	460 $\pm$ 136‡	
Phosphatidyl ethanolamine (163 $\mu$ mol/L)	257 $\pm$ 108		478 $\pm$ 117‡
Phosphatidyl serine (412 $\mu$ mol/L)	973 $\pm$ 72	425 $\pm$ 147‡	
Phosphatidyl serine (270 $\mu$ mol/L)	221 $\pm$ 71		447 $\pm$ 158*
Phosphatidyl glycerol (487 $\mu$ mol/L)	806 $\pm$ 189	141 $\pm$ 56*	
Phosphatidyl glycerol (488 $\mu$ mol/L)	821 $\pm$ 95		552 $\pm$ 294

Thrombin generation supported by unmodified phospholipids, phospholipids incubated with annexin V, and phospholipids modified by oxidation. Phospholipid concentrations were chosen to ensure that either reductions (with annexin V) or increases (with oxidation) would be measurable on the thrombin generation standard curve. The presented results were obtained after incubation with 10  $\mu$ mol/L annexin V (similar results were obtained with 5  $\mu$ mol/L annexin V) and 2.5  $\mu$ mol/L cupric sulfate. Comparisons of mean thrombin concentrations, by Student's *t*-test, were undertaken for phospholipids: (1) incubated with or without annexin V or (2) incubated with and without cupric sulfate.

\* $P = .015$ .

† $P = .02$ .

‡ $P = .03$  (mean  $\pm$  SEM, all experiments were undertaken on three to eight separate occasions).

$\mu$ mol/L) was  $\leq 25$  nmol/L, lysophosphatidyl choline (1008  $\mu$ mol/L) was 55 nmol/L, and phosphatidyl choline (654  $\mu$ mol/L) was 38 nmol/L (results are means of two separate experiments). Other components of lipoproteins including cholesterol, triglyceride, apo B, and apo E were also examined for their capacity to support thrombin generation, but none of these components supported thrombin generation beyond the lower limit of detection of the assay. Thrombin generation supported by cholesterol (1292  $\mu$ mol/L), triglyceride (1293  $\mu$ mol/L), apo B (0.15  $\mu$ mol/L), and apo E (0.2  $\mu$ mol/L) was  $\leq 25$  nmol/L. A threefold increase in concentration of sphingomyelin, lysophosphatidyl choline, phosphatidyl choline, cholesterol, triglyceride, apo B, or apo E did not significantly increase thrombin generation.

#### Effect of Oxidation and Nonenzymatic Glycosylation on Lipoprotein-supported Thrombin Generation

The effect of modification of lipoproteins by oxidation and nonenzymatic glycosylation on lipoprotein-supported thrombin generation was then examined. Oxidation of LDL produced a 12-fold increase in lipoprotein-supported thrombin generation, compared to thrombin generation by unmodified LDL ( $P < .0001$ ). No significant increase in lipoprotein-supported thrombin generation was observed with oxidation of any of the other

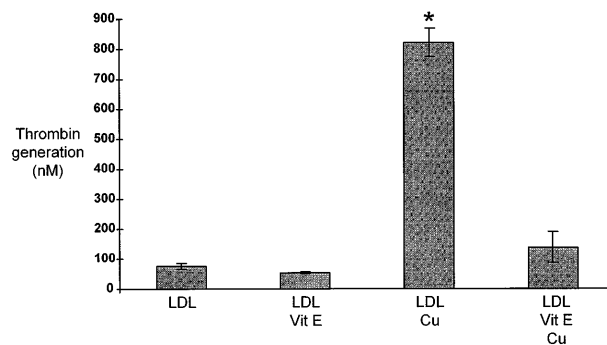
lipoprotein classes (Table 1). LDL and VLDL lipoproteins were then studied after modification by nonenzymatic glycosylation. A range of glucose concentrations were chosen from physiological (5 mmol/L) to pathological (100 mmol/L). Incubation of VLDL and LDL with glucose concentrations over this range of concentrations did not increase lipoprotein-supported thrombin generation.

