Glycoxidized HDL, HDL enriched with oxidized phospholipids and HDL from diabetic patients inhibit platelet function

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ABSTRACT

Context: High-density lipoproteins (HDL) possess atheroprotective properties including anti-thrombotic and antioxidant effects. Very few studies relate to the functional effects of oxidized HDL on platelets in type 2 diabetes (T2D).

Objective: The objective of our study was to investigate the effects of in vitro glycoxidized HDL, and HDL from T2D patients on platelet aggregation and arachidonic acid signaling cascade. At the same time, the contents of hydroxylated fatty acids were assessed in HDL.

Results: Compared to control HDL, in vitro glycoxidized HDL had decreased proportions of linoleic (LA) and arachidonic (AA) acids in phospholipids and cholesteryl esters, and increased concentrations of hydroxy-octadecadienoic acids (9-HODE and 13-HODE) and 15-hydroxy-eicosatetraenoic acid (15-HETE), derived from LA and AA respectively, especially hydroxy derivatives esterified in phospholipids. Glycoxidized HDL dose-dependently decreased collagen-induced platelet aggregation by binding to SR-BI. Glycoxidized HDL prevented collagen-induced increased phosphorylation of platelet p38 MAPK and cytosolic phospholipase A₂, as well as intracellular calcium mobilization. HDL enriched with oxidized phospholipids, namely PC(16:0/13-HODE) dose-dependently inhibited platelet aggregation. Increased concentrations of 9-HODE, 13-HODE and 15-HETE in phospholipids (2.1, 2.1 and 2.4-fold increase respectively) were found in HDL from patients with T2D, and these HDL also inhibited platelet aggregation via SR-BI.

Conclusions: Altogether, our results indicate that in vitro glycoxidized HDL as well as HDL from T2D patients inhibit platelet aggregation, and suggest that oxidized LA-containing phospholipids may contribute to the anti-aggregatory effects of glycoxidized HDL and HDL from T2D patients.
Abbreviations:

AA, arachidonic acid; BHT, butylated hydroxytoluene; CE, cholesteryl esters; cPLA₂, cytosolic phospholipase A₂; DHA, docosahexaenoic acid; DMA, dimethylacetal; EPA, eicosapentaenoic acid; HDL, high-density lipoproteins; HEDE, hydroxy-eicosadienoic acid; HETE, hydroxy-eicosatetraenoic acid; HODE, hydroxy-octadecadienoic acid; LA, linoleic acid; LDL, low-density lipoproteins; MDA, malondialdehyde; NaBH₄, sodium borohydride; PC, phosphatidylethanolamine; PL, phospholipids; PRP, platelet-rich plasma; PUFA, polyunsaturated fatty acids; SR-BI, scavenger receptor BI; TAG, triacylglycerols; TBA, thiobarbituric acid; TLC, thin-layer chromatography; TxA₂, thromboxane A₂; T2D, type 2 diabetes.
INTRODUCTION

There is growing evidence that HDL composition determines its functional properties besides the levels of HDL cholesterol (1). HDL are highly heterogeneous particles consisting of two hundred individual molecular lipid species and a hundred of proteins (2,3). Amongst lipids, the presence of oxidizable polyunsaturated fatty acids (PUFAs) substrates renders these particles sensitive to oxidative attack. In particular, HDL are the major carriers of primary and terminal end-products of non-enzymatic lipid peroxidation such as lipid hydroperoxides (4) and isoprostanes (5) in human blood plasma. HDL also remove seeding molecules from LDL such as hydroperoxy-octadecadienoic and hydroperoxy-eicosatetraenoic acids (6). Oxidatively modified HDL have been detected \textit{in vivo} (7) in the intima of atherosclerotic plaques in human abdominal aortae (8) and in patients with type 2 diabetes (T2D) (9), however the molecular mechanisms involved were not investigated compared to the extensive studies on oxidized LDL in atherogenesis. Functional properties of oxidized HDL in T2D, a disease associated with increased risk for atherothrombosis and oxidative stress, have been investigated but gave contradictory results. Most studies showed that HDL might lose their protective properties in T2D (10,11) while other studies provided evidence for beneficial effects such as increased cholesterol efflux from macrophages in patients with T2D (12,13). Studies on the effects of oxidatively modified HDL on platelet functions are few and contradictory, reporting either a stimulation (14) or inhibition (15) of platelet aggregation by \textit{in vitro} oxidized HDL. To clarify this issue in the frame of T2D, we undertook two approaches. On one hand, HDL from control subjects were modified \textit{in vitro} by glycoxidation in order to mimic changes occurring in HDL particles of T2D patients, and their effects on platelet activation were determined and compared to those induced by unmodified HDL. On the other hand, HDL from T2D patients were compared to HDL from healthy control subjects as far as platelet aggregation was concerned. We hypothesized that some elective lipid peroxides in HDL phospholipids (PL) might exert anti-aggregating properties.
SUBJECTS AND METHODS

