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Research Article

Fungal siderophores function as protective agents of LDL oxidation and are promising anti-atherosclerotic metabolites in functional food

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ONLINE PROOF

Iron-mediated oxidation of low-density lipoprotein has been implicated in the pathogenesis of vascular disorders such as atherosclerosis. The present investigations were performed to test whether hydrophobic fungal siderophores – hexadentate trihydroxamates desferricoprogen, desferrichrome, desferrirubin, and desferrichrysin – might suppress heme-catalyzed LDL oxidation and the toxic effects of heme-treated LDL on vascular endothelium. Indeed, two of these – desferricoprogen and desferrichrome – markedly increased the resistance of LDL to heme-catalyzed oxidation. In similar dose–response fashion, these siderophores also inhibited the generation of LDL products cytotoxic to human vascular endothelium. When iron-free fungal siderophores were added to LDL/heme oxidation reactions, the product failed to induce heme oxygenase-1, a surrogate marker for the noncytocidal effects of oxidized LDL (not in the case of desferrichrysin). Desferricoprogen also hindered the iron-mediated peroxidation of lipids from human atherosclerotic soft plaques *in vitro*, and was taken up in the gastrointestinal tract of rat. The absorbed siderophore was accumulated in the liver and was secreted in its iron-complexed form in the feces and urine. The consumption of mold-ripened food products such as aged cheeses and the introduction of functional foods and food additives rich in fungal iron chelators in diets may lower the risk of cardiovascular diseases.

Keywords: Atherosclerosis / Functional food / Heme oxygenase / LDL oxidation / Siderophores

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1 Introduction

Reactive oxygen species and transition metals are thought to be involved in the pathogenesis of numerous vascular disorders such as atherosclerosis, vasculitis, reperfusion injuries, and neonatal retinopathy of prematurity [1]. Environmental factors greatly influence the onset and course of these diseases as proved by epidemiological studies [2]. Important among these factors are infection, inflammation and diet, all of which modulate oxygen and iron metabolism [3–5]. Several epidemiological studies have suggested that elevated tissue iron levels may increase the risk for atherosclerosis [6–8], although there are some contrasting views [9, 10]. Intralesional accumulation of iron has been observed in atherosclerosis [11, 12] and studies revealed

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Abbreviations: HO, heme oxygenase; **HUVEC,** human umbilical vein endothelial cells; **MTT,** 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; **TBAR,** thiobarbituric acid-reactive substance

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that ferritin coupled to heme oxygenase-1 (HO-1) is highly expressed in human atherosclerotic plaques [13, 14] possibly reflecting a cellular response directly to heme- or iron exposure or an indirect response to heme- or iron-generated lipid peroxidation products [15, 16]. Infection generates free radical stress through leukocyte activation and cytokine production, which, while effective in killing pathogens, may cause secondary damage to the host.

The severity of inflammation-associated damage is powerfully influenced by the presence of "loose" iron not bound to iron-binding proteins such as transferrin and ferritin [17, 18]. In many cases, chelators which suppress the reactivity of iron have been found to ameliorate tissue damage arising from inflammatory reactions [19, 20]. However, the utility of the only clinically used high affinity chelator, desferrioxamine B, is limited by its evanescence, potential toxicity and lack of cell membrane penetration [21, 22]. Desferrioxamine was initially isolated from a bacterium (*Streptomyces pilosus*), which uses the chelator as a means for wresting iron from iron-poor environments [23 – 25]. Numerous other unicellular organisms also produce siderophores but many of these have little promise for clinical use. For example, a siderophore produced by *Pseudomonas aeruginosa*, pyochelin, does not suppress iron-mediated oxidation/reduction reactions and may even augment oxidant cell injuries [26, 27].

Fungi also produce iron chelators, including hydroxamates such as rhodotorulic acid, coprogens, ferrichromes, and fusarinines [28]. Although the pharmacology of these chelators is less well known than that of desferrioxamine B, these compounds may be absorbed following the ingestion of food containing fungi such as fungi-ripened food [29]. This raises the possibility that fungal siderophores may influence the iron metabolism in humans and possibly modify the severity of iron-mediated pathological processes.

In many situations, the oxidation of PUFAs appears to be a crucial event in oxidative cell damage. Recently it has been shown that oxidation of LDL might occur not only in the interstitial fluid but also within lysosomes of macrophages in the atherosclerotic lesion [30]. Such oxidation can destroy the barrier function of membranes through oxidation of unsaturated fatty acids in phospholipids and it has been shown that prevention of fatty acid oxidation will, at least in some experimental circumstances, prevent oxidant-mediated cell death [1, 19, 31, 32]. The oxidation of PUFAs almost always requires the presence of reactive transition metals, notably iron. In the human body, one abundant and potentially dangerous source of reactive iron is heme. Heme is particularly hazardous because, being a hydrophobic iron "chelate", it readily crosses cell membranes and cells exposed to heme become extraordinarily sensitive to oxidant damage [33]. Heme also promotes the conversion of low-density lipoprotein to cytotoxic oxidized products [34]. Cells respond to an influx of heme [35 – 38] and oxidative stress [39 – 41] by inducing both HO-1 and ferritin. The former cleaves the heme [42] and the latter represents a safe storage site for the released iron [38]. The importance of the heme – HO – ferritin system in human vascular disorders was emphasized by the recent description of severe vasculopathy in the case of an HO-1 deficient child [43, 44].

However, the heme – HO – ferritin system may not be the only effective way to deal with the challenges presented by the combination of oxidants and iron sources. Because of the above-mentioned drawbacks of the only clinically used high affinity iron chelator, in the present study we have investigated the possible beneficial (or deleterious) effects of fungal siderophores in iron-catalyzed low-density lipoprotein (LDL) peroxidation. We selected the hexadentate trihydroxamates desferricoprogen, desferrichrome, desferrirubin, and desferrichrysin (Fig. 1) for this study, which are produced by *Penicillium* and *Aspergillus* species used frequently in the food industry (*e.g.*, *P. roqueforti*) or are important in the spoilage of different food products (*e.g.*, *Aspergillus ochraceus*). Our results show that these fungal iron chelators, often present in the human diet, variably suppress heme-mediated oxidative modification of LDL, prevent the formation of cytotoxic oxidized LDL and diminish the homeostatic upregulation of HO-1 expression in human vascular endothelial cells exposed to heme-treated LDL. Moreover, one of the siderophores produced frequently by *Penicilium* spp. – desferricoprogen – hindered considerably the iron-mediated oxidation of the lipid cores of human atheromas, and was absorbed in the gastrointestinal tract of rat. We are speculating that the consumption of either traditional mold-ripened food or new-type functional food rich in fungal hexadentate trihydroxamate siderophores may be beneficial in the prevention of cardiovascular disorders like atherosclerosis.

2 Materials and methods

2.1 Purification of coprogen, ferrichrome, and related compounds

Fungal hexadentate siderophores (Fig. 1) were purified from culture fluids of *Penicillium chrysogenum* (coprogen, ferrichrome), *Neurospora crassa* (coprogen) cultivated in defined low-iron minimal media [45 – 47]. The purification schemes included Amberlite XAD-2, Kieselgur G, and Bio-Gel P-2 liquid chromatographies and preparative HPLC on a Supelcosil-Si matrix [47, 48]. The purity of ferri-siderophores was checked by HPLC using a C-18 RP column [49, 50], and pure ferri-siderophores were deferrated using methanolic 8-hydroxyquinoline [51, 52]. Yields for desferricoprogen were 35 mg/L culture medium with *P. chrysogenum* and 66 mg/L culture medium with *N. crassa*. Ferrichrome, ferrichrysin and ferrirubin were in part a kind gift of Dr. G. Winkelmann (University of Tübingen) or were bought from Biophore Research Products (Tübingen, Germany). The bacterial hexadentate siderophore desferriox-

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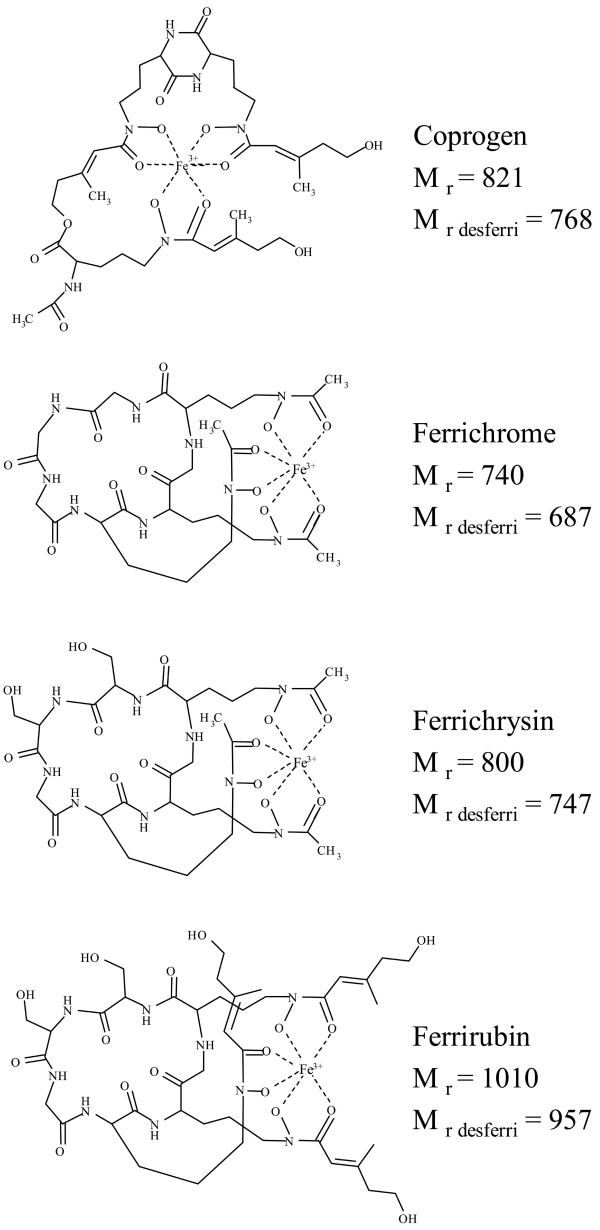

Figure 1. Chemical structure of coprogen, ferrichrome, ferrichrysin, and ferrirubin. Desferricoprogen belongs to the family of linear trihydroxamates while desferrichrome, desferrichrysin, and desferrirubin are cyclic modified hexapeptides.

amine B, which was used as a control in the same experiements, was purchased as Desferal® from Novartis (Basel, Switzerland).

2.2 Isolation of low-density lipoprotein

LDL was isolated from plasma derived from EDTA (1 mg/mL)-anticoagulated venous blood taken from healthy overnight-fasted volunteers [53, 54]. Density of plasma was adjusted to 1.3 g/mL with KBr, and a two-layer gradient was made in a Quick-Seal polyallomer ultracentrifuge tube (Beckman Instruments) by layering 0.9% NaCl on 10 mL of density adjusted plasma, which was then centrifuged at 302 000 × *g* for 3 h at 4°C (VTi 50.2 rotor, Beckman Instruments, Brea, CA, USA). Purity of the LDL fraction was checked by agarose gel electrophoresis. The LDL samples were kept at 4°C and protected from light, and the protein content was determined by the BCA protein assay (Pierce, Rockford, IL, USA).

2.3 Oxidative resistance of LDL

Heme-mediated oxidation of LDL was monitored by both measuring conjugated diene formation and heme degradation spectrophotometrically at 234 and 405 nm, respectively. Reaction mixtures contained LDL (200 mg/L protein), heme (5 μM), hydrogen peroxide (75 μM), and HEPES buffer (10 mM, pH 7.4) [34, 53, 54]. When siderophores were employed, we preincubated LDL samples with them to allow their incorporation into the LDL. In heme-catalyzed oxidation of LDL, heme degradation occurs in concert with formation of lipid oxidation products including conjugated dienes and lipid hydroperoxides. Thus, heme degradation reflects the progress of lipid peroxidation. The kinetics of heme disappearance was monitored at 405 nm in an automated microplate reader (model EL340, Bio-Tek Instruments, Winooski, VT, USA). LDL oxidation was monitored by the time (ΔT) required for the process to achieve maximum velocity (V_{max}) of heme degradation in minutes. Reaction mixtures were supplemented as indicated with desferri-siderophores at concentrations of 5, 10, and 25 μM.

2.4 Saturation of LDL with coprogen and desferricoprogen

The physical incorporation of both coprogen and desferricoprogen into LDL was demonstrated *in vitro*. Isolated LDL was incubated with coprogen and desferricoprogen within the concentration range of 10 – 250 μM for 2 h at 37°C in a volume of 2.0 mL containing 1 mg of LDL protein. The samples were dialyzed exhaustively against physiological saline (3 × 2 h), and one series of desferricoprogen-treated LDL samples were supplemented with $FeCl_3$ in a molar ratio of desferricoprogen/$FeCl_3$ 1:5. All samples were freeze-dried and extracted with 1.0 mL of ice-cold methanol/double-distilled water (1:2) by vigorous mixing for 2 min. After centrifugation at 10 000 × *g* for 5 min, the supernatants were analyzed using both analytical HPLC and TLC [47, 49, 50].

2.5 Endothelial cell isolation and culture

Human umbilical vein endothelial cells (HUVECs) were removed from human umbilical veins by exposure to dis-

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pase and cultured in medium 199 containing 15% fetal calf serum, penicillin (100 U/mL), streptomycin (100 U/mL), and heparin (5 U/mL) supplemented with L-glutamine, sodium pyruvate, and endothelial cell growth factor [55]. Endothelial cells were identified by cell morphology and by the presence of von Willebrand factor.

2.6 Endothelial cell cytotoxicity assay

Confluent endothelial cells grown in 24-well tissue culture plates were washed three times with Hank's balanced salt solution (HBSS), and then exposed to a reaction mixture containing LDL (200 mg/L), heme (5 μM), H_2O_2 (75 μM) with or without the addition of iron-free or iron-saturated siderophores (20 μM). After an incubation period of 4 h, the test solutions were replaced with 500 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) (0.5 mg/mL) dissolved in HBSS, and endothelial cell monolayers were incubated for another 6 h. The reduced MTT was measured spectrophotometrically at 570 nm after the formazan was dissolved in 100 μL of 10% SDS and 500 μL of hot isopropanol containing 20 mM HCl.

2.7 Heme oxygenase enzyme activity assay

Heme oxygenase activity in endothelial cell microsomes was measured by bilirubin generation [55]. The induction of HO activity was determined in endothelial cells grown in 10 cm-diameter tissue culture dishes and treated with a reaction mixture containing LDL (50 mg/L), heme (1.25 μM), H_2O_2 (18.75 μM) with or without the addition of iron-free or iron-saturated siderophores (5 μM) for 60 min followed by an 8 h incubation with complete media alone. Endothelial microsomes were incubated with hepatic cytosol (2 mg), hemin (20 μmol/L), glucose-6-phosphate dehydrogenase (0.2 units), and NADPH (0.8 mmol/L). The formed bilirubin was extracted with chloroform and Δ OD of 464 and 530 nm was measured (extinction coefficient 40 $M^{-1} \cdot cm^{-1}$). Heme oxygenase activity is expressed as pmol bilirubin formed/mg cell protein/60 min.

2.8 Heme oxygenase-1 mRNA analysis

HO-1 mRNA content was analyzed in confluent HUVECs incubated with control medium or LDL test solutions as described above for the measurement of enzyme activity. Cellular RNA was isolated using RNAzol (TEL-TEST, Friendswood, TX, USA), and 20 μg quantities of total RNA were run on agarose gels and transferred to nylon membrane. The 28S and 18S ribosomal RNAs, and equal loading of samples were checked by ethidium bromide staining. Alternatively, 2 μg quantities of total RNA were subjected to dot blot analysis. After crosslinking to the nylon membranes, RNAs were hybridized with biotin-labeled cDNA for HO-1 (Bioprime DNA Labeling System, Life Technologies) [32], and the HO-1-active bands and dots were visualized by a chemiluminescent detection system (Photogene System 2.0, Life Technologies). Autoradiographs were quantified by computer-assisted videodensitometry.

2.9 Effect of desferricoprogen on heme-mediated oxidation of atherosclerotic plaques

Specimens of human atherosclerotic lesions were obtained from aorta or its primary branches of deceased heart-beating donors for organ transplantation. Removal of tissue samples from deceased heart-beating donors was approved by the Scientific and Research Ethic Comity of Scientific Council of Health of the Hungarian Government. Entire vessels and complete lesions were removed during donation, washed with saline, and dried. Small pieces of collected vessel samples were dispersed in phosphate buffer containing heme (10 μM) and desferricoprogen (25 and 50 μM) and then incubated for 24 or 48 h at 37°C. For the measurement of thiobarbituric acid-reactive substances (TBARs), 250 μL of reaction mixture was combined with 250 μL thiobarbituric acid reagent (0.375 g 2-thiobarbituric acid, 2.08 mL cc. HCl, 15 mL 10% trichloroacetic acid and distilled water adjusted to a final volume of 100 mL). After incubating at 90°C for 20 min, the samples were cooled to room temperature and extracted with 250 μL *n*-buthanol. The concentration of TBARs was determined spectrophotometrically at 532 nm (extinction coefficient = 1.56×10^5 $M^{-1} \cdot cm^{-1}$) and was expressed as nmol/(mg tissue).

2.10 Uptake, secretion, and tissue accumulation of desferricoprogen and coprogen administered orally or intravenously in rat

FLF_1 hybrid rats (m = 150–350 g; fed on a normal chow diet) were treated with either desferricoprogen or coprogen intravenously through femoral vein at a concentration of 50 mg/(kg body mass) ($c_{\text{stock solution}}$ = 20 mg/mL, dissolved in sterile filtered saline), or orally at a concentration of 100 mg/(kg body mass), through orogastric tube. Rats were kept in separate cages with free access to water and food. A selected group of animals were also given 0.5 mL 20% ethanol or 0.5 mL olive oil immediately after the oral administration of the siderophore to estimate the influence of alcohol and lipids on the absorption of siderophores in the gut. All the animal experiments were approved by the Animal Care and Use Committee of the University of Debrecen.

Urine and feces specimens were collected for 24 h intervals on the first, second, third, and sixth days following siderophore administration. Urine samples were frozen at –80°C and lyophilized meanwhile feces samples were shaken in 40 mL aliquots of water for 30 min, were centrifuged (1000 × *g*, 15 min), and the supernatants were frozen (–80°C) and lyophilized.

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To map the accumulation of orally administered desferricoprogen and coprogen in different organs and tissues, a separate group of experimental animals were sacrificed after 4, 12, and 24 h siderophore treatments after taking blood samples (2.0 – 2.5 mL), which were fractionated into blood cells and plasma by centrifugation. The liver, spleen and intestinal epithelium of the animals were excised, weighed, frozen at −80°C and freeze-dried.

To extract and quantify siderophore contents of the collected specimens, all lyophilized samples were homogenized with a glass rod, suspended in 20 mL aliquots of 50% methanol and agitated vigorously on a test tube spinner for 1 min. After centrifugation, the ferri-siderophore contents of the supernatants were analyzed by HPLC as described above. In the case of the desferricoprogen treatments, the supernatants were divided into two equal, approximately 10 mL aliquots, one of which was supplemented with $FeCl_3$ (with six-fold molar equivalents of the administered siderophore). The difference between the ferri-siderophore contents of $FeCl_3$-treated and nontreated samples was considered as the quantity of the iron-free uncomplexed fraction of the siderophore accumulated in the analyzed tissues and organs.

2.11 Statistical analysis

Unless otherwise indicated, data are presented as means ± SD. The Student's *t*-test was employed to test statistical significance.

3 Results

3.1 *In vitro* studies demonstrating the beneficial effects of fungal siderophores on heme-mediated LDL-peroxidation

The peroxidation of isolated LDL was followed by the generation of conjugated dienes at 234 nm and by heme degradation at 405 nm. Neither iron-free nor iron-saturated fungal chelators induced LDL lipid peroxidation alone. However, in the presence of heme the iron-free siderophores inhibited the formation of conjugated dienes (Fig. 2). As shown on Fig. 2 heme and hydrogen peroxide-mediated LDL oxidation – which was followed by conjugated diene formation – was inhibited by desferricoprogen which prolonged the initiation phase of lipid peroxidation. In contrast, under the same conditions iron-saturated coprogen had no effect on the heme-mediated oxidative modification of LDL. Importantly, ferricoprogen did not initiate oxidative modification of LDL. Oxidative modification of LDL does not occur when LDL is treated with ferri-siderophores and H_2O_2 suggesting that ferri-siderophores can not provide catalytically active iron to the reaction therefore we can say that ferri-siderophores are inert in the presence of H_2O_2. Simultaneous measurements of heme degradation (followed at 405 nm) yielded similar results to that of we have got by measuring conjugated diene formation.

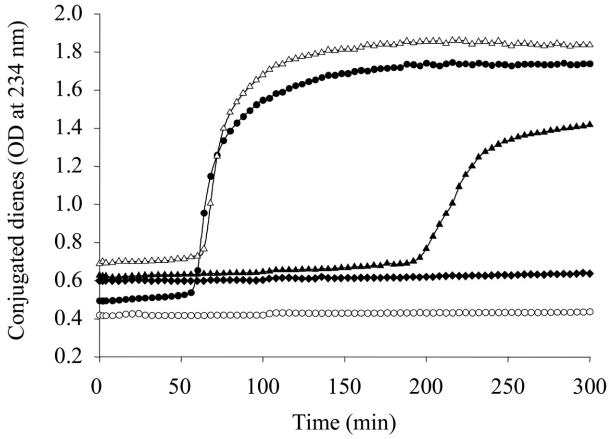

Figure 2. Kinetics of lipid peroxidation of LDL catalyzed by heme. The standard reaction mixture contained LDL (200 μg/mL protein), heme (5 μM), H_2O_2 (75 μM) and was also supplemented with 100 μM desferricoprogen or 100 μM coprogen as required Formation of conjugated dienes were monitored at 234 nm. Symbols stand for: ○, native LDL alone; ●, LDL + heme + H_2O_2; △, LDL + coprogen + heme + H_2O_2; ▲, LDL + desferricoprogen + heme + H_2O_2; ◆, LDL + coprogen + H_2O_2.

Table 1. Dose dependence of the protective effect of desferricoprogen on LDL against oxidative modification triggered by heme-H_2O_2

Concentration of desferricoprogen (μM)	ΔT at V_{max} [a)] (min)	Relative ΔT at V_{max} [a)] (% of control)
0	57 ± 1	100
10	109 ± 1	191 ± 2
50	206 ± 1	361 ± 2
100	225 ± 6	395 ± 10
250	>240	>420

a) ΔT at V_{max} values were calculated from three independent experiments. The protective effects were significant ($p < 0.001$) for all the desferricoprogen concentrations tested.

As shown in Table 1, heme-catalyzed oxidation of LDL was inhibited by desferricoprogen in a dose-dependent manner. Pretreatment of LDL with 10 μM of desferricoprogen then challenged to heme and H_2O_2 prolonged the ΔT at V_{max} value from 57 ± 1 to 109 ± 1 min, resulting in a 91% increase in the resistance of LDL against heme-catalyzed oxidation. Similarly to heme degradation the formation of conjugated dienes and the accumulation of TBARs were inhibited in a dose-dependent fashion (data not shown). Desferricoprogen at the concentration of 250 μM and above completely inhibited the oxidative modification of LDL over a period of 7 days. The inhibition of peroxidation by desferricoprogen was compared to those of other iron-free fungal hexadentate chelators and to that of the bacterial hexadentate desferrioxamine B (Table 2). Interestingly, desferrirubin and desferrichrysin, although structurally similar

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Table 2. Relative increases in the ΔT at V_{max} values due to the Fe^{3+}-squevenging effect of desferri-siderophores in heme-catalyzed oxidative modification of LDL by H_2O_2

ΔT at V_{max}[a,b] (%)	Concentration of desferri-siderophores		
	5 μM	10 μM	20 μM
Desferricoprogen	134.8	191.3	341.3
Desferrirubin	134.8	186.9	254.3
Desferrichrysin	104.3	119.6	139.1
Desferrichrome	152.2	191.3	302.2
Desferrioxamine B	136.9	200.0	386.9

a) ΔT at V_{max} values were normalized with ΔT at V_{max} measured in heme-H_2O_2 LDL modification systems with no siderophore supplementation.
b) A typical set of data is shown.

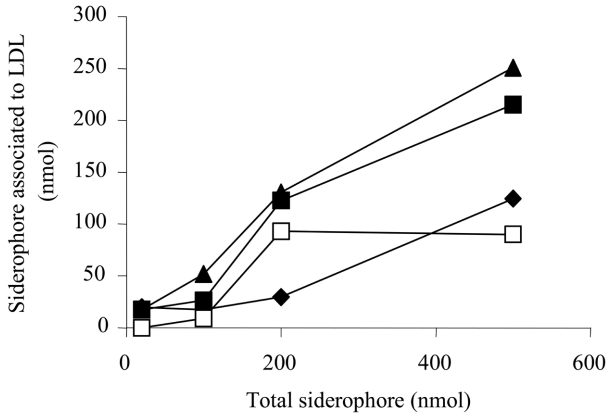

Figure 3. Saturation of LDL with desferricoprogen and coprogen *in vitro*. LDL was incubated with coprogen or desferricoprogen in the presence or absence of iron as described in Section 2. After removal of unincorporated siderophores by dialysis, coprogen content of LDL was measured. Symbols represent coprogen treatment (▲), desferricoprogen treatment + - $FeCl_3$ added (■), desferricoprogen treatment without extra iron added (♦), desferricoprogen content of LDL after desferricoprogen treatment (difference between coprogen levels with and without $FeCl_3$ addition) (□).

to desferrichrome (Fig. 1), were significantly less effective in preventing LDL oxidation. In contrast, the ΔT at V_{max} was greatly prolonged by 20 μM concentrations of desferricoprogen, desferrichrome, and desferrioxamine B.

The effectiveness of desferricoprogen in suppressing heme-catalyzed LDL oxidation (Fig. 2, Tables 1 and 2) suggested that this chelator might enter LDL. Indeed, as shown in Fig. 3, incubation of LDL with coprogen or desferricoprogen led to substantial amounts of LDL-associated chelators. The incorporation of both forms into LDL was dose-dependent and saturable. Incubating 200 μg/mL LDL protein and 250 μM coprogen (total coprogen content in the reaction buffer was 500 nmol) for 2 h led to the incorporation of 50.1 and 43.0% of the added coprogen and desferricoprogen (251 and 215 nmol/mg LDL protein), respectively.

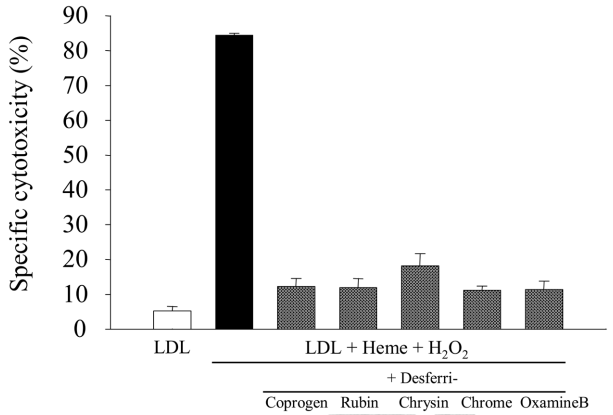

Figure 4. Protective effects of desferri-siderophores on endothelial cells exposed to oxidatively modified LDL. LDL (200 μg/mL) was oxidized with heme (5 μM) and H_2O_2 (75 μM) in the presence of desferricoprogen, desferrirubin, desferrichrysin, desferrichrome or desferrioxamineB at the concentration of 20 μM. HUVECs were exposed to the reaction mixtures for 4 h, and then cell cytotoxicity was measured by MTT assay. Figure represents means and SD values calculated from three independent experiments.

These results raised the question of whether the attenuation of LDL oxidation by fungal iron chelators might diminish the vascular endothelial cell cytotoxicity of treated LDL. Oxidized LDL contains products of lipid peroxidation, among them lipid hydroperoxides directly toxic to endothelial cells. LDL at 200 μg/mL concentration was incubated in a standard lipid peroxidation reaction mixture containing 5 μM heme and 75 μM H_2O_2 in the presence or absence of 20 μM concentrations of iron-free fungal siderophores. After exposing endothelial cell monolayers to the reaction mixtures for 4 h, the cytotoxic effect was measured. In these experiments, 20 μM desferrioxamine B was used as a positive control [34]. As expected, LDL treated with heme and hydrogen peroxide was markedly cytotoxic whereas iron-free siderophores hindered the generation of cytotoxic LDL in a pattern quite similar to that observed in the kinetic analysis of LDL lipid peroxidation (Table 2). The protective effect exerted by desferrichrysin was significant but less than those of the other chelators tested (Fig. 4).

We previously found that oxidized LDL at sublethal concentrations markedly induced the expression of HO-1 gene and increased HO enzyme activity in vascular endothelial cells *in vitro* [40]. As a further test of the effectiveness of the anti-oxidant activities of fungal siderophores, LDL solutions were oxidized by heme and H_2O_2 for 1 h in the standard reaction mixture supplemented with siderophores at a final concentration of 20 μM and then diluted to a final LDL concentration of 50 μg/mL. Endothelial cells treated with native LDL exhibited low levels of HO-1 mRNA expression and HO enzyme activity demonstrated by Northern blot analysis (Figs. 5A and B, first lane and bar) and bilirubin generation (Fig. 5D, first bar). Oxidized LDL

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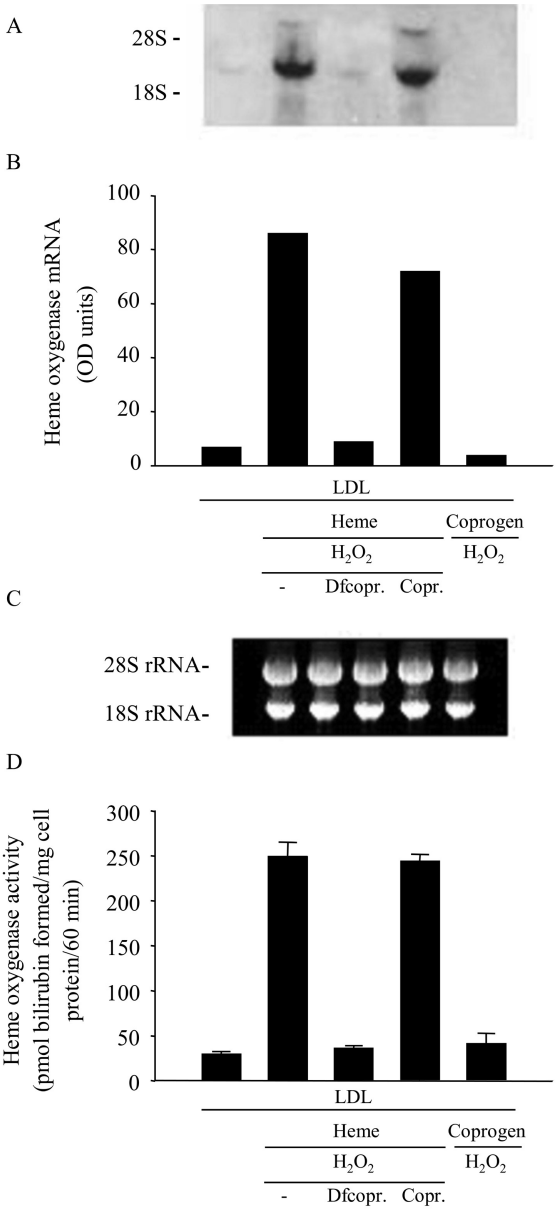

Figure 5. Correlation between intracellular HO-1 mRNA levels and specific HO activities. LDL (200 μg/mL) was treated with heme (5 μM) and H_2O_2 (75 μM) supplemented with 20 μM desferricoprogen or coprogen as indicated, or LDL was treated with coprogen and H_2O_2. After a 2 h pre-incubation, samples were diluted four times, and HUVECs were treated with the reaction mixtures. Heme oxygenase-1 mRNA levels were determined by Northern blot (A), and mRNA concentrations were quantified by videodensitometry (B). Panel C shows ethidium bromide staining of the gel to prove equal loading of the samples. HO-1 activity (D) was measured by bilirubin generation as described in Section 2. Specific HO-1 activities are shown as means ± SD calculated from three independent experiments.

caused a >40-fold increase in HO-1 mRNA expression (Figs. 5A and B, second lane and bar) together with a tenfold increase in enzyme activity in human vascular endothelial cells (Fig. 5D, second bar). When LDL oxidation was inhibited by 20 μM desferricoprogen, increases in HO-1 mRNA levels (Figs. 5A and B, third lane and bar) and HO enzyme activities (Fig. 5D, third bar) were almost totally prevented. In contrast, coprogen did not inhibit LDL oxidation and did not prevent HO-1 induction in endothelial cells exposed to the products of the reaction (Fig. 5, fourth lane and bars). As shown in Fig. 6, among the iron-free siderophores only desferrichrysin was unable to prevent the induction of the HO-1 gene (dot B4) in agreement with its relatively weak protective effect against heme-mediated LDL lipid peroxidation. The other fungal siderophores together with the positive control desferrioxamine B efficiently inhibited both the oxidation of LDL and the induction of HO-1 (dots B1 – 3, B5). Not surprisingly, iron-complexed siderophores did not prevent the induction of HO-1 gene (dots C1 – 5), in agreement with their lack of effect on LDL-oxidation. Importantly, when LDL was pretreated with iron-complexed siderophores and H_2O_2 for 1 h in the absence of heme and then incubated with endothelial cell monolayers for 8 h at sublethal concentration neither HO-1 gene induction (Fig. 6, dots D1 – 5) nor increase in enzyme activity (Fig. 5, fifth lane and bars) was observed.

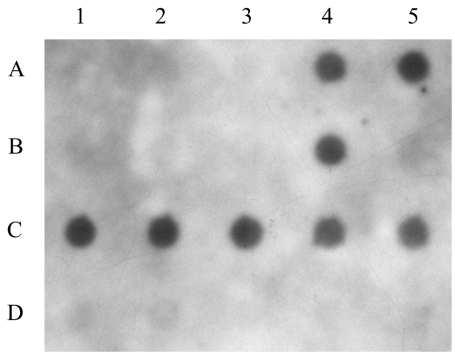

Figure 6. Dot blot analysis of the changes in the gene expression levels of HO-1 in the presence of desferri- and ferri-siderophores. A(4 – 5): positive control, upregulation of HO-1 after LDL + heme + H_2O_2-treatment of endothelial cells. B(1 – 5): protection of HUVECs with 20 μM desferrioxamine B, desferricoprogen, desferrirubin, desferrichrysin, and desferrichrome. C (1 – 5): supplementation of reaction mixtures with 20 μM iron-saturated ferrioxamine, coprogen, ferrirubin, ferrichrysin, and ferrichrome. D (1 – 5) Treatment of HUVECs with LDL + ferri-siderophore + H_2O_2 mixtures without any heme. Siderophore – iron complexes were the same as in row C.

3.2 The fungal siderophore desferricoprogen prevents peroxidation of lipids from atherosclerotic soft plaques

As previously described [34, 55], oxidative modification of LDL is a key event in the development of atherosclerotic lesions, and lipid cores of the atheromas are as sensitive for oxidative modifications as LDL. Heme-mediated oxidation

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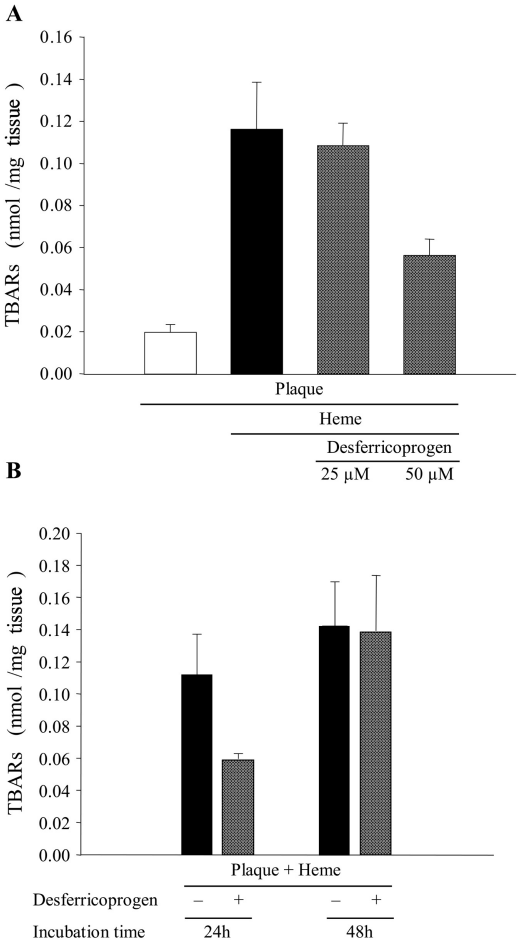

Figure 7. Desferricoprogen protects atherosclerotic plaques against heme-mediated oxidation. Atherosclerotic plaques were incubated with heme in the presence or absence of desfericoprogen, and concentration of TBARs were measured. Significant ($p < 0.1\%$) protective effect was observed at 50 μM desferri-siderophore concentration even after 24 h incubation at in the presence of heme (A), but a prolonged (48 h) heme-mediated oxidation eliminated this effect (B). Means ± SDs calculated from five independent experiments are presented.

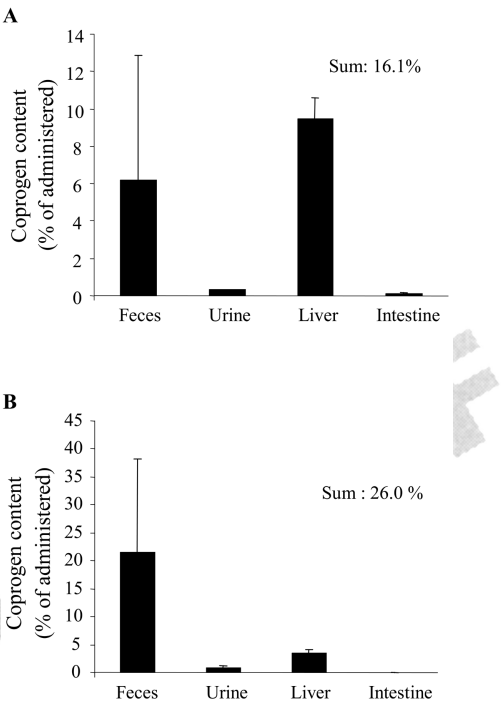

Figure 8. Distribution of orally administered desferricoprogen (A) and coprogen (B) between feces, urine, liver and intestinal epithelium 1 day after dosage. Each animal was dosed with 100 mg/(kg body mass) siderophore. The data presented here are means ± SDs calculated from five independent experiments and stand for mean total (desferri + ferri) siderophore contents.

of lipids originated from atherosclerotic soft plaques takes place after about 12 – 18 h, which time was increased dose dependently by desferricoprogen up to 24 h (Fig. 7A). In contrast, desferricoprogen had no protective effect when oxidation last longer (48 h; Fig. 7B).

3.3 Uptake, accumulation, and secretion of desferricoprogen and coprogen in rat

Considering uptake and accumulation of orally administered desferricoprogen and coprogen, substantial quantities of siderophore appeared in the liver 1 day after administration (8.5 – 9.5%; Figs. 8 and 9) meanwhile there was no detectable amount of siderophore in the spleen. Interestingly, when desferri- and ferri-siderophores were dosed intravenously small amounts (0.05 – 0.07%) of siderophore was detected in the spleen. Approximately 0.1% of the orally administered siderophore remained in the intestinal epithelium (Fig. 8) while there was no detectable siderophore in the intestinal epithelial cells when desferricoprogen was added intravenously. No coprogen was detected in blood cells separated from any blood specimen and the serum concentration of the siderophore was always about 0.01 mg/mL (12 μmol/L in coprogen equivalents), which was around the LOD of the HPLC procedure used. Importantly, 3.5 times more (21.5% *vs*. 6.2%) siderophore was detected in feces and 2.6 times more (0.90% *vs*. 0.34%) siderophore was secreted in urine when coprogen was added in iron-complexed form (Fig. 8). Surprisingly, approximately 84% of orally administered desferricoprogen and 74% of orally administered coprogen were neither secreted into the feces and urine nor accumulated in the organs and tissues studied 1 day after dosage. Secretion of orally administered siderophores was followed up to 6 day after dosage but the recorded quantities were always low (≤1%; data not shown). Coprogen was secreted into both feces and urine exclusively in its iron-complexed form independently of that ferri- or desferri-siderophore was administered.

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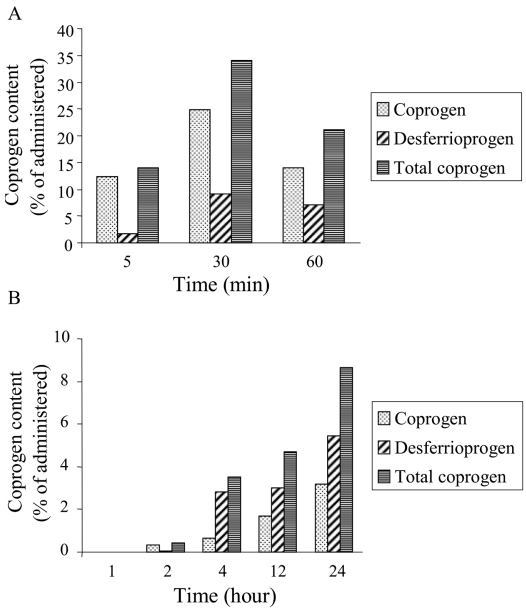

Figure 9. Accumulation of coprogen in the liver after intravenous (A) and oral (B) administrations of desferricoprogen. All experimental animals were treated with 50 (intravenous) or 100 (oral) mg/(kg body mass) desferri-siderophore. The data presented here are typical datasets.

The hepatic accumulations of desferricoprogen and its iron complex were studied in more details after both intravenous and oral applications. As shown in Fig. 9, about 14% (1.7% in desferri form) of intravenously added siderophore localized in the liver within 5 min after administration and this amount increased further to 21 – 34% (7.2 – 9.1% were not complexed with iron) 30 min – 1 h post-administration. During oral application, the coprogen content of the liver increased steadily and reached 8.6% (5.5% in desferri form) of total administered coprogen 1 day after dosage in this set of experiments (Fig. 9).

Co-administration of ethanol or olive oil did not affect the accumulation of either desferricoprogen or coprogen in the liver (Fig. 10). Secretion of siderophores in either feces or urine as well as the low siderophore contents of intestinal epithelium and spleen remained unaffected by ethanol and oil (data not shown).

4 Discussion

In many experimental and clinical situations, it has been shown that suppression of iron-catalyzed oxidation reactions can have significant protective effects [1, 56, 57]. For example, oxidation of LDL particles by free iron released during the oxidative degradation of heme inside the hydrophobic domains of lipoprotein has been implicated in vascular endothelial cell damage [32, 34, 58] and the bacterial Fe^{3+}-chelator desferrioxamine B has been found to effec-

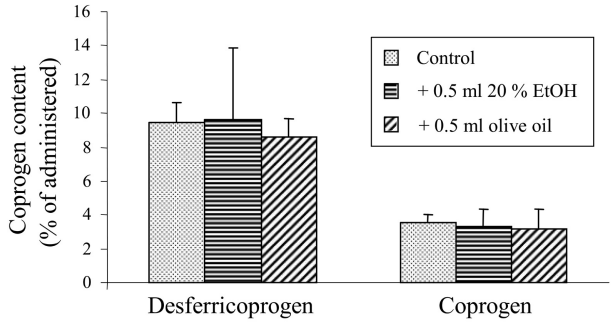

Figure 10. Effect of ethanol and olive oil on the accumulation of orally administered desferricoprogen and coprogen in the liver of rats. Experimental animals were dosed with 100 mg/(kg body mass) siderophore in each case. The data presented here are means ± SDs calculated from five independent experiments and represent mean total (desferri + ferri) siderophore contents recorded 24 h post-administration.

tively inhibit such LDL oxidation. Li *et al*. [59] have demonstrated that oxLDL-induced damage of macrophages is associated with iron-mediated intralysosomal oxidative reactions, leading to lysosomal rupture and subsequent apoptosis, that can be prevented by the iron-chelator, desferrioxamine. More recently, Minqin *et al.* [57] demonstrated that desferrioxamine B inhibited atherosclerotic lesion formation in rabbits on cholesterol-rich diet, and reduced iron content of plaques. Importantly, iron chelation improved endothelial function in patients with coronary artery disease [22].

Unfortunately, desferrioxamine B has some serious drawbacks *in vivo*: it has to be administered intravenously [60], does not last very long in the circulation [20] and can have serious side effects [61]. The present investigations were carried out to estimate the potential of several fungal siderophores to similarly suppress heme/iron-driven oxidation reactions and cytotoxicity and to study their absorption in the gastrointestinal tract. The results may have additional interest inasmuch as fungi which make these siderophores are used in making processed foods and exist as contaminants on commonly eaten foodstuffs [29].

All of the four fungal iron chelators tested here inhibited heme-catalyzed oxidation of LDL and attenuated damage to vascular endothelium caused by products of this LDL oxidation. In contrast, iron-saturated siderophores failed to provide cytoprotection against lipoprotein-mediated oxidative injury. Furthermore, we demonstrated that upregulation of redox sensitive HO-1 in response to oxidative stress was also blunted. Interestingly, a significant difference was observed in the extent of the inhibitory effects of the chelators with desferricoprogen exhibiting the strongest inhibitory potential in all of the three experiments (Table 2, Figs. 4 – 6). It is noteworthy that the weaker protective effect of desferrichrysin and, at least in part that of desferrirubin (Table 2), cannot be explained simply by differences in iron-chelate stability because the stability constants of tri-

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hydroxamate–iron complexes are 30–31 (log β_{110} values for the reactions of $L^{3-} + Fe^{3+}_{aq} \rightarrow FeL$) independent of the side-chains [24].

In heme-mediated peroxidation of LDL, heme is buried in the lipoprotein surface monolayer with the carboxyl groups in contact with positive regions of the protein and the solvent [62]. Hence, the primary site for heme degradation, iron release and lipid peroxidation is the surface monolayer itself [62, 63]. The iron-scavenging capability of the siderophores tested may therefore be connected to their partition coefficient and orientation within the surface monolayer. The 2-hydroxymethyl moieties in the chemical structures of ferrirubin and ferrichrysin (Fig. 1) may be disadvantageous for effective inhibition of iron-catalyzed lipid peroxidation (Table 2). However, further studies are needed to elucidate if satisfactory incorporation or proper orientation in the surface monolayer is disturbed in the case of ferrirubin and ferrichrysin. Importantly, coprogen was proven to incorporate into LDL particles in its desferri-form (Fig. 3). This implies that free, redox-active iron, released during oxidative reactions between heme, LDL, and H_2O_2, may be captured *in situ* by desferricoprogen.

Ferri-siderophores were completely inert in all the LDL modification and endothelial cell cytotoxicity experiments (Figs. 2, 4–6), which is in good accordance with the low redox potentials of Fe^{3+}–trihydroxamate siderophore complexes ($E_{1/2}$ = –0.400–0.468 V) in aqueous solutions [24]. They also did not alter endothelial HO-1 expression, although using coprogen, the incorporation of the chelator into LDL particles is equivalent to that of desferricoprogen (Fig. 3). When LDL was treated by coprogen in the presence of heme, LDL oxidation progressed, and the HO-1 gene was induced. In the absence of heme, the inclusion of coprogen into the reaction mixtures did not generate LDL oxidation, and did not result in HO-1 induction either. This means that, similar to the bacterial desferrioxamine B, iron ions buried in fungal hexadentate trihydroxamates are not "redox-active" unlike the tridentate *P. aeruginosa* siderophore pyochelin [26, 27]. Two of the tested four siderophores, coprogen, and ferrichrome – both produced by cheese-ripening *Penicillium* spp. – were approximately as powerful as desferrioxamine B in preventing heme-catalyzed LDL peroxidation and preventing the accumulation of cytotoxic LDL oxidation products. Both fungal siderophores may be a viable option to desferrioxamine B for the *in vivo* suppression of iron-catalyzed oxidation reactions and, perhaps, prevention of oxidant damage to tissues.

Catalytic iron – which can be derived from macrophages by exocytosis [64] – plays a role in the severity of atherosclerosis [65] *via* oxidation of the lipid cores of atheromas [34, 55, 65]. Desferricoprogen hindered considerably the heme-mediated oxidation of human atherosclerotic soft plaque lipids although this effect was temporary (Fig. 7). It is worth noting that desferrioxamine B only had a significant effect on atherosclerotic lesion development and lesion iron content in cholesterol-fed rabbits when the administration of siderophore was extended to 12 wk [57]. The contribution of iron to the initiation and development of atherosclerosis can be controlled only by long-term application of iron-removing agents [57].

Our findings may have one further implication. To the extent that these fungal siderophores may be absorbed by the gastrointestinal tract, the possibility exists that diets rich in fungal siderophores may provide protection against *in vivo* lipid peroxidation, and atherogenesis. According to our measurements, the coprogen content of *P. roqueforti*-processed cheese can be as high as 38 mg/kg, >30% of which is in the desferri-form.

The distribution of orally administered desferricoprogen and coprogen in organs and feces and urine in the rat clearly indicated that both the iron-complexed and the iron-free forms of the siderophore was taken up in the gastrointestinal tract and was accumulated primarily in the liver (Fig. 8). Although the uptake and accumulation of desferricoprogen was coupled to complexation of iron and coprogen was only secreted in its ferri-form about 64% of liver-bound siderophore was uncomplexed after 1 day of administration (Fig. 9). When dosed intravenously, 21–34% of the administered desferricoprogen appeared in the liver 0.5–1.0 h post-administration but the ratio of the iron-uncomplexed form was considerably less, 27–34% of total absorbed siderophore (Fig. 9). Orally administered desferricoprogen was eliminated from the body at a much lower rate than coprogen (Fig. 8) and similar observations were made during intravenous administrations (data not shown). Hence, desferricoprogen was retained meanwhile coprogen was secreted more effectively than their iron-complexed and iron-uncomplexed counterparts, respectively, influencing negatively the body iron status. Although the total amount of siderophore detected in blood was negligible in comparison to those found in liver or spleen the serum concentration of coprogen was about 0.01 mg/mL (12 μmol/L), about one third of the total serum iron concentration recorded in rats (35.67 ± 5.46 μmol/L) [66] and was comparable to that published for humans (18.2 ± 5.4 μmol/L) [67]. Frequent blood donation lowering body iron may protect against accelerated atherosclerosis [67, 68] but the hypothesized deleterious effects of dietary iron uptake and body iron stores on the atherosclerotic process have been questioned most recently [69, 70]. Therefore, the beneficial effects of dietary siderophore uptake on the vascular system is expected to come primarily from scavenging heme-released, biologically active free iron in the serum and LDL particles (Fig. 3) and extracting iron from atherosclerotic plaques (Fig. 7) [34, 55, 58, 65] and not from the overall reduction of iron stores [69, 70].

It is important to note that huge quantities of orally administered desferricoprogen (84%) and coprogen (74%) did not appear in either feces or urine and were not localized in the tested organs either (Figs. 8 and 9). This may indicate

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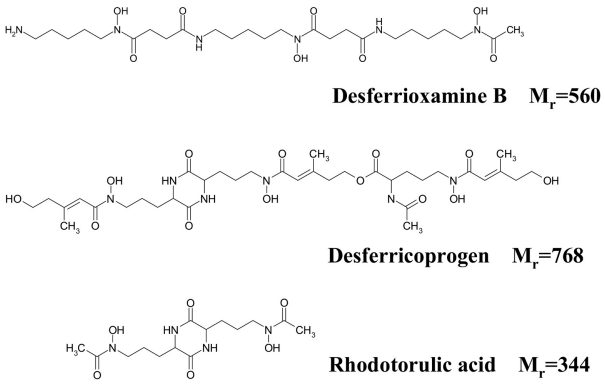

Figure 11. Chemical structure of desferrioxamine B, desferricoprogen, and rhodotorulic acid. The tetradentate rhodotorulic acid is structurally related to the coprogen family.

(i) chemical and microbial degradation of the compounds in the gastrointestinal tract, (ii) accumulation and storage of siderophores in organs other than liver, spleen and intestinal epithelium, or (iii) fast degradation of the chelator and/or its iron-chelate in the liver. All three options will be taken into consideration in forthcoming physiological and pharmacokinetic investigations but at least one not-yet-identified coprogen derivative with HPLC retention time shorter than that of coprogen has been detected in liver under both oral and intravenous administrations of either desferricoprogen or coprogen (data not shown). As the chemical composition and molar extinction coefficient of this siderophore derivative has remained yet to be elucidated its concentration, which increased steadily in time, could not be estimated. Important to know that this metabolite was not dimerumic acid, a tetra-dentate iron-chelator, which is formed by hydrolysis of the ester bond of coprogen (Figs. 1 and 11) [50, 71], and it also appeared in feces specimens in various concentrations.

Although no information has been available thus far on the gastrointestinal absorption and bioavailability [72] of coprogen-type and ferrichrome-type siderophores in humans hexadentates were considered in general as orally inactive chelators [73]. This false conclusion arose from the poor uptake of desferrioxamine B (Fig. 11) in the gastrointestinal tract [74]. Nevertheless, this bacterial hexadentate trihydroxamate possesses a positively charged free amino group (pK = 10.84; Fig. 7) [75] at physiologically relevant pH values, which may influence profoundly its migration through biological membranes [76]. Taking into consideration the relatively high acidic dissociation constants of the hydroxamate groups of desferrioxamine B (pK_1 = 8.30, pK_2 = 9.00, pK_3 = 9.46) we can establish that this siderophore is a cation, *i.e.*, an electrolyte, under physiological conditions including the intestinal lumen.

Unlike desferrioxamine B, desferricoprogen with acidic dissociation constants of pK_1 = 8.00, pK_2 = 9.00, and pK_3 = 9.84 [77] is an uncharged nonelectrolyte under physiological conditions (Fig. 11). Therefore, desferricoprogen possesses a more hydrophobic character, which was demonstrated by its effective incorporation into LDL particles (Fig. 3), and which may facilitate its migration across biological membranes too, including those of the epithelium of the gastrointestinal tract. Considering the very different physico-chemical characteristics of desferrioxiamine B and coprogen it is now understandable why fungal nonelectrolyte hexadentate chelators are orally available. Of course, the elucidation of the mechanism of the unexpected uptake of desferricoprogen and coprogen in the gut needs further studies.

It is worth noting that rhodotorulic acid, a tetradentate dihydroxamate, which is structurally related to the coprogen family (Fig. 11), increased moderately urinary iron excretion (up to 50 – 200 μg/kg/day) in iron-overloaded rats when it was administered orally [78]. This observation also indicated that there was some limited absorption of rhodotorulic acid in the gastrointestinal tract, which, of course, was not satisfactory for a potential iron-chelating drug for oral application [78].

Finally, in the particular case of LDL oxidation and atherogenesis, the mind wonders to the famous "French paradox" wherein people who eat high lipid (often cheese-rich) diets and drink wine are protected against atherogenesis. This has been explained with the possible anti-oxidant effects of wine, especially, red wine. But hold on – maybe it is the cheese! The authors propose that traditional mold-ripened foods as well as the introduction of new-type functional foods and food additives rich in hexadentate trihydroxamate fungal siderophores might give us a new and powerful tool in preventing vascular diseases such as atherosclerosis.

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The authors have declared no conflict of interest.

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