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Análise da expressão da enzima heme oxigenase I durante a diferenciação eritróide.

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"...I am so glad that this has taken me so long, cause it is the journey that made me so strong..."

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Resumo

A enzima heme oxigenase I cataliza a reação de clivagem da molécula heme, gerando como produtos ferro, monóxido de carbono e biliverdina. Essa enzima pode ser induzida por diversos estímulos, tais como heme, metais pesados, xenobióticos, UV, fatores endócrinos e metaloporfirinas. HO-1 tem sido descrita como protetora uma vez que remove as moléculas de heme livre, extramente danosas para a célula quando em excesso, liberando em troca produtos com alta capacidade antioxidante. As diversas funções desempenhadas pelo grupo heme dentro da célula fazem da atividade da enzima HO-1 uma etapa fundamental para o controle da homeostase celular. A primeira parte do presente trabalho tem por objetivo a análise da expressão da HO-1 durante a diferenciação de células eritróides. Obstante ao fato dessas células possuírem uma alta taxa de síntese de heme, nada se sabe sobre o comportamento da HO-1 durante o processo de diferenciação das células vermelhas. Através de uma série de experimentos, demonstramos de forma clara que a enzima HO-1 tem sua expressão regulada de forma positiva durante o processo de diferenciação. Além disso, demontramos que a modulação da expressão dessa enzima pode interferir no processo de hemoglobinização. Por fim, na segunda parte desse trabalho, elaboramos uma hipótese sustentando que alelos específicos da enzima HO-1 estariam sendo selecionados em regiões endêmicas de malária. Alelos diferentes para HO-1 resultam em uma atividade catalítica maior ou menor da enzima, o que em ultima análise estaria interferindo na remoção do excesso de heme acumulado em patologias caracterizadas por alta hemólise. Portanto, o presente trabalho destaca importância da enzima HO-1 em aspectos até então pouco observados na literatura, sempre destacando a importância da molécula heme, uma vez que a mesma desempenha inúmeras funções em nível celular.

Abstract

The enzyme heme oxygenase I catalyzes the reaction of heme cleavage generating iron, carbon monoxide and biliverdin. This enzyme is induced by a wide variety of stimuli such as heme, heavy metals, xenobiotics, UV, endocrine factors and metaloporphirins. HO-1 is described as a protector factor once it removes potentionally toxic free heme and releasing in exchange products with high antioxidant proprieties. The heme molecule has multiple celullar functions and, as a consequence, the reaction catalyzed by HO-1 plays a fundamental role controlling cellular homeostasis. The first part of the present study has the objective to analyze the expression of HO-1 during the erythroid differentiation. Although red blood cells show the highest rate of heme synthesis in the organism, nothing is known about HO-1 pattern of expression during the differentiation of these cells. Our results clearly show HO-1 being positively regulated during red blood cell developing. Furthermore, modulation in the HO-1 expression resulted in alterations of the hemoglobinization process. Finally, in the second part of this study, we elaborate a hipothesis supporting that HO-1 specific alleles are being selected on malaria endemic regions. HO-1 allelic variants confer different enzymatic activities, which in turn interfer on the clearance of the heme accumulated during the development of certain pathologies like hemolitic disorders. Therefore, our study stress the importance of HO-1 regarding aspects poorly investigated in the literature so far, always considering the HO-1 substrate, heme, as the main responsible for the wide variety of functions displayed by this enzyme in the organism.

CAPÍTULO I

Introdução

Introdução:

Grupo Heme

O grupo heme é um complexo essencial para a função de todas as células aeróbicas, sendo formado por ferro e protoporfirina IX. O heme serve como grupo prostético de numerosas hemoproteínas (por exemplo, hemoglobina, mioglobina, citocromos, guanilato ciclase, e óxido nítrico sintase) e desempenha um importante papel controlando a síntese protéica e a diferenciação celular (Ponka, 1999; Ponka, 1997). As células sanguíneas vermelhas (RBC) contêm grande parte do heme presente no organismo (75-80%). Os precursores dessas células sangüíneas apresentam uma taxa de síntese de heme que é de uma ordem de magnitude superior a das células do fígado, as quais correspondem ao segundo maior produtor de heme no organismo.

A biosíntese da molécula heme é um processo que envolve 8 passos, sendo que 4 deles ocorrem dentro do citosol, enquanto as 4 etapas restantes dentro da mitocôndria. Na matriz mitocondrial, δ-ácido aminolevulinico sintase (ALAS) cataliza a primeira etapa da rota de biosíntese de heme onde ocorre a condensação entre uma glicina e a succinil coenzima A (COA) resultando no ácido aminolevulinico (ALA) (Kikuchi *et al.*, 1958). ALA é transportada para o citosol onde as próximas 4 etapas ocorrem. ALA desidrogenase converte 2 moléculas de ALA no monopirrol, porfobilinogênio (PBG). As próximas duas etapas envolvem as enzimas PBG desaminase e a uroporfirinogênio III sintase, convertendo 4 moléculas de PBG em um tetrapirrol cíclico, uroporfirinogênio III. Este produto é então descarboxilado para formar o coproporfirinogênio III (CoPIII), o qual é transportado para a mitocondria através de um mecanismo até então desconhecido. A enzima, CoPIII oxidase, localizado no espaço intermembranas da mitocôndria, catalisa a descarboxilação oxidativa do CoPIII formando o protoporfirinogênio IX (Ponka, 1997). Protoporfirinogênio III oxidase, proteina integral presente na membrana interna da mitocôndria, catalisa o penúltimo passo da rota de síntese de heme, gerando protoporfirina IX (PIX). A última etapa da rota envolve a inserção de um átomo de Fe^{+2} na PIX através da ação da enzima ferroquelatase (Napier *et al.*, 2004).

Diferenças no metabolismo de ferro e em genes que codificam a enzima ácido 5-aminolevulínico sintase (ALA-S, a primeira enzima na rota de biossíntese de heme) são responsáveis por diferenças na regulação e taxas de síntese de heme em células eritróides e não eritróides. Existem dois genes que codificam a enzima ALA-S, um dos quais é expressado de forma ubíqua (ALA-S1), e outro que é expresso especificamente em células eritróides (ALA-S2). A disponibilidade de ferro controla os níveis de protoporfirina IX nas células eritróides, uma vez que o RNAm de ALA-S2 apresenta, em sua região 5' não traduzida (UTR), um elemento responsivo a ferro (IRE), agindo de forma a induzir a tradução do RNAm de ALA-S2 na presença de ferro. Em células não eritróides, a etapa limitante do processo de produção de heme é catalizada pela enzima ALA-S1, a qual tem sua síntese regulada negativamente pelo grupo heme. Por outro lado, em células eritróides, o grupo heme não apresenta efeito inibitório seja na atividade ou na síntese da enzima ALA-S2 (Ponka, 1997; Morgan, 1981). Esses mecanismos regulatórios distintos são responsáveis pela grande diferença nas taxas de síntese de heme em RBC não diferenciadas quando comparadas com células não eritróides. Essas células por sua vez, apesar da baixa taxa relativa de síntese de heme, ainda produzem quantidades suficientes de heme para incorporação nas hemoproteínas.

Heme Oxigenase (HO)

O único mecanismo fisiológico relacionado à degradação do grupo heme é desempenhado pela enzima HO. A clivagem oxidativa do grupo heme resulta na formação de monóxido de carbono (CO), Fe^{+2} e biliverdina, sendo essa última subsequentemente convertida em bilirubina citosólica através da ação da enzima biliverdina redutase (fig. 1)(Maines, 1988; Shibahara, 1988).

Duas isoformas da HO já foram identificadas em mamíferos: HO-1 e HO-2. (Maines, 1988; Shibahara, 1988; McCoubrey *et al.*, 1997). HO-1 é a isoforma indutível, apresentando 32 KDa. A HO-1 está dentro do grupo das proteínas de choque térmico (*heat shock protein, HSP32*), e sua expressão é desencadeada como resultado de diversos estímulos de estresse, entre eles hipóxia, metais pesados, radiação UV, ROS (como

peróxido de hidrogênio - H_2O_2) e espécies reativas de nitrogênio como óxido nítrico (NO) (Motterlini *et al.*, 2000; Mitani *et al.*, 1993; Keyse & Tyrrell, 1989; Hara *et al.*, 1999; Hartsfield *et al.*, 1997; Dói *et al.*, 1999; Tanaka *et al.*, 2003; Zamora *et al.*, 2002).

As rotas de síntese e degradação do grupo heme afetam o metabolismo oxidativo uma vez que ambas estão estreitamente relacionadas com o ciclo celular do ferro. A função determinante na quebra dos grupos heme é desempenhada pela HO-1.

A maioria dos grupos heme existentes no organismo de um mamífero é utilizada para o transporte de oxigênio na proteína hemoglobina. O destino do grupo heme presente na hemoglobina é bem definido: a proteína é sintetizada nos eritrócitos e degradada no sistema reticuloendotelial. A hemoglobina presente nas células vermelhas intactas, mas senescentes, sofre degradação no sistema reticuloendotelial do fígado, do rim e principalmente do baço, onde a atividade da HO-1 é alta. A hemoglobina livre e o grupo heme liberados podem então entrar na circulação sanguínea durante a hemólise. O heme liberado circula no sangue complexado a proteínas do soro (hemopexina e albumina), enquanto as formas livre de hemoglobina formam complexos com a haptoglobina. As cadeias de hemoglobina e grupo heme são então ligadas e importadas para o parênquima do fígado, através de endocitose mediada por receptor, sendo sua degradação realizada pela HO-1 (Ryter & Tyrrell, 2000).

O grupo heme, quando livre, pode promover reações dependentes de ferro que podem ser deletérias para o organismo, uma vez que geram espécies reativas de oxigênio (ROS) e/ou a peroxidação de lipídeos de membrana, os quais podem levar ao rompimento das membranas celulares. O grupo heme é hidrofóbico e pode facilmente entrar nas membranas celulares, agindo então como um catalisador para a oxidação da lipoproteína de baixa densidade (LDL) e gerando produtos que são tóxicos para o endotélio (Balla *et al.*, 1991; Miller & Shaklai, 1994; Camejo *et al.*, 1998). Os efeitos tóxicos do grupo heme livre são importantes em uma série de patologias, particularmente em condições agudas como a hemólise intravascular (a qual pode resultar em falha renal), e em processos de maior frequência, como a aterogênese, no qual depósitos de ferro dentro das lesões podem ser observados (Hunter *et al.*, 1991; Jeney *et al.*, 2002). Em condições fisiológicas normais o *pool* de heme livre é reduzido (0,1-0,2μM no fígado) (Granick *et al.*, 1975). Esse *pool* é resultado tanto dos novos grupos heme sintetizados com o objetivo de serem

incorporados nas hemoproteínas, existindo transientemente como heme livre, quanto o grupo heme liberado durante o *turnover* natural ou farmacologicamente induzido das hemoproteínas, também existindo transientemente como heme livre antes de sua degradação pela HO. Esse *pool* seria também a fonte de grupos heme a serem reutilizados pela célula. Dessa forma, o *pool* de heme livre pode aumentar, como, por exemplo, em situações em que se acelera o *turnover* das hemoproteínas. Considera-se, então, que situações de estresse oxidativo podem modificar a estrutura de proteínas, causando fragmentação, ligação cruzada (interligação), mudanças de carga, hidrofobicidade e solubilidade (Neuzil *et al.*, 1993; Davies & Delsignore, 1986). Tais modificações se ocorrerem em uma hemoproteína, podem levar à liberação do grupo heme. Como o grupo heme em sua forma livre é potencialmente tóxico para a célula, sua concentração deve ser controlada para a manutenção da homeostase celular. Sendo assim, a indução da HO-1 seria uma estratégia para degradar o grupo heme liberado das hemoproteínas intracelulares em condições de estresse oxidativo, a fim de compensar o aumento transiente da quantidade de heme livre necessário para síntese de hemoproteínas, prevenindo assim a acumulação de heme nas membranas (Ryter & Tyrrell, 2000).

As funções biológicas da heme oxigenase estão associadas com a resposta celular a diferentes tipos de estresse (Willis *et al.*, 1996; Siow *et al.*, 1999). Muitas evidências indicam um papel vital da HO-1 tanto no crescimento quanto na morte celular, principalmente através de seu envolvimento na regulação da apoptose (Tanaka *et al.*, 2003). A HO-1 estimula o crescimento celular e a proliferação de vários tipos celulares, como indicam experimentos onde a administração de seu inibidor anulou totalmente o efeito proliferativo (Clark *et al.*, 1997). Existe também indicação de envolvimento da HO-1 em angiogenese (Deramaudt *et al.*, 1998), reforçando ainda mais seu papel proliferativo. A HO-1 é amplamente expressa em células tumorais, incluindo adenocarcinoma, hepatoma, sarcoma, gliobastoma e melanoma (Doi *et al.*, 1999; Goodman *et al.*, 1997; Tsuji *et al.*, 1999; Deininger *et al.*, 2000; Torisu-Itakura *et al.*, 2000), as quais possuem alta capacidade proliferativa. A expressão dessa enzima parece suportar também o crescimento celular. Evidências indicam que tal fato só é possível visto que o crescimento é um balanço entre a proliferação e a morte celular. Logo, como

a HO-1 está em estreito contato com a rota que determina a apoptose, sua regulação pode ser a chave para a determinação da acentuada proliferação celular (Fang *et al.*, 2003).

Os produtos originados da quebra do grupo heme pela HO-1 são CO, ferro e biliverdina (que é convertida em bilirubina). Diversos estudos indicam que o CO é um dos principais efetores das funções protetivas e anti-inflamatórias desempenhadas pela HO-1 (Lee & Chau, 2002; Lee *et al.*, 2003). No entanto, muita controvérsia existe a este respeito.

Existem 2 fontes de CO em sistemas biológicos: uma é heme dependente (80%) e outra é independente de heme (~20%) (Abraham & Kappas, 2008). No entanto, o rápido aumento de CO que ocorre *in vivo* é somente devido à indução da enzima HO-1. É descrito na literatura que CO e óxido nítrico (NO) apresentam propriedades similares (Solari *et al.*, 2003; Snyder & Baranano, 2001; Motterlini *et al.*, 1996), onde ambos os gases se comportam como mensageiros e moléculas de sinalização. CO e NO são capazes de induzir o relaxamento dos vasos sanguíneos através de vasodilatação e inibição da proliferação das células vasculares lisas musculares (VSMC) (Stanford *et al.*, 2003). Da mesma forma que o NO, CO derivado da atividade catalítica da HO-1 influencia as rotas que envolvem a gualinato ciclase (sGC) e guanosina monofosfato cíclico (cGMP), as quais tem como função a regulação tanto da pressão quanto da contratibilidade vascular (Ndisang *et al.*, 2004). Experimentos envolvendo transplantes cardíacos em camundongos e ratos demonstraram que a expressão de HO-1 pelo sistema vascular do enxerto é crítica para se obter a sobrevivência do mesmo. Nesse contexto, observou-se que a inibição da HO-1 resulta na rejeição do enxerto em um prazo de 3-7 dias. A rejeição foi associada com uma ampla agregação plaquetária, trombose das arteríolas coronárias, infarto do miocárdio, e apoptose tanto das células do miocárdio como de células endoteliais. Mantendo a HO-1 inibida e fornecendo CO, suprimiu-se a rejeição do enxerto, restaurando sua longa sobrevivência. Esse efeito do CO foi associado com a inibição da agregação plaquetária e a proteção das células endoteliais contra apoptose (Sato *et al.*, 2001). Recentemente, demonstrou-se também que o CO confere um potente efeito anti-proliferativo em células musculares lisas vasculares e em células das vias aéreas, de forma dependente das rotas de sinalização de MAPK (*mitogen-activated protein kinase*) e cGMP (*cyclic-GMP*) (Song *et al.*, 2002; Otterbein *et al.*, 2003). Em

outro estudo, CO inibiu a proliferação de linfócitos T ativados, através de uma rota independente de MAPK e cGMP. Com a inibição das caspase-3 e caspase-8, mostrou-se que somente com a inibição da caspase-8 houve uma diminuição no efeito antiproliferativo do CO, evidenciando uma dependência de CO em relação à caspase-8 para suprimir a proliferação (Song *et al.*, 2004).