Study subjects
Eight T2D patients (5 men and 3 women, aged 66 ± 3.5 years) and eight healthy subjects (6 men and 2 women, aged 25 ± 2.9 years) were included. The patients had poorly controlled diabetes (fasting glycemia: 8.7 ± 1.4 mmol/L; glycated hemoglobin HbA1C: 8.3 ± 0.6%, 67 mmol/mol). They had mild hypertriglyceridemia (triglycerides: 1.9 ± 0.4 mmol/L), normal LDL-cholesterol (2.4 ± 0.2 mmol/L) and low HDL-cholesterol (1.1 ± 0.1 mmol/L). The patients were recruited at the Department of Endocrinology and Metabolic Diseases, Cardiovascular Hospital, Lyon Bron. The protocol was approved by the Local Ethics Committee (CPP Sud-Est IV, Hospices Civils de Lyon) and the study was conducted in accordance with the principles of the Helsinki declaration. Written informed consent was obtained from each of the participants.

Isolation of HDL by ultracentrifugation
Blood was collected on EDTA and HDL were immediately isolated from plasma by potassium bromide stepwise ultracentrifugation (16). HDL were extensively dialyzed against PBS (pH 7.35) in the presence of 1 mmol/L EDTA. The concentration of proteins was estimated using a Lowry assay (17).

Preparation of HDL modified by glycoxidation
Glycoxidized HDL consisted of HDL incubated with 50 mmol/L glucose for 5 days at 37°C, dialyzed in PBS to remove excess glucose, and treated with 5 μmol/L CuCl2 for 1 day at 37°C. Control HDL were prepared by incubating native HDL in the presence of butylated hydroxytoluene (BHT) (5μmol/L) and EDTA (1mmol/L) for 6 days at 37°C. All HDL were finally dialyzed against EDTA-free PBS just before their interaction with platelets.

Platelet isolation
Venous blood was collected on citrate-phosphate-dextrose (19.6 mmol/L citric acid, 89.4 mmol/L sodium citrate, 16.1 mmol/L NaH2PO4, 128.7 mmol/L dextrose, pH 5.6) from healthy volunteers who
had not ingested any aspirin or anti-inflammatory drugs in the previous ten days. Platelet-rich plasma (PRP) was prepared by centrifugation of the blood at 200g for 15 min at 20°C. Platelets were isolated as previously described (18).

**Synthesis of 1-palmitoyl, 2-(13-hydroxy-octadecadienoyl)-sn-glycero-3-phosphocholine**

1-palmitoyl,2-(13(S)-hydroperoxy-octadecadienoyl)-sn-glycero-3-phosphocholine (PC(16:0/13(S)-HpODE) was enzymatically synthesized from 1-palmitoyl,2-linoleoyl-sn-glycero-3-phosphocholine (PLPC or PC(16:0/18:2), Cayman Chemicals, Ann Arbor, MI, USA) following incubation of PC(16:0/18:2) with soybean 15-lipoxygenase (type V) in the presence of deoxycholate for 30 min under a continuous flux of oxygen (19). Phosphatidylcholine hydroperoxide formed was reduced by sodium borohydride (NaBH₄) into phosphatidylcholine hydroxide. 1-palmitoyl,2-(13(S)-hydroxy-octadecadienoyl)-sn-glycero-3-phosphocholine (PC(16:0/13-HODE)) concentration was determined spectrophotometrically by UV absorbance at 235 nm and its purity was checked by HPLC.

**Synthesis of 1-palmitoyl,2-(15(S)-hydroxy-eicosadienoyl)-sn-glycero-3-phosphocholine**

Firstly, 1-palmitoyl,2-eicosadienoyl-sn-glycero-3-phosphocholine PC(16:0/20:2n-6) was chemically synthesized. To a solution of 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (0.2 mmol), eicosa-11,14-dienoic acid (20:2n-6) (0.08 mmol) was added in anhydrous chloroform (1 mL). A solution of freshly recrystallized 4-Pyrrolidinopyridine (PPyr) (20) (0.09 mmol) and dicyclohexylcarbodiimide (DCC) (0.09 mmol) in 1 mL of chloroform was then added drop wise (21). After 40 h, the reaction mixture was concentrated and the product was then purified by flash chromatography on silica gel (chloroform/methanol/H₂O, 65:35:4, by vol.). Fractions containing the product were further purified by ion exchange chromatography using Amberlyst resin (chloroform/methanol/H₂O, 65:35:4, by vol.) to give PC(16:0/20:2). 1-palmitoyl,2-(15(S)-hydroperoxy-eicosadienoyl)-sn-glycero-3-phosphocholine (PC(16:0/15(S)-HpEDE) was then enzymatically synthesized from PC(16:0/20:2) as described above and reduced into PC(16:0/15(S)-HEDE), and used as an internal standard in relevant analyses.
**In vitro enrichment of HDL with oxidized phospholipids**

To prepare HDL enriched with oxidized PL, native HDL were incubated with dried PC(16:0/13-HODE) for 24 hours at 37°C in a shaking bath. Unbound PL were removed by dialysis in PBS and using PD-10 desalting columns. Similar procedure was applied to prepare HDL enriched with PC(16:0/18:2).