#### *Effect of Oxidation on Thrombin Generating Capacity of Specific Phospholipids*

In view of the effect of oxidation on LDL-supported thrombin generation, we examined the effect of oxidation of individual phospholipids on phospholipid-supported thrombin generation. Phospholipids contained within lipoproteins, identified by HPTLC, were tested before and after modification by oxidation. A variety of phospholipids were examined for their capacity to support thrombin generation. Oxidation increased phospholipid-supported thrombin generation with phosphatidyl inositol, phosphatidyl ethanolamine, and phosphatidyl serine (Table 2). Another phospholipid, phosphatidyl glycerol, that was not detected in lipoproteins by HPTLC was also tested and oxidation did not increase phosphatidyl glycerol-supported thrombin generation. Both oxidation and nonenzymatic glycosylation of apo B100, apo E, cholesterol, and triglyceride did not significantly increase thrombin generation, with thrombin levels remaining close to the lower limit of detection of the assay. The ability of both lipoproteins and phospholipids to support thrombin generation was  $\text{Ca}^{2+}$ -dependent, and omission of any of the three coagulation factors reduced the signal to background levels confirming that all three coagulation factors are necessary for prothrombinase complex assembly.

#### *Effect of Vitamin E on Thrombin Generation Capacity of Oxidized Lipoproteins*

On a separate occasion, LDL from nine subjects was preincubated with vitamin E then subjected to copper oxidation. The LDL was washed and thrombin generating capacity was tested (Fig 1). In accordance with the results noted above, there was a dramatic increase in the thrombin generation potential when the LDL had been oxidized (unmodified LDL =  $76 \pm 12$  nmol/L  $\nu$  oxidized LDL =  $822 \pm 57$  nmol/L;  $P < .0001$ ). The effect of copper oxidation alone on lipoprotein-supported thrombin generation was markedly reduced by preincubating LDL with vitamin E before oxidation ( $138 \pm 47$  nmol/L  $\nu$   $822 \pm 57$ ;  $P < .0001$ ). Thrombin generation supported by LDL that had been incubated with vitamin E only was  $53 \pm 2$  nmol/L, whereas incubation with vitamin E without lipoprotein was below the limit of detection of the assay, suggesting that vitamin E and its solvent had little effect on baseline thrombin generation. The LDL samples were subsequently analyzed by agarose gel electrophoresis (Fig 2). Native LDL and LDL incubated with vitamin E migrated the least from the wells. In comparison, oxidized LDL migrated further down the gel. LDL that had been preincubated with vitamin E before oxidation migrated an intermediate distance. This result suggests a difference in charge between these forms of LDL with oxidation increasing net negative charge. In addition, the poorly defined bands in both the copper oxidized and copper oxidized after vitamin E preincubation fractions suggests heterogeneity within



**Fig 1.** The effect of vitamin E on thrombin generation supported by LDL. LDL from 9 subjects was incubated with or without vitamin E for 30 minutes before oxidation for 17 to 20 hours at 37°C. LDL with no additions and LDL incubated only with vitamin E were also prepared. Thrombin generation potential was quantified as described previously. The results are expressed as nmol/L thrombin, calculated from the standard curve generated with  $\alpha$  thrombin. \*Thrombin generation supported by oxidized LDL was increased compared to each of the other three groups (LDL + copper  $\nu$  LDL,  $P < .0001$ ; LDL + copper  $\nu$  LDL and vitamin E,  $P < .0001$ ; LDL + copper  $\nu$  LDL + vitamin E + copper,  $P < .0001$ ).

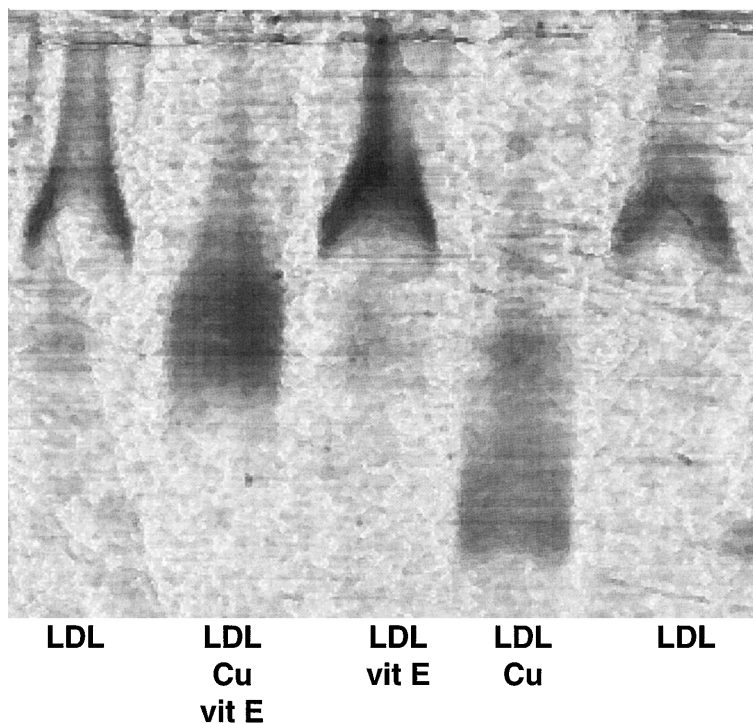
the oxidized species of LDL formed. Results obtained with the conjugate diene assay supported the observation that vitamin E diminished LDL oxidation. Thus, these results suggest that vitamin E prevents the copper-mediated increase in LDL-supported thrombin generation by decreasing LDL oxidation.