Por outro lado, classicamente atribui-se ao ferro originado da degradação do grupo heme um papel antioxidante, uma vez que ele estimularia a expressão da proteína ferritina responsável por estocar o ferro celular, não permitido assim que o mesmo ficasse disponível para participar das reações oxidativas danosas à célula. Essa regulação ocorreria em nível de mRNA, onde uma proteína regulatória (IRP) se ligaria ao mRNA da ferritina inibindo sua tradução até o momento em que o ferro se tornasse disponível no citoplasma, esse ferro se ligaria a IRP, liberando Fe-IRP do mRNA da ferritina e desreprimindo sua tradução (Eisenstein & Munro, 1990). Camundongos nocaute para o gene da HO-1 desenvolveram anemia associada com níveis baixos de ferro no soro, com acumulação renal e hepática de ferro que contribui para dano oxidativo macromolecular, dano no tecido e inflamação crônica. Tais resultados indicam que a HO-1 tem um importante papel na reciclagem de ferro facilitando sua liberação das células hepáticas e renais (Poss & Tonegawa, 1997).

Um ser humano adulto produz cerca de 300mg de bilirubina (BR) por dia (Abraham *et al.*, 1983; Schacter, 1988). 80 a 85% da BR produzida *in vivo* é derivada do catabolismo do grupo heme derivado da hemoglobina liberada de eritrócitos senescentes ou danificados (Abraham *et al.*, 1983; Schacter, 1988). As ações biológicas da bilirubina podem ser particularmente relevantes na prevenção de ações oxidativas vasoconstritoras mediadas pelo fator de necrose tumoral (TNF) e angiotensina II (AngII) (Kushida *et al.*, 2002a; Kushida *et al.*, 2002b). Bilirubina, em baixas concentrações, atua na remoção de espécies reativas de oxigênio *in vitro*, reduz o dano celular mediado por oxidação e atenua o estresse oxidativo *in vivo* (Stocker *et al.*, 1987a; Stocker *et al.*, 1987b). Entre essas características antioxidantes da BR estão a prevenção da oxidação de ácidos graxos polinsaturados. A BR funciona como redudora de radicais peroxil, e também quando complexada à albumina (como é encontrada no sangue) previne a peroxidação da albumina. A albumina está ligada a ácidos graxos e acaba também protegida da

degradação mediada pela ação de radicais superóxido (Stocker *et al.*, 1987b; Neuzil & Stocker, 1993; Stocker & Ames, 1987c; Stocker & Peterhans, 1989a; Stocker & Peterhans, 1989b). Existem poucas evidências que sugerem que a BR seja seletivamente retida no plasma ou nas membranas intracelulares para servir como antioxidante de membranas. As evidências experimentais dessas propriedades antioxidantes vêm em sua maioria de estudos *in vitro*. O excesso e o acúmulo de BR não conjugada no plasma leva à ocorrência de icterícia em recém-nascidos e altas concentrações desse metabólito podem conduzir ao desenvolvimento de encefalopatia. No entanto, o exato mecanismo pelo qual BR leva à neurotoxicidade é desconhecido (Ryter & Tyrrell, 2000).

Como pode-se observar, os produtos da reação da HO-1 possuem múltiplos efeitos na homeostase celular. Destacar um desses produtos como o principal efetor das funções desempenhadas pela HO-1 dentro do organismo é uma missão difícil. No entanto, não podemos deixar de vislumbrar a possibilidade de que dependendo do tipo celular haverá um produto com maior importância, ou até mesmo que esses três produtos produzam um efeito sinérgico, somando suas capacidades antioxidantes, e colocando a HO-1 em uma posição central dentro das rotas celulares que determinam a sobrevivência celular diante de diferentes tipos de estresse oxidativo.

Heme Oxigenase I e Bach 1

A análise da sequência e organização da heme oxigenase I de humanos, ratos, camundongos e galinha revelou que o controle transcricicional dessa enzima ocorre através de elementos regulatórios localizados na região flanqueadora 5' do promotor da HO-1 (Alam *et al.*, 1999; Mignotte *et al.*, 1989a; Nishizawa *et al.*, 1989; Zhang *et al.*, 2004). Esses elementos regulatórios correspondem a sítios de ligação para diversos fatores de transcrição sensíveis ao estado redox celular, como fator nuclear kB (NFkB), proteina ativadora 1 e 2 (AP1/2) (Milot *et al.*, 1996), CCAAT/*enhancer-binding protein* (Trimarchi & Lees, 2002), fator indutível por hipóxia 1 (HIF-1) (Kanezaki *et al.*, 2001; Morita *et al.*, 1997), adenosina 3', 5'- cíclica monofosfato (cAMP) responsiva ao elemento de ligação proteica (CREB) (Lee *et al.*, 2002) e o fator nuclear E2 relacionado

ao fator 2 (Nrf2) (Alam *et al.*, 2000; Andrews *et al.*, 1993). O gene da HO-1 humana contem um possível elemento de reconhecimento a Maf (MARE) imediatamente a 3'do elemento responsivo a cadmium. AP-1 e NF-kB são os principais fatores de transcrição que podem transativar a HO-1 através da ligação à região promotora do gene (Alam *et al.*, 1995; Sun *et al.*, 2004; Hoshino & Igarashi, 2002; Milot *et al.*, 1996; Steiner *et al.*, 1996; Mignotte *et al.*, 1989b).

Apesar do elemento antioxidante (ARE) localizado na região promotora do gene da HO-1 ter sido considerado inicialmente como mediador no processo de resposta a estresse através da interação com AP-1, a mesma sequência foi posteriormente descrita como local de ligação para o fator de transcrição Nrf2 (Alam *et al.*, 2000; Andrews *et al.*, 1993). A significância funcional do Nrf2 na resposta adaptativa a estresse oxidativo/nitrosativo foi observada em estudos conduzidos com camundongos nocaute para Nfr2, os quais demonstraram ser mais suscetíveis à toxicidade química induzindo dano tecidual (Civil *et al.*, 2002; Creusot *et al.*, 1988; Grosveld *et al.*, 1987). Nfr2 é sequestrado no citoplasma como um complexo inativo junto com o seu repressor Keap1 (*kelch-like ECH-associated protein-1*). A dissociação do Nrf2 de sua proteína inibitória Keap1 é o pré-requisito para a translocação nuclear do Nrf2. Após formar um heterodímero com a proteína *small* Maf, a forma ativa de Nfr2 liga-se a elementos *cis* que apresentam sequências comuns, chamadas MARE ou ARE (Monson *et al.*, 1992). Tal processo, desencadeia a expressão de diversos genes alvo, incluindo a HO-1. Keap1 e Bach1 são as proteínas responsáveis pela regulação da ligação Nrf2/ARE.

Bach1 é uma proteína que se liga a heme (Ogawa *et al.*, 2001), característica inicialmente constatada pela pigmentação amarronzada observada após purificação da proteina Bach1 recombinante da bactéria *Escherichia Coli*. Bach1 possui seis motivos cistina-prolina (CP) e estudos de deleção demonstraram que a região de ligação à heme encontra-se dentro dessa região de motivos CP. Heme afeta Bach1 de duas formas: Heme reduz consideravelmente a atividade de ligação a DNA desempenhada por Bach1 (Ogawa *et al.*, 2001); além disso, a inibição da síntese de heme promove a acumulação nuclear de Bach1, por outro lado, o tratamento de células com hemina resulta na exclusão nuclear de Bach1 (Igarashi & Sun, 2006) bem como sua ubiquitinação e consequente degradação (Zenze-Kawasaki *et al.*, 2007).

A indução da expressão do gene *hmox-1* de camundongo através de heme e outros oxidantes é regulada principalmente por dois elementos reforçadores (*enhancers*) localizados a 5'do gene, E1 e E2 , os quais funcionam de uma forma cooperativa (Alam *et al.*, 1989; Alam *et al.*, 1994; Alam *et al.*, 1995; Alam *et al.*, 2000). Ambas as regiões reforçadoras contêm múltiplas cópias do elemento responsivo a estresse (StRE) (Inamdar *et al.*, 1996). Os elementos StRE e MARE são estruturalmente e funcionalmente relacionados um ao outro (Igarashi & Sun, 2006), da mesma forma que o elemento responsivo antioxidante (ARE) é também relacionado a MARE (Itoh *et al.*, 1997). Entre os fatores que se ligam a MARE, Nrf2 e Bach1 apresentam um papel crítico na regulação da hmox-1 (Alam *et al.*, 1999; Sun *et al.*, 2002). Experimentos em camundongos revelaram que na ausência de Bach1, HO-1 é expressada de forma constitutiva, em altos níveis, em varios tecidos, em condições fisiológicas normais (Sun *et al.*, 2002). Por outro lado, células deficientes em Nrf2 apresentam expressão de HO-1 reduzida quando estimuladas através de vários indutores (Ishii *et al.*, 2000; Ishii *et al.*, 2004; Kwak *et al.*, 2001). A relação genética entre esses dois fatores (Nrf2 e Bach1) foi confirmda bioquimicamente. Ensaios de imunoprecipitação de cromatina (ChIP) em células NIH3T3 e timócitos revelaram que, em condições normais, Bach1 e Maf heterodímeros ocupam as regiões E1 e E2 da *hmox-1* (Sun *et al.*, 2002; Sun *et al.*, 2004). Heme promove o desligamento de Bach1 das regiões reforçadoras, seguida pela ligação de Nfr2 às mesmas regiões. Dessa forma, regulação de hmox-1 involve diretamente a detecção dos níveis de heme celulares pelo fator de transcrição Bach1, gerando um simples mecanismo de *feedback* onde o substrato afeta o antagonismo repressor/ativador (Sun *et al.*, 2002; Sun *et al.*, 2004).

HO-1 e o Metabolismo do Ferro

A quantidade de ferro necessária para a produção diária de 300 bilhões de células vermelhas no sangue é fornecida na sua maioria pela reciclagem do ferro presente nos grupos heme pelos macrófagos, no processo de fagocitose de eritócitos senescentes (Iolascon *et al.*, 2009). Esse processo permite a reciclagem de cerca de 20-25mg de ferro por dia, quantidade essa quase que suficiente para suprir a necessidade diária de ferro

requerido para eritropoiese na medula óssea (Knutson *et al.*, 2003). Em condições normais, o ferro absorvido na dieta, pelos enterócitos do duodeno (1-2mg/dia), é suficiente para compensar as perdas diárias resultantes da descamação de células epiteliais e de pequenas perdas de sangue.

Os eritrócitos senescentes e danificados são reconhecidos pelos macrófagos presentes no fígado e baço, através de uma série de mecanismos específicos (Lutz et al., 1988; Bratosin et al., 2001), sendo então fagocitados. Dentro dos macrófagos, as células vermelhas se encontram nos chamados fagossomos, os quais sofrem uma série de fusões com vesículas intracelulares e com o retículo endoplasmático a fim de adquirir a maquinaria necessária para a degradação dos constituintes das células vermelhas (Desjardins, 2003). A molécula heme é catabolizada por um complexo enzimático ancorado a membrana do retículo endoplasmático, o qual compreende a NADPH-citocromo c redutase, HO1 e biliverdina redutase. O ferro liberado pela catabolismo do heme pode ser tanto reciclado de volta ao plasma, através do transportador de membrana chamado ferroportina (o qual exporta o ferro na sua forma +2), ou então ser retido na célula dentro da proteina ferritina. Eritrofagocitose induz mudança na expressão gênica dos macrófagos, incluindo HO-1, ferroportina e ferritina, através de vários mecanismos. Heme, como já mencionado anteriormente, é um potente ativador transcripcional da HO-1 (Furuyama *et al.*, 2007) e o ferro liberado do catabolismo de heme regula a expressão da ferroportina e tradução do mRNA que codifica para ferritina (Delaby *et al.*, 2008) através do sistema IRE/IRP. A quantidade de ferro exportado pelos macrófagos para o plasma é controlada pelo hormônio chamado hepcidina e sua interação com o transportador ferroportina (Nemeth *et al.*, 2004; Delaby *et al.*, 2005). Qualquer alteração nesse processo de reciclagem do ferro pode resultar em diversas patologias: aumento na ativação de macrófagos por citocinas e consequente aumento na eritrofagocitose são características da anemia crônica e síndrome hemofagocítica (Weiss & Goodnough, 2005). Portanto, eritrofagocitose e reciclagem do ferro presente no grupo heme aparecem como processos centrais responsáveis pelo equilíbrio do ferro no organismo.

A HO-1 catalisa o único mecanismo fisiológico de degradação de heme e por consequência se posiciona como parte fundamental do mecanismo de manuntenção do equilíbrio de ferro dentro do organismo. A deficiência de HO-1 em humanos exibe como

sintomas inflamação e desregulação do equilíbrio de ferro no corpo, como por exemplo, acumulação de ferro nos rins e fígado (Yachie *et al.*, 1999; Kawashima *et al.*, 2002). Similarmente, o camundongo nocaute para a enzima HO-1 desenvolve severo grau de inflamação e anemia associada com baixa transferrina ligada a ferro e também acumulação de ferro no fígado e nos rins (Poss & Tonegawa, 1997). Portanto, foi proposto que além da ativa participação no processo de liberação do ferro presente no grupo heme, a enzima HO-1 possa agir de forma a facilitar o efluxo de ferro nas células hepáticas e renais. Foi observado que tanto em humanos quanto em camundongos que apresentam deficiência de HO-1, os níveis de hepcidina são baixos (Kartikasari *et al.*, 2008). Hepcidina é um hormônio produzido no fígado e liberado, quando maduro, no plasma e eliminado na urina. Esse hormônio age de forma a reduzir a quantidade de ferro circulante no organismo através da prevenção da saída desse metal de dentro das células, especialmente enterócitos e macrófagos. Para limitar a saída do ferro das células, a hepcidina se liga à ferroportina induzindo a internalização e degradação da mesma (Delaby *et al.*, 2005; Nemeth *et al.*, 2005). Na ausência da hepcidina, ocorre aumento na absorção intestinal e efluxo de ferro nos macrófagos o que resulta na acumulação desse metal (De Domenico *et al.*, 2008). A enzima HO-1 não age de forma direta na modulação da expressão do hormônio hepcidina pelo fígado, no entanto, o grupo heme regula transcricionalmente a expressão do gene da ferroportina 1 (*FPN1*), alvo do hormônio hepcidina, em um mecanismo envolvendo Bach1 e Nfr2. Dessa forma, heme seria suficiente para aumentar a atividade transcricional da FPN1, enquanto que o ferro liberado pela clivagem do grupo heme controla a tradução da FPN1 em um processo envolvendo um elemento IRE presente na extremidade 5' não traduzida do transcrito desse transportador (Marro *et al.*, 2010). Portanto, as quantidades de heme livre, que em ultima análise são controladas pela enzima HO-1, são também responsáveis pela maior ou menor presença do exportador de ferro nas membranas de macrófagos e enterócitos. Tais fatos colocam a enzima HO-1 como uma das grandes responsáveis pelo correto fluxo do ferro dentro do organismo, promovendo sua adequada reciclagem nos macrófagos e também agindo de forma indireta na disponibilização desse ferro no plasma.

Heme Oxigenase e Eritropoiese

Como salientado anteriormente, quase a totalidade do ferro resultante da reciclagem do grupo heme é destinado ao processo de eritropoiese que ocorre na medula óssea. Durante o processo de diferenciação, as células eritróides adquirem ferro do complexo ferro-transferrina (Tf) presente no plasma, em uma rota envolvendo o receptor de transferrina (TfR). Todas células em proliferação expressam receptores de transferrina na membrana em níveis que variam entre 10^3 e 10^5 moléculas por célula. Precursores eritróides, os quais possuem uma alta necessidade de ferro a fim de permitir a síntese de hemoglobina, podem apresentar cerca de 10^6 receptores de transferrina por célula (Ponka *et al.*, 1998). O complexo Fe(III)-transferrina é transportado para o interior das células principalmente através do receptor de transferrina do tipo 1 (TfR1), sendo o mesmo presente em todos as células em estado de divisão e especialmente abundante em precursores eritróides.

O pH da superfície celular permite a ligação específica apenas entre o TfR1 e a transferrina diférrica. O complexo Tf-Fe(III)/TfR1 é internalizado em uma vesícula chamada endossomo, a qual é revestida pela proteína clatrina. O complexo formado pela proteína adaptadora AP-2 (Conner & Schmid, 2003), auxilia na redução do pH dentro do endossomo. A redução no pH permite a liberação do ferro da transferrina, o qual é subsequentemente reduzido a Fe(II) e transportado através da membrana endossomal pelo Transportador Divalente de Metal 1 (DMT1) (Canonne-Hergaux *et al.*, 2001). A molélula de transferrina "vazia" retorna à superfície celular, onde, sob pH de 7,4, ocorre a liberação da mesma e o início de um novo ciclo. O ciclo Tf-TfR é completado em poucos minutos, e durante seu tempo na circulação uma molécula de Tf realiza de 100 a 200 ciclos (Katz, 1961).

