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EDITORIAL

Heme in intestinal epithelial cell turnover, differentiation, detoxification, inflammation, carcinogenesis, absorption and motility

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Abstract

The gastrointestinal tract is lined by a simple epithelium that undergoes constant renewal involving cell division, differentiation and cell death. In addition, the epithelial lining separates the hostile processes of digestion and absorption that occur in the intestinal lumen from the aseptic environment of the internal milieu by defensive mechanisms that protect the epithelium from being breached. Central to these defensive processes is the synthesis of heme and its catabolism by heme oxygenase (HO). Dietary heme is also an important source of iron for the body which is taken up intact by the enterocyte. This review describes the recent literature on the diverse properties of heme/HO in the intestine tract. The roles of heme/HO in the regulation of the cell cycle/apoptosis, detoxification of xenobiotics, oxidative stress, inflammation, development of colon cancer, heme-iron absorption and intestinal motility are specifically examined.

Key words: Absorption; Heme; Uptake; Release; Heme oxygenase; Oxidant; Cytoprotection; Inflammation; Cancer; Detoxification

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INTRODUCTION

The lumen of the intestine mucosa is predominately covered with epithelial cells called enterocytes which are responsible for the terminal digestion and absorption of nutrients. These cells have a limited lifespan before being replaced by cells derived from the crypt region[1]. There is also evidence of apoptosis within the crypt, presumably in response to excess cellular proliferation, cytotoxicity or genomic damage[2]. Surviving cells undergo apical migration, limited cell replication, commitment and differentiation[1]. The process of differentiation is gradual, characterised by the accumulation of cell-specific products in the upper crypt region and attaining the mature phenotype in the lower to middle-villus region. Recent evidence indicates that heme is important in intestinal development as well as maintaining the mucosal barrier and protecting the body from invasion and the damaging consequences of ingested xenobiotics. However, heme in the colon may irritate the mucosa and derange the normal rates of proliferation/exfoliation, circumstances that raise the probability of colon cancer. Heme is also an important source of body iron and how it is absorbed by the enterocyte is considered in this article, as well as the role heme plays in intestinal motility. It needs to be recognised that an in depth focus on each of these components is outside the scope of this review, rather it is our intention to provide the general reader with evidence and interpretations supporting the markedly varied involvement of heme in intestinal function.

HEME BIOSYNTHESIS AND HEME OXYGENASE (HO) (EC 1.14.99.3)

Heme biosynthesis

Heme biosynthesis involves 8 enzymes, four localised to the cytoplasm and the others in the mitochondrial matrix[3-5] and is regulated by the first enzyme in its synthesis aminoleuvilinic acid synthase[6] (Figure 1A). Heme biosynthesis also requires iron, which in the intestinal crypt is derived from the plasma by the activity of the transferrin receptor operating in collaboration with the hemochromatosis protein (HFE)[7] (Figure 1B). Although heme synthesis is highest in the crypt epithelium it continues along the length of the crypt-villus axis. As the cells leave the crypt region iron appears to be acquired from the diet since dietary iron deficiency reduces the heme content of villus enterocytes, and in villus cells transferrin receptor has 25% the activity of crypt epithelium[8,9] (Figure 1B).

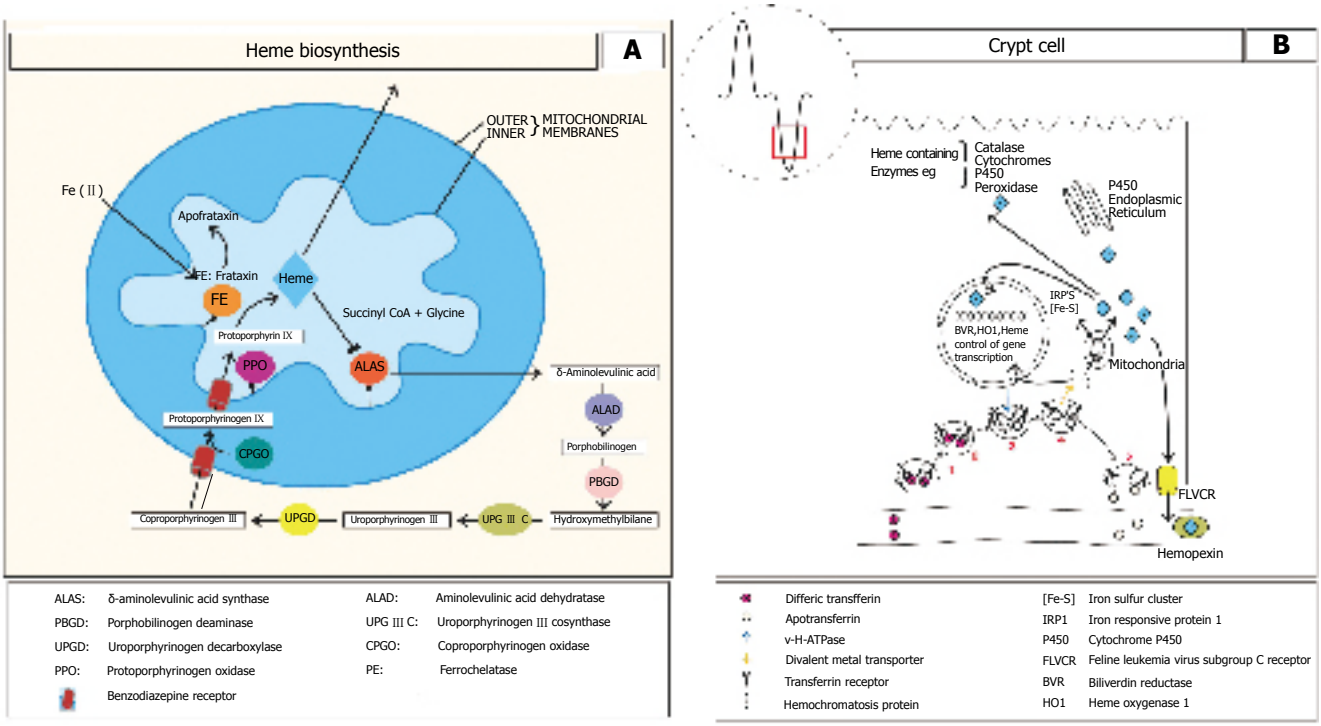

Figure 1 **A**: The heme biosynthetic pathway. Mitochondrial and cytosolic locations of the eight enzymes are shown circled and coloured. Commencing synthesis is ALAS on the inner mitochondrial membrane of the first intermediate as well as subsequent intermediates. Heme synthesis is regulated by heme at the level of ALAS *via* feedback repression. It has been suggested that frataxin may donate ferrous iron to protoporphyrin in the formation of heme; **B**: In the intestinal crypts the uptake of plasma transferrin-iron occurs by the transferrin receptor (TfR). In iron deficiency HFE complexes with TfR1 and to a much lesser extent with iron loading. (1) TfR binds to plasma diferric transferrin. (2) TfR is internalised by receptor mediated endocytosis. (3) In the cytoplasm a v-H+ATPase fuses with the endosome and acidifies it to release the iron from transferrin. Following ferrireduction Fe(II) is transported to the cytoplasm by a metal transporter. (4) possibly divalent metal transporter 1 (DMT1). The iron is then transported into the mitochondria where it is incorporated into heme. The mitochondria are also a major producer of iron sulphur clusters. (5) The transferrin receptor - apotransferrin complex then return to the cell membrane where at the neutral pH, apotransferrin dissociates. Heme, heme oxygenase and BVR may regulate gene transcription during enterocyte differentiation. FLVCR functions to export excess heme.