**Characterization of HDL**

**Fatty acid compositions of lipid classes**

Fatty acid methyl esters and fatty dimethylacetals were analyzed by gas chromatography as detailed in the Supplemental Methods.

**Quantification of monohydroxylated fatty acids**

Hydroxylated fatty acids were separated and quantified by reverse-phase HPLC according to the amount of appropriate internal standards, PC(16:0/15-HEDE) and 15-HEDE. For the detailed procedure, please refer to Supplemental Methods.

**Stereochemical analysis of hydroxylated fatty acids**

Optical isomers of HODE and HETE were separated by chiral phase HPLC as detailed in the Supplemental Methods.

**Malondialdehyde (MDA) determination**

Overall lipid peroxidation was assessed by quantitation of thiobarbituric acid (TBA)-MDA adducts by reverse-phase HPLC with fluorimetric detection. For details, please refer to Supplemental Methods.

**Vitamin E determination**

Tocopherol isomers were separated by reverse-phase HPLC and measured fluorimetrically containing tocol as an internal standard. For details, please refer to Supplemental Methods.
Platelet aggregation

Aggregation was measured in isolated platelets in a Chrono-log dual-channel aggregometer (Coulter, Margency, France) according to the method of Born (22). Platelet suspensions were pre-incubated for 5 min at 37°C in the presence or absence of different preparations of HDL and then stimulated with threshold concentrations of collagen (Nycomed, Linz, Austria) with continuous stirring at 1000 rpm. The threshold concentration of collagen was defined as the concentration of collagen that induced approximately a 60% increase in light transmission. The extent of platelet aggregation was expressed in terms of percentage of change in light transmission 4 min after the addition of collagen.

Platelet p38 MAPK and cytosolic phospholipase A2 activation

Chemiluminescent Western blotting detection of p38 MAPK, phospho-p38 MAPK and phospho-cPLA2 was performed as described in the Supplemental Methods.

Determination of intracellular Ca^{2+} concentrations

Intracellular calcium concentrations were measured fluorimetrically in Fura-2-loaded platelets as described in the Supplemental Methods.

Statistical analysis

Results are expressed as the means ± SEM. Comparisons between groups were performed using paired Student t-test.

RESULTS

Characterization of glycoxidized HDL compared to control HDL

Fatty acid composition of phospholipids and cholesteryl esters (CE)

Following in vitro glycoxidation, proportions of the main PUFAs, linoleic (LA, 18:2n-6) and arachidonic (AA, 20:4n-6) acids, significantly decreased in PL and CE of glycoxidized HDL compared to control HDL (Table). In PL from glycoxidized HDL, the proportions of LA, AA,
eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids decreased by 54%, 91%, 56% and 91% respectively. The proportions of 16:0 dimethylacetals (DMA), 18:0 DMA and 18:1 n-9 DMA issued from the alkenyl residue of alkenyl,acyl-glycero-phosphoethanolamine (ethanolamine plasmalogens) decreased in PL from glycoxidized HDL compared to those from control HDL. By contrast, proportions of palmitic (16:0), stearic (18:0) and oleic (18:1n-9) acids increased in modified HDL. In CE, glycoxidation of HDL also led to decreased proportions of LA, AA and EPA, by 53%, 92% and 86% respectively, and increased proportions of palmitic, stearic and oleic acids.

Vitamin E concentration
The concentrations of alpha-tocopherol strongly decreased in glycoxidized HDL compared to control HDL (0 in glycoxidized HDL vs. 2.04 ± 0.28 nmol/mg HDL protein in control HDL, n=5, P<0.001). Gamma-tocopherol concentrations decreased by 75% in glycoxidized HDL (0.08 ± 0.01 in glycoxidized HDL vs. 0.32 ± 0.02 nmol/mg HDL protein in control HDL, n=5, P<0.001).

MDA concentration
The concentrations of MDA, a marker of overall lipid peroxidation, were more than 10-fold higher in glycoxidized HDL compared to control HDL (1.93 ± 0.32 nmol/mg protein in glycoxidized HDL vs. 0.18 ± 0.13 nmol/mg protein in control HDL, n=4, P<0.01).