## DISCUSSION

The novel and potentially important findings of our study are that atherogenic lipoproteins have the capacity to support assembly of the prothrombinase complex and to generate thrombin. Thrombin generation supported by VLDL is significantly greater than for any other unmodified lipoprotein species and oxidation specifically increases the capacity of LDL to support thrombin generation. Vitamin E is able to attenuate the increased capacity of oxidized LDL to support thrombin generation and this result suggests a unique mechanism by which vitamin E may protect against the development of CHD.

Although it is well established that oxidized LDL is incorporated into an atheromatous plaque via the macrophage scavenger receptor,<sup>31,32</sup> the mechanisms linking VLDL and plaque formation are poorly understood. Our results suggest a unique association between VLDL, coagulation factors, and atherosclerosis. Furthermore, evidence is accumulating from in vitro, clinical, and epidemiological studies to support the notion that VLDL is an atherogenic lipoprotein particle. VLDL is retained in the intimal lining of arteries in humans<sup>33</sup> and in the arteries of genetically hyperlipidemic rabbits.<sup>34</sup> Subjects with type III hyperlipidemia (Frederickson classification) have increased plasma VLDL concentrations and an increased risk of CHD. Epidemiological studies show that fasting triglyceride levels are an important CHD risk factor<sup>35,36</sup> and in the fasting state VLDL is the major triglyceride-containing lipoprotein.

At present the explanation for the differences in thrombin generating capacity between lipoprotein classes is unknown. In this study, lipoprotein-supported thrombin generation was mediated predominantly by the presence of certain phospholipids.



**Fig 2.** Effect of vitamin E on the oxidation state of LDL. LDL from 9 subjects was incubated with or without vitamin E before copper oxidation. The samples were subjected to gel electrophoresis and stained with Sudan Black as described previously. This is a representative photograph showing unmodified LDL, LDL preincubated with vitamin E before copper oxidation, LDL incubated with vitamin E, oxidized LDL, and unmodified LDL.

Phosphatidyl inositol, phosphatidyl ethanolamine, and phosphatidyl serine supported thrombin generation, whereas thrombin generation supported by sphingomyelin, phosphatidyl choline, and lysophosphatidyl choline was close to the limit of detection of the assay. Recently the relative contribution of factor Va, prothrombin, and factor Xa in the generation of thrombin supported by phospholipid bilayers has been determined.<sup>37</sup> Thrombin generation increased with the amount of phosphatidyl serine in the phospholipid bilayer, with the concentration of factor Va, and with the prothrombin concentration. Incorporation of phosphatidyl ethanolamine into the phospholipid bilayer also increased the steady-state rate of thrombin production. Our results, however, show that the presence of phospholipid alone is not sufficient to explain the differences in thrombin generation supported by different lipoproteins. It is possible that specific phospholipids within these lipoprotein particles may be inaccessible and therefore are unable to bind components of the prothrombinase complex. Steric hindrance by apolipoproteins on the lipoprotein surface may prevent assembly of the prothrombinase complex on phospholipid surfaces in HDL and unmodified LDL. Alternatively, the differences in thrombin generating capacity between lipoproteins may be mediated by the presence of a certain protein(s) associated with the lipoproteins, such as tissue factor pathway inhibitor (TFPI), which is known to inhibit factor Xa activity.<sup>38,39</sup>

It is widely accepted that oxidized LDL is an atherogenic lipoprotein and is internalized into a developing atherosclerotic plaque via the macrophage scavenger receptor, creating a foam cell.<sup>31,32</sup> Oxidation may be caused by a number of initiators including copper or iron ions found in high concentrations in the plaque, perhaps associated with microthrombi.<sup>40</sup> Our data suggest a novel mechanism by which oxidized LDL participates in atherogenesis. Oxidation of LDL markedly increased throm-

bin generation (compared with native LDL) and it is possible that local production of thrombin in the vicinity of a developing atherosclerotic plaque increases accumulation of fibrin from proteolytic cleavage of fibrinogen. Interestingly, with respect to these results it has been shown that the lipid-rich region of the atheromatous plaque is also the most thrombogenic. This region of the plaque was approximately sixfold more thrombogenic than the sclerotic collagen-rich component.<sup>41</sup>

