SUPPLEMENTAL METHODS

Chemicals

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

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were cultured as previously described.[1,2]

Hemoglobin and hemopexin preparation

Purified ferrohemoglobin was prepared from lysed fresh human red cells using ion-exchange chromatography.[3] Human hemopexin was isolated following procedures described by Vretblad and Hjorth.[4]

Preparation and oxidation of low density lipoprotein

LDL was isolated from plasma derived from EDTA-anticoagulated venous blood of healthy volunteers by gradient ultracentrifugation (Beckman Instruments, Palo Alto CA).[5] Oxidation of LDL was carried out by the addition of heme (5 μmol/L) and H_2O_2 (75 μmol/L).[1]

Determination of neutral lipids and fatty acid.

Lipid extracts were redissolved in chloroform and spotted onto a Silica gel G (Merck, Darmstadt, Germany) plate. Neutral lipids were determined by thin layer chromatography as described.[6] Quantification was performed using QuantiScan software (Biosoft, Cambridge, UK). Neutral lipids and phospholipids were identified and quantitated using analytical grade standards (Sigma). For fatty acid analyses, the lipid extracts were hydrolyzed and methylated.[7] Fatty acid methylesters were injected into a Hewlett Packard 5890 gas

chromatograph coupled to a Hewlett Packard 5970 mass spectrometer (Palo Alto, CA, USA). Results are expressed as mean ± SD of mol% from three independent experiments.

Measurement of lipid peroxidation parameters, heme and iron in blood vessel samples

Conjugated diene content, total lipid hydroperoxides, thiobarbituric acid-reactive substances (TBARs), iron and heme were determined spectrophotometrically as described earlier.[1]

Lymphoblastoid cell culture and cytotoxicity assay

Human HO-1 deficient and control immortalized lymphoblastoid cell lines (LCLs) were generous gifts from Akihiro Yachie (Kanazawa University, Japan). LCLs were cultured in RPMI 1640 medium containing 15% FBS.

Lymphoblastoid cell suspensions were pretreated with heme (5 μmol/L, 60 min), then exposed to lipid suspensions (2 mg/mL in HBSS) for 16 hours. Following this, the cell suspension was centrifuged (400 x g, 10 min), the supernatant was removed and 0.5 mg/mL MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium-bromide) in HBSS was added in order to assess cell viability. After an additional 6 hours, the MTT solution was removed, dimethyl-sulfoxide (DMSO) was added and absorbance at 570 nm was measured.

Real time RT-PCR to determine HO-1 mRNA

Confluent endothelial cells grown in 6-well plates were exposed to lipid suspensions (1 mg/mL) for 1 hour. Total RNA was isolated from cells using the RNAzol STAT-60 (TEL-TEST Inc., Friendswood, TX). HO-1 and cyclophilin (housekeeping gene) levels were measured by real time PCR using fluorescent TaqMan probes following reverse transcription.[8]

Western blot for HO-1 protein

Confluent endothelial cells grown in 6-well plates were exposed to pretreated lipid suspensions described above for 1 h. HO-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein amounts were determined.[9]

HO enzyme activity assay

HO activity in endothelial cell microsomes was measured by bilirubin formation after treating the cells grown in Petri dishes as above.[2]

SUPPLEMENTAL RESULTS

Lipid extracts from atheromatous lesions exposed to heme also underwent lipid peroxidation as reflected by the accumulation of TBARs and lipid hydroperoxides. Concentrations of TBARs and lipid hydroperoxides reached a maximum at 12 hours and remained high for 24 hours (supplemental Fig I A and B). In the course of lipid peroxidation, the heme absorbance decreased (supplemental Fig I C) accompanied by an increase of 'free' iron of 0.62±0.27 nmol/mg indicating (probably oxidative) scission of the porphyrin ring. In contrast, no significant increases in either TBARs or lipid hydroperoxides occured in lipid extracts from control blood vessels and heme was not destroyed.

The chemical changes exerted by heme on lipids isolated from atheromatous lesions were attenuated by antioxidants such as BHT, α-tocopherol, the iron chelator deferoxamine, and the heme binding protein, hemopexin. Inhibition of heme-induced lipid oxidation (supplemental Fig II A) reduced both cytotoxicity (supplemental Fig II B) and induction of HO-1 expression at the mRNA and protein levels (supplemental Fig II C and D).

We have previously shown that HO-1 has a crucial role in preventing human lymphoblastoid cells from oxidized LDL mediated cytotoxicity.[8] Therefore, we tested whether HO-1 deficient cells might be more sensitive to heme-oxidized atheromatous lesion lipid as well. We have compared the cytotoxic effect of oxidized lipid on a lymphoblastoid cell line derived from a HO-1 deficient child and from control individuals. Indeed, heme-treated lipid from atheroma was more cytotoxic to the HO-1 deficient cells than to the control lymphoblast cells at all examined concentrations (supplemental Fig III).