Concentrations of hydroxylated fatty acids in lipid classes
The concentrations of the stable primary products of PUFA peroxidation, 13-HODE and 9-HODE, derived from LA, and 15-HETE, derived from AA, were assessed in HDL. In control HDL, most hydroxylated fatty acids were present in CE, followed by PL and TAG, while most of them were present in PL of glycoxidized HDL (Figure 1). HODE concentrations increased strongly in PL of glycoxidized HDL compared to those of unmodified HDL (Figures 1A and 1B). 13-HODE concentrations tended to increase (1.8-fold) and 9-HODE concentrations significantly increased in CE from glycoxidized HDL. In TAG, 13-HODE concentrations increased in glycoxidized HDL (2.7-fold) and 9-HODE concentrations tended to increase (1.8-fold) compared to control HDL. 15-HETE
concentration increased in PL from glycoxidized HDL (8.5-fold) while 15-HETE concentration tended to increase in CE and TAG from glycoxidized HDL compared to control HDL (Figure 1C).

**Effects of control and glycoxidized HDL on platelet activation**

Compared to platelets incubated with collagen, pre-incubation of platelets with glycoxidized HDL for 5 min at 37°C resulted in a dose-dependent inhibition of collagen-induced platelet aggregation with near complete inhibition at 100 µg/mL (Figure 1D). Control HDL had no effect at 25 µg/mL but significantly inhibited collagen-induced platelet aggregation at concentrations greater than or equal to 50 µg/mL. Because scavenger receptor SR-BI binds modified HDL and is expressed in platelets (23), its involvement in the mechanism of action of HDL on these cells was sought. Pre-incubation of platelets with anti-SR-BI blocking antibody for 5 min at 37°C fully prevented the inhibitory effects of glycoxidized HDL on collagen-induced platelet aggregation (Figure 1D). Pre-incubation of platelets with anti-SR-BI also prevented the inhibition of collagen-induced platelet aggregation in presence of control HDL (aggregation rate: 65% in platelets incubated with anti-SR-BI antibody and 100 µg/ml control HDL vs. 12% in platelets incubated with 100 µg/ml control HDL). Anti-SR-BI blocking antibody had no effect on collagen-induced platelet aggregation, and pre-incubation with non-immune isotype control antibody did not reduce the inhibitory effect of glycoxidized HDL on platelet aggregation (data not shown).

To determine the effects of glycoxidized HDL on key enzymes involved in the release of AA from membrane PL, phosphorylation of p38 MAPK and cPLA₂ was determined in platelet suspensions incubated in the absence or presence of HDL for 5 min and further stimulated with collagen for 4 min. As shown in Figures 2A and 2B, addition of collagen to platelets resulted in increased amounts of phosphorylated p38 MAPK and cPLA₂. Pre-incubation of collagen-stimulated platelets with glycoxidized HDL fully prevented the collagen-induced increased phosphorylation of both enzymes.

Since Ca²⁺ is a key second messenger downstream of most signaling pathways and is essential for the translocation of cPLA₂ to membranes, the effects of control and glycoxidized HDL on the kinetics of collagen-induced Ca²⁺ release were determined (Figure 2C). Addition of collagen to Fura2-AM loaded platelets resulted in a rapid and transient increase in intracellular Ca²⁺ levels. Glycoxidized HDL
inhibited collagen-induced intracellular Ca\(^{2+}\) increase in platelets by 47% while control HDL
decreased it by 32%. Pre-incubation of platelets with anti-SRBI blocking antibody for 5 min at 37°C
alleviated the inhibitory effects of glycoxidized HDL on phosphorylation of p38 MAPK and cPLA\(_2\),
and Ca\(^{2+}\) mobilization (data not shown).

**Effects of HDL enriched with 1-palmitoyl,2-(13-hydroxy-octadecadienoyl)-sn-glycero-3-
phosphocholine on platelet aggregation**

To determine the role of oxidized LA-containing PL for anti-aggregating effects of HDL, HDL were
pre-loaded with two different concentrations of PC(16:0/13-HODE) prepared by 15-lipoxygenation of
LA esterified to phosphatidylcholine, followed by chemical reduction with NaBH\(_4\) (Figure 3A). As
expected, higher concentrations of 13-HODE, close to those found in glycoxidized HDL, were present
in enriched HDL compared to control HDL, while concentrations of 9-HODE were similar in control
and enriched HDL, confirming the absence of non enzymatic lipid peroxidation during the preparation
of enriched HDL particles. As shown in Figure 3B, HDL enriched with PC(16:0/13-HODE) inhibited
collagen-induced platelet aggregation. The more HDL were enriched with PC(16:0/13-HODE), the
more platelet aggregation was inhibited. HDL enriched with native PC(16:0/18:2) had no significant
effect on collagen-induced platelet aggregation compared to platelets incubated with control HDL and
stimulated with collagen (data not shown).

**Effects of glycoxidized HDL obtained from patients with T2D on platelet aggregation**

The concentrations of hydroxylated fatty acids were assessed in HDL PL from poorly controlled T2D
patients and control subjects. 13-HODE and 9-HODE concentrations were two-fold higher in HDL PL
from T2D patients compared to those in control subjects. 15-HETE concentration was 2.4-fold higher
in patient HDL PL compared to its concentration in control HDL (Figure 4A). To determine whether
hydroxylated fatty acid products originated from enzymatic or non enzymatic lipid peroxidation, chiral
phase HPLC was carried out on each isomer. Concerning hydroxylated linoleic acid metabolites, 9(R)-
HODE and 9(S)-HODE enantiomers were equally present in HDL from T2D patients (51% 9(S)-
HODE and 49% 9(R)-HODE) suggesting that the great majority of 9-HODE originated from non-
enzymatic lipid peroxidation. 13-HODE consisted of 78% S isomer and 22% R isomer indicating the involvement of enzymatic lipid peroxidation, presumably by 15-/ω6-lipoxygenase, together with non-enzymatic lipid peroxidation in the formation of 13-HODE. 15-HETE comprised 84% S and 16% R isomer suggesting again the involvement of 15-lipoxygenase in the oxidation of AA. By comparison, unmodified HDL from control healthy subjects contained equal ratios of 9(R/S)-HODE, 60% S isomer and 40% R isomer for 13-HODE and 15-HETE, suggesting that the lipoxygenation was a minor process compared to what occurred in T2D.

The pre-incubation of platelets with HDL from T2D patients resulted in an inhibition of collagen-induced platelet aggregation compared with control platelets incubated with collagen (Figure 4B). The inhibitory effect of patients HDL on collagen-induced platelet aggregation was stronger than the one induced by control HDL because 50 µg/mL patients HDL inhibited collagen-induced platelet aggregation by 63% whereas 50 µg/mL control HDL inhibited it by 28%. To establish the role of oxidized PL in the anti-aggregating effects of HDL, HDL were enriched with PC(16:0/13-HODE) to obtain similar amounts of hydroxylated fatty acid as those found in T2D HDL (Figure 4C). PC(16:0/13-HODE) enriched HDL inhibited collagen-induced platelet aggregation to a similar extent as patient HDL (Figure 4D).