Em células eritróides, a maior parte do ferro que deixa os endossomos é direcionada para a mitocôndria a fim de participar dos processos de síntese de heme e montagem dos *clusters* ferro-enxofre (Napier *et al.*, 2004). A precisa forma química do ferro durante seu trânsito entre endossomo e mitocôndria é ainda assunto de grande debate (Sheftel *et al.*, 2007). A hipótese de um contato direto entre endossomos e

mitocôndria é fortemente sustentada na lieratura, em um modelo chamado de "*kiss and run*" (Sheftel *et al.*, 2007). A proteína Mitoferrina (SLC25a37) é a responsável pelo transporte do ferro através das membranas mitocondriais (Shaw *et al.*, 2006; Muhlenhoff *et al.*, 2003). Como já mencionado anteriormente, a enzima Ferroquelatase, última enzima da rota de síntese do grupo heme, localizada na membrana interna da mitocôndria, catalisa a inserção do Fe(II) na protoporfirina IX (PPIX). Após sua síntese, o grupo heme é exportado da mitocôndria a fim de ser associado com as cadeias de globinas, resultando então na molécula de hemoglobina.

O excesso de ferro livre pode promover a geração de espécies reativas de oxigênio (Martin *et al.*, 2006) e, por outro lado, quantidades reduzidas de ferro pode resultar no bloqueio das atividades metabólicas e respiratórias da mitocôndria o que prejudica a formação da hemoglobina em precursores eritróides. Dessa forma, diferentes mecanismos operam para coordenar a aquisição de ferro e a síntese de heme, especialmente em células eritróides (Ponka, 1997). Existem duas proteínas regulatórias de ferro (IRP1 e IRP2) que atuam como sensores de ferro nas células de mamíferos e regulam a estabilidade e tradução dos mRNAs que codificam para proteínas relacionadas ao metabolismo de ferro (Muckenthaler *et al.*, 2008). A ligação entre as IRPs e os elementos IREs presentes na região 5' não traduzida dos mRNAs da cadeias pesada (H) e leve (L) da ferritina, bem como com o mRNA do transportador ferroportina e a enzima ALAS2 resultam em repressão da tradução dos mesmos. A ligação dos IRPs aos múltiplos elementos IREs presentes na região 3' não traduzida do mRNA que codifica o receptor TfR1 promove a estabilização do mesmo. Quando na presença de excesso de ferro, as proteínas IRPs se desligam dos elementos IREs. Considerando a situação específica das células eritróides, a entrada de ferro promove a tradução da enzima ALAS2 e consequente aumento na síntese de heme, além da desestabilização do mRNA que codifica para TfR1, reduzindo a entrada de ferro na célula e evitando qualquer tipo de excesso de ferro.

Durante a diferenciação eritróide, é essencial uma balanceada produção de cadeias α e β de globina bem como moléculas de heme, garantindo, dessa forma, a formação da molécula de hemoglobina (2 α - 2 β - 4heme). As cadeias de globina tendem a assumir conformações anômalas e precipitar na ausência da quantidade correta de

heme. Logo, em situações de deficiência de heme, uma enzima quinase é ativada, essa proteina é chamada inibidor regulado por heme (HRI), a qual fosforila o fator iniciador de tradução eIF2a (Kramer *et al.*, 1976; McEwen *et al.*, 2005). Quando eIF2 é fosforilado, o mesmo mantém-se ligado a uma proteína de ligação, o que previne a regeneração do GDP em GTP resultando no desligamento da tradução dos mRNAs celulares. Considerando que em uma célula eritróide em processo de diferenciação boa parte dos mRNAs em processo de tradução correspondem aos mRNAs das globinas, tal mecanismo resulta na inibição da tradução das globinas, evitando excesso e consequente precipitação dessas cadeia proteicas. Além disso, a transcrição dos genes que codificam para as globinas também é controlada por heme. O mecanismo pelo qual heme promove essa regulação ocorre através do fator de transcrição Bach1 (Tahara *et al.*, 2004a; Tahara *et al.*, 2004b), em um mecanismo semelhante ao que ocorre no promotor da HO-1 já destacado anteriormente no tópico 1.3 dessa introdução.

Portanto, os mecanismos acima destacados evitam que a célula eritróide absorva mais ferro do que o necessário para síntese de heme, da mesma forma que evita quantidades de globina que excedam as quantidades de heme sintetizadas. Tal situação, indica que os níveis "heme livre", se existem, são muito baixos durante o processo de diferenciação. Apesar da expressão da enzima HO-1 não ter sido extensivamente estudada em células eritróides, foi demonstrado que os níveis do mRNA que codifica para HO-1 diminuem durante o processo de diferenciação eritróide em um linhagem celular eritroleucêmica de camundongo, chamada MEL (Fujita *et al.*, 1991; Fujita *et al.*, 1989). No entanto, esses resultados foram obtidos usando apenas a linhagem celular MEL, sendo essa diferenciada durante um período de tempo (48h) onde os níveis de heme acumulados não se mostravam significativamente altos. Além disso, estudos de proteômica demonstraram que, durante o processo de diferenciação, as células MEL apresentam uma redução na ligação do fator de transcrição Bach1 aos elementos MARE, situação essa importante para a indução da expressão dos genes que codificam para as globinas (Brand *et al.*, 2004). Essa redução na ligação de Bach1 aos genes alvo poderia determinar uma indução na expressão do gene que codifica para a enzima HO-1. Todas essas alterações no padrão transcricional das células eritróides são em grande parte um reflexo do aumento nas quantidades de heme sendo sintetizadas e destinadas ao processo

de hemoglobinização. Portanto, considerando essa situação de altas quantidades de heme juntamente com uma situação transcricional de desrepressão, favorecem a hipótese que a enzima HO-1 possa ter seus níveis regulados positivamente e assumindo uma posição mais ativa no processo de diferenciação eritróide.

A presente tese apresenta os resultados obtidos da análise da expressão da enzima HO-1 durante o processo de diferenciação eritróide. Esses dados estão apresentados no capítulo II, e demonstram que a enzima HO-1 tem seus níveis aumentados durante o processo de diferenciação eritróide, além de apresentar indícios da participação dessa enzima de forma ativa na modulação do processo de hemoglobinização. No capítulo III apresentamos e defendemos a hipótese que alelos específicos de hmox1 estariam sendo selecionados de forma diferencial em áreas endêmicas de malária, sendo esses alelos fatores importantes na determinação de resistência a essa doença. Portanto, os capítulos II e III refletem a grande importância da reação catalisada pela enzima HO-1 dentro da homeostase do organismo, assim como a diversidade de funções desempenhadas pelo seu subtrato, o grupo heme.

CAPÍTULO II

The increase of heme oxygenase I expression levels during erythroid differentiation

The increase on heme oxygenase I expression levels during erythroid differentiation.

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Abstract

Heme is indispensable to the function of all aerobic cells. The degradation of heme is solely accomplished through the heme oxygenases (HO) pathway. There are two isoforms described so far: HO-1 and HO-2. HO-1 is the inducible form and it is induced by heme, various metals, xenobiotics, endocrine factors, and synthetic metalloporphyrins. The activity of this enzyme is considered protective once it promotes the removal of potential pro-oxidant heme and realising antioxidant molecules such as biliverdin and cabon monoxide (CO). The major source of heme destined for catabolism are red blood cells (RBC), which contain the highest amounts (75-80%) of organismal heme. HO-1 has been extensively studied in non-erythroid cells, however nothing is known about its expression in erythroid cells. The present study investigates the expression of HO-1 on murine erithroleukemia (MEL) and primary erythroid (FL) cells. Our results indicate that HO-1 is up-regulated at mRNA and protein levels during erythroid differentiation on both cell models. Furthermore, either the induction or suppression of HO-1 expression resulted in alterations in the hemoglobinization process on DMSO-induced MEL cells. These observations support that HO-1 is able to access the heme synthesized during the erythroid maturation and interfere with the differentiation process. Therefore, our results strongly suggest HO-1 might act as a co-regulator of the RBC development by controlling the heme levels.

Introduction

Heme is indispensable to the function of all aerobic cells. It is a complex containing iron (Fe) at the center of a protoporphyrin IX ring. Heme operates as a prosthetic group in many hemoproteins such as, myoglobin, cytochromes, guanylate cyclase, and nitric oxide synthase. It is also involved in protein synthesis and cellular differentiation [1,2]. As essential as it is to aerobic cells, if it is left unguarded, with no protein attachment, it can induce free radical formation and lipid peroxidation which will lead to cellular damage and tissue injury[3]. For this reason, the rate of heme biosynthesis and heme catabolism must be kept well in balance and tightly controlled.

Erythropoiesis is the process by which multipotencial hematopoietic stem cells differentiate into mature, non-nucleated erythrocytes. During this process, the hormone erythropoietin (EPO) acts upon erythroid progenitors in the early stages of the differentiation, from colony-forming unit-erythroid to the earliest of basophilic erythroblasts (BasoEB), where this hormone, produced in the kidneys, prevents apoptosis in the progenitors[4]. In the late stages of differentiation, from BasoEB to final differentiated RBC, there is an intense production of hemoglobin, which needs in consequence high amounts of heme[5]. The high heme levels in the late stages of differentiation triggers the expression of the α- and β-globin[6,7] genes as well as its protein translation[8].

It is not surprising that the major source of heme destined for catabolism are red blood cells (RBC) considering they contain the highest amounts (75-80%) of organismal heme. On a per cell basis, RBC precursors' rates of generation of heme are at least one order of magnitude above hepatic cells, which are next in line in the body for heme producing activity. Heme from RBC is fated to be catabolized in splenic and hepatic macrophages, following erythrophagocytosis of senescent RBC.

The degradation of heme is solely accomplished through the heme oxygenases (HO) pathway. Heme oxygenase's oxidation of heme to biliverdin, ferrous ions and carbon monoxide represents the first and rate limiting step of heme degradation[9-11]. There are two isoforms described so far: HO-1 and HO-2. The constitutive form, HO-2 is membrane protein bound found at highest levels in the brain and testis[12]. The inducible form, HO-1, is a 32.8 kDa membrane-bound enzyme found at highest concentrations in the liver and spleen. HO-1 is

increased in whole animal tissues after treatment with its natural substrate heme, as well as various metals, xenobioticis, endocrine factors, and synthetic metalloporphyrins[13] .This enzyme is a heat shock protein, and also a stress protein induced by several agents that cause oxidative damage[14-17]. HO-1 promotes protection through the removal of the potential dangerous pro-oxidant free heme, generate in stress conditions, and release of bilirubin and biliverdin, metabolites with antioxidant proprieties[18]. The HO-1 activity also releases Fe(III), which is rapidly stored in ferritin[19], and carbon monoxide (CO) that has important cellular signalling functions such as in neurotransmission and vascular relaxation[20-22].

Previously, HO, in particular HO-1, has been extensively studied in hepatocytes and many other non-erythroid cells[9,10]. Ironically, even though RBC possesses the greatest amounts of organismal heme, virtually nothing is known about the expression of HO-1 in the developing RBC. Moreover, it is unknown whether HO-1 plays any role in erythroid cell development under physiological or pathological conditions. Here we demonstrate that HO-1 is present in erythroid cells, and can be easily induced in the presence of heme. More importantly, our results indicate that HO-1 expression is increased during erythroid differentiation. Interestingly, we observed that modulation on HO-1 expression interfered with the hemoglobinization process induced by DMSO in murine erytroleukemia cells (MEL). Our results show importance of the expression of the enzyme HO-1 during the erythroid differentiation, suggesting a role of this enzyme as a co-regulator of the hemoglobinization process.

Material and Methods

Cell culture and chemicals

Mouse eythroleukemia cells, established by Friend in 1966, undergo dramatic changes in their morphological and biochemical characteristics following exposure to various agents, such as dimethyl sulfoxide (DMSO) and sodium butyrate. MEL cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS), to which penicillin (100 units/mL of medium) and streptomycin (100 µg/mL of medium) is added (all from Invitrogen, Burlington, Ontario). All the experiments were conducted in both uninduced MEL cells (control cells) as well as cells treated with 2% DMSO (Me_2SO_2) to promote differentiation and hemoglobinization[23]. Hemin and tin-protoporphyrin (SnPP) were obtained from Frontier Scientific (Logan, UT) and dissolved in 100mM sodium hydroxide (NaOH) . Sodium arsenite ($NaAsO_2$) (Fisher Chemical, Ottawa, ON) was dissolved in water.

Primary erythroid cells were cultures as described[24]. Briefly, cells were grown from fetal livers from E12.5 embryos (wild-type, MF1 background) of mice and resuspended in serum-free StemPro-34 medium plus Nutrient Supplement (Invitrogen-Gibco, Carlsbad, CA) plus 2 U/mL human recombinant erythropoietin (Epo; 100 ng/mL), murine recombinant stem cell factor (SCF; 100 ng/mL), the synthetic glucocorticoid dexamethasone (Dex; 106 M), and insulin-like growth factor 1 (IGF-1; 40 ng/mL). Cell number and size distribution of cell populations were monitored daily in an electronic cell counter (CASY-1, Scha¨rfe-System, Reutlingen, Germany). Dead or differentiating cells were removed by Ficoll purification.

siRNA Transfection

MEL cells were seeded in 6 well plates at the concentration of $0.5x10^6$ cells per well in 2 mL of DMEM with 10% FBS and without antibiotics. The cells were transfected using Lipofectamine™ transfecting agent (Invitrogen™, Burlington, Ontario), according to the manufacturer's instructions. Briefly, 100 ng of ON-TARGETplus Hmox siRNA (Dharmacon, Inc, Chicago, USA) was transfected and after 18 h the cells were washed and the desired treatment applied. The control siRNA transfections were done either using Lipofectamine2000 alone ("mock") or scrambled siRNA, 5' GUAGACAGACUCCAAACCA 3', generated considering the Hmox siRNA sequence.

Northern blotting

Total RNA was isolated using Trizol (Invitrogen) following the manufacturer protocol. 15µg of total RNA were separated in denaturing 1% formaldehyde-agarose gels. Equal loading was controlled by ethidiumbromide staining. RNA was transferred to nylon membranes (Hybond-XL, GE Healthcare, Buckinghamshire, UK), and fixed by UV irradiation (1200mJ; UV-Crosslinker, Stratagene, LaJolla, CA). Membranes were sequentially hybridized with [^{32}P]-labeled cDNA-probes generated by random-primed labeling (DecaLabel,Fermentas, Burlington, Canada) specific for mouse HO-1 and mouse beta-actin. Results were digitized using Storm840 phosphor imaging system (GE Healthcare, Buckinghamshire, UK) and quantitated using ImageQuant software. Each Northern was repeated at least three times.

Western blotting

Cells were harvested and lysed using Munro's lysis buffer (10mM Hepes [pH 7.6], 3mM $MgCl_2$, 40mM KCl, 5% glycerol, and 0.2% NP-40). Protein content was determined using Bradford reagent (BioRad, Mississauga, Ontario). 30ug of protein were separated on a 15% SDS-polyacrylamide gel and then transferred to a nitrocellulose blotting membrane. Membranes were blocked with blocking solution (5% milk powder in TBS/0.2% Teen20). Then they were first incubated overnight with the indicated primary antibody: β–actin (Sigma, Sigma-Aldrich, Inc., Oakville, Canada; 1:5000) ; HO-1 (Stressgen, Ontario; 1:5000); Ferritin (Sigma-Aldrich, Inc., Oakville, Canada; 1:500); globin rabbit (MP Biomedicals, Inc ; 1:10000) and then with the rabbit-IgG secondary antibody in a 1:20000 dilution in blocking solution for 1h at RT. The western blot is developed using HyBlot CLTM autoradiography film (Denville Scientific Inc, Metuchen, New Jersey). HO1, ferritin and globin protein levels were quantified using the software ImageJ 1.4 (Wayne Rasband, National Institutes of Health, USA) and normalized according to β–actin levels in each sample well. Each western was repeated at least three times.

Heme Assay

Assay was performed as described[25,26]. Briefly, 2-5 x10^5 cells were transferred in triplicates into 96 well microtiter plate with conical bottom and washed with 100ul PBS. Cells were lysed in 50ul H2O. Thereafter, we added 125 ul dye solution (0.5 mg/mL o-phenylene-diamine-dihydrochloride (Sigma-Aldrich, St. Louis, MO,), 50 mM citric acid, 0.1 M Na_2HPO_4; add 1 µL/mL of 30% H_2O_2). The reaction was stopped with 25ul 8N H_2SO_4 and samples read at OD492nm.