SUPPLEMENTAL REFERENCES

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SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure I.

Heme induces peroxidation of lipid extract derived from atheromatous lesions; in parallel heme degradation occures. Lipid extracts (1 mg/ml) derived from atheromatous lesion and control were reacted with heme (5 μmol/L) at 37 ^{o}C. TBARs **(A)** and LOOH **(B)** contents of lipids and heme **(C)** for atheromatous (●) and control (○) lesions were measured. Figure is a representative of five separate experiments.

Supplemental Figure II.

Oxidation of lipids of atheromatous lesions and subsequent endothelial reactions are inhibited by antioxidants and by hemopexin. Lipids from atheromatous lesions (n=11), were treated with heme alone or in the presence of BHT, α-tocopherol, DFO or hemopexin (n=5) for 16 hours. Lipid hydroperoxide **(A)**, cytotoxicity **(B)**, HO-1 mRNA **(C)** and protein **(D)** in endothelial cells were measured.

Supplemental Figure III.

HO-1 deficient cells are more sensitive to oxidative damage caused than control cells. Lymphoblastoid cells of a HO-1 deficient patient and control were treated with oxidized atheroma lipids and cytotoxicity was measured.

SUPPLEMENTAL TABLES

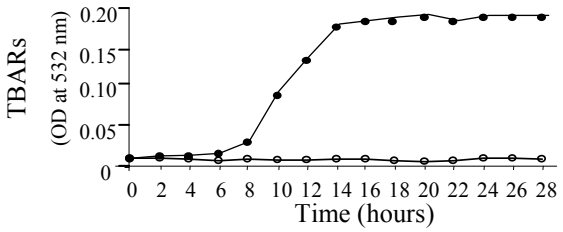
Table I. Chemical analysis of parameters indicative of oxidative stress in vessel wall samples. Products of lipid peroxidation, iron and hemoglobin contents of controls and atheromatous lesions were measured.

	Control (n=20)	Atheroma (n=33)	Significance ($p<0.05$)
Conjugated dienes (A_{234}/mg tissue)	0.006 (±0.002)	0.021 (±0.003)	0.002
LOOH (nmol/mg tissue)	0.003 (±0.003)	0.248 (±0.106)	0.016
TBARs (nmol/mg tissue)	0.002 (±0.001)	0.005 (±0.001)	0.021
Iron (nmol/mg tissue)	0.059 (±0.027)	0.185 (±0.096)	0.048
Hemoglobin(nmol/mg tissue)	not detectable	not detectable	n.s.

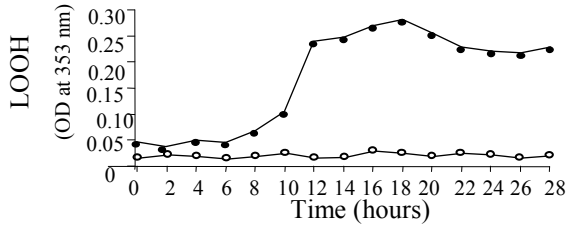
Table II. Lipid and fatty acid composition of lipid extracts derived from control and atheromatous lesions. Amounts of neutral lipids, phospholipids, and fatty acids (saturated fatty acids – SFA, monounsaturated fatty acids – MUFA, polyunsaturated fatty acids - PUFA) were measured.

		Control (n=20)	Atheroma (n=33)	Significance ($p<0.05$)
Lipid composition (mol%)	Cholesterol	16.9±4.3	42.6±11.2	0.01
	Oxy-sterol	2.05±0.5	5.14±1.2	0.04
	Diacylglycerol	0	1.17±0.2	0.04
	Triacylglycerol	54.4±12.5	35.8±18.2	n.s.
	Free fatty acids	17.3±5.4	8.06±4.5	n.s.
	Lyso-phospholipid	2.61±0.6	4.42±0.8	0.03
	Phosphatidylserine	6.61±1.3	2.85±2.2	0.04
Fatty acid composition (mol%)	C14:0	0	0.63±0.6	0.03
	C16:0	28.7±5.6	27.1±8.2	n.s.
	C18:0	10.98±4.3	10.1±7.6	n.s.
	C16:1	0	2.56±0.92	0.04
	C18:1	18.31±5.2	28.84±8.3	0.02
	C20:1	0.1±0.2	3.91±1.1	0.04
	C18:2	39.58±8.9	21.9±7.6	0.02
	C20:3	0.78±0.5	1.54±1.2	n.s.
	C20:4	1.54±0.9	1.21±1.1	n.s.
	Total SFA	39.68	37.83	n.s.
	Total MUFA	18.41	35.31	0.02
	Total PUFA	41.9	24.65	0.02