The mechanism by which oxidation of LDL caused such a marked increase in lipoprotein-supported thrombin generation is uncertain. It is possible that the increased negative charge of oxidized surface phospholipids mediates more efficient binding of the prothrombinase complex to these phospholipids with a subsequent increase in thrombin generation. Thus, phospholipids that are known to bind components of the prothrombinase complex relatively poorly, such as phosphatidyl ethanolamine, may be converted on oxidation into phospholipids with similar binding characteristics to anionic phospholipids, such as phosphatidyl serine, that are known to bind components of the prothrombinase complex more avidly. Alternatively, it is known that modification of LDL by oxidation produces fusion of lipoprotein particles,<sup>42</sup> similar to those found in atherosclerotic lesions, and fusion of several LDL particles may modulate the surface of accessible phospholipids for assembly of the prothrombinase complex.

Vitamin E, or  $\alpha$ -tocopherol, is the most abundant antioxidant in LDL and is probably located within the phospholipid bilayer. It converts the highly reactive peroxy free radical to the less damaging hydroperoxide, and thus presumably protects tissues and lipoproteins from oxidative damage. It is known that vitamin E delays oxidation of LDL in vitro and while taking oral supplements.<sup>43</sup> In addition, large prospective epidemiological studies<sup>44,45</sup> and a randomized control trial in subjects with

CHD<sup>46</sup> suggest that vitamin E is protective against CHD, although it is not known whether this effect is solely attributable to a reduction in macrophage uptake of LDL. Our results show that vitamin E dramatically reduced the vastly enhanced thrombin generation potential associated with oxidized LDL and suggest a novel mechanism by which vitamin E protects against development of CHD. This result suggests that the charge of a lipoprotein may be important in mediating the capacity to support thrombin generation because preincubation with vitamin E not only markedly reduced the capacity of LDL to support thrombin generation, but also partially protected the lipoprotein from oxidation.

This study was designed to define the thrombin generating capacity of different lipoprotein classes. Although the number of volunteers studied was too small to show significant differences between men and women or between subgroups of volunteers, there were indications that lipoprotein-supported thrombin generation differed in certain subjects compared to the mean thrombin generating capacity of the group as a whole. At present it is uncertain whether atherogenic lipoproteins from individuals with CHD, or individuals at increased risk of CHD, are capable of supporting increased thrombin generation.

Our results have raised several questions that now require addressing in other experimental models. Does the phenomenon of lipoprotein-supported thrombin generation:

(1) Explain the association between increased plasma triglyceride concentrations and a procoagulant state?

(2) Promote retention of both oxidized LDL and VLDL within atheromatous plaques; possibly mediated by thrombin providing the ligand for binding of both of these lipoproteins to either atherosclerotic lesions<sup>5</sup> or neointima<sup>6</sup> expressing the thrombin receptor?

(3) Explain some of the advantageous effects of vitamin E in the treatment of CHD?

In addition, it is necessary to elucidate the mechanisms by which VLDL and oxidized LDL support vastly increased amounts of thrombin generation compared with unmodified LDL and HDL. These results are potentially important because they may give insight into a novel mechanism by which lipoproteins and coagulation factors interact to cause atherosclerosis.

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#### REFERENCES

1. Miller GJ: Lipoproteins and the haemostatic system in atherothrombotic disorders. *Bailliere's Clin Haematol* 7:713, 1994
2. Fager G: Thrombin and proliferation of vascular smooth muscle cells. *Circ Res* 77:645, 1995
3. Wenzel UO, Fouqueray B, Grandaliano G, Kim YS, Karamitsos C, Valente AJ, Abboud HE: Thrombin regulates expression of monocyte chemoattractant protein-1 in vascular smooth muscle cells. *Circ Res* 77:503, 1995
4. Li X-N, Varma VK, Parks JM, Benza RL, Koons JC, Grammer JR, Grenett H, Tabengwa EM, Booyse FM: Thrombin decreases the

urokinase receptor and surface-localized fibrinolysis in cultured endothelial cells. *Arterioscler Thromb Vasc Biol* 15:410, 1995

5. Nelken NA, Soifer SJ, O'Keefe J, Vu TK, Charo IF, Coughlin SR: Thrombin receptor expression in normal and atherosclerotic human arteries. *J Clin Invest* 90:1614, 1992