Function of HO

HO catalyses the mixed function oxidation of heme using cytochrome P-450, NAPDH and molecular oxygen[10-12]. HO functions in the oxidative cleavage of heme specifically at the α-methane bridge, resulting in the formation of biliverdin IXα which is rapidly reduced to bilirubin IXα by soluble biliverdin reductase (BVR). Since tissue BVR activity is 30-50 times greater than HO activity, this suggests that it is unlikely to limit heme breakdown, and that the rate limiting component is HO[12]. Recently, the crystal structure of HO in complex with heme and biliverdin-iron has been solved[13]. HO binds heme and oxygen between two helical folds with the proximal fold binding heme while the distal helix contains an oxygen binding site[13].

Isoforms of HO

HO is expressed as two isoforms designated HO-1[14] and HO-2[14,15] which are products of different genes[14]. HO-1 shares substantial homology with HO-2[15]. The molecular mass of HO-1 is 32 kD, while HO-2 is 36 kD. HO-1 expression is induced by numerous factors, including oxidative stress, inflammation, cytokines, nitric oxide, prostaglandins, an elevated level of substrate[16], iron deficiency[17], metals including Cd, Co, Cr, Cu, Fe, Hg, Ni, Pd, Pt, Sn, Zn[3,16,18,19], hyperoxia[20] and UV light[21]. The induction of HO-1 by hyperthermia has led to use of an alternate name, heat shock protein 32 (HSP-32)[22]. Unlike the inducible expression of HO-1, HO-2 expression is relatively constant.

HO and re-utilization of heme

HO-1 is mainly involved in the reutilization of heme-iron from hemoglobin and the expulsion of iron from tissue stores as evidenced by HO-1 knockout mice which develop anaemia because of progressive tissue iron retention particularly within macrophages[23]. A previous study shows that less than 50% of endogenous hepatic heme degradation in rats is accounted for by HO-1 activity as evidenced by the generation of CO from heme[24]. Therefore there appear two separate fates for catabolized heme-iron. Firstly a HO-1 dependent pathway, where iron from heme passes efficiently from the macrophage to the plasma, probably by the iron transporter ferroportin[25], and secondly, a HO-1 independent pathway which results in retention of the freed iron.

HO and oxidative stress

HO-1 functions to diminish cellular oxidative stress because HO-1 reduces the levels of the pro-oxidant heme and produces the antioxidant bilirubin[26]. Supporting this, humans deficient in HO-1[27] and individuals with impaired transcription due to a microsatellite polymorphism in the HO-1 promoter region[28,29] present with a phenotype similar to HO-1 knockout mice[30]. Interestingly, HO-2 is unable to compensate for the loss of HO-1, probably

because its expression is restricted to a select group of cells or it is unable to be induced to the levels of activity required to produce the effects seen with HO-1 expression[27-30]. HO-1 and intestinal oxidative stress is discussed in a later section.

INTESTINAL HEME BIOSYNTHESIS AND HEME OXYGENASE

Heme biosynthesis

The synthesis of heme and heme-containing proteins is crucial for intestinal function. These hemoproteins include electron carrying proteins such as cytochrome (CYP) P450 (see section on detoxification), mitochondrial localised cytochromes, the ferrireductase Dcytb[31], catalase and peroxidases which catalyse the reaction of hydrogen peroxide (H_2O_2) to water and oxygen (see section on oxidative stress). In addition to biosynthesis, heme can also be acquired by the enterocyte *via* intestinal absorption. This will be discussed in detail below with respect to the intestine.

HO gene expression

In the human intestinal cell lines CaCo-2 and HT-29, internalisation of heme increased HO-1 expression, indicating that the heme responsive element in the promoter region of the HO-1 gene was accessible and functional[32,33]. Duodenal HO-1 expression is also increased in iron deficiency[17] and by conditions that lead to oxidative stress including heavy metals and inflammation (see below with respect to the intestine). Up-regulation of HO-1 gene expression *via* the estrogen receptor β[34], octreotide, a somatostatin analogue[35] and glutamine[36] has been established. HO-2 expression is constitutive and mainly confined to the enteric nervous system and interstitial cells of Cajal, although it is possible that HO-2 is expressed by enterocytes[37]. This will be addressed later in this review.

Heme turnover along the crypt-villus axis

Heme turnover is the balance between heme synthesis and its destruction by heme oxygenase. It is subject to variation along the crypt-villus length, being highest in the crypt and least at the villus tip[38]. Thus the crypt region has the highest activity of both heme biosynthesis and heme oxygenase activity. As the cells migrate the rate of heme synthesis decreases but destruction decreases to a lesser extent, therefore total heme content is highest at the villus enterocytes compared with crypt epithelium.

HO-1 and intestinal cell proliferation and differentiation in the crypts

Cell turnover and differentiation is a function of crypt epithelium. Similar to that seen in the crypt epithelium, HO-1 activity is highest in undifferentiated intestinal epithelial Caco-2 cells[39]. This suggests that HO-1 and cell proliferation/apoptosis may be linked[40]. Supporting this, inhibiting HO-1 activity reduced cell proliferation and increased cell death[40,41]. Conversely, in the human intestinal cell line HT-29 cells induction of HO-1 activity reduced expression of the pro-apoptotic gene caspase-3 and inhibited apoptosis. This supports the idea that HO-1 activity is anti-apoptotic[42]. It is possible that HO-1 mediates these effects indirectly on gene transcription *via* the activity of BVR (Figure 2).

HO/BVR in intestinal cell signalling

BVR (EC 1.3.1.24) must undergo auto-phosphorylation in order to convert biliverdin to bilirubin[43]. This property of phosphorylation/dephophorylation during the conversion of biliverdin to bilirubin is similar to that seen with signalling kinases. Recent evidence indicates that BVR functions as a serine/threonine kinase that operates in the insulin receptor/MAPK pathways[44] and a transcription factor with a bZip domain involved in ATF-2/CREB and HO-1 regulation[45]. These additional roles suggest that BVR may have a broader function in regulating cellular activity[46]. Since BVR immunoreactivity is seen in nuclei of epithelium lining the GI tract, this suggests a possible role in the regulation of gene transcription[47].

HO-1 acts as a guardian of the genome during differentiation

It is possible that HO-1 may modulate proliferation by scavenging and/or preventing the formation of reactive oxygen metabolites (ROM) and reactive nitrogenous metabolites (RNM), since ROM inhibit Caco-2 cell proliferation[48] and stimulate apoptosis[49]. This is particularly relevant to the intestinal crypt region where proliferation exists and the levels of antioxidant detoxifying enzymes such as superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase are low[50]. If this is true then HO-1 level in the crypt region may act in defence against oxidative stress to limit mutation of DNA. HO-1 may therefore be one guardian of the genome, limiting mutations of DNA and promoting deletion of aberrant cells (Figure 2).