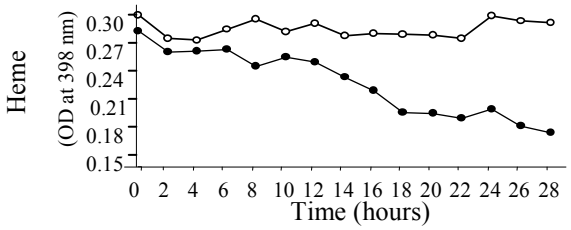
A

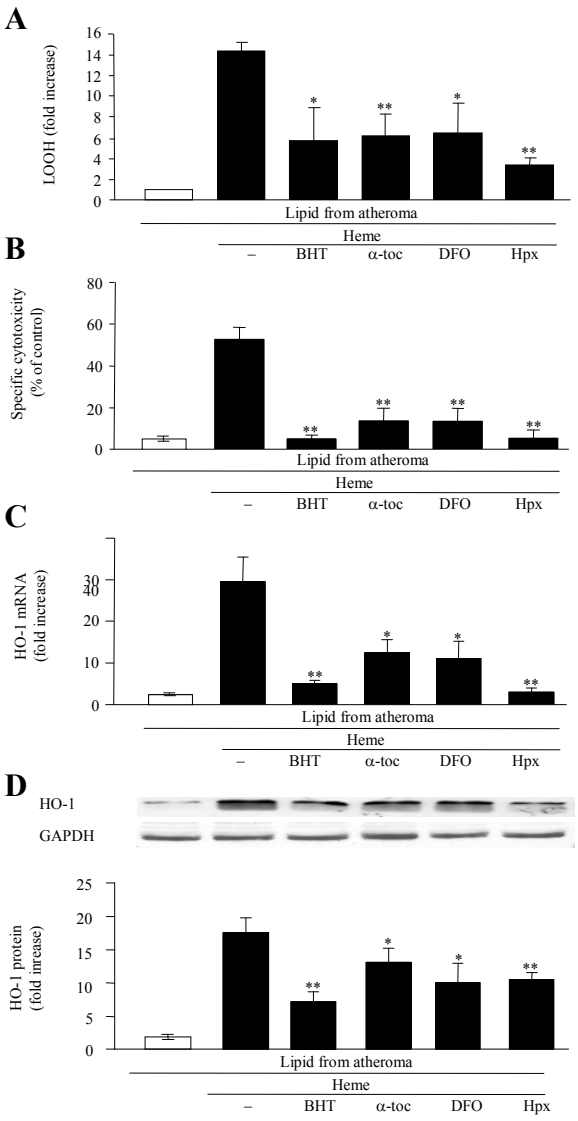

TBARs
(OD at 532 nm)
0.20
0.15
0.10
0.05
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (hours)

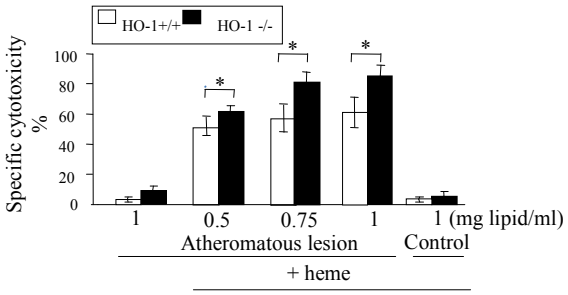
B


LOOH
(OD at 353 nm)
0.30
0.25
0.20
0.15
0.10
0.05
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (hours)

C


Heme
(OD at 398 nm)
0.30
0.27
0.24
0.21
0.18
0.15
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (hours)


A
LOOH (fold increase)
16
14
12
10
8
6
4
2
0
*
**
*
**
Lipid from atheroma
Heme
–
BHT
α-toc
DFO
Hpx
B
Specific cytotoxicity (% of control)
80
60
40
20
0
**
**
**
**
Lipid from atheroma
Heme
–
BHT
α-toc
DFO
Hpx
C
HO-1 mRNA (fold increase)
30
40
20
10
0
**
*
*
**
Lipid from atheroma
Heme
–
BHT
α-toc
DFO
Hpx
D
HO-1
GAPDH
HO-1 protein (fold inrease)
25
20
15
10
5
0
**
*
*
**
Lipid from atheroma
Heme
–
BHT
α-toc
DFO
Hpx


HO-1+/+
HO-1 -/-
Specific cytotoxicity %
100
80
60
40
20
0
*
*
*
1
0.5
0.75
1
1 (mg lipid/ml)
Atheromatous lesion
Control
+ heme