6. Wilcox JN, Rodriguez J, Subramanian R, Ollerenshaw J, Zhong C, Hayzer DJ, Horaist C, Hanson SR, Lumsden A, Salam TA, Kelly AB, Harker LA, Runge M: Characterization of thrombin receptor expression during vascular lesion formation. *Circ Res* 75:1029, 1994

7. McVey JH: Tissue factor pathway. *Bailliere's Clin Haematol* 7:469, 1994

8. Papahadjopoulos D, Hanahan DJ: Observations on the interaction of phospholipids and certain clotting factors in prothrombin activator formation. *Biochem Biophys Acta* 90:436, 1964

9. Hemker HC, Esnouf MP, Hemker PW, Swart ACW, MacFarlane RG: Formation of the prothrombin converting activity. *Nature* 215:248, 1967

10. Govers-Riemslog JW, Janssen MP, Zwaal RF, Rosing J: Prothrombin activation on dioleoylphosphatidylcholine membranes. *Eur J Biochem* 220:131, 1994

11. Bradley WA, Song JN, Gianturco SH: Thrombin/prothrombin interactions with very low density lipoproteins. *Ann NY Acad Sci* 485:159, 1986

12. Bradley WA, Gianturco SH: Vitamin K-dependent proteins bind to very low-density lipoproteins. *Semin Thromb Hemost* 14:253, 1988

13. Bajaj SP, Harmony JAK, Martinez-Carrion M, Castellino FJ: Human plasma lipoproteins as accelerators of prothrombin activation. *J Biol Chem* 251:5233, 1976

14. Davi G, Ganci A, Averna M, Giammarresi C, Barbagallo C, Catalano I, Cala A, Notarbatolo A: Thromboxane biosynthesis, neutrophil and coagulative activation in type IIa hypercholesterolemia. *Thromb Haemost* 74:1015, 1995

15. Broijersén A, Hamsten A, Silveira A, Fatah K, Goodall AJ, Eriksson M, Angelin B, Hjendahl P: Gemfibrozil reduces thrombin generation in patients with combined hyperlipidaemia, without influencing plasma fibrinogen, fibrin gel structure or coagulation factor VII. *Thromb Haemost* 76:171, 1996

16. Wilkes HC, Meade TW, Barzegar S, Foley AJ, Hughes LO, Bauer KA, Rosenberg RD, Miller GJ: Gemfibrozil reduces plasma prothrombin fragment F<sub>1+2</sub> concentration, a marker of coagulability in patients with coronary heart disease. *Thromb Haemost* 67:503, 1992

17. Bröijersén A, Eriksson M, Leijd B, Angelin B, Hjendahl P: No influence of simvastatin treatment on platelet function *in vivo* in patients with hypercholesterolemia. *Arterioscler Thromb Vasc Biol* 17:273, 1997

18. Yokode M, Hammer RE, Ishibashi S, Brown MS, Goldstein JL: Diet-induced hypercholesterolaemia in mice-prevention by overexpression of LDL receptors. *Science* 250:1273, 1990

19. Schacterle GR, Pollack RL: A simplified method for the quantitative assay of small amounts of protein in biologic material. *Anal Biochem* 51:654, 1973

20. Byrne CD, Wang TWM, Hales CN: Control of Hep G2-cell triacylglycerol and apolipoprotein B synthesis and secretion by polyunsaturated non-esterified fatty acids and insulin. *Biochem J* 288:101, 1992

21. Hemker HC, Béguin S: Thrombin generation in plasma: its assessment via the endogenous thrombin potential. *Thromb Haemost* 74:134, 1995

22. Lee LH, Baglin T: Altered platelet phospholipid-dependent thrombin generation in thrombocytopenia and thrombocytosis. *Brit J Haematol* 89:131, 1995

23. Vecchini A, Chiaradia E, Covalovo S, Binaglia L: Quantitation of phospholipids on thin layer chromatographic plates using a desk-top scanner. *Mol Cell Biol* 145:25, 1995

24. Hoff HF, Whitaker TE, O'Neil J: Oxidation of low density

lipoprotein leads to particle aggregation and altered macrophage recognition. *J Biol Chem* 264:602, 1992

25. Wallin B, Rosengren B, Shertzer HG, Camejo G: Lipoprotein oxidation and measurement of thiobarbituric acid reacting substances formation in a single microtitre plate: its use for evaluation of antioxidants. *Anal Biochem* 208:10, 1993

26. Lyons TJ, Li W, WellsKnecht MC, Jokl R: Toxicity of mildly modified low-density lipoproteins to cultured retinal capillary endothelial cells and pericytes. *Diabetes* 43:1090, 1994

27. Pigault C, Folleniuswund A, Schmutz M, Freyssinet JM, Brisson A: Formation of two-dimensional arrays of annexin V on phosphatidylserine-containing liposomes. *J Mol Biol* 236:199, 1994

28. Andree HAM, Reutelingsperger CPM, Hauptmann R, Hemker HC, Hermens WT, Willems GM: Binding of vascular anticoagulant alpha (VACalpha) to planar phospholipid bilayers. *J Biol Chem* 265:4293, 1990

29. Meers P, Daleke D, Hong K, Papahadjopoulos D: Interactions of annexins with membrane phospholipids. *Biochemistry* 30:2903, 1991

30. Meers P, Mealy T: Phospholipid determinants for annexin V binding sites and the role of tryptophan 187. *Biochemistry* 33:5829, 1994

31. Steinberg D, Parathasarathy S, Carew TE, Khoo JC, Witztum JL: Beyond cholesterol: Modifications of low density lipoprotein that increase its atherogenicity. *N Engl J Med* 320:915, 1989

32. Witztum JL: The oxidation hypothesis of atherosclerosis. *Lancet* 344:793, 1994

33. Rapp JH, Lespine A, Hamilton RL, Colyvas N, Chaumeton AH, Tweedie-Hardman J, Kotite L, Kunitake ST, Havel RJ, Kane JP: Triglyceride-rich lipoproteins isolated by selected-affinity anti apolipoprotein B immunosorption from human atherosclerotic plaque. *Arterioscler Thromb* 14:1767, 1994

34. Nordestgaard, BG, Wootton R, Lewis B: Selective retention of VLDL, IDL and LDL in the arterial intima of genetically hyperlipidemic rabbits in vivo, molecular size as a determinant of fractional loss from the intima-inner media. *Arterioscler Thromb Vasc Biol* 15:534, 1995

35. Assman G, Schulte H, von Eckardstein A: Hypertriglyceridemia

and elevated lipoprotein(a) are risk factors for major coronary events in middle-aged men. *Am J Cardiol* 77:1179, 1996

36. Hokanson JE, Austin MA: Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: A meta-analysis of population-based prospective studies. *J Cardiovasc Risk* 3:213, 1996

37. Billy D, Willems GM, Hemker HC, Lindhout T: Prothrombin contributes to the assembly of the factor Va-factor Xa complex at phosphatidylserine-containing phospholipid membranes. *J Biol Chem* 270:26883, 1995

38. Hubbard AR, Jennings CA. Inhibition of the tissue factor-factor VII complex: Involvement of factor Xa and lipoproteins. *Thromb Res* 46:527, 1987

39. Sandset PM, Bendz B. Tissue factor pathway inhibitor: Clinical deficiency states. *Thromb Haemost* 78:467, 1997

40. Bruckdorfer KR: Oxidized lipoproteins. *Bailliere's Clin Endocrinol Metab* 9:721, 1995

41. Fernandez-Ortiz A, Badimon J, Falk E, Fuster V, Meyer B, Mailhac A, Weng D, Shah PK, Badimon L: Characterization of the relative thrombogenicity of atherosclerotic plaque components: Implications for consequences of plaque rupture. *J Am Coll Cardiol* 23:1562, 1994

42. Pentikäinen MO, Lehtonen EMP, Kovanen PT: Aggregation and fusion of modified low density lipoprotein. *J Lipid Res* 37:2638, 1996

43. Jessup W, Rankin SM, de Whalley CV, Hoult JRS, Scott J, Leake DS: Alpha-tocopherol consumption during low-density lipoprotein oxidation. *Biochem J* 265:399, 1990

44. Rimm ER, Stampfer MJ, Ascherio A, Giovannucci E, Colditz GA, Willett WC: Vitamin E consumption and the risk of coronary heart disease in men. *N Engl J Med* 328:1450, 1993

45. Stampfer MJ, Hennekens CH, Manson JE, Colditz GA, Rosner B, Willett WC: Vitamin E consumption and the risk of coronary heart disease in women. *N Engl J Med* 328:1444, 1993

46. Stephens NG, Parsons A, Schofield PM, Kelly F, Cheeseman K, Mitchinson MJ, Brown MJ: Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet* 347:781, 1996