**DISCUSSION**

The present results indicate that HDL modified by glycoxidation inhibited platelet aggregation via SR-BI in a dose-dependent manner and displayed higher anti-aggregatory potency than control HDL. Regarding the platelet signaling cascade involved in the mechanism of action of HDL, we present new data showing that in vitro glyoxidized HDL prevented both the collagen-induced increased phosphorylation of p38 MAPK, the stress kinase responsible for the phosphorylation of cPLA₂ (24) and that of cPLA₂, the key enzyme involved in the release of AA from membrane PL (25) which constitutes a rate-limiting step in the biosynthesis of biologically active eicosanoids. Supporting our in vitro results, we show for the first time that HDL from T2D patients also inhibited platelet aggregation via SR-BI at concentrations as low as 50 µg/mL. Our results are in line with our previous results reporting anti-aggregatory properties of severely oxidized HDL isolated from patients with
abetalipoproteinemia, an orphan metabolic disease characterized by the absence of apolipoprotein B-containing lipoproteins (26), and extend them in T2D, a common and growing disease known to be associated with chronic oxidative stress (27). T2D patients, with (28) or without cardiovascular complications (29), show platelet hyperactivation evidenced by increased platelet adhesion and aggregation as well as increased thromboxane $A_2$ production. Oxidatively modified lipoproteins present in plasma from T2D patients may represent important contributing factors modulating platelet activation, as shown with oxidized LDL from T2D patients which activate platelets (30). Decreased plasma levels of HDL in T2D patients lead to lower levels of oxidized HDL, and it is likely that the anti-aggregatory effects of HDL from T2D may be reduced and might indirectly contribute to platelet hyperactivation in T2D. So far, few studies have investigated the effects of oxidized HDL on platelet function. While it has been shown that hypochlorite-oxidized HDL may stimulate platelet aggregation via CD36 (31), HDL oxidatively modified by copper sulfate or myeloperoxidase strongly inhibit agonist-induced platelet activation and aggregation via SR-BI (15). Besides effects of HDL on platelet function, oxidative modification of HDL (as a result of in vitro oxidation or diabetes) may decrease cholesterol efflux capacity (32,33), impair their capacity to protect LDL from oxidation (10) and alter their anti-inflammatory properties (34). Nevertheless, a few studies reported beneficial effects of HDL modified by oxidative tyrosylation on the efflux of cholesterol in fibroblasts and macrophages (35). Altogether, these discrepant results might be related to differences in the extent and type of HDL oxidation which may determine the binding of HDL to either SR-BI or CD36 platelet receptors and the capacity of HDL to inhibit or stimulate platelet aggregation by binding either to SR-BI or CD36 respectively (36). Our results are also in agreement with studies demonstrating that copper-oxidized HDL showed better binding to platelet SR-BI than native HDL (15), and showing that oxidized HDL were more effective competitors than native HDL in cultured endothelial cells (8). In addition, it has been reported that the abundance of human SR-BI was reduced on the surface of platelets from patients with atherosclerotic disease and that its expression level correlated negatively with platelet aggregation (23), which might impair the anti-aggregatory properties of HDL in T2D patients.
We also establish the contribution of PL esterified with oxidized LA, especially PC(16:0/13-HODE), in the anti-aggregatory properties of glycoxidized HDL. First, HODEs and 15-HETE were strongly increased in PL from *in vitro* glycoxidized HDL. It was associated with decreased proportions of LA and AA in this class of lipids, increased MDA levels and vitamin E consumption. Increased concentrations of HODE and 15-HETE, originating from both enzymatic and non enzymatic lipid peroxidation, were also observed in HDL PL from T2D patients compared to control HDL. It should be underlined that the concentrations of HODEs and HETE in HDL PL were negatively associated with collagen-induced platelet aggregation ($r = -0.65$, $P = 0.004$). This confirms PL esterified with oxidized LA as important biologically active components supporting protective effects of HDL against platelet aggregation. Second, *in vitro* enrichment of HDL in phosphatidylcholine carrying out 13(S)-HODE inhibited platelet aggregation whereas enrichment of HDL with non-oxidized phosphatidylcholine had no significant effect on platelet aggregation. Similar increases of 13-HODE, 9-HODE and 15-HETE have been recently described in HDL total lipids from patients with diabetes compared with those from patients without diabetes (37) but this is the first evidence of increased content of hydroxylated fatty acids in the phospholipid class of HDL from T2D patients correlated with atheroprotective activities of HDL. Moreover, the association of higher levels of oxidized PL in HDL and lower response to aggregation are in line with the Cardiovascular Risk in Young Finns study suggesting that an elevated cardiovascular risk profile was associated with lower oxidized HDL lipids levels (38). Due to their localization at the surface of HDL particles, it is conceivable that PL are more accessible targets of free radicals compared to CE and TAG localized in the core of HDL, and are more likely to interfere with platelets following SR-BI interaction. In addition, the structure of lipoproteins in the physiological conditions might increase the preferential oxidation of linoleate into HODE over cholesterol as shown in human plasma (39). A limitation of our study is that lipidomic analyses were limited to HODE and HETE species, including their stereo-isomers. The contribution of other oxidized lipid species should be explored in order to decipher the molecules involved in the protective role of glycoxidized HDL on platelet aggregation, although inhibition of platelet aggregation by exogenous PC(16:0/13-HODE) was very similar to that observed by endogenous ones in glycoxidized HDL as well as in HDL from DT2 patients.
We conclude from our experiments that, as far as platelet aggregation is concerned, the ability of HDL to decrease platelet aggregation is not impaired in glycoxidized HDL, but even increased compared to control HDL. Supporting our in vitro results, we also show that glycoxidized HDL from T2D patients retain their anti-aggregating properties. We show that the HDL content of hydroxylated fatty acids esterified in PL could contribute to the inhibitory effects of glycoxidized HDL on platelet aggregation and associated signaling pathways through SR-BI (Figure 5). Our consistent findings challenge the concept of the systematic detrimental effects of oxidized lipids on atherothrombosis.

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REFERENCES


Table. Fatty acid composition of phospholipids and cholesteryl esters in control and glycoxidized HDL.

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Results, expressed as mol % of main fatty acids, are means ± SEM of 5 independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001 vs. control HDL. Dimethylacetal (DMA) sum corresponds to the sum of 16:0 DMA, 18:0 DMA and 18:1 n-9 DMA.
Figure 1. Hydroxylated fatty acids in control and glycoxidized HDL. Effects of respective HDL on collagen-induced platelet aggregation. Concentrations of 13-HODE (A), 9-HODE (B), 15-HETE (C) in control and glycoxidized HDL. Results are the means ± SEM of 5 independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001 vs. control HDL. (D) Platelet aggregation monitored in platelets isolated from healthy donors, following pre-incubation for 5 min at 37°C in the absence or presence of anti-SR-BI antibody, incubation with either control or glycoxidized HDL (25, 50 or 100 µg/mL) for 5 min at 37°C, and stimulation with collagen (2.5-5 µg/mL). Results are the means ± SEM of up to 10 experiments performed with 10 independent preparations of HDL and 10 independent platelet suspensions. Asterisks indicate significant differences (*, P<0.05; **, P <0.01; ***, P<0.001). NS, not significant.

Figure 2. Effects of glycoxidized HDL on platelet p38 MAPK and cPLA2 phosphorylation, and on collagen-induced increases of calcium concentrations in platelets. Phosphorylation levels of p38 MAPK (A) and cPLA2 (B) in platelets pre-incubated for 5 min at 37°C in the absence or presence of anti-SR-BI blocking antibody, then incubated with control or glycoxidized HDL (either 25 or 50 µg/mL) for 5 min at 37°C, and stimulated with collagen (2.5-5 µg/mL). Results, expressed as percentages of control, represent the means ± SEM of 5 independent experiments. Asterisks indicate significant differences (*, P<0.05; **, P <0.01; ***, P<0.001). NS, not significant. (C) Intracellular concentrations of calcium in Fura 2-AM loaded platelets pre-incubated in the absence or presence of 50 µg/mL control or glycoxidized HDL for 5 min at 37°C and stimulated with collagen (2.5-5 µg/mL). Tracings are representative of 4 independent experiments. a, P < 0.001 vs. (platelets + collagen) ; b, P < 0.05 vs. (platelets + control HDL + collagen).

Figure 3. Effects of HDL enriched with oxidized phosphatidylcholine on collagen-induced platelet aggregation. (A) Concentrations of HODE in HDL samples enriched or not with [PC(16:0/13-HODE)] or 2x [PC(16:0/13-HODE)]. **, P < 0.01 HDL enriched with PC(16:0/13-
HODE) vs. control HDL. (B) Collagen-induced aggregation obtained in platelets pre-incubated for 5 min at 37°C in the absence (control HDL) or presence of HDL (50µg/mL) enriched with [PC(16:0/13-HODE)] or 2x [PC(16:0/13-HODE)], and stimulated with collagen (2.5-5 µg/mL). Results are the means ± SEM of 4 different preparations of various HDL and 4 different suspensions of platelets. Asterisks indicate significant differences (*, P<0.05; **, P<0.01). NS, not significant.

Figure 4. Hydroxylated fatty acids in HDL phospholipids from control healthy subjects and from T2D patients. Effects of respective HDL on collagen-induced platelet aggregation. (A) Concentrations of 13-HODE, 9-HODE and 15-HETE in HDL PL from 5 control healthy subjects and 5 patients with T2D. Results are the means ± SEM. **, P < 0.01 vs. HDL from control subjects. (B) Platelet aggregation monitored in platelets isolated from healthy donors, pre-incubated for 5 min at 37°C in the absence or presence of HDL (50 µg/mL) from control subjects and patients with T2D for 5 min at 37°C, and then stimulated with collagen (2.5-5 µg/mL). Results are the means ± SEM of 8 independent experiments performed with 8 different preparations of HDL and platelets. Asterisks indicate significant differences (*, P<0.05; ***, P<0.001). (C) Concentrations of 13-HODE, 9-HODE and 15-HETE in HDL samples enriched with PC(16:0/13-HODE) (“enriched HDL”) or not (control HDL), n=2. (D) Collagen-induced aggregation obtained in platelets pre-incubated for 5 min at 37°C in the absence (control HDL) or presence of HDL enriched with [PC(16:0/13-HODE)] (“enriched HDL”) and stimulated with collagen. Results are the means ± SEM of 4 different preparations of control and enriched HDL. Asterisks indicate significant differences (*, P<0.05).

Figure 5. Summary diagram for the effects of glycoxidized HDL on platelet signaling pathways. Binding of glycoxidized HDL or HDL enriched with PC(16:0/13-HODE) to SR-BI receptor on platelet membranes led to the inhibition of signaling pathways through decreased phosphorylated p38 MAPK and cPLA2 levels and inhibited collagen-induced platelet aggregation.
Figure 1
Figure 2
Figure 3
Phospholipids from control HDL
Phospholipids from T2D HDL

pmol/mg protein

Platelets
Platelets + control HDL
Platelets + T2D HDL

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Figure 4
Glycoxidized HDL or HDL+ PC(16:0/13-HODE)

TP receptor

Ca^{2+}

Dense tubules

Arachidonic Acid

platelet

membrane

Aggregation

Figure 5
Supplemental Methods

Fatty acid compositions of lipid classes

Following the addition of appropriate internal standards (1,2-diheptadecanoyl-sn-glycero-3-phosphocholine, 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine, 1,2,3-triheptadecanoyl-sn-glycerol and heptadecanoyl cholesteryl ester) into HDL preparations and extraction with ethanol/chloroform (1:2, v/v) in the presence of BHT (50 μmol/L), lipid classes were separated by thin-layer chromatography (TLC) with hexane/diethylether/acetic acid (80:20:1, by vol.) to separate total PL, cholesteryl esters (CE) and triacylglycerols (TAG) (1). Corresponding silica zones were scraped off and treated with trifluoride boron/methanol (1.3 mol/L, 10%) for 90 min at 100°C. The derivatized fatty acid methyl esters and fatty dimethylacetals were extracted twice with isooctane and separated by gas chromatography using an HP 6890 gas chromatograph equipped with a SP 2380 capillary column (0.25μm, 30m × 0.25mm, Supelco, Bellefonte, PA, USA) and a flame ionization detector.

Quantification of monohydroxylated fatty acids

Following lipid extraction in the presence of appropriate standards (PC(16:0/15-HEDE) and 15-HEDE) and separation of lipid classes by TLC as previously described, dried extracts were reduced by NaBH₄. Ester bonds were hydrolyzed with 0.5 mol/L potassium hydroxide for 30 min at 60°C followed by an acidification to pH 3 using acetic acid. Non esterified hydroxylated fatty acids and fatty acids were first extracted on an Oasis Sep-Pak cartridge column (Waters, Milford, MA) or were extracted with hexane. Then, non-esterified hydroxylated fatty acids were separated by TLC with hexane/diethylether/acetic acid (60:40:1, by vol.). The spots were scraped off and then extracted with methanol, separated by reverse-phase HPLC on X Bridge C₁₈ column (3.5 μm, 4.6 × 150 mm, Waters, Milford, MA) using a gradient solvent of acetonitrile and water (pH 3) and measured at 235 nm (2).
**Stereochemical analysis of hydroxylated fatty acids**

Hydroxylated fatty acids isomers (13(R,S)-HODE, 9(R,S)-HODE and 15(R,S)-HETE) were firstly separated by reverse-phase HPLC as previously described, collected, evaporated and transmethylated with diazomethane for 15 min at room temperature to obtain methyl esters of hydroxylated fatty acids. Each fraction was injected to chiral phase HPLC on a CHIRALCEL ®OD-H column (5 µm, 250 x 4.6 mm, Daicel), eluted isocratically with hexane/2-propanol/acetic acid (85:15:0.1 by vol) at a flow rate of 1 mL/min at 25°C. Hydroxylated fatty acids were measured at 235 nm with a diode array detector.

**Malondialdehyde (MDA) determination**

HDL samples were mixed with thiobarbituric acid (TBA) (10 mmol/L), acetic acid and BHT (5 mmol/L) and the mixture was heated at 95°C for 60 min. The TBA-MDA adducts were extracted with ethyl acetate, separated onto a Nucleosil C18 column (5µm, 4.6 × 250 mm, Macherey-Nagel, Hoerdt, France) by reverse-phase HPLC and measured fluorimetrically (excitation 515 nm, emission 553 nm) (3).

**Vitamin E determination**

HDL samples (1 vol.), containing tocol as an internal standard, were extracted twice with hexane (4 vol.) following the addition of ethanol (1 vol.). Tocopherol isomers were separated by reverse-phase HPLC onto a Nucleosil C18 column (5µm, 4 × 150 mm) and measured fluorimetrically (excitation 295nm, emission 340nm) (4).

**Platelet p38 MAPK and cytosolic phospholipase A2 activation**

Following platelet lysis, proteins were denatured, electrophoresed in 12% bis-Tris and transferred to nitrocellulose membranes. The membranes were incubated with either 1:2500 anti-p38 MAPK or antiphospho-p38 MAPK, or anti-phospho-cytosolic phospholipase A2 (cPLA2) polyclonal antibodies, washed, and incubated with 1:5000 goat anti-rabbit horseradish peroxidase conjugate. P38 MAPK,
phospho-p38 MAPK and phospho-cPLA₂ were visualized by enhanced chemiluminescence, and bands were quantified by densitometry.

**Determination of intracellular Ca²⁺ concentrations**

PRP was acidified to pH 6.4 with citric acid and incubated with 1 µmol/L Fura 2-AM for 45 min at 37°C in a water bath protected from light (5). Platelets were then isolated and suspended in Tyrode-HEPES buffer, and left at room temperature for at least 1 hour in the dark. Fura 2-AM loaded platelets were pre-incubated with 50 µg/ml control or glycoxidized HDL for 5 min then stimulated with collagen. The external Ca²⁺ concentration in platelet suspensions was adjusted to 1 mmol/L using CaCl₂. Platelets were excited alternately at 340 and 380 nm and fluorescence emission was recorded at 510 nm. Intracellular Ca²⁺ concentrations were calculated from the ratio of fluorescence emission to excitation.

**References**