Differentiation is likely to result in elimination of cellular heme

As discussed previously the production of heme for enzymes, electron transport and as substrate for activity of HO1 and BVR is likely to be finely balanced since excess heme leads to oxidative stress and subsequent cell damage. Therefore as differentiation concludes heme production must fall. This may be achieved through reduced heme biosynthesis, increased HO-1 activity or increased heme export. With respect to heme export, a human heme exporter with homology to Feline leukaemia virus, subgroup C receptor (FLVCR) has recently been identified which has a clear function in erythropoiesis at the CFU-E stage of development[51]. Impairment of FLVCR leads to the loss of CFU-E cells and impairs erythroid differentiation by inducing apoptosis. FLVCR is also expressed by Caco-2 cells, suggesting that it may be involved in intestinal differentiation by reducing the cellular heme concentration as the cell differentiates[51]. This would reduce the oxidative burden on the stem/progenitor cell and potentially limit genomic damage[52]. Supporting the existence of the FLVCR in the intestine, Caco-2 cells internalised heme by an active transport process and transcytosed it from apical to basal surfaces[53].

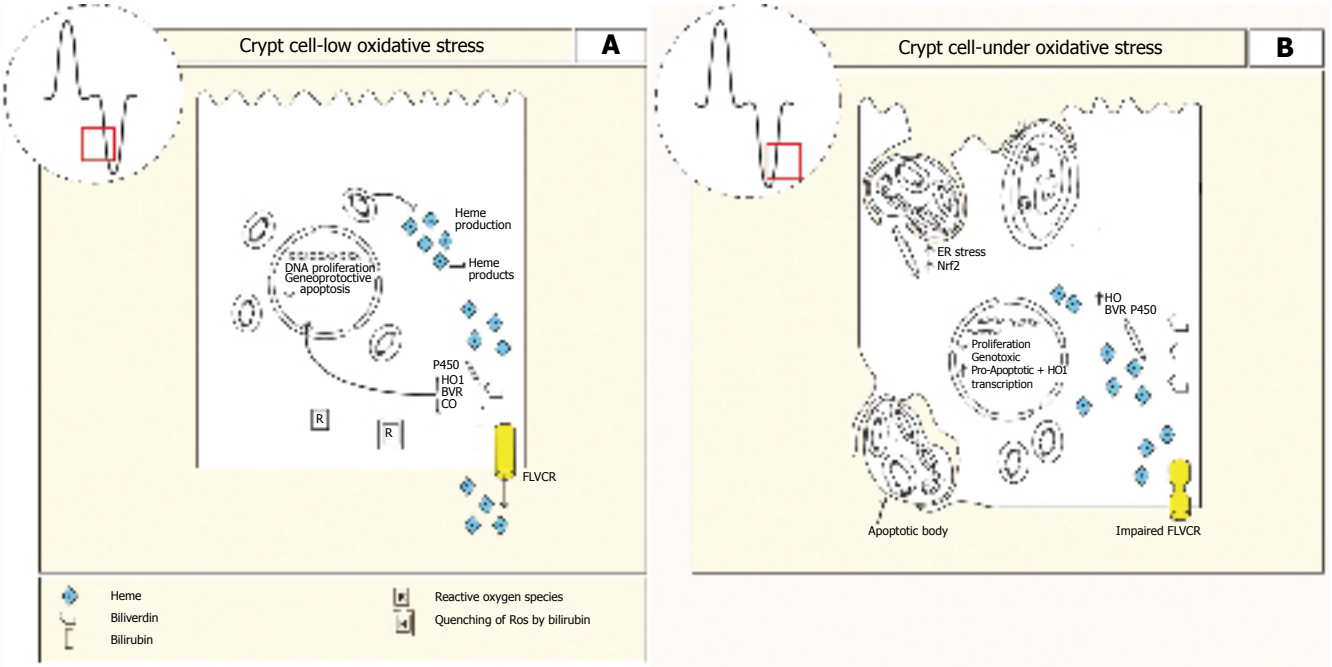

Figure 2 **A**: Epithelium of the crypt region is active in cell proliferation and differentiation. Heme production is required for the synthesis of heme containing enzymes. In these cells there are also high levels of heme oxygenase activity suggesting that heme breakdown is required for the production of bilirubin and carbon monoxide to maintain appropriate proliferation, differentiation and apoptosis. If the oxygen tension of the cell should increase or production of heme exceeds use, as would be seen as differentiation proceeds, then excess heme may be exported *via* FLVCR to limit oxidative stress. Increased oxidative stress may also be buffered by the antioxidant bilirubin; **B**: In the presence of increased oxidative stress caused by excess heme production, impaired FLVCR transport or increased oxygen tension, heme increases to levels that are genotoxic and the cell is predisposed to pro-apototic gene expression placing the cell into a death programme. Normal intestinal growth and differentiation would be impaired.

The converse was also true. Exposing the membranes to trypsin selectively increased the rate of uptake across the apical membrane only. Taken together these results raise the possibility that heme can be actively secreted from the cell in either direction possibly involving FLVCR (Figure 2).

HO activity along the length of the intestinal tract correlates with heme-iron absorption

HO activity is highest in the duodenum and lowest in the terminal ileum[54-56]. This pattern of HO activity appears to correlate with the uptake of ingested xenobiotics and heme-iron absorption along the length of the intestinal tract (see below). In fact, treating rats with phenobarbital increased microsomal P450 enzyme activity, and absorption of iron from hemoglobin[57]. Conversely, when an inhibitor of intestinal HO activity was given, intestinal heme-iron absorption decreased[58] (see below).

HO and CYP450 activities in xenobiotic metabolism

The intestine makes an important contribution to the detoxification of many ingested xenobiotics (food additives, industrial chemicals, pesticides, plant toxins and pharmaceutical agents)[59-61]. The heme containing P450 enzymes in particular the CYP3A superfamily are an integral component of xenobiotic detoxification. P450 levels are highest in the proximal duodenum, falling to lowest levels at the ileum[62,63]. This correlates with the gradient of exposure to ingested xenobiotics. The highest activity of the P450 enzymes studied to date is the villus region[64-67]. Interestingly, ingested xenobiotics induce greater CYP activity in the crypt epithelium compared with villus enterocytes[66]. Since the crypt cells do not absorb nutrients, this suggests that they passively absorb the drug or that the drug is actively absorbed by enterocytes and then taken up from the plasma by crypt cells. This interpretation is consistent with highest levels of heme biosynthesis in crypt epithelium.

Detoxification involves three phases, firstly the CYP450s and its mixed function oxidases adds a reactive group to the xenobiotic, secondly the molecule is made water soluble by conjugation to glucuronic acid, sulphates, glutathione or amino acids by UDP-glucuronosltransferases [UGT], sulfotransferases [SULT] or glutathione *S*-transferases [GST], respectively, thirdly the metabolite is excreted from the enterocyte into the lumen by a transporter such as the ATP binding cassette transporters (ABC), P-glycoprotein[59,62,63]. This "first pass" detoxification of xenobiotics is most active in the upper villus where absorption of nutrients and xenobiotics are greatest[64-67].

To perform optimal detoxification the enterocyte must express appropriate levels of CYP450 and this is in part determined by heme turnover. Therefore for the enterocyte to express appropriate CYP450, adequate absorption of iron from the diet is required for heme synthesis along with conditions that limit HO-1 expression[68-70]. If HO-1 activity is induced, for example by ingestion of environmental contaminants such as cadmium, organotins and heavy metals increased destruction of CYP will take place and first pass detoxification will be compromised. Similarly, iron deficiency reduces the ability to synthesise heme and therefore detoxify xenobiotics[64,65,71]. This may

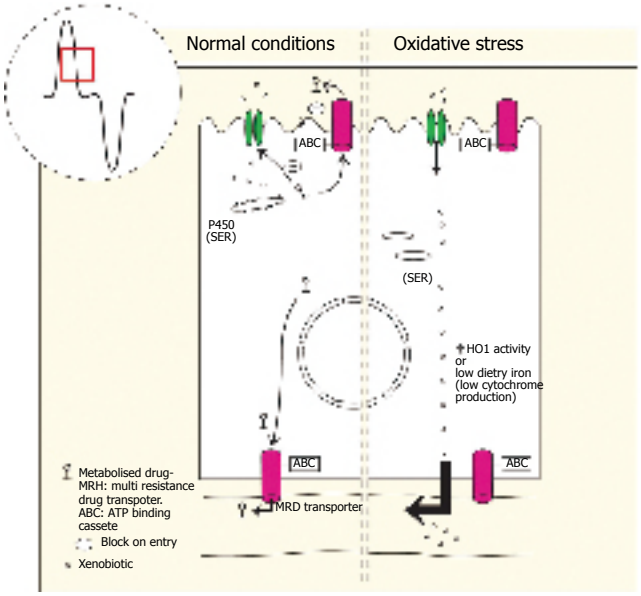

Figure 3 Left: Xenobiotics in the diet enter the enterocyte *via* facilitated diffusion or a specific transport process. Appropriate P450 expression on smooth endoplasmic reticulum (SER) enables first pass metabolism including phase I, and phase II metabolizing enzymes. Phase III multi drug resistance transporters (MDR) transport the conjugated-xenobiotic compound to the lumen or blood stream where increased hydrophilicity impairs re-entry into the enterocyte and leads to its elimination from the body directly. *De novo* synthesis of P450 occurs in the enterocytes and is dependent on appropriate levels of dietary iron. Right: In the presence of oxidative stress caused by high dietary intake of metals or compounds that induce heme oxygenase 1 (HO-1), heme containing P450 are broken down leading to increased entry of xenobiotics to the body. Dietary iron deficiency leads to reduced P450 activity and reduced detoxification capabilities.

therefore predispose an individual to cancer and ulceration of the colon[72] (Figure 3).

HO and hyperbilirubinaemia

Several metalloporphyrins are competitive inhibitors of HO-1 activity because they have the capacity to interact with the heme binding site in HO-1, but are unable to activate the enzyme. This leads to a loss of heme degradation[73-76]. This strategy has been used in the correction of human neonatal hyperbilirubinemia[77-79]. Treatment with tin-protoporphyrin/mesoporphyrin, two structurally related heme analogues are effective in lowering serum bilirubin levels in many animals by competitively inhibiting HO[73-79]. In addition, the use of short interfering RNAs targeting HO-1 mRNA expression has also been proposed to treat hyperbilirubinemia[80]. Although there is a recognised loss of endogenous heme through the bile during metalloporphyrin administration[81-83] that has been linked to an iron deficient state[84], the iron deficiency has been shown to be readily reversible.

In the enterocyte, bilirubin is conjugated to glucuronic acid by bilirubin glucuronyl-transferase and excreted into the intestinal lumen[85], or passed into the plasma where it non-covalently binds albumin and is transported to the liver, conjugated and excreted into the bile. However, early in perinatal life the luminal activity of secreted lysosomal-derived glucuronidase is high suggesting that enterocyte and biliary excreted conjugated-bilirubin can be deconjugated within the intestinal lumen enabling bilirubin to be reabsorbed *via* the enterohepatic circulation[86]. This would contribute to neonatal hyperbilirubinemia.

Glutamine increases HO-1 expression

Glutamine is a major source of energy for the enterocyte and has been shown to promote intestinal growth and maintain intestinal integrity particularly when the intestine is heat stressed and starved[36,87-91]. Glutamine stimulates intestinal proliferation and acts synergistically with epidermal growth factor to induce the mitogen-activated protein kinases and Jun nuclear receptor kinases. These in turn phosphorylate nuclear transcription factors such as AP-1 which activate transcription of target genes involved in cell proliferation and repair, including HO-1[36,88]. Recently it was shown that glutamine stimulation of HO-1 expression was protective against endotoxic shock of the lower intestine[90].

The inflammatory response and the role of HO-1

The epithelium lining the gastrointestinal tract presents a "mucosal barrier" to the migration of pathogens into the lamina propria that reside within the lumen of the gastrointestinal tract. In addition to the epithelium which is selectively permeable to nutrient absorption, the mucosal barrier comprises tight junctions that prevent migration of pathogens between cells. Breaching the mucosal barrier elicits an inflammatory response which first involves the innate immune system. Toll like receptors (TLR) expressed on the basolateral surface of enterocytes and the cell membrane of macrophages are activated[92] and these in turn activate intracellular signalling pathways that induce NF-κB dependent transcription of genes involved in the pro-inflammatory response such as cytokines, chemokines, immune receptors, nitric oxide synthase, prostaglandins and cell surface adhesion molecules[93-95]. The pro-inflammatory mediators initially function to increase blood flow and edema. Concomitant with this, endothelial cell membranes express cell adhesion receptors including ICAM-1 that enable white blood cells to adhere and extravasate[34]. The further release of pro-inflammatory chemokines (CINC-1, -3) may lead to hemostasis and organ failure[34].

Inflammation is known to induce HO-1 gene expression and in turn its activity. The bilirubin and CO produced are thought to have restorative effects on impaired tissue function, in the case of bilirubin it is a potent anti-oxidant[26,96-98]. There was increased oxidized bilirubin in the urine of patients following invasive surgery, supporting the idea that bilirubin acts as an antioxidant to scavenge reactive oxygen species[97].

The second metabolite of HO-1 activity, CO has been shown to relax vascular smooth muscle by binding to the heme moiety of soluble guanyl cyclase (sGC). Activation of sGC increases blood flow to the site of intestinal injury[99,100], inhibits platelet aggregation[101], reduces microvascular fibrin accumulation[102] and restricts leukostasis in postcapillary venules[93,103]. Reduced leukostasis by CO is thought to occur *via* inhibition of the expression of the adhesion molecules, P-, E- selectins, and ICAM although some contribution by bilirubin is also thought responsible for the leukostasis[104-106]. CO exerted additional cytoprotection by inhibiting components of the pro-inflammatory pathway including TNF-α, IL-1β, IL-2,

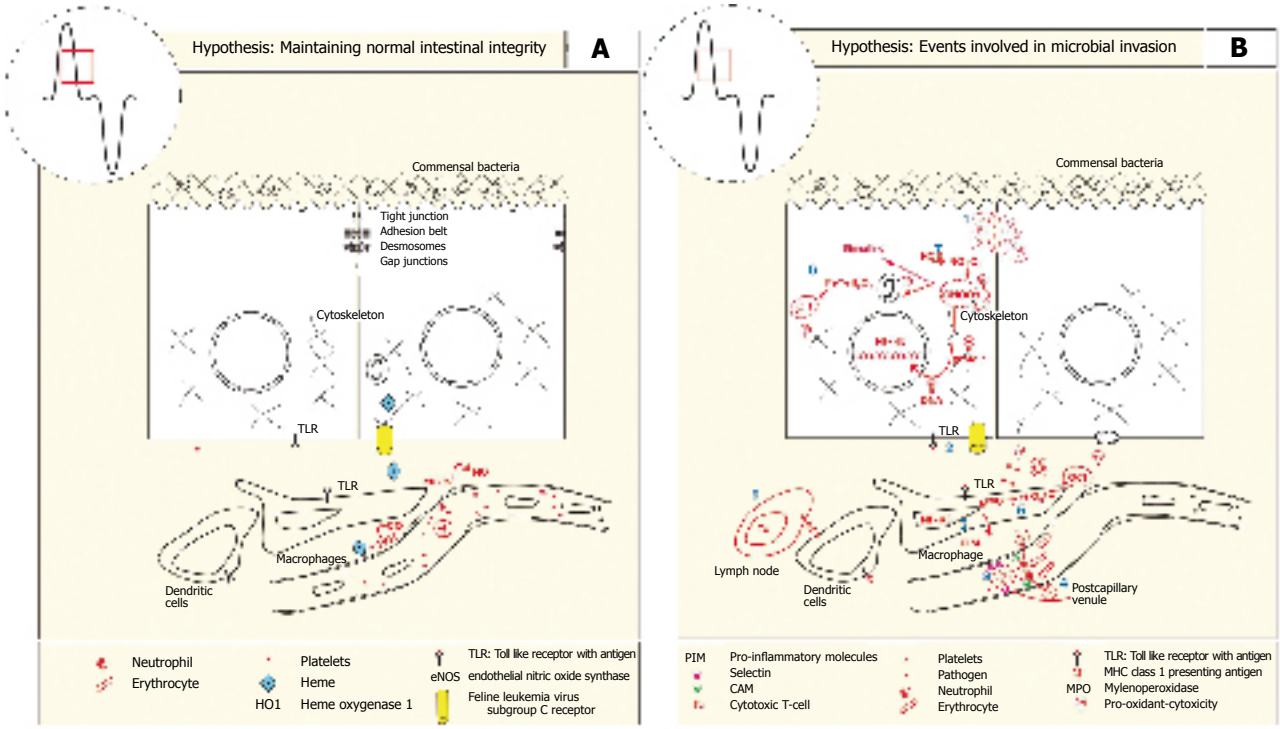

Figure 4 **A**: The intestinal mucosal barrier is maintained by a series of lateral membrane specialisations near the apical pole of the epithelial cell. It comprises tight junctions, adhesion belts, desmosomes and gap junctions that prevent the movement of pathogens across the epithelial monolayer. Constitutive expression and activities of endothelial nitric oxide synthase (eNOS) and heme oxygenase 1 (HO-1) is important for maintaining adequate blood flow, anti-inflammatory, anti-thrombotic (-) and anti-apoptotic effects on endothelium, neutrophils, platelets, and enterocytes, respectively. HO-1 activity produces the antioxidant bilirubin to limit oxidative damage; **B**: The loss of mucosal integrity results in the translocation of pathogens and establishment of an inflammatory response by the following series of events. (1) Initiation of synthesis of proteases by bacteria erode tight junction complexes between epithelial cells. (2) Binding of bacterial motifs activates toll like receptors, initiating the NF-kB pathway. (3) Increased expression of pro-inflammatory cytokines, chemokines and endothelial cell surface adhesion molecules. (4) Leukocytes extravasate and increased permeability of capillaries increases fluid accumulation (5) Phagocytic cells produce myeloperoxidase which combines with peroxide to form hypochlorous acid that damages pathogen and host systems alike. (6) Peroxide produced by enterocytes in combination with ferrous iron can produce superoxide anions that damage lipids, DNA and proteins. (7) NO is produced at high concentrations that combines with peroxide to form the pro-oxidant peroxynitrite. (8) Platelets also bind to the endothelial surface to induce hemostasis. (9) Presentation of antigens by dendritic cells *via* major histocompatibility class 1 to cytotoxic T-cells leads to antibody presentation and destruction of infected epithelial cells.

IL-6, interferon-γ and cyclo-oxygenase, while stimulating the anti-inflammatory cytokine IL-10[42,107-114] (Figure 4).

The third metabolite resulting from HO-1 activity is Fe(II). If this reaches the labile iron pool it will induce oxidative stress by participating in Fenton and Haber Weiss driven reactions and this would exacerbate inflammation. However, this is avoided by sequestration by ferritin[21].

Endothelial nitric oxide synthase (eNOS) maintains mucosal integrity

Nitric oxide synthase (NOS) is a heme-containing enzyme that converts L-arginine to nitric oxide (NO) and citrulline. Similar to CO, NO binds the heme moiety of guanylate cyclase to produce vascular smooth muscle relaxation. Under normal circumstances eNOS/NO is important in maintaining mucosal integrity by modulating intestinal blood supply. NO at low concentrations stimulates mucous production, electrolye secretion and decreases pro-inflammatory responses of mast cells, neutrophils, platelets and endothelial cells[115-117] (Figure 4A).

Induction of nitric oxide synthase (iNOS) damages mucosal integrity

During inflammation cytokines activate NF-κB dependent gene expression of iNOS by intestinal epithelial cells, neutrophils and macrophages. This leads to production of NO[116-118] at considerably higher levels than by eNOS activity. At this concentration NO reacts with superoxide anions to form the cytotoxic reactive nitrogen metabolite, peroxynitrite[119-123]. Although peroxynitrite destroys micro organisms, it also reversibly inhibits heme containing proteins including cytochrome C, catalase, cytochrome P-450 and cytoskeletal proteins[120,122]. It was suggested that inhibition of iNOS during endotoxin-induced gut mucosal dysfunction was beneficial because mitochondrial oxidative metabolism was unimpaired[119]. This leads to maintenance of mucosal barrier integrity that resists bacterial translocation[124] (Figure 4B).

Collectively, these findings indicate that at low concentrations NO maintains mucosal integrity, but at high concentrations NO induces reactive nitrogen metabolites which impair intestinal function.

The role of HO-1 versus iNOS in intestinal inflammation

During intestinal inflammation HO-1 mRNA expression increases in response to the activity of NO[125]. It is likely that this is due to increased transcription and stabilization of existing transcripts[125]. In addition, induction of HO-1 in a human intestinal cell line resulted in the degradation of cytokine-induced NOS. This reduced the production of NO and therefore peroxynitrite[124]. Heme was also shown to reduce the NOS mRNA[124]. The inhibition of NOS

activity by HO-1 was lost when tin protoporphyrin was given, indicating the direct effect of HO-1 in regulating NOS activity[126]. These findings are consistent with a role for HO-1 in limiting the deleterious effects of excessive iNOS by directly inhibiting its transcription, degrading existing NOS and scavenging excess ROM/RNM with bilirubin.

NUTRITION AND MECHANISM OF HEME-IRON ABSORPTION

In western civilisations, 40% of the average non-vegetarian person's total body iron is derived from heme products. However, iron from these substances only constitutes 15% of ingested iron[127,128], suggesting that heme-iron is more efficiently absorbed than non-heme iron. This observation also explains why vegetarians are more prone to iron deficiency than meat eaters. Despite the importance of the contribution of heme to body iron stores, how it is absorbed is still poorly understood.

Mechanism of Heme-Iron Absorption

It is generally recognised that in omnivorous animals, heme is not transferred into the blood as an intact metalloporphyrin, instead absorption of iron from heme involves three steps (1) Uptake of luminal metalloporphyrin [Fe(II)-protoporphyrin-IX] by the enterocyte (2) catabolism within the enterocyte, combining of pools of ingested iron from non-heme and heme sources and (3) release of elemental iron to the bloodstream by the enterocyte[129-133]. A large number of proteins are thought to be involved in the mechanism of heme iron absorption and these are tabulated along with their sites of expression and function (Table 1). Most of these proteins will be discussed individually in the following sections and is also summarised in Figure 5.

Worthington and co-workers used immunofluorescent methods to show that the uptake of a heme analog was temperature and time dependent, could be inhibited by heme competition and augmented by inhibitors of heme synthesis[134]. It is likely that Worthington and co-workers identified a heme transport process by Caco-2 cells that may be a transporter and/or possibly a heme receptor.

Heme uptake by a heme transporter

Heme is taken into the enterocyte intact as evidenced by the recovery of ^{59}Fe-heme from the small intestinal mucosa following the gavage of radiolabelled hemoglobin[130-133]. This process is energy dependent indicating an active process[135]. The finding that absorption of iron from hemiglobin and hemoglobin were equivalent suggests that uptake of heme is independent of the redox state of the heme-iron[136,137]. Alternatively there is an oxidoreductive mechanism on the cell surface that is capable of converting the iron redox state before internalization.

A microvillus membrane transporter that imports heme from the lumen into enterocytes of mice was recently characterised[138]. This protein was expressed in the duodenum but not the ileum, consistent with expression at the site of highest heme-iron absorption. Heme carrier protein 1 (HCP1) encodes a protein with strong homology to bacterial tetracycline-resistance proteins, which are characterised as having 12 transmembrane domains and are members of the major facilitator superfamily[138]. Functional characterisation of HCP1 using *Xenopus* oocytes revealed selectivity for the transport of heme but not tetracycline or non-heme iron. *In vitro* studies involving HCP1 siRNA and *in vivo* studies blocking HCP1 activity by antibodies indicated that the uptake of heme fell. HCP1 also required energy but the source of energy is presently unknown. Collectively, these findings indicate the first functional characterisation of a heme specific transporter.

Interestingly, during conditions known to increase non-heme iron absorption such as hypotransferranemia and iron deficiency, HCP1 mRNA expression remained constant although it was increased by hypoxia. Similarly, the extent of HCP1 protein expression remained constant with respect to the iron content of the enterocyte, although the protein translocated from the microvillus membrane to the basal cytoplasm during iron loading. The lack of increased expression of HCP1 by iron deficiency may in part explain the limited ability to increase heme-iron absorption. It may also indicate that HCP1 needs additional modulating proteins in order to regulate heme-iron absorption (Figure 5).

Table 1 Proteins involved in intestinal heme-iron absorption along with their function, location and whether they are regulated by iron

Protein	Function	Location	Regulation by Fe
Heme receptor	Receptor for heme	?	Inversely
HCP1	Transporter of heme	AM -> BC	Constant
FLVCR	Heme exporter	?	Unknown
Ferritin	Iron storage	C	Directly
DMT1	Fe(II) importer	AM+Lys	Inversely
Ferroportin	Fe(II) exporter	BL AM	Inversely
Hephaestin	Ferroxidase + ?	SN, BL	Constant
HO 1	Degradation of heme	C	Inversely
HO 2	Degradation of heme	SMC, EN	Constant
HFE	Regulator	TW	Inversely
TfR1	Tf:Fe endocytosis	BL, SN	Constant
Transferrin	Endosomal iron transport	C	Inverse

DMT1 = divalent metal transporter 1; HO = heme oxygenase; HCP1 = heme carrier protein 1; FLVCR = Feline leukaemia virus, subgroup C receptor; HFE = hemochromatosis protein; TfR1 = transferrin receptor 1; AM = apical membrane; BL = basolateral membrane; SN = supranuclear; LM = lateral membrane; Lys = Lysosomes; TW = terminal web; C = cytoplasm; BC = basal cytoplasm; SMC = smooth muscle cells, EN = enteric nerves; ? = putative; Tf:Fe = transferrin iron.

Heme uptake by a heme receptor

Previous studies have reported a 50% increase in heme binding to microvillus membrane preparations during iron deficiency, raising the possibility of a brush border localised heme receptor[139-142]. This is based on the measurements of binding [^{14}C]-heme to semi-purified brush border preparations[139-142]. Subsequent solubilisation of the brush border microvillus membranes identified

the size of the heme binding substances, one with a molecular mass of about 250 kDa the other about 60 kDa. Displacement of the [^{14}C]-heme by unlabelled heme was seen with the 250 kDa complex, but not the about 60 kDa complex[139-141], suggesting the larger peak represented a heme receptor complex, while the smaller peak was thought to be polymerised heme[140]. Based on the capacity of the large complex to be saturated with heme and having an Ka of 10^{-6} to 10^{-7} mol/L this suggests that it is a relatively high affinity heme receptor.

In addition to the identification of a putative heme receptor in the intestine, others have identified a heme binding protein that is distinct from the hemopexin receptor[143] with similar binding characteristics to the intestinal heme receptor. Since the heme binding protein and HCP1 have molecular weights of about 250 kDa and about 50 kDa, respectively, it is unlikely they are the same protein, unless HCP1 forms part of a larger complex. The finding that erythroleukaemic cells internalise heme coated latex beads[144,145] and that trypsin treatment eliminates heme binding[146,147] supports the existence of a heme receptor-mediated, endocytotic pathway. It therefore appears that there are at least two defined pathways involved in the uptake of heme into the enterocyte, one involving HCP1[138] and the other a receptor-mediated endocytotic process[139-142,144-147]. Despite considerable characterisation of the heme receptor almost thirty years ago there has been little progress made since (Figure 5).

Intracellular processing of heme

Morphological studies show that following ingestion of a heme-rich meal by rodents, heme was first seen along the microvillus membrane, then in tubulovesicular structures of the apical cytoplasm and finally in secondary lysosomes[148,149]. Based on time course studies, DAB (3,3-Diaminobenzidine tetrahyhydrochide) disappeared from lysosomes suggesting that heme was either transported from these structures or that it was degraded within them. In either case heme degradation involves HO activity but whether this is HO-1 or HO-2 is presently unknown.

Alcohol and heme-iron absorption

In rats treated with alcohol there was increased absorption of iron from heme as well as the entire hemoglobin complex where it was transported to the liver as a haptogobin-hemoglobin complex[150,151]. Thus, absorption of iron from hemoglobin also appears to contribute to the iron over loading caused by excessive alcohol consumption.

Limitations in iron absorption from heme

The intracellular sites where restrictions to the absorption of iron from heme occur have been studied in dogs given radiolabelled hemoglobin and then measuring the progression of radioactivity through mucosal compartments[133]. The most likely sites where the rate of iron absorption was limited appears to be at the stage of heme breakdown and/or the release of iron from the cell. This might involve the steps where HO operates, where iron is released out of an intracellular compartment, or from the cell (see below).

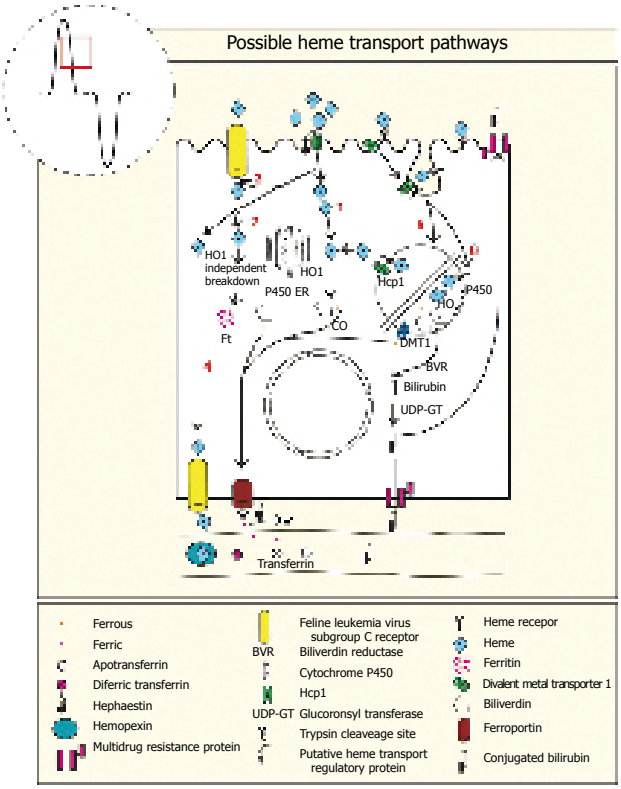

Figure 5 Six steps in the uptake of heme by intestinal enterocytes. Heme taken up by heme carrier protein (HCP1) is internalised and broken down in the cytoplasm by HO-1 (1), by a HO-1 independent enzyme(s) (2), some is released intact back into the lumen (3) or plasma by FLVCR (4). Heme may also bind to a heme receptor and with HCP1 be internalised by receptor mediated endocytosis. The heme may be released to the cytoplasm by HCP1 (5), or the heme may be broken down in the lysosome and the released iron transported to the cytoplasm by the divalent metal transporter (DMT1) (6). The iron released from heme passes to the basal cytoplasm and is transported across the basal membrane by ferroportin in the ferrous state, oxidized to ferric-iron by hephaestin and transported in the plasma by transferrin.

Other proteins possibly involved in the transport of Fe(II) from heme

In view of the likely convergence of iron derived from sources of non-heme and heme iron what is known for non-heme iron is described.

Divalent Metal Transporter 1 (DMT1)

The Microcytic mouse (*mk*) and anaemic Belgrade rat (*b*) have an autosomal recessive inherited, hypochromic, microcytic anaemia associated with a well-characterised defect in the transferrin cycle in erythroid cells[152], as well as a defective intestinal non heme-iron transport that is manifest at the site of uptake at the microvillus membrane[153]. The similar phenotypes are explained by an identical mutation in DMT1 at G185R[154,155]. Deletion of DMT1 also resulted in loss of iron transport by the intestine but not the liver or placenta[156]. The finding that heme is broken down intracellularly and a portion of DMT1 is found inside the enterocyte could suggest that DMT1 is involved in heme-iron absorption. There is an absolute requirement for DMT1 in the uptake of iron by the intestine[156], suggesting that intestinal absorption of iron from heme also requires DMT1 but this remains to be determined (Figure 5).

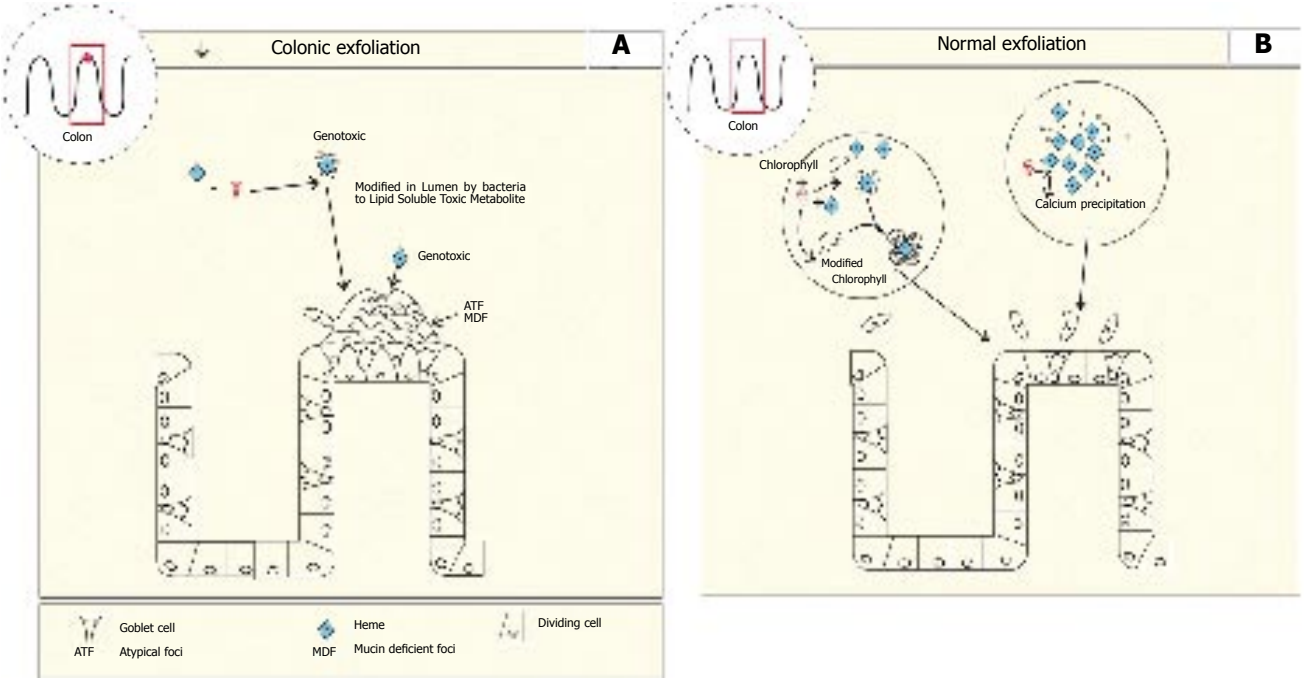

Figure 6 **A**: In the colon excess heme is metabolised into a lipid soluble heme metabolite possibly by commensal bacteria. Heme itself is also genotoxic. This results in the formation of aberrant atypical foci, that are mucin deficient (ATF, MDF). Apoptosis is inhibited which could lead to increased survival of mutant cells; **B**: In the presence of calcium or chlorophyll heme precipitates into biological inactive compounds which inhibit the heme factor or binds the heme factor rendering it inert, respectively leading to normal colon growth.

Hemochromatosis protein (HFE)

Intestinal expressed HFE is recognised to regulate iron absorption *via* the uptake of transferrin bound iron by crypt cells. The finding that HFE is expressed along the terminal web of enterocytes during iron deficiency where it co-localised with DMT1, raises the possibility that HFE may function directly in iron absorption and this may include heme-iron[157]. This is also supported by the finding that HFE expression is inversely proportional to iron absorption[157]. If this is the case then HFE is positioned to interact with HCP1, the putative heme receptor and DMT1. Whether DMT1 and HFE work intracellularly (such as in lysosomes) at levels that cannot be detected by immunofluorescent microscopy remains to be determined.

Ferroportin

Basolateral transport of non-heme iron involves ferroportin/Ireg-1/MTP-1/SLC40A1, most often referred to as ferroportin[25]. This is based on the study showing that over-expression of ferroportin in macrophages during erythrophagocytosis increased release of non-heme iron, but not heme[158]. This observation is likely to apply to the enterocyte but this needs to be determined. Also selective deletion of ferroportin in mice resulted in non-heme iron accumulation within enterocytes[159] which provides support for the hypothesis that ferroportin functions with non-heme iron (Figure 5).

Mammalian iron-ATPase

Baranano and co-workers have identified a microsomal membrane Fe(Ⅱ) transporter from the spleen which presumably represents an iron transporter expressed by macrophages. It is induced by heme, and depends on hydrolysable triphosphate, magnesium and temperature[160]. It is proposed that following heme catabolism by macrophages, Fe(Ⅱ) is shunted into the lumen of the endoplasmic reticulum. Others have found a similar transporter in liver microsomes[161]. Whether this transporter functions in enterocytes remains to be determined.

HEME AND COLON CARCINOGENESIS

Although heme-iron is more bio-available than non-heme iron it has limited ability to be absorbed. Therefore, unabsorbed heme reaches the colon. Luminal heme is also derived from the blood *via* extravasation and from desquamation. Previous studies have shown that heme irritates the epithelium of the colon as evidenced by mild diarrhoea[162,163]. It was shown that feeding heme but not non-heme iron to rats results in significant increased proliferation of colonic mucosa[162]. In addition, the incidence of aberrant atypical foci (ATF) and mucin-depleted foci (MDF)[164] increased as the heme content of the diet increased suggesting that heme is carcinogenic[164,165]. In fact, it was demonstrated that heme was genotoxic in the human colon tumour cell line HT29[166].

It has been shown that a heme breakdown product rather than heme or iron *per se* was responsible for the inflammation and ATF formation[162,163]. In the colon some heme breakdown products are produced by the presence of colonic bacteria[167], and it has been suggested the heme is converted to a cytotoxic factor, although it has not been fully characterised[162,163]. Gene microarray analysis of 365 genes expressed by the colon revealed that feeding heme

down-regulated mucosal pentraxin 30-fold[168,169]. Since pentraxin is involved in the recognition and clearance of dying cells, a process that is normally ongoing in the intestinal tract, downregulation of this gene by heme infers that apoptosis of colonic mucosal cells may be inhibited. If this is true then it might explain the increased carcinogenic potential if cells with mutated genomes cannot be eliminated[168]. In support of this, De Vogel et. al., showed that heme supplementation decreased colonic exfoliation[170] (Figure 6).

The cytotoxic affect of heme on the colon was lost when the diet was supplemented with green vegetables[170]. It was hypothesised that chlorophyll in green vegetables inhibited the formation of the heme factor by competing for solubilisation with heme in the large intestine. Alternatively, chlorophyll and heme could form a complex that blocks the site of covalent modification of the heme and reduces the formation of the heme factor[170]. Calcium was also shown to protect against the effects of heme on colonic proliferation and normalising pentraxin expression, presumably because calcium precipitates heme, thereby preventing the formation of the soluble heme induced cytotoxic factor[169,171,172]. This conclusion is consistent with the inhibitory effect that calcium has on heme bioavailability for its absorption in the small intestine[171] (Figure 6A and B).

HEME AND HO-2 IN INTESTINAL MOTILITY

Peristaltic contractions are controlled by stellate shaped non-neuronal interstitial cells of Cajal (ICC) situated within the myenteric plexus (ICC-MY)[173-177]. Clusters of spindle shaped bipolar ICC found throughout the circular and longitudinal muscle layers (ICC-DMP) generate pacemaker potentials spontaneously but these are modified by neural input[177]. Adjacent to the submucosa and within the circular muscle layer ICC also appear to synapse with nerves (ICC-IM)[174-177]. Loss of ICC leads to markedly impaired neurotransmission and typical gastrointestinal motor patterns indicating their importance in co-ordinating neural modulation of intestinal motility. In the small intestine ICC-MY appear important for pacemaker ICC but in other regions of the bowel this is regulated by ICC-IM.

The network is connected to the smooth muscle syncytium *via* either gap junctions or peg in socket junctions. These membrane specialisations provide a means of conducting pacemaker currents to intestinal smooth muscle[174-177]. It is thought that pacemaker potentials originate from unitary potentials caused by the release of calcium from mitochondrial stores[177,178] which in turn cause a rise in membrane potential generated by opening of Ca^{2+} permeable channels. The plateau component observed in pacemaker potentials is generated by opening Ca^{2+} activated Cl^- channels[179]. Repolarisation involves removal of cytosolic Ca^{2+} to stores and K^+ transport *via* activated K^+ channels[179]. The frequency of these events establishes the pacemaker potential of a particular region of the intestine. Muscle contraction will occur providing the membrane potential is capable of activating L-type Ca^{2+} channels and depolarising the cell[180]. The resulting increase in cytosolic Ca^{2+} levels is coupled to contraction. Contraction is limited by activation of large-conductance Ca^{2+}-activated K^+ channels and L-type Ca^{2+} inactivation[180].

It has been shown that HO-2 but not HO-1 is present in all classes of ICC (-MY, -IM & -DMP), although HO-2 expression was greater in ICC-MY than in ICC-IM. Enteric neurons also express HO-2[180-192]. In the gastric fundus and in particular mucosal epithelial cells, neurons of the submucosal and myenteric plexus and ICC co-express HO-2 and BVR indicating that these cells have the capacity to generate bilirubin[47]. Since ICC have numerous mitochondria it is hypothesised they produce heme to serve as substrate for HO-2 activity and the CO produced may regulate membrane potential and in turn affect intestinal contraction[186]. In the genetic absence of ICC and in HO-2 knockout mice the membrane potential of intestinal smooth muscle is depolarised compared with wild type controls[174,185,188]. Studies have shown that the HO-2 mediated hyperpolarisation is probably due to the effect of CO on activation of K^+ currents in smooth muscle[181,184], and that exogenous CO given to HO-2 knockout mice hyperpolarises the resting membrane potential[191]. Supporting this, the membrane is more hyperpolarised near the submucosa and these cells have higher HO-2 activity and CO production than cells near the myenteric plexus where the membrane is more depolarised[191]. Taken together it suggests that CO produced from ENS and ICC function in maintaining membrane potential and the gradient that exists along the longitudinal and across the circular musculature[184,191]. It would be expected that increased CO production would result in a greater level of smooth muscle relaxation because the membrane potential is further away from threshold. The mechanism by which CO reduces the resting membrane potential is unclear[188].

CONCLUSIONS

Within the intestine heme serves important roles in energy production, in enzymes involved in detoxification, in the generation of the second messenger gases NO and CO and the antioxidant bilirubin. The products of heme breakdown namely CO and bilirubin restrict oxidative stress, inflammation, and regulate the cell cycle and differentiation in the crypt region. Excess heme may also promote the development towards colon cancer. Dietary heme is an important source of iron for the body and the absorption of iron from heme differs from non-heme. The molecular mechanism operating in the early phases of absorption appears to involve a transporter although there is evidence of a receptor mediated process and numerous other proteins may function in heme-iron as in non-heme iron absorption. The ability of HO to perform these varied functions within the enterocyte probably depends on different compartments within the cell which are differentially accessed by heme and HO. Future studies will determine how heme-iron is absorbed and the mechanisms by which HO regulates the cell cycle and differentiation, limits the inflammatory process.

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