Results

HO-1 mRNA levels increased during erythroid differentiation

We determined the HO-1 mRNA levels from MEL cells either kept in an undifferentiated stage or differentiated for up to 3 days. The results obtained are depicted in figure 1a and b and demonstrate a markedly increase in HO-1 mRNA levels after 72h of differentiation. Positive controls indicate induction of HO-1 when MEL cells were treated either with hemin (figure 1a) or $NaAsO_2$ (figure 1a). We tested the possible correlation between high levels of heme biosynthesis and increased expression of HO-1 in differentiating MEL cells by using succinyl acetone (SA) as a specific inhibitor of aminoleculinic acid dehydratase and thus an inhibitor of heme biosynthesis. As shown in figure 1a and b, the addition of SA 24h prior to harvest, resulted in a clear reduction in the HO-1 mRNA levels, after 72h of differentiation, when compared with no treated cells (figure 1a and b).

Erythroleukemic cells lines share common drawbacks with respect to erythroid differentiation such as non-physiological stimuli to initiate differentiation and mature incompletely or aberrantly (insufficient hemoglobin accumulation, abnormal morphology, lack of enucleation)[27]. For these reasons, we adopted primary erythroblasts derived from mouse fetal liver (FL), which is considered a more "physiological" erythroid model, to corroborate our fidings regarding HO-1 pattern of expression during differentiation. Similar results were obtained when we analysed the expression of HO-1 in FL (figure 2a and b). These primary cells even clearer demonstrated the increase in HO-1 mRNA levels during differentiation. Again, SA treatment resulted in a significant decrease in the HO-1 mRNA levels, mainly after 48 and 72h of differentiation when compared with no-treated controls (figure 2a and b). We measured the heme levels from the same samples (figure 2c) and there was a clear correlation between levels of heme and HO-1 mRNA. Together, these results clearly show a clear upregulation suggest a positive regulation of HO-1 expression during the erythroid differentiation. This increase in HO-1 expression is probably a result of the increased cellular heme levels.

HO-1 protein levels increased during erythroid differentiation

Similarly to our study, Fujita *et al.*[28] also analyzed HO-1 mRNA expression in MEL cells. The authors did not investigate HO-1 protein levels and if it displays similar pattern of expression as observed at mRNA level. So, in a next step, we analysed the expression of HO-1 on the protein level. Immunoblotting with an HO-1 specific antibody indicate that HO-1 was highly induced by addition of hemin either in presence or absence of DMSO (figure 3b). In concordance with the mRNA levels (figure 1a and b), HO-1 protein levels display a peak of expression at 72h of differentiation (figure 3a). The presence of SA was able to reduce the HO-1 protein levels when compared with no-treated controls (figure 3a).

To further evaluate the HO-1 protein expression pattern in erythroid cells, we differentiated FL during 48h, and analyzed the protein levels of this enzyme by western blotting. Accordingly to the results obtained from MEL cells also highly hemoglobinizing FL cells (48h of differentiation) manifested an up-regulation of HO-1 (figure 4). Moreover, this increase in HO-1 protein levels was efficiently abrogated by addition of SA to the medium. These results underline the results obtained from HO-1 mRNA expression studies showing that also HO-1 protein is up-regulated during erythroid differentiation. Moreover, inhibiton of heme biosynthesis decreases the protein levels.

Induction of HO-1 by treatment with $NaAsO_2$ impairs hemoglobinization in DMSO-treated MEL cells

MEL cells were differentiated with DMSO and treated with increasing concentrations of $NaAsO_2$. We observed a dose-dependent increase in HO-1 protein expression (figure 5a and b) and a correlating impairment in hemoglobinization due to a reduction of globin protein expression (5a and b). Moreover, there is a clear up-regulation of ferritin protein levels probably as a consequence from augmented “free” iron due to the increase in HO-1-mediated heme catabolism (figure 5a and b). To exclude any unspecific effect of $NaAsO_2$, we treated the MEL

cells with tin-protoporphyrin (SnPP), a HO-1 specific chemical inhibitor[29]. SnPP treatment was able to inhibit the effect of $NaAsO_2$ over globin and ferritin levels in DMSO-treated MEL cells (figure 5a and b). Therefore, $NaAsO_2$ treatment resulted in a reduced hemoglobinization in DMSO-treated MEL cells. Futhermore, this impairment of the hemoglobinization process was mediated by HO-1 overexpression once treatment with SnPP was enough to restore the globin and ferritin control levels.

HO-1 siRNA promotes increased hemoglobinization in DMSO-treated MEL cells

To further elucidate the role of HO-1 in developing RBC, we tested if not only the induction of HO-1 but also its suppression affects the differentiation process. For this purpose we transfected MEL cells with ON-TARGETplus HO-1 siRNA. Control experiments show that the HO-1 specific siRNA reduced HO-1 expression by about 50% in $NaAsO_2$treated cells (figure 6a). After transfection, the cells were treated for 48h with DMSO, and then harvested and subjected to western blot analysis. Surprisingly, the MEL cells transfected with HO-1 siRNA and differentiated with DMSO displayed an increase in the globin levels when compared with mock and scramble siRNA controls (figure 6b and c). These results indicate that MEL cells with reduced expression of HO-1 are able to accumulate more hemoglobin. HO-1 mght be acting as a regulator of the differentiation process, controlling any possible excess of heme.

Discussion

The results obtained show that HO-1 is induced in erythroid cells treated with either hemin or sodium arsenite. More importantly, this induction occurs in a physiological manner at mRNA and protein level during the process of erythroid differentiation. This induction follows the accumulation of heme as demonstrated using the heme synthesis inhibitor, SA. The modulation of HO-1 expression either by induction with $NaAsO_2$ or suppression using specific siRNA decreased and increased the hemoglobinization of differentiating MEL cells, respectively.

During differentiation, mammalian red blood cells synthesize enormous quantities of hemoglobin, which consists of a tetramer of two α- and two β-globin polypeptides associated with a heme group. Heme not only is incorporated as a structural component of hemoglobin but also causes an increase in the expression of globin as well as enzymes of the heme biosynthetic pathway in erythroid cells [30-32]. The transcription repressor Bach1 is a sensor and effector of heme[33]. Bach1 forms heterodimers with the small Maf proteins to bind to the Maf recognition element (MARE), thus repressing the expression of its target genes[34]. As sensor of heme, Bach1 binds heme through its multiple heme regulatory motifs (HRMs)[35], thereby losing its activity as a repressor. The net effect of heme is a derepression of the Bach1 target genes, which include α- and β-globin [6,7,36,37] and HO-1 [37,38]. We observed that HO-1 mRNA expression is up-regulated during erythroid differentiation, and this increase is more evident after 72h of differentiation (figures 1a and 1b ; figures 2a and 2b). Moreover, the HO-1 levels follow the same pattern of increase displayed by heme during differentiation (figure 1c and 2c), where high heme levels correlates with high HO-1 mRNA levels. Block of heme biosynthesis by addition of SA also results in a reduction of HO-1 expression (figures 1a-b and 2 a-b). Therefore, we suppose that derepression of HO-1 is mediated by heme, possibly via Bach1.

Fujita *et al.*[28] described a decrease in HO-1 mRNA levels in differentiating MEL cells during the first 48h of DMSO treatment when compared to control. However, Fujita *et al.* did not look at later time points of differentiation where MEL cells start to hemoglobinize extensively. At those stages we observed a clear rise in the expression of HO-1 mRNA (figure 1a-c). Furthermore, our findings in MEL cells were reinforced from very similar results obtained from

primary erythroid cells. FL cells represent a more physiological model than MEL cells[39]. When induced for differentiation FL cells undergo more prominent morphological changes than MEL cells characterized by size decrease, hemoglobin accumulation and nuclear condensation with enucleation at the end stage of maturation[39]. Although there are a wide variety of chemicals[14-17] which induce HO-1 expression, there is no evidence so far that also DMSO has this capability. Nevertheless, our results using a DMSO-independent model (FL cells, figures 2a, 2b and 4) undermines that the induction of HO-1 during erythroid differentiation is a physiological process.

Arsenite, the trivalent form of inorganic arsenic, is a potent inducer of oxidative stress. Many of the effects of arsenite are attributable to its affinity for soft nucleophiles, particularly cysteine residues in glutathione and proteins[40]. In response to oxidative stress, such as that mediated by arsenite, cells induce a battery of protective antioxidant enzymes of which HO-1 is a well recognized member. We observed that increasing concentrations of arsenite induced HO-1 in MEL cells in a dose-dependent manner (figure 5a-b). Together with HO-1 up-regulation, arsenite increases ferritin protein levels (figure 5a-b). In murine macrophages, only induction of HO-1 via heme caused an increase in the synthesis rate of the iron storage protein, ferritin[41]. The treatment of macrophages, with similar amounts of arsenite, did not increase ferritin levels. According the authors, heme levels, and not HO-1, limit cellular heme catabolism[41] The treatment of MEL cells with DMSO induces the accumulation of heme, and consequently the production of hemoglobin. Induction of HO-1 by arsenite would interfere with the synthesis of haemoglobin by cleaving a significant fraction of the accumulating heme. On one hand, this would activate the expression of ferritin in order to neutralize the toxic “free” iron (figures 1c and 5a-b). On the other hand, the decrease in the heme-pool would lead to a decrease in the globin expression (figure 5-b). This was also indicated by the reduced red color of the arsenite-treated MEL cells after 72h of differentiation when compared to control cells (data no shown). The specific role of HO-1 in this observation is demonstrated by co-treatment with SnPP. Additon of this HO-1 inhibitor neutralizes the effect seen with arsenite alone, i.e. the protein expression of ferritin and globin remains similar to control levels. (figure 5a-b).

As mentioned above, we observed that DMSO-treated MEL cells as well as FL cells show an up-regulation in the HO-1 mRNA and protein levels during erythroid differentiation.

This induction can be, at least partially, inhibited by blocking the biosynthesis of heme with SA. Therefore we postulate that the induction of HO-1 is a consequence of the increase in heme levels. This endogenous induction occurring during the differentiation process seems not to impair the hemoglobinization of either MEL or FL cells. However, the levels of HO-1 induced by arsenite were notably higher and were able to decrease the hemoglobinization of differentiating cells (figure 5a-b). Altogether, these data indicate that induction of HO-1 expression during erythroid differentiation must be tidily controlled to avoid interference with the synthesis of hemoglobin. Our hypothesis is further corroborated by the use of HO-1 siRNA in DMSO-treated MEL cells. The bisection of HO-1 protein expression (figure 6c-d) was enough to promote an increase in globin protein levels (figure 6a-b). Therefore, both up-regulation of HO-1 or down-regulation has a direct effect on the hemoglobinization of differentiating erythroid cells (figures 5a-b and 6a-b).

The erythroid cells tightly regulate the differentiation process. As mentioned before, heme regulates positively the expression of the globin genes[6,7]. Heme also regulates the translation of globin mRNA through the protein kinase heme regulated inhibitor (HRI). Heme deficiency activates HRI which phosphorylates the translational initiator factor eIF2a. When phosphorylated, eIF2a is unable to promote mRNAs translation. This mechanism affects mainly globin mRNAs, once it is the most abundant transcript at the last stages of erythroid differentiation[8,42]. Therefore, heme regulates globin expression at transcriptional and translational level, guarantying enough globin for the newly synthesized heme. All the mentioned heme-mediated regulatory mechanisms support the idea of the virtual absence of "uncommitted" heme during the differentiating process. However, the importance of heme as a regulatory molecule suggests the existence of a "heme regulatory pool" (HRP), which would be responsible for the modulation of molecules such as Bach1 and HRI. This HRP probably reflects the changes in the heme amounts during the differentiation process. HO-1 would play a role as a guard of the HRP, removing any cell damaging excess of heme.

The increase or reduction of HO-1 protein, as we observe in our results, should not interfere with the hemoglobinization process, unless the enzyme can access the HRP. The cellular localization of HO-1 in erythroid cells is not known, but it was first identified as an integral type I membrane protein of the smooth endoplasmic reticulum (sER)[43] of bovine

macrophages. After its release from mitochondria, heme is directed towards its target proteins in the cytosol. Thus, the localization of HO-1 as a membrane bound protein of the sER with the active site facing the cytoplasm would facilitate the access to heme and its cleavage before it gets bind to the globins. However, there are also more recent reports showing the presence of HO-1 in the nucleus[44] and mitochondria[45] in non-erythroid cells. Therefore, we can not rule out that HO-1 is active somewhere else in the erythroid cell or even might change its localization during the differentiation process.

We hypothesized that HO-1 controls the levels of the heme pool during erythroid differentiation. Similar function might be accomplished by the putative heme exporter, FLVCR[46], which would be responsible for pumping out of the cell the excess of heme. However, FLVCR is expressed in immature erythroblasts[47], whereas HO-1 is induced in highly hemoglobinizing erythroid cells. On the other hand, our results showing increased levels of globins, when HO-1 is partially suppressed, indicate an action of this enzyme, not cleaving some virtual excess of heme, but affecting the availability of this molecule. In this situation, the HO-1 would be acting over heme molecules which are “commited” with the hemoglobinization process.

Additional experiments are necessary to address the question regarding a possible action of HO-1 as a co-regulator of the differentiation process. The gradual increase in HO-1 levels observed in our results might also suggest that this enzyme works, when it reaches high levels, at late stages of differentiation, as a signal to erythroid cell to terminate the hemoglobinization process.

Figure 1

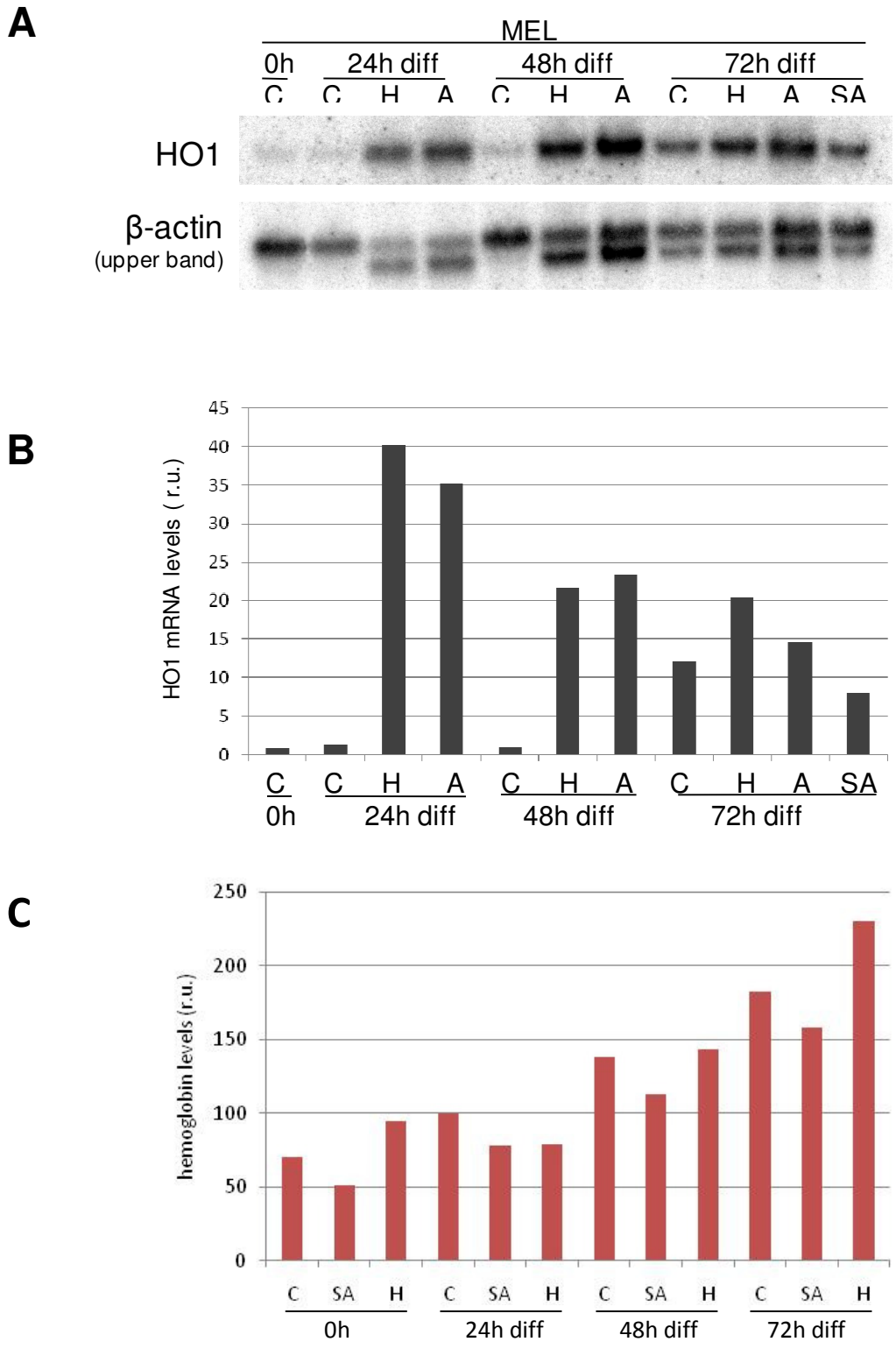

A
MEL
0h
24h diff
48h diff
72h diff
C C H A C H A C H A SA
HO1
β-actin
(upper band)
B
HO1 mRNA levels (r.u.)
45
40
35
30
25
20
15
10
5
0
C C H A C H A C H A SA
0h
24h diff
48h diff
72h diff
C
hemoglobin levels (r.u.)
250
200
150
100
50
0
C SA H C SA H C SA H C SA H
0h
24h diff
48h diff
72h diff

Figure 2

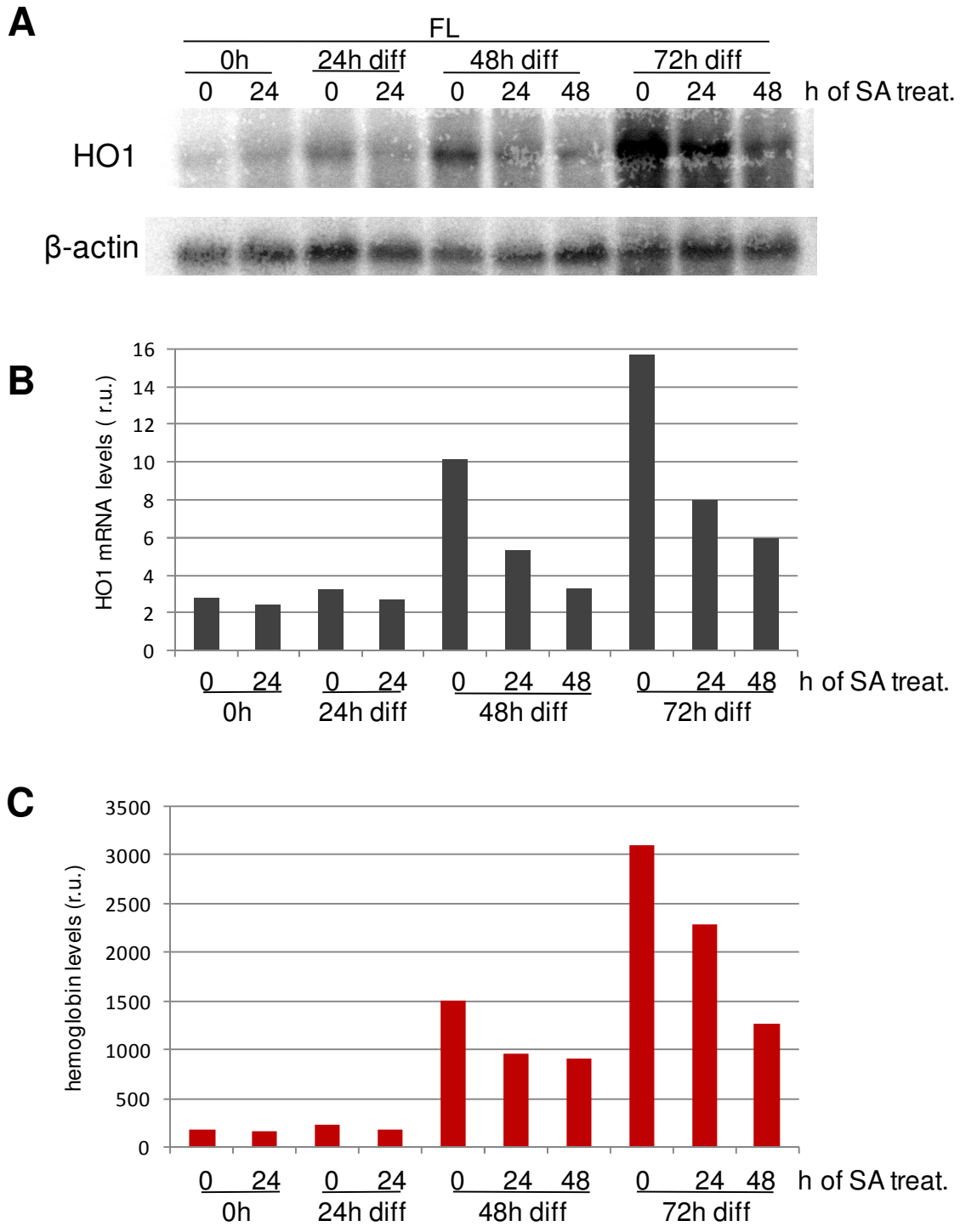

A
FL
0h
24h diff
48h diff
72h diff
0 24 0 24 0 24 48 0 24 48 h of SA treat.
HO1
β-actin
B
HO1 mRNA levels (r.u.)
16
14
12
10
8
6
4
2
0
0 24 0 24 0 24 48 0 24 48 h of SA treat.
0h
24h diff
48h diff
72h diff
C
hemoglobin levels (r.u.)
3500
3000
2500
2000
1500
1000
500
0
0 24 0 24 0 24 48 0 24 48 h of SA treat.
0h
24h diff
48h diff
72h diff

Figure 3

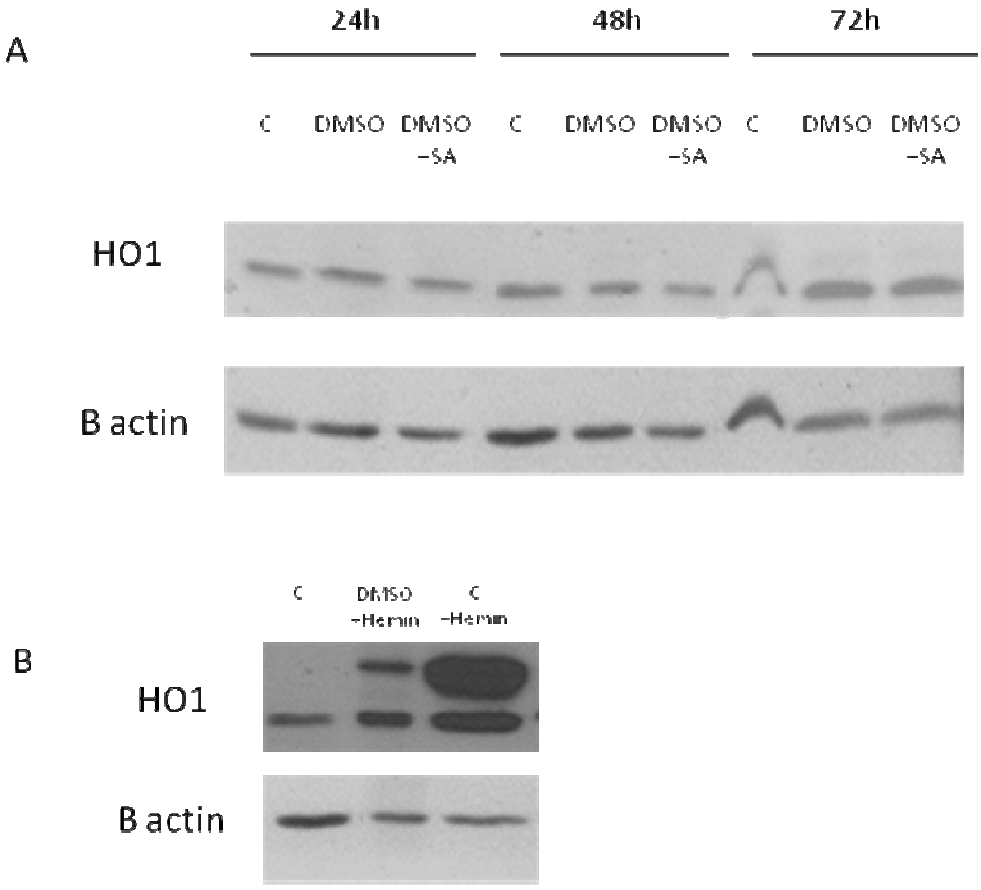

A
24h
48h
72h
C
DMSO
DMSO -SA
C
DMSO
DMSO -SA
C
DMSO
DMSO -SA
HO1
B actin
B
C
DMSO -Hemin
C -Hemin
HO1
B actin

Figure 4

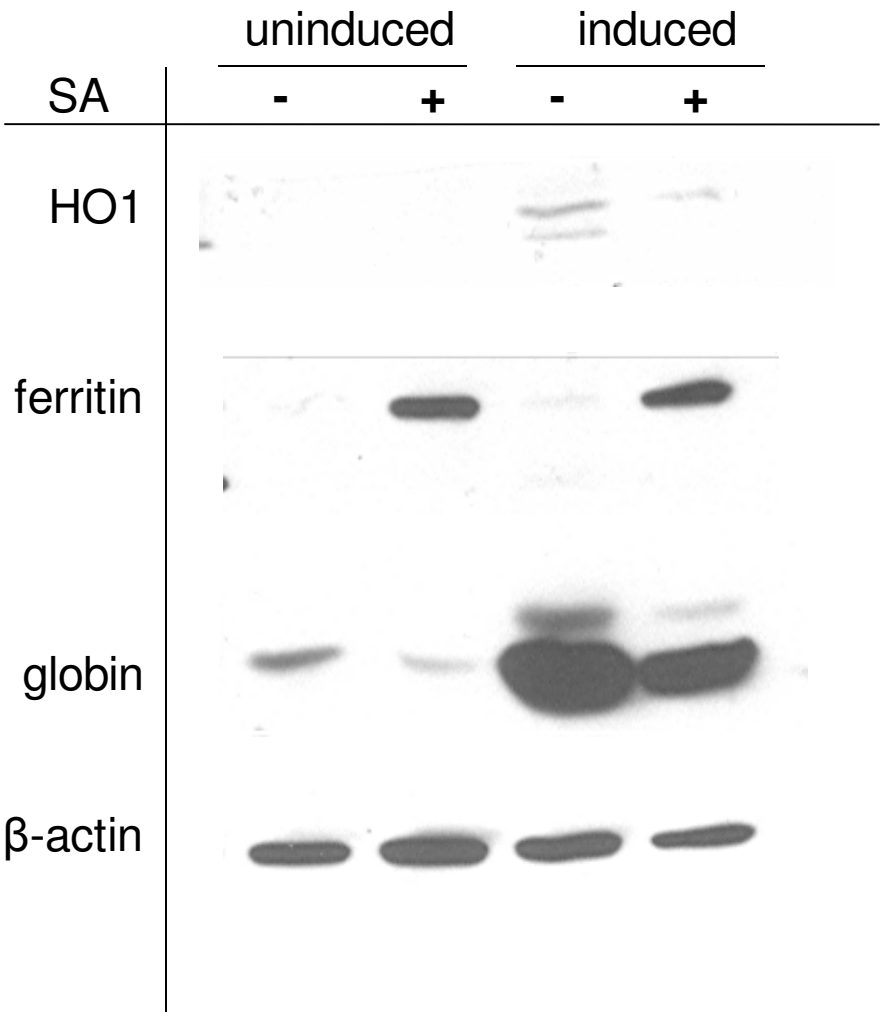


Figure 5

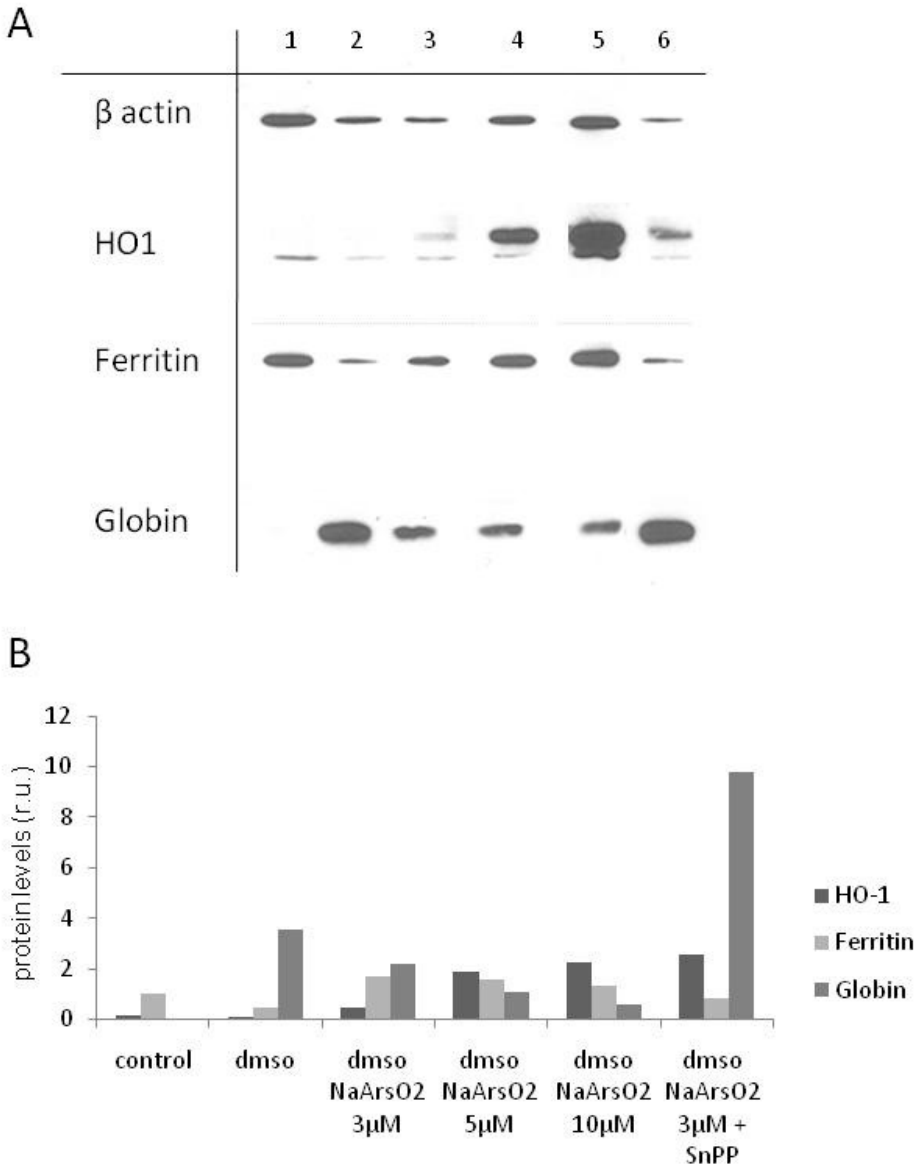
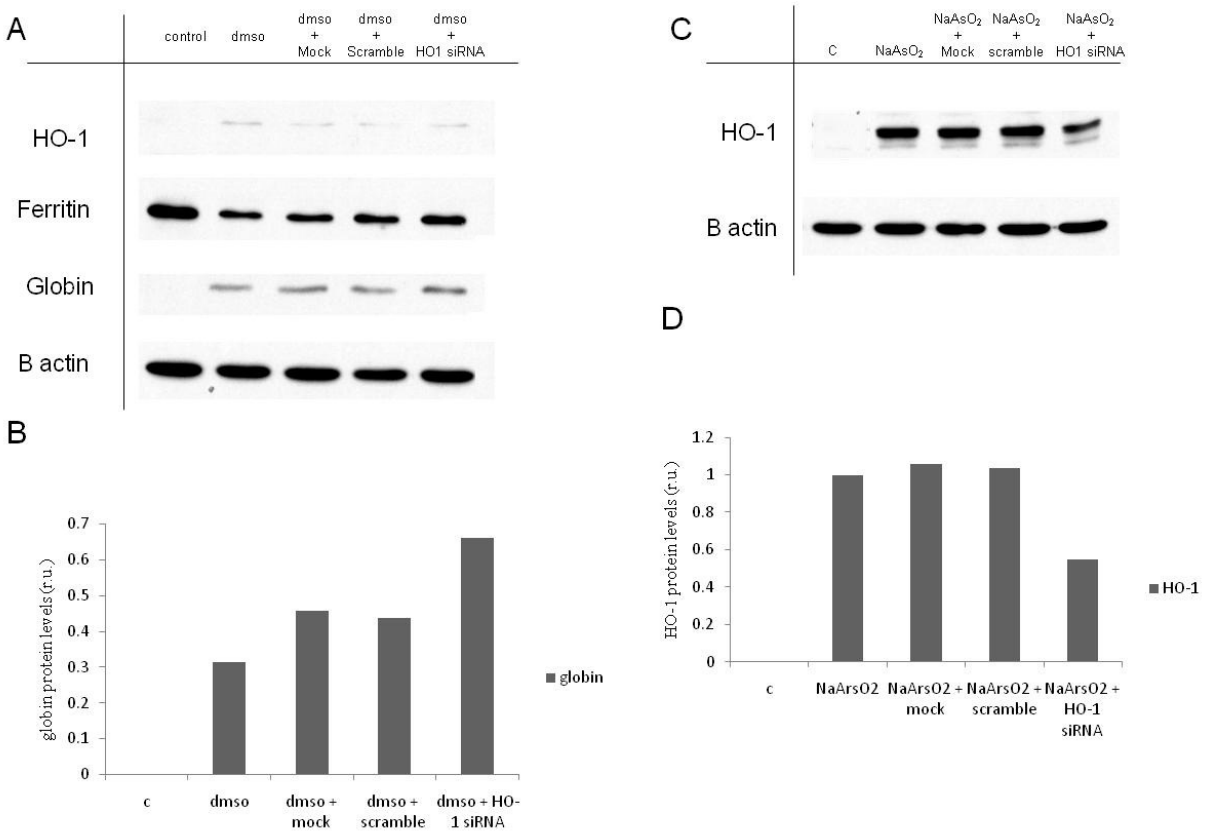

A
1
2
3
4
5
6
β actin
HO1
Ferritin
Globin
B
protein levels (r.u.)
12
10
8
6
4
2
0
control
dmso
dmso NaArsO2 3µM
dmso NaArsO2 5µM
dmso NaArsO2 10µM
dmso NaArsO2 3µM + SnPP
HO-1
Ferritin
Globin

Figure 6


A
control
dmso
dmso + Mock
dmso + Scramble
dmso + HO1 siRNA
HO-1
Ferritin
Globin
B actin
B
globin protein levels (r.u.)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
c
dmso
dmso + mock
dmso + scramble
dmso + HO-1 siRNA
globin
C
C
NaAsO2
NaAsO2 + Mock
NaAsO2 + scramble
NaAsO2 + HO1 siRNA
HO-1
B actin
D
HO-1 protein levels (r.u.)
0
0.2
0.4
0.6
0.8
1
1.2
c
NaArsO2
NaArsO2 + mock
NaArsO2 + scramble
NaArsO2 + HO-1 siRNA
HO-1

Legends

Figure 1. Expression of heme oxygenase (HO1) mRNA during DMSO-induced differentiation of MEL cells. (A) RNA levels of heme oxygenase (HO-1) and β-actin (loading control) were analyzed by northern blotting. MEL cells were either kept in an undifferentiated state (0h) or induced for differentiation for 24h, 48h or 72h. Cells were either kept untreated "C" or treated either with 10 μM $NaAsO_2$ "A" or 50μM heme "H" or 0.4mM succinyl acetone "SA" 24h prior to harvesting. (B) Quantification of HO1 RNA signal depicted in the upper panel (A). HO1 levels had been normalized to β-actin signal. (C) Heme measurement of the differentiation induced by DMSO on MEL cells at different time points. The shown values are means of three measurements, normalized by the cell numbers and volume.

Figure 2. Expression of HO-1 mRNA during differentiation of FL cells. (A) RNA levels of heme oxygenase (HO1) and β-actin (loading control) were analyzed by northern blotting. Primary erythroblasts derived from fetal livers (FL) were either kept in an undifferentiated state (0h) or induced for differentiation for 24h, 48h and 72h. Cells were treated with 0.2mM succinly acetone (SA) for either 24h or 48h prior to harvesting. (B) Quantification of HO1 RNA signal depicted in the upper panel (A). HO1 levels had been normalized to β-actin signal. (C) Heme measurement of the differentiation induced on FL cells at different time points. The shown values are means of three measurements, normalized by the cell numbers and volume, considering possible loss during centrifugation.

Figure 3 – Expression of HO-1 protein during differentiation of MEL cells. Western blot against HO1 and B actin. (A) MEL cells were incubated with "DMSO" or without "C" 2% DMSO during different time points (24, 48, 72h) and SA (succinyl acetone, heme syntesis inhibitor) for 24h. (B) MEL cells were cultivated in the presence or absence of 2% DMSO for 48h and 50 μM of Hemin for 24h.

Figure 4. Expression of HO-1 protein during differentiation of FL cells. Western blot against HO1, ferritin, globin and β-actin. Primary erythroid cells derived from mouse fetal livers day E13 were kept in a non-differentiating stage till about day 12 and then either induced for differentiation or kept in a non-differentiating stage for 48h. 24h prior to harvest the cells were either kept in absence (-) or presence (+) of 0.2mM final concentration of SA.

Figure 5 – Influence of HO-1 induction, by $NaAsO_2$, on DMSO-differentiated MEL cells. Western blot against HO1, Ferritin, Globin and β actin. The cells were plated in medium with or without (control) 2% DMSO and incubated for 72h. 24h prior to harvest the cells were treated with 3, 5 and 10μM of $NaAsO_2$ or/and 100μM of SnPP during 48h. (A) 1, control; 2, DMSO; 3, DMSO + $NaAsO_2$ 3μM; 4, DMSO + $NaAsO_2$ 5μM; 5, DMSO + $NaAsO_2$ 10μM; 6, DMSO + $NaAsO_2$ 3μM + SnPP 100μM. (B) Quantification protein signal depicted in the upper panel. Protein levels had been normalized to β-actin signal.

Figure 6 – Influence of HO-1 supression, by siRNA, on DMSO-differentiated MEL cells. Western blot against HO-1, Ferritin, Globin and B actin. (A) and (C) Mel cells were transfected with HO-1 siRNA, after 18h the cells were washed and plated in fresh medium with or without 2% DMSO during 48h. 24h prior to harvest the cells were treated with 10μM of $NaAsO_2$. (B) Quantification globin signal depicted in the upper panel. Protein levels had been normalized to β-actin signal. (D) Quantification of HO-1 signal depicted in the upper panel. Protein levels had been normalized to β-actin signal.

References

1. Ponka P. Tissue-specific regulation of iron metabolism and heme synthesis: distinct control mechanisms in erythroid cells. Blood. 1997;89:1-25.
2. Ponka P. Cell biology of heme. Am J Med Sci. 1999;318:241-256.
3. Balla J, Jacob HS, Balla G, Nath K, Eaton JW, Vercellotti GM. Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage. Proc Natl Acad Sci U S A. 1993;90:9285-9289.
4. Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell. 1995;83:59-67.
5. Koury MJ, Ponka P. New insights into erythropoiesis: the roles of folate, vitamin B12, and iron. Annu Rev Nutr. 2004;24:105-131.
6. Tahara T, Sun J, Igarashi K, Taketani S. Heme-dependent up-regulation of the alpha-globin gene expression by transcriptional repressor Bach1 in erythroid cells. Biochem Biophys Res Commun. 2004;324:77-85.
7. Tahara T, Sun J, Nakanishi K, et al. Heme positively regulates the expression of beta-globin at the locus control region via the transcriptional factor Bach1 in erythroid cells. J Biol Chem. 2004;279:5480-5487.
8. McEwen E, Kedersha N, Song B, et al. Heme-regulated inhibitor kinase-mediated phosphorylation of eukaryotic translation initiation factor 2 inhibits translation, induces stress granule formation, and mediates survival upon arsenite exposure. J Biol Chem. 2005;280:16925-16933.
9. Maines MD. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. FASEB J. 1988;2:2557-2568.
10. Maines MD. The heme oxygenase system: a regulator of second messenger gases. Annu Rev Pharmacol Toxicol. 1997;37:517-554.
11. Maines MD. The heme oxygenase system: update 2005. Antioxid Redox Signal. 2005;7:1761-1766.
12. Trakshel GM, Kutty RK, Maines MD. Purification and characterization of the major constitutive form of testicular heme oxygenase. The noninducible isoform. J Biol Chem. 1986;261:11131-11137.
13. Abraham NG, Kappas A. Pharmacological and clinical aspects of heme oxygenase. Pharmacol Rev. 2008;60:79-127.
14. Shibahara S, Muller RM, Taguchi H. Transcriptional control of rat heme oxygenase by heat shock. J Biol Chem. 1987;262:12889-12892.

15. Mitani K, Fujita H, Sassa S, Kappas A. Heat shock induction of heme oxygenase mRNA in human Hep 3B hepatoma cells. Biochem Biophys Res Commun. 1989;165:437-441.

16. Keyse SM, Tyrrell RM. Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. Proc Natl Acad Sci U S A. 1989;86:99-103.

17. Nascimento AL, Luscher P, Tyrrell RM. Ultraviolet A (320-380 nm) radiation causes an alteration in the binding of a specific protein/protein complex to a short region of the promoter of the human heme oxygenase 1 gene. Nucleic Acids Res. 1993;21:1103-1109.

18. Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. Science. 1987;235:1043-1046.

19. Eisenstein RS, Garcia-Mayol D, Pettingell W, Munro HN. Regulation of ferritin and heme oxygenase synthesis in rat fibroblasts by different forms of iron. Proc Natl Acad Sci U S A. 1991;88:688-692.

20. Prabhakar NR, Dinerman JL, Agani FH, Snyder SH. Carbon monoxide: a role in carotid body chemoreception. Proc Natl Acad Sci U S A. 1995;92:1994-1997.

21. Ollinger R, Yamashita K, Bilban M, et al. Bilirubin and biliverdin treatment of atherosclerotic diseases. Cell Cycle. 2007;6:39-43.

22. Li M, Kim DH, Tsenovoy PL, et al. Treatment of obese diabetic mice with a heme oxygenase inducer reduces visceral and subcutaneous adiposity, increases adiponectin levels, and improves insulin sensitivity and glucose tolerance. Diabetes. 2008;57:1526-1535.

23. Friend C, Scher W, Holland JG, Sato T. Hemoglobin synthesis in murine virus-induced leukemic cells in vitro: stimulation of erythroid differentiation by dimethyl sulfoxide. Proc Natl Acad Sci U S A. 1971;68:378-382.

24. Schranzhofer M, Schifrer M, Cabrera JA, et al. Remodeling the regulation of iron metabolism during erythroid differentiation to ensure efficient heme biosynthesis. Blood. 2006;107:4159-4167.

25. Dolznig H, Kolbus A, Leberbauer C, et al. Expansion and differentiation of immature mouse and human hematopoietic progenitors. Methods Mol Med. 2005;105:323-344.

26. Kowenz E, Leutz A, Doderlein G, Graf T, Beug H. ts-oncogene-transformed erythroleukemic cells: a novel test system for purifying and characterizing avian erythroid growth factors. Haematol Blood Transfus. 1987;31:199-209.

27. Dean A, Erard F, Schneider AP, Schechter AN. Induction of hemoglobin accumulation in human K562 cells by hemin is reversible. Science. 1981;212:459-461.

28. Fujita H, Sassa S. The rapid and decremental change in haem oxygenase mRNA during erythroid differentiation of murine erythroleukaemia cells. Br J Haematol. 1989;73:557-560.

29. Maines MD, Trakshel GM. Differential regulation of heme oxygenase isozymes by Sn- and Zn-protoporphyrins: possible relevance to suppression of hyperbilirubinemia. Biochim Biophys Acta. 1992;1131:166-174.

30. Charnay P, Maniatis T. Transcriptional regulation of globin gene expression in the human erythroid cell line K562. Science. 1983;220:1281-1283.

31. Fukuda Y, Fujita H, Taketani S, Sassa S. Haem is necessary for a continued increase in ferrochelatase mRNA in murine erythroleukaemia cells during erythroid differentiation. Br J Haematol. 1993;85:670-675.

32. Sassa S, Nagai T. The role of heme in gene expression. Int J Hematol. 1996;63:167-178.

33. Igarashi K, Sun J. The heme-Bach1 pathway in the regulation of oxidative stress response and erythroid differentiation. Antioxid Redox Signal. 2006;8:107-118.

34. Oyake T, Itoh K, Motohashi H, et al. Bach proteins belong to a novel family of BTB-basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site. Mol Cell Biol. 1996;16:6083-6095.

35. Ogawa K, Sun J, Taketani S, et al. Heme mediates derepression of Maf recognition element through direct binding to transcription repressor Bach1. EMBO J. 2001;20:2835-2843.

36. Brand M, Ranish JA, Kummer NT, et al. Dynamic changes in transcription factor complexes during erythroid differentiation revealed by quantitative proteomics. Nat Struct Mol Biol. 2004;11:73-80.

37. Sun J, Brand M, Zenke Y, Tashiro S, Groudine M, Igarashi K. Heme regulates the dynamic exchange of Bach1 and NF-E2-related factors in the Maf transcription factor network. Proc Natl Acad Sci U S A. 2004;101:1461-1466.

38. Sun J, Hoshino H, Takaku K, et al. Hemoprotein Bach1 regulates enhancer availability of heme oxygenase-1 gene. EMBO J. 2002;21:5216-5224.

39. Dolznig H, Boulme F, Stangl K, et al. Establishment of normal, terminally differentiating mouse erythroid progenitors: molecular characterization by cDNA arrays. FASEB J. 2001;15:1442-1444.

40. Spuches AM, Kruszyna HG, Rich AM, Wilcox DE. Thermodynamics of the As(III)-thiol interaction: arsenite and monomethylarsenite complexes with glutathione, dihydrolipoic acid, and other thiol ligands. Inorg Chem. 2005;44:2964-2972.

41. Sheftel AD, Kim SF, Ponka P. Non-heme induction of heme oxygenase-1 does not alter cellular iron metabolism. J Biol Chem. 2007;282:10480-10486.

42. Kramer G, Cimadevilla JM, Hardesty B. Specificity of the protein kinase activity associated with the hemin-controlled repressor of rabbit reticulocyte. Proc Natl Acad Sci U S A. 1976;73:3078-3082.

43. Yoshinaga T, Sassa S, Kappas A. Purification and properties of bovine spleen heme oxygenase. Amino acid composition and sites of action of inhibitors of heme oxidation. J Biol Chem. 1982;257:7778-7785.

44. Lin Q, Weis S, Yang G, et al. Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress. J Biol Chem. 2007;282:20621-20633.

45. Slebos DJ, Ryter SW, van der Toorn M, et al. Mitochondrial localization and function of heme oxygenase-1 in cigarette smoke-induced cell death. Am J Respir Cell Mol Biol. 2007;36:409-417.

46. Keel SB, Doty RT, Yang Z, et al. A heme export protein is required for red blood cell differentiation and iron homeostasis. Science. 2008;319:825-828.

47. Quigley JG, Yang Z, Worthington MT, et al. Identification of a human heme exporter that is essential for erythropoiesis. Cell. 2004;118:757-766.

CAPÍTULO III

HO-1 polymorphism as a genetic determinant behind the malaria resistance afforded by haemolytic disorders

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HO-1 polymorphism as a genetic determinant behind the malaria resistance afforded by haemolytic disorders

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SUMMARY

Malaria affects thousands of people around the world representing a critical issue regarding health policies in tropical countries. Similarly, also haemolytic diseases such as sickle cell disease and thalassemias are a concern in different parts of the globe. It is well established that haemolytic diseases, such as sickle cell disease (SCD) and thalassemias, represent a resistance factor to malaria, which explains the high frequencies of such genetic variants in malaria endemic areas. In this context, it has been shown that the rate limiting enzyme heme oxygenase I (HO-1), responsible for the catabolism of the free heme in the body, is an important resistance factor in malaria and is also important in the physiopathology of haemolytic diseases. Here, we suggest that allelic variants of HO-1, which display significant differences in terms of protein expression, have been selected in endemic malaria areas since the HO-1 enzyme can enhance the protection against malaria conferred by haemolytic diseases This protection apply mainly in what concerns protection against severe malaria forms. Therefore, HO-1 genotyping would be fundamental to determine resistance of a given individual to lethal forms of malaria as well as to common clinical complications typical to haemolytic diseases and would be helpful in the establishment of public health politics.

Heme oxygenase and the endothelial system

Heme oxygenase (HO) is the rate limiting enzyme in the catabolism of free heme into biliverdin, releasing free iron and carbon monoxide (Fig. 1). There are two isoforms described so far: HO-1 and HO-2. The activity of HO-1, the inducible isoform of HO, is increased in tissues of the whole animal after treatment with its natural substrate heme, as well as by various metals, xenobiotics, endocrine factors, and synthetic metalloporphyrins [1]. This enzyme is a heat shock protein, and also a stress protein induced by several agents that cause oxidative damage [2–5]. HO-1 promotes protection through the removal of the potential dangerous pro-oxidant free heme, generated in stress conditions, and release of bilirubin and biliverdin, metabolites with anti-oxidant properties [6]. The HO-1 activity also releases iron II, which is rapidly stored in ferritin [7], and carbon monoxide (CO) that has important cellular signalling functions such as in neurotransmission and vascular relaxation [8–10].

HO-1 has been extensively described as a protective factor in different disorders. For example, both the pharmacological induction of HO-1 as well as adenovirus mediated gene transfer of HO-1 decrease the lesion formation in murine models of atherosclerosis, whereas the inhibition of HO-1 expression promotes lesion development [11]. A reduction in anti-oxidant reserves has been related to endothelial cell dysfunction in diabetes [12,13].

Endothelial dysfunction is a common response to cardiovascular risk factors and precedes the development of atherosclerosis. Endothelial dysfunction is involved in early and late mechanisms of atherosclerosis such as up-regulation of adhesion molecules, increased chemokine secretion and leukocyte adherence, increased cell permeability, enhanced LDL oxidation, platelet activation, cytokine elaboration, and vascular smooth cell (VSMC) proliferation and migration [14]. Endothelial HO-1 overexpression significantly attenuates the production of inflammatory mediators and stimulates cell cycle progression and proliferation in the vascular endothelium [15–17]. HO-derived CO influences the sGC and cGMP pathways, which act to regulate both blood pressure and vascular contractility [18], resulting in vasodilatation and lower blood pressure levels. The protective effects mediated by HO-1 are related, in most of cases, to the strong anti-inflammatory potential displayed by this enzyme. Therefore, it is more than reasonable consider that HO-1 has extreme importance in infections, where different tissues are involved in inflammatory responses aiming to pathogen clearance.

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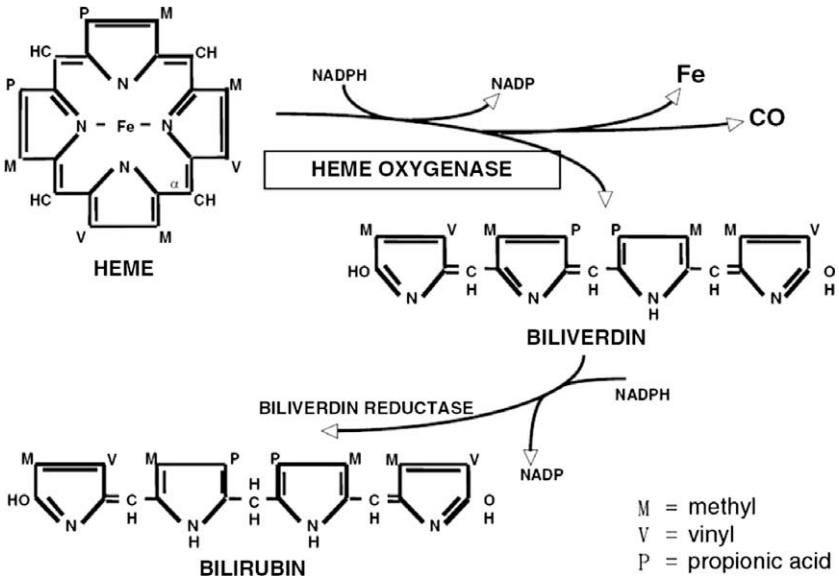

Fig. 1. Heme cleavage mediated by heme oxygenases.

Malaria, a worldwide spread disease

All over the world, malaria affects an estimated 300–500 million new individuals every year [19]. The individuals infected with Plasmodium, the causative agent of malaria, display a clinically silent "liver stage" [20,21] and thereafter a "blood stage" [21,22], which is associated with the appearance of the clinical symptoms including prostration, respiratory distress, pulmonary edema, convulsions, circulatory collapse, abnormal bleeding, jaundice, hemoglobinuria, severe anemia, and/or impaired consciousness [23,24]. Severe malaria is defined by the appearance of one or more of these symptoms in individuals with no apparent cause of disease other than Plasmodium infection [25]. Lethal forms of severe malaria, including cerebral malaria, account for the death of more than one million children (less than 5 year old) per year, mainly in sub-Saharan Africa [19].

Although highly prevalent among human populations, less than 1–2% of Plasmodium infected individuals succumb due to the infection [19]. This low rate of death suggests a co-evolution between the parasite and the human host, reaching an evolutionary "trade-off", where the life cycle of the parasite, most of the time, does not compromise that of its host [25]. Similar to other infection agents, Plasmodium expresses pathogen-associated molecular patterns (PAMPs) that can be recognized by host germline-encoded pattern recognition receptors (PRR) [26]. This pathway in innate immune cells, such as monocytes/macrophages, triggers a potent pro-inflammatory response [27,28]. Polymorphisms in members of the human toll-like receptor family (TLRs), such as TLR4 and TLR9, are associated with susceptibility to severe malaria in children [29] and with the outcome of Plasmodium infections during pregnancy [30]. Additional mechanisms are involved in this evolutionary "trade-off" between the Plasmodium and the human host. In this context, the limitation of the deleterious effects of the Plasmodium over the red blood cell (RBC) (hemolysis) is an important mechanism. Hemolysis is associated with the release of an estimated 20–40% of the initial pool of RBC haemoglobin (Hb) into the circulation [31]. Once free, Hb tetramers associate forming dimers, which can be oxidized by ROS or reactive nitrogen species (RNS) resulting in methemoglobin (metHb). MetHb easily releases its heme prosthetic group and this free heme can be highly cytotoxic to endothelial cells [32,33]. These events exposes the prothrombotic sub-endothelial matrix to the coagulation cascade, leading to the formation of more or less extensive microvascular thrombi with concomitant vaso-occlusion and tissue ischemia [25].

Different mechanisms have evolved to handle the free Hb and heme in humans. The free Hb is rapidly scavenged by haptoglobin (Hp), a tetrameric plasma protein. The complexes Hb/Hp are recognized by specific receptors (CD163), expressed by monocytes/macrophages in the red pulp of the spleen, internalized and processed by the HO system [34]. The gene that encodes the alpha chain of the Hp has two allele variants that produce Hp tetramers with different affinities for Hb and CD163 receptor. Interestingly, it was already been show that genetic polymorphisms can affect the susceptibility to malaria. Individuals carrying the phenotype that displays high affinity for Hb and CD163 are less susceptible to develop severe malaria [35]. In situations of high amounts of free Hb, the Hp is rapidly depleted, and metHb is formed with consequent release of heme. In this situation, the monomeric protein hemopexin (Hpx) is responsible to bind the free heme. The complexes heme/Hpx are recognized by the specific receptor CD91, which is expressed in hepatocytes, macrophages, fibroblasts, adipocytes, neurons, and trophoblasts, internalized by endocytosis and degraded by the HO system [36]. Once the capacity of the plasmatic Hpx is exhausted, free heme can still be scavenged by plasma albumin, and directed to HO degradation [25]. In situations where all the previous discussed systems are depleted, the HO cellular catabolism is a last resource to remove the excess of free heme.

Plasmodium infection, both in mice and humans, results in increased expression of HO-1 in different cells and tissues [37–40]. Interestingly, mouse strains that express higher levels of HO-1 in response to plasmodium infection do not succumb to severe and/or cerebral malaria, while those that express low levels of HO-1 do so [37]. Moreover, deletion of Hmox1 locus by homologous recombination is sufficient per se to promote the onset of severe and/or cerebral malaria [37]. The mechanism of action of HO-1 does not interfere with the parasite load in the host RBC, which means that HO-1 does not change the immunological response of the host to the Plasmodium infection [37].

The inflammatory nature of malaria, which is characterized by a large accumulation of free heme, confers to HO-1 a central position in all the mechanisms related to removal of this harmful pro-inflammatory molecule. Haemolytic disorders such as sickle cell disease or α- and β-thalassemias are highly prevalent in areas of

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malaria occurrence and confer, at different degrees, resistance to the onset of severe and/or cerebral malaria.

Sickle cell anemia and thalassemias

Sickle cell disease (SCD) is a haemolytic disease characterized by recurrent episodes of painful vaso-occlusion, leading to ischemia/reperfusion injury and organ damage. Moreover, SCD is currently being considered as a chronic inflammatory disease [41]. A major source of oxidative stress in SCD is heme iron. The high rates of RBC hemolysis expose the SCD patients endothelium to large amounts of ROS generated by the presence of Hb, heme and free iron in the plasma [42]. Similar to Plasmodium infection, metHb is generated and heme is released, which rapidly intercalates into the cell membranes [43,44]. The vasculature activates the HO catabolism system as a defence mechanism to protect against the injury mediated by the free heme. Belcher et al. [45] have shown that HO-1 expression is significantly increased in the lungs, liver, and spleen of two different SCD mice models as compared to control mice. When the HO-1 expression is further up-regulated by hemin injections, the SCD mice displays no stasis after events of hypoxia and reoxygenation and a reduction in inflammatory markers such as NF-kb, VCAM-1 and ICAM-1 compared with no injected controls. The importance of the HO-1 system as a heme scavenger and anti-inflammatory mediator in SCD is not limited to mice models. Lanaro et al. [46] observed altered levels of cytokines and inflammatory mediators in SCD patients. Moreover, in those patients they also observed increased levels of HO-1 in monocytes and neutrophils, suggesting that the HO-1 catabolic activity plays a vital role as a protective mechanism of the vasculature against heme-mediated injury. Once the toxic heme is degraded it may facilitate the resolution of vaso-occlusion events. Moreover, with the heme-catabolism, CO is released and acts as vasodilator, biliverdin is also produced playing an important role as an anti-oxidant molecule reducing the ROS levels, and ferritin is induced by iron release, removing all the potential harmful free iron [47–50].

Thalassemias occurs due to a large number of different mutations causing abnormal globin gene expression and resulting in total absence or reduction of globin chain synthesis [51]. α-thalassemia is usually due to deletions within the α-globin gene cluster, leading to loss of function of one or both α-globin genes in each locus [52]. β-thalassemia is caused mainly by point mutations either at the promoter region or at the coding sequence of the β-globin gene. As a consequence of these mutations, there is a major defect in the β-globin gene expression [51]; so far it has been described more than 200 different point mutations associated with β-thalassemias [51,53]. Clinical symptoms of these syndromes include moderate-to-severe anemia, high infection susceptibility, iron overload, hemolysis, extra-medullary erythropoieses, hypoxemia, pulmonary hypertension, leg ulcers and spleen and liver enlargement [54]. In β-thalassemic patients, where vascular complications are frequent, there is strong evidence of endothelial cell activation, and impaired endothelial function is indicated by increased levels of circulating activated endothelial cells, as well as by elevated TNF-α, IL-1B and endothelial vascular growth (VEGF) levels [55–57]. Evidence points to a role for aberrant endothelial function in β-thalassemia syndromes, where iron overload and hemolysis may contribute for these alterations. The extent of these alterations and their implications in β-thalassemia are not yet clear, however, the endothelial cell activation and accompanying inflammatory process may play an important role in some of the major complications of thalassemia, including cardiovascular alterations, pulmonary hypertension and thrombotic events [58]. The clinical symptoms of the thalassemic patients are similar to those of SCD patients, where hemolysis and thrombosis are prominent events. Free heme toxicity and consequent endothelial dysfunction are common pathologic events in both thalassemia and SCD. Therefore, although there is no evidence so far, it is reasonable to argue an importance of HO-1 in thalassemia outcome, as a defence mechanism of endothelium compartment against free heme.

Hypothesis: HO-1 variants and hemoglobin variants concur to protect against Plasmodium infection

As previously described, HO-1 plays an important role in Plasmodium infection. Also, SCD as well as thalassemic patients, display a natural resistance to malaria. Population surveys have related that α^1-thalassemia is by far the most common of the thalassemic syndromes [59]. α^1-thalassemia occurs in all malarial areas, overlapping the regions affected by β-thalassemia, or α^0-thalassemia (in Mediterranean and, additionally with Hb E, in Southeast Asia) and with Hb S in Africa [60]. Case control studies have consistently shown that α^1-thalassemia protects against severe life-threatening malaria [61–64] and that this protective effect is most pronounced in homozygotes [61–63,65]. There is no evidence in the literature indicating that the protection afforded by α^1-thalassemia is related with reduction of parasite prevalence or density. The results obtained so far strongly support the idea that α^1-thalassemia prevents disease progression through mechanisms other than limiting parasite replication and that protection is limited to severe manifestations of the malarial disease [66,67]. It was already proposed that α^1-thalassemia limits the decline in the hemoglobin concentration that is associated with a febrile infection, particularly those that are accompanied by inflammation [68].

The HO-1 catabolism system is important as a protection factor in malaria [37] and SCD [45]. Considering that thalassemias share similar clinical manifestations with malaria and SCD (free heme toxicity and endothelial dysfunction) it is reasonable to hypothesize a role for the HO-1 system in thalassemias. All these hemoglobin variants have been submitted to a strong selective pressure once they confer protection against malaria. As a consequence of this process the mutated genes responsible for thalassemias and SCD are present in high frequencies in specific regions of the globe (Mediterranean, the Middle East, the Indian Subcontinent, Southeast Asia, and sub-Saharan-Africa). Therefore, a genetic component that determines the HO-1 levels of expression would be strongly selected in that same specific regions, with the presence of high frequencies of genotypes that confer high HO-1 expression in the population.

HO-1 gene polymorphism

To date, three polymorphisms in the 5′ flaking region of the heme oxygenase-1 gene have been described: a (GT)n dinucleotide length polymorphism [69,70] and two single nucleotide polymorphisms (SNPs), G(−1135)A and T(−413)A [71,72]. Variable numbers of tandem repeats polymorphisms often have alleles that are highly variable in length and therefore are useful to genetic studies [73]. The purine-pyrimidine alternating sequence has the potential to assume Z-DNA conformation, which is thermodynamically unfavourable as compared to the B-DNA conformation [74]. Furthermore, Z-DNA conformation has been described as negatively affecting transcriptional activity [75,76]. The influence of the GT repeats in the HO-1 promoter activity has been experimentally demonstrated by luciferase promoter constructs and transient transfection in different cell lines where constructs with lengths of <25 repeats showed an increased HO-1 basal promoter activity [77] or increased transcriptional up-regulation in response to various stimuli as compared to >25 repeats [69]. The biological

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Table 1
Association between HO-1 polymorphisms and different diseases described in the literature. Modified from Exner et al. [116].

Disease	Author	Polymorphism	Sample size	Polymorphism associated with disease
Pulmonary disease				
Presence of emphysema in smokers	[69]	(GT)n	201	Yes
Rapid decline in lung function in smokers	[91]	(GT)n	621	No
Airway obstruction in smokers	[92]	(GT)n	749	Yes
Acute respiratory distress syndrome	[93]	(GT)n	437	Yes
Chronic obstructive pulmonary disease	[89]	(GT)n	452	Yes
Cardiovascular disease				
Hypertension in women	[71]	T(−413)A	1998	Yes
CAD in patients with risk factors	[94]	(GT)n	577	Yes
CAD in type II diabetic patients	[77]	(GT)n	796	Yes
Abdominal aortic aneurysms	[95]	(GT)n	271	Yes
Myocardial infarction and stable CAD	[96]	(GT)n	649	No
CAD and myocardial infarction	[97]	(GT)n/T(−413)A	3219	No
CAD	[72]	T(−413)A	2569	Yes
CAD in diabetic patients	[98]	(GT)n	986	Yes
Kawasaki disease	[99]	(GT)n	61	No
Inflammation after balloon angioplasty	[100]	(GT)n	317	Yes
Restenosis after coronary stenting	[101]	(GT)n	323	Yes
Restenosis after coronary stenting	[102]	(GT)n	1807	No
Restenosis after peripheral angioplasty	[103]	(GT)n	96	Yes
Restenosis after peripheral angioplasty	[104]	(GT)n	381	Yes
Peripheral artery disease	[81]	(GT)n	472	Yes
Renal impairment				
IgA nephropathy	[105]	(GT)n	916	Yes
Polycystic kidney disease and IgA nephropathy	[86]	(GT)n	160	No
Renal transplantation				
Kidney allograft function	[106]	(GT)n	101	Yes
Kidney allograft function	[107]	(GT)n	384	Yes
Kidney allograft function	[108]	(GT)n	181	Yes
Arthritis				
Rheumatoid arthritis	[82]	(GT)n/T(−413)A	736	Yes
Rheumatoid arthritis	[84]	(GT)n	325	Yes
Obstetrics				
Idiopathic recurrent miscarriage	[109]	(GT)n	291	Yes
Cancer				
Gastric adenocarcinoma and lymphovascular tumor invasion	[110]	(GT)n	183	Yes
Melanoma	[111]	(GT)n	152	Yes
Gastrointestinal stromal tumor	[112]	(GT)n	44	Yes
Neurological disease				
Alzheimer and Parkinson disease	[70]	(GT)n	429	No
Parkinson disease	[87]	(GT)n	827	No
Hematological/serological disorders				
Susceptibility to apoptosis	[78]	(GT)n	–	Yes
Neonatal hyperbilirubinemia	[113]	(GT)n	211	No
Iron status in type II diabetes mellitus	[114]	(GT)n	189	Yes
Stem cell				
Stem cell transplantation	[115]	(GT)n	92	Yes

CAD, Coronary artery disease.

relevance of these *in vitro* assays was demonstrated in experiments with lymphoblastoid cell lines established from subjects with known GT repeat lengths [78], where, in situations of oxidative stress, HO-1 mRNA expression was higher in lymphoblastoid cell lines with short repeats when compared to those with long repeats.

The polymorphisms in the 5′ flanking region of HO-1 have been associated with outcome in different diseases such as cancer, pulmonary and cardiovascular disease, neurological disease and haematological/serological disorders as well as in renal transplantation (Table 1). Concerning malaria and HO-1, an elegant set of experiments by Epiphanio et al. [79], suggested that, although HO-1 expression might initially favour the generation of liver merozoites and their progression into the blood, this expression thereafter prevent the onset of severe malaria. Therefore, it suggests that Plasmodium interaction with the human host might have evolved in a way to subvert the HO-1 in order to allow the host survival [79].

HO-1 polymorphisms, malaria and hemoglobin variants

Testing through human population analyses

To date, there are no studies in the literature approaching HO-1 polymorphisms in populations affected with haemolytic disorders such as thalassemias and SCD. Considering the important role of HO-1 as a protection factor in malaria, SCD and in extension to thalassemias, we expect a high frequency of alleles with short repeats in populations that belong to the regions where these haemolytic diseases are endemic. We suggest that individuals from these regions would suffer a strong selective pressure to carry short GT repeats once, concomitant with high HO-1 expression, they will have protection against the lethal outcome of malaria and resistance against vasculature damage in sickle cell disease and thalassemias. The vasculature injury and thrombotic events are

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common features on malaria, SCD and thalassemias [25,58,80] and the same physiopathologic scenario is common to coronary artery disease (CAD), which has been extensively associated with HO-1 length polymorphism in the literature. For example, Dick et al. [81] have shown that homozygous and heterozygous individuals for short repeats (<25 repeats) had lower rates of myocardial infarction, percutaneous coronary interventions and coronary bypass operations compared to patients with longer repeats (>25 repeats), and the presence of long (GT)n repeats was associated with a 4.7-fold increased risk for CAD in Chinese type II diabetic patients [77]. The high amounts of free heme present in haemolytic diseases triggers different pro-inflammatory cascades and the heme degradation, mediated by HO-1, plays a central function as an anti-inflammatory factor [45,46]. Considering other pro-inflammatory conditions, Rueda et al. [82], have shown that short HO-1 alleles (<25 repeats) confers protection against Rheumatoid Arthritis (RA). Although the exact etiology of RA remains elusive, it is thought that oxidative and inflammatory stress is involved in RA joint damage [83,84]. Therefore, as extensively described in the literature, HO-1 length polymorphism is strongly associated with thrombotic and inflammatory diseases.

The thalassemias occur at high frequency throughout the Mediterranean, the Middle East, the Indian Subcontinent, and Southeast Asia. They are probably the world's most common monogenic diseases, and in some regions they reach gene frequencies as high as 70%. Another remarkable feature is the geographic distribution of the thalassemias and other common hemoglobinopathies (Hb S, C, and E); rather than having a fairly uniform distribution, particular groups of mutations (typically, two or three common variants and a few minor types) are more frequent in particular geographic regions. This implied that these mutations have only reached high frequencies quite recently (since population movements have so far failed to disperse them uniformly) [60]. The local pressure of selection is the malaria infection, and the protection conceived by thalassemias and SCD might be further enhanced by high HO-1 expression. The efficiently removal of the uncommitted heme, mediated by HO-1, and generated during the course of the hemoglobinopathies, is a main responsible of endothelium function improvement and, in last instance, for the survival of the individual affected by the disease.

Taking into account the previous considerations, we propose that, in order to keep the "trade-off" between parasite and host, the HO-1 short alleles would be positively selected in malaria endemic areas, allowing protection to the host against severe forms of the disease. Simultaneously, individuals with hemoglobinopathies and high levels of HO-1 expression would be further protected against the disease itself and severe malaria. In this way, short alleles repeats would be a major factor selected by malaria in individuals that carry haemoglobin variants. Preliminary observations already point to this situation: data from the literature concerning HO-1 length polymorphism, suggest that the most prevalent short alleles in human populations (22 and 23 GT repeats) reach maximal frequencies of around 20% [85–89], although these same alleles, in the only study concerning Plasmodium infected individuals in an endemic malaria region reach a frequency of around 26% [90].

Testing through in vitro and in vivo infection assays

Our hypotheses stress the necessity of further investigation of HO-1 genotypes in populations with high incidence of malaria and the relation of these genotypes with thalassemias and SCD. Moreover, *in vitro* studies will be important to address a direct relation between different HO-1 alleles and the outcome of the Plasmodium infection. These experiments could be performed by *in vitro* infection of a specific cell type (macrophages, for example) carrying a HO-1 allele with a known number of repeats, with Plasmodium, and followed by the analysis of different inflammatory markers. Also, similar experiments could be performed using different animal models of thalassemia and SCD available. The results obtained could indicate the real influence of the HO-1 genotypes in the outcome of these diseases. In conclusion, investigations on HO-1 genotypes (both in controls as well as in individuals carriers of hemoglobin variants) would allow to determine the real role of this putative important genetic protector factor not only in malaria, but also in other disorders which have haemolytic events as part of the disease outcome.

Conflicts of interest statement

None declared.

References

[1] Abraham NG, Kappas A. Pharmacological and clinical aspects of heme oxygenase. Pharmacol Rev 2008;60:79–127.
[2] Shibahara S, Muller M, Taguchi H. Transcriptional control of rat heme oxygenase by heat shock. J Biol Chem 1987;262:12889–92.
[3] Mitani K, Fujita H, Sassa S, Kappas A. Heat shock induction of heme oxygenase mRNA in human Hep3B hepatoma cells. Biochem Biophys Res Commun 1989;165:437–41.
[4] Keyse SM, Tyrrell RM. Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. Proc Natl Acad Sci USA 1989;86:99–103.
[5] Nascimento AL, Luscher P, Tyrrell RM. Ultraviolet A (320–380 nm) radiation causes an alteration in the binding of a specific protein/protein complex to a short region of the promoter of the human heme oxygenase 1 gene. Nucleic Acids Res 1993;21:1103–9.
[6] Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. Science 1987;235:1043–6.
[7] Eisenstein RS, Garcia-Mayol D, Pettingell W, Munro HN. Regulation of ferritin and heme oxygenase synthesis in rat fibroblasts by different forms of iron. Proc Natl Acad Sci USA 1991;88:688–92.
[8] Prabhakar NR, Dinerman JL, Agani FH, Snyder SH. Carbon monoxide: a role in carotid body chemoreception. Proc Natl Acad Sci USA 1995;92:1994–7.
[9] Ollinger R, Yamashita K, Bilban M, et al. Bilirubin and biliverdin treatment of atherosclerotic diseases. Cell Cycle 2007;6:39–43.
[10] Li M, Kim DH, Tsenovoy P, et al. Treatment of obese diabetic mice with an heme oxygenase inducer reduces visceral and abdominal adiposity increases adiponectin levels and improves insulin sensitivity and glucose tolerance. Diabetes 2008;57:1526–35.
[11] Juan SH, Lee TS, Tseng KW, et al. Adenovirus-mediated heme oxygenase-1 gene transfer inhibits the development of atherosclerosis in apolipoprotein E-deficient mice. Circulation 2001;104:1519–25.
[12] Baynes JW. Role of oxidative stress in development of complications in diabetes. Diabetes 1991;40:405–12.
[13] Zou MH, Shi C, Cohen RA. High glucose via peroxynitrite causes tyrosinenitration and inactivation of prostacyclin synthase that is associated with thromboxane/prostaglandin H2 receptor-mediated apoptosis and adhesion molecular expression in cultured human aortic endothelial cells. Diabetes 2002;51:198–203.
[14] Hadi HA, Carr CS, Al Suwaidi J. Endothelial dysfunction: cardiovascular risk factors, therapy, and outcome. Vasc Health Risk Manage 2005;1:183–98.
[15] Kawamura K, Ishikawa K, Wada Y, et al. Bilirubin from heme oxygenase-1 attenuates vascular endothelial activation and dysfunction. Arterioscler Thromb Vasc Biol 2005;25:155–60.
[16] Dulak J, Loboda A, Zagorska A, Jozkowicz A. Complex role of hemeoxygenase-1 in angiogenesis. Antioxid Redox Signal 2004;6:858–66.
[17] Bussolati B, Mason JC. Dual role of VEGF-induced heme-oxygenase-1 in angiogenesis. Antioxid Redox Signal 2006;8:1153–63.
[18] Ndisang JF, Tabien HE, Wang R. Carbon monoxide and hypertension. J Hypertens 2004;22:1057–74.
[19] World Health Organization. Expert committee on malaria: 20th report; <http://www.rbm.who.int/docs/ecr20.pdf> 2000 [accessed 2009].
[20] Prudencio M, Rodriguez A, Mota MM. The silent path to thousands of merozoites: the Plasmodium liver stage. Nat Rev Microbiol 2006;4:849–56.
[21] Schofield L, Grau GE. Immunological processes in malaria pathogenesis. Nat Rev Immunol 2005;5:722–35.
[22] Haldar K, Murphy SC, Milner DA, Taylor TE. Malaria: mechanisms of erythrocytic infection and pathological correlates of severe disease. Annu Rev Pathol 2007;2:217–49.
[23] Idro R, Jenkins NE, Newton CR. Pathogenesis, clinical features, and neurological outcome of cerebral malaria. Lancet Neurol 2005;4:827–40.
[24] Miller LH, Baruch DI, Marsh K, Doumbo OK. The pathogenic basis of malaria. Nature 2002;415:673–9.

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[25] Ferreira A, Balla J, Jeney V, Balla G, Soares MP. A central role for free heme in the pathogenesis of severe malaria: the missing link? J Mol Med 2008;86:1097–111.
[26] Gazzinelli RT, Denkers EY. Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. Nat Rev Immunol 2006;6:895–906.
[27] Parroche P, Lauw FN, Goutagny N, et al. Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9. Proc Natl Acad Sci USA 2007;104:1919–24.
[28] Coban C, Ishii KJ, Uematsu S, et al. Pathological role of Toll-like receptor signaling in cerebral malaria. Int Immunol 2007;19:67–79.
[29] Mockenhaupt FP, Cramer JP, Hamann L, et al. Toll-like receptor (TLR) polymorphisms in African children: common TLR-4 variants predispose to severe malaria. Proc Natl Acad Sci USA 2006;103:177–82.
[30] Mockenhaupt FP, Hamann L, von Gaertner C, et al. Common polymorphisms of toll-like receptors 4 and 9 are associated with the clinical manifestation of malaria during pregnancy. J Infect Dis 2006;194:184–8.
[31] Francis SE, Sullivan Jr DJ, Goldberg DE. Hemoglobin metabolism in the malaria parasite *Plasmodium falciparum*. Annu Rev Microbiol 1997;51:97–123.
[32] Jeney V, Balla J, Yachie A, et al. Pro-oxidant and cytotoxic effects of circulating heme. Blood 2002;100:879–87.
[33] Balla J, Jacob HS, Balla G, Nath K, Eaton JW, Vercellotti GM. Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage. Proc Natl Acad Sci USA 1993;90:9285–9.
[34] Kristiansen M, Graversen JH, Jacobsen C, et al. Identification of the haemoglobin scavenger receptor. Nature 2001;409:198–201.
[35] Atkinson SH, Mwangi TW, Uyoga SM, et al. The haptoglobin 2–2 genotype is associated with a reduced incidence of *Plasmodium falciparum* malaria in children on the coast of Kenya. Clin Infect Dis 2007;44:802–9.
[36] Hvidberg V, Maniecki MB, Jacobsen C, Hojrup P, Moller HJ, Moestrup SK. Identification of the receptor scavenging hemopexin–heme complexes. Blood 2005;106:2572–9.
[37] Pamplona A, Ferreira A, Balla J, et al. Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. Nat Med 2007;13:703–10.
[38] Schluesener HJ, Kremsner PG, Meyermann R. Heme oxygenase-1 in lesions of human cerebral malaria. Acta Neuropathol (Berl) 2001;101:65–8.
[39] Medana IM, Mai NT, Day NP, et al. Cellular stress and injury responses in the brains of adult Vietnamese patients with fatal *Plasmodium falciparum* malaria. Neuropathol Appl Neurobiol 2001;27:421–33.
[40] Clark IA, Awburn MM, Harper CG, Liomba NG, Molyneux ME. Induction of HO-1 in tissue macrophages and monocytes in fatal falciparum malaria and sepsis. Malar J 2003;2:41.
[41] Chies JA, Nardi NB. Sickle cell disease: a chronic inflammatory condition. Med Hypotheses 2001;57:46–50.
[42] Balla J, Vercellotti GM, Jeney V, et al. Haem, haem oxygenase and ferritin in vascular endothelial cell injury. Nephrol Dial Transplant 2003;18:v8–v12.
[43] Bunn HF, Jandl JH. Exchange of heme among hemoglobins and between hemoglobin and albumin. J Biol Chem 1968;243:465–75.
[44] Hebbel RP, Morgan WT, Eaton JW, Hedlund BE. Accelerated autooxidation and heme loss due to instability of sickle hemoglobin. Proc Natl Acad Sci USA 1988;85:237–41.
[45] Belcher JD, Mahaseth H, Welch TE, Otterbein LE, Hebbel RP, Vercellotti GM. Heme oxygenase-1 is a modulator of inflammation and vaso-occlusion in transgenic sickle mice. J Clin Invest 2006;116:808–16.
[46] Lanaro C, Franco-Penteado CF, Albuqueque DM, Saad ST, Conran N, Costa FF. Altered levels of cytokines and inflammatory mediators in plasma and leukocytes of sickle cell anemia patients and effects of hydroxyurea therapy. J Leukoc Biol 2009;85:235–42.
[47] Otterbein LE, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. Trends Immunol 2003;24:449–55.
[48] Wagener FA, Volk HD, Willis D, et al. Different faces of the heme–heme oxygenase system in inflammation. Pharmacol Rev 2003;55:551–71.
[49] Stocker R, McDonagh AF, Glazer AN, Ames BN. Antioxidant activities of bile pigments: biliverdin and bilirubin. Methods Enzymol 1990;186:301–9.
[50] Balla G, Jacob HS, Balla J, et al. Ferritin: a cytoprotective antioxidant strategem of endothelium. J Biol Chem 1992;267:18148–53.
[51] Steinberg MH, Forget BG, Higgs DR, et al. Disorders of hemoglobin: genetics, pathophysiology and clinical management. Cambridge, UK: Cambridge University Press; 2001.
[52] Vichinsky EP. Changing patterns of thalassemia worldwide. Ann NY Acad Sci 2005;1054:18–24.
[53] Giardine B, van Baal S, Kaimakis P. HbVar database of human hemoglobin variants and thalassemia mutations: 2007 update. Hum Mutat 2007;28:206.
[54] Taher AT, Otrock ZK, Uthman I, Cappellini MD. Thalassemia and hypercoagulability. Blood Rev 2008;22:283–92.
[55] Aggeli C, Antoniades C, Cosma C, et al. Endothelial dysfunction and inflammatory process in transfusion-dependent patients with betathalassemia major. Int J Cardiol 2005;105:80–4.
[56] Butthep P, Bunyaratvej A, Funahara Y, et al. Alterations in vascular endothelial cell-related plasma proteins in thalassaemic patients and their correlation with clinical symptoms. Thromb Haemost 1995;74:1045–9.
[57] Butthep P, Rummavas S, Wisedpanichkij R, Jindadamrongwech S, Fucharoen S, Bunyaratvej A. Increased circulating activated endothelial cells, vascular endothelial growth factor, and tumor necrosis factor in thalassemia. Am J Hematol 2002;70:100–6.
[58] Conran N, Costa FF. Hemoglobin disorders and endothelial cell interactions. Clin Biochem 2009;42:1824–38.
[59] Flint J, Harding RM, Boyce AJ, Clegg JB. The population genetics of the haemoglobinopathies. In: Higgs DR, Weatherall DJ, editors. Baillière's clinical haematology haemoglobinopathies. London: Baillière Tindall and WB Saunders; 1993.
[60] Clegg JB, Weatherall DJ. Thalassemia and malaria: new insights into an old problem. Proc Assoc Am Physicians 1999;111:278–82.
[61] Williams TN, Wambua S, Uyoga S, et al. Both heterozygous and homozