THESIS FOR THE DEGREE OF Ph.D.

INDUCTION OF OXIDATIVE STRESS AND STRESS-ADAPTATON BY FERRIPORPHYRINS AND HEME PROTEINS

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Debrecen, 2002

1. INTRODUCTION

Oxidative cell damage implicated in various human diseases. Much of the cellular damage caused by activated oxygen involves the collaboration of intracellular iron. One critical feature of highly damaging iron to endothelium is permeation of the metal into cells. Chelation of iron by lipophilic iron chelators results in endothelial cell associated catalytically active iron chelates accumulating in endothelial lipid compartments, and the endothelium becomes extremely sensitive to both exogen and endogen oxidant stress.

One physiologically ubiquitous hydrophobic iron chelate is heme. Heme readily concentrates within the hydrophobic milieu of intact endothelium and greatly amplifies cellular damage arising from reactive oxygen species.

Heme serves as a catalyst for the oxidation of low-density lipoprotein (LDL). Oxidative modification of LDL has been implicated in the pathogenesis of atherosclerosis. Oxidatively modified LDL is cytotoxic to endothelium, chemoattractant for circulating monocytes. Enhanced uptake of LDL by macrophages through acetyl LDL receptor, leading to the generation of foam cells. Oxidatively modified LDL is highly immunogenic.

Endothelial cells can respond to chronic heme exposure or heme mediated lipid peroxidation by upregulating heme oxygenase (HO) and ferritin. Heme oxygenase catalyzes the initial reaction in heme catabolism; opens the porphyrin ring producing biliverdin and carbon monoxide and releasing iron. Three genes encode for 3 izoenzymes for heme oxygenase. Heme-oxygenase-1 identified as a 32.8 kD stress protein, is the form transcriptionally inducible by a variety of agents, such as heme, heavy metals, oxidants and cytokines. Heme oxygenase-2 and heme oxygenase-3 are constitutively expressed. Ferritin is the major intracellular depot of nonmetabolic iron. This multimeric protein consist of 24 subunits of 2 types (heavy chain and light chain) and has a very high capacity for storing iron (up to 4500 mol of iron per mol of ferritin). The heavy chain of ferritin manifests ferroxidase activity. Ferritin synthesis is regulated posttranscriptionally by intracellular iron. The central importance of HO-1 was recently highlighted by the discovery of a child with HO-1 deficiency who exhibited extensive endothelial damage. Similar damage to endothelium as well as hepatic and renal cytotoxicity has been observed in transgenic knockout mice deficient in HO-1. In both human and mice very high concentration of circulating heme were present.

Balla et al described that methemoglobin (MetHb) similarly to free heme sensitizes vascular endothelial cells to oxidant injury. If the endothelium is exposed to methemoglobin for a more

prolonged period, it accumulates large amounts of ferritin and heme oxygenase, and the endothelium converts from hypersusceptible to hyperresistant to oxidative damage. Both oxidative stress induction and stress adaptation are significantly reduced if heme release from methemoglobin is inhibited.

Although a number of heme proteins have been reported to act as oxidants of LDL, the mechanisms involved are by no means clear. Some authors postulated that tyrosyl radical species on the globin surface induce oxidative modification of LDL. Our studies offer an alternative pathway for modification of LDL by hemoglobin in plasma involving heme release from methemoglobin.

2. AIMS

Acute hepatic porphyrias, a failure of iron-protoporphyrin IX biosynthesis and accumulation of porphyrin precursors cause a myriad of disabling symptoms. Therapeutically, infusion of heme has proven efficacious by correcting heme metabolism, but has been accompanied by considerable toxicities, including vasculitis and coagulopathy.

Finnish investigators successfully introduced heme arginate in the treatment of acute hepatic porphyrias without evidence of vasculopathy or thrombotic side effects.

We hypothesize that heme arginate lack of vascular side effects in vivo may be due to its inefficient catalyses of oxidant-mediated endothelial cell injury. In order to test whether various heme compounds used clinically in the treatment of porphyria are vasculotoxic in vitro, we examined the effects of ferriporphyrins with different hydrophobic properties on oxidant susceptibility of endothelial cells or LDL. We also tested ferriporphyrin's ability to induce heme oxygenase and ferritin in endothelium.

Numerous pathologies may involve toxic side effects of free hem and heme-derived iron. Deficiency of the heme-catabolizing enzyme, heme oxygenase-1 in both human and transgenic knockout mice leads to an abundance of circulating heme and damage to vascular endothelium. Although heme can be directly cytotoxic, the present investigations examine the possibility that hemoglobin-derived heme and iron might be indirectly toxic through the generation of oxidized forms of low-density lipoprotein. We wanted to test the effect of other in vivo relevant heme proteins on oxidative modification of LDL in whole plasma. We hypothesize that hemoglobin in plasma, when oxidized to methemoglobin by oxidants such as leukocyte-derived reactive oxygen, causes oxidative modification of LDL, and endothelial

cell cytotoxicity. We planned to characterize the LDL of a HO-1 deficient child, described by Yachie et al.

3. METHODS

Isolation and culture of endothelial cells

Human umbilical vein endothelial cells were removed from human umbilical vein by exposure to dispase. Human aortic and microvascular endothelial cells were isolated using type I collagenase.

Preparation of human polymorphonuclear leukocytes (PMN)

PMNs were isolated from human volunteers by centrifugation.

Preparation of hemoglobin

Purified ferrohemoglobin was prepared using ion-exchange chromatography. Ferrihemoglobin was achieved by incubation of hemoglobin with $K_3Fe(CN)_6$. Cyanoferrihemoglobin was formed by the addition of 2-fold excess NaCN to ferrihemoglobin followed by gel filtration.

Preparation of low density lipoprotein

Plasma LDL was prepared from Na_2EDTA anticoagulated venous blood employing single spin gradient ultracentrifugation.

Measurement of oxidative resistance of LDL

Oxidative resistance of LDL was characterized by in seconds, the time period passing till the maximal velocity of hemin disappearance in the propagation phase of heme mediated lipid peroxidation.

Detection of lipid peroxidation

Conjugated dienes were determined spectrophotometrically at absorbance of 232 nm. Lipid hydroperoxide in LDL was detected using the Ferrous Oxidation of Xylenol orange assay. Thiobarbituric acid reactive substances in LDL were also measured. Reactive amino groups in LDL were estimated with fluorescamine using lysine as a standard. Electrophoretic mobility of lipoprotein was determined by agarose gel electrophoresis.

Iron and heme determinations

Iron was measured spectrophotometrically as ferrozine-iron complex. LDL associated heme was determined spectrophotometrically at 398 nm after formic acid was added to LDL samples.

Endothelial cell cytotoxicity assay

Cytotoxicity was determined by ^{51}Cr release or MTT assay in cultured endothelial cells.

Measurement of heme oxygenase enzyme activity

Heme oxygenase enzyme activity was measured by bilirubin generation. Microsomal pellet of endothelial cells were added to the reaction mixture containing rat liver cytosol, hemin, glucose-6-phosphate, gluose-6-phosphate-dehydrogenase, and NADPH for 60 min. The formed bilirubin was extracted with chloroform, and ΔOD 464-530 nm was measured.

Measurement of ferritin content

Endothelial cells were solubilized and ferritin was determined with the use of the IMx ferritin enzyme immunoassay (Abbott Laboratories).

Northern blot analysis of heme oxygenase and ferritin mRNAs

Endothelial cell RNA was isolated by the RNAzol method. RNA was electrophoresed, and transferred to nylon membranes. The membranes were hybridized with ^{32}P- or biotin-labeled cDNA probes. Autoradiographs were quantified by computer-assisted videodensitometry.

4. RESULTS

4.1. FERRIPORPHYRIS AND ENDOTHELIUM

In acute life-threatening porphyric attacks, heme has been successfully used although there may develop harmful effects on the vasculature. Heme arginate treatment for acute porphyric attacks has been very effective without evidence of vascular side effects. In our study we tested substituted derivatives of heme, and heme arginate. In derivatives the vinyl side chains of heme were substituted by hidrogen (iron deuteroporphyrin IX), sulfonate (iron deuteroporphyrin IX, 2,4-bis-sulfonate), propionate (iron coproporphyrin III) or glycol (iron deuteroporphyrin IX, 2,4-bis-glycol).

4.1.1. Sensitization of endothelial cells to oxidant damage by ferriporphyrins

In our studies, heme arginate unlike heme did not amplify hydrogen peroxide or activated polymorphonuclear leukocyte-mediated endothelial cytotoxicity. Nonpermeant heme analogues, iron deuteroporphyrin IX, 2,4-bis-sulfonate, iron coproporphyrin III, and iron deuteroporphyrin IX, 2,4-bis-glycol, also failed to sensitize endothelial cells to oxidants. In contrast brief exposure of endothelium to the lipid soluble ferriporphyrin iron deuteroporphyrin IX sensitized cells to oxidant injury mediated by hydrogen peroxide or activated neutrophils.

4.1.2. Induction of cytoprotectants by ferriporphyrins

Heme arginate did not provoke oxidant-mediated endothelial damage, but nevertheless entered endothelial cells similarly to heme and iron deuteroporphyrin IX; heme oxygenase mRNA level and enzyme activity were markedly increased in cells exposed to heme arginate as in heme- or iron deuteroporphyrin IX-treated cells. This substantial induction of heme oxygenase gene was accompanied by increased endothelial ferritin synthesis. In spite of the similar enhancement of heme oxygenase mRNA and enzyme activity, heme arginate only doubled endothelial ferritin content, while in endothelium exposed to heme or iron deuteroporphyrin IX, ferritin increased 20-fold and 13-fold respectively. To assess differences in degradation of the porphyrin ring, heme arginate or heme was used as a substrate of heme oxygenase. Less bilirubin was generated when heme arginate was used as a substrate for heme oxygenase than when heme was used. The lower level of iron release from the heme arginate porphyrin ring may explain the blunted ferritin response. Heavy- and light-chain ferritin mRNA levels were not altered by heme arginate, heme, or iron deuteroporphyrin IX, demonstrating that in these cell systems, iron-mediated regulation of ferritin synthesis occurred primarily at the posttranscriptional level.

Prolonged incubation of endothelial cells with heme rendered them markedly resistant to oxidant challenge via the induction of heme oxygenase and ferritin. The ultimate cytoprotectant against iron-driven oxidant injury was identified as ferritin in a wide range of experimental conditions. Endothelial cells, when incubated with iron deuteroporphyrin IX, were also markedly induced increase heme oxygenase mRNA and enzyme activity as well as their ferritin content. The remarkably increased synthesis of heme oxygenase and ferritin proteins were associated with resistance to oxidative stress imposed by the highly damaging combination of heme and hydrogen peroxide. However no associated cytoprotection against iron-driven oxidant injury was afforded if only a high level of heme oxygenase enzyme activity was present without a substantial increase in ferritin content as seen after endothelial cells were exposed to heme arginate. Nonpermeant ferriporphyrins, iron deuteroporphyrin IX, 2,4-bis-sulfonate, iron coproporphyrin III, and iron deuteroporphyrin IX, 2,4-bis-glycol, were noninducers for heme oxygenase and ferritin in endothelium and did not provide cytoprotection against oxidant damage.

4.1.3. Comparison of heme and heme arginate as a catalyst of oxidative modification of LDL

Heme can also threaten vascular endothelial cells integrity by its ability to catalyze the oxidation of LDL. LDL oxidized by heme is extremely cytotoxic to endothelial cells. The kinetics of LDL lipid peroxidation mediated by heme arginate in the presence of hydrogen peroxide was characterized by a longer ΔT at Vmax as well as a slower propagation phase

compared with heme-mediated lipid peroxidation of LDL as judged by conjugated diene formation. The results of several independent assays for LDL oxidation all support the conclusion that heme arginate promoted LDL oxidation less efficiently. Accordingly, heme arginate/H_2O_2 –conditioned LDL was less cytotoxic to cultured human endothelial cells than heme/H_2O_2 –conditioned LDL.

4.2. PRO-OXIDANT AND CYTOTOXIC EFFECTS OF HEME PROTEINS

The pronounced vascular pathologies described for both an HO-1 deficient human and mice in which this enzyme has been knocked out suggest that defective heme catabolism (and, by implication, heme itself) can have serious pathologic effects. Whereas heme may be directly cytotoxic, the present investigations represented an attempt to determine whether the observed in vivo effects of HO-1 deficiency might, at least in part, represent an indirect process. Specifically, we hypothesized that extensive; heme/heme iron mediated oxidation of LDL might produce oxidized forms of LDL with appreciable cytotoxicity. In our study we examined heme and some important heme proteins in the oxidative modification of LDL in whole plasma.

4.2.1. Cytotoxic effect of LDL isolated from heme protein-pretreated plasma

LDL isolated from plasma pre-incubated with either heme or metHb is markedly cytotoxic to cultured endothelial cells. Conversion of ferrohemoglobin (ferroHb) to metHb appears to be essential for the ensuing oxidation of LDL, presumably because metHb readily releases heme whereas ferroHb does not. This observation prompted us to hypothesize that, following dissociation of heme from metHb; the free heme may readily enter lipoprotein particles. Indeed, LDL particles successfully compete for heme released from metHb in plasma despite the presence of specific and aspecific heme-binding proteins. Haptoglobin or cyanide was shown to strengthen heme-globin liganding preventing heme release from metHb. Preincubation of metHb with sodium cyanide or stoichiometric amounts of haptoglobin prevented the generation of toxic species of LDL.

4.2.2. Detection of oxidative modification of LDL separated from plasma pre-incubated with heme proteins

The results of several independent assays for LDL lipid peroxidation support the conclusion that the presence of heme or methemoglobin in plasma promotes LDL oxidation. Shortening of ΔT at Vmax by methemoglobin is paralleled by a rapid decrease in the α-tocopherol content of LDL, which was followed by the formation of conjugated dienes, lipid-hydroperoxides (LOOHs) and thiobarbituric reactive substances (TBARS). Ferrohemoglobin

in plasma did not have the capacity to increase the susceptibility of LDL to oxidative modification, but if ferroHb-containing plasma is exposed to activated PMNs for 90 min, the separated LDL was oxidatively modified.

4.2.3. In vivo oxidative modification of LDL

Spectral analysis of plasma from the heme oxygenase-1 deficient child revealed that plasma hemoglobin was predominantly methemoglobin. The proportion of total hemoglobin present as methemoglobin was around 80% (~60 μM). LDL isolated from the plasma of this child showed the same cytotoxic effects as were obtained with LDL isolated following pre-incubation of normal plasma with methemoglobin. The oxidative modification of the child's LDL was demonstrated by several independent assays for lipid peroxidation. Conjugated dienes, LOOHs and TBARS accumulated in his lipoprotein, and the child's LDL had increased electrophoretic mobility, decreased oxidative resistance, and decreased α-tocopherol content.

4.2.4. Induction of cytoprotectants by LDL isolated from plasma pre-treated with heme proteins

Exposure of endothelial cells to sublethal amounts of oxidized LDL isolated from plasma containing heme or methemoglobin markedly induced HO-1 mRNA a sensitive marker for oxidative stress. Accompanying this mRNA induction, HO activity was also significantly enhanced. In contrast, LDL isolated from ferrohemoglobin treated plasma did not alter HO-1 mRNA level and enzyme activity in endothelial cells. Similar effects were observed in the case of ferritin; LDL from plasma exposed to methemoglobin or heme caused a doubling of endothelial ferritin content whereas ferrohemoglobin failed to induce ferritin synthesis. If ferrohemoglobin-containing plasma is exposed to activated PMNs for 90 min and then the LDL is isolated and added to endothelial monolayers, induction of HO-1 mRNA and enzyme activity occur. Likewise, under the same conditions, endothelial ferritin accumulates.

Exposure of endothelial cells derived from healthy subjects to sublethal stress of the child's LDL led to marked increase in enzyme activity for HO, and doubled ferritin content.

5. SUMMARY

- **Ferriporphyrins with hydrophobic property threaten vascular endothelial cells to oxidant stress and catalyze the oxidative modification of LDL.**
- **Heme-arginate does not sensitize endothelial cells to oxidant challenge, and promote LDL oxidation less efficiently than heme does.**
- **Ferriporphyrins with hydrophobic property induce cytoprotectants; heme oxygenase and ferritin in human endothelial cells.**
- **LDL particles successfully compete for heme released from heme proteins in plasma despite the presence of heme-binding proteins.**
- **LDL isolated from plasma pre-incubated with methemoglobin but not ferrihemoglobin was found to be markedly cytotoxic to endothelial cells.**
- **Pre-treatment of plasma with methemoglobin in contrast to ferrohemoglobin increase the susceptibility of LDL to oxidative modification.**
- **Sublethal dose of LDL isolated from methemoglobin but not ferrohemoglobin pre-incubated plasma induces cytoprotectants in endothelial cells.**
- **LDL isolated from ferrohemoglobin and activated PMNs containing plasma is cytotoxic, or at sublethal concentration induce heme oxygenase and ferritin.**
- **Spectral analysis of plasma from heme oxigenase-1 deficient child revealed that the proportion of total hemoglobin present as methemoglobin was around 80% (~60 μM).**
- **LDL isolated from the heme oxygenase-1 deficient child was cytotoxic, and oxidatively modified. Sublethal stress of the child's LDL led to marked increase in enzyme activity for HO, and doubled ferritin content in endothelial cells.**

6. LIST OF PUBLICATION

ORIGINAL ARTICLES THE THESIS IS BASED ON

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2. Balla J, Balla G, Kakuk G, Jeney V, Ujhelyi L, Jacob HS and Vercellotti GM: Iron-protoporphyrin IX associated with arginate does not serve as a free radical catalyst but induces cytoprotectants in endothelial and tubular epithelial cells. *Nephrol Dial Transplant,* 15:19A, 2000.

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3. Jeney V, Balla J, Yachie A, Varga Z, Balla G: Az LDL oxidációja a hemoxigenáz-1 deficiencia patomechanizmusában. *A Magyar Biokémiai Egyesület Molekuláris Biológiai Szakosztálya 6. Munkaértekezlete,* Sárospatak, 2001.

OTHER ABSTRACTS

4. Balla J, Balla G, Kakuk G, Jeney V, Ujhelyi L, Nath K and Vercellotti GM: Hemoglobin induced upregulation of heme oxygenase and ferritin in rat lung and kidney in vivo to prevent oxidant damage. *Nephrol Dial Transplant,* 14:41A, 1999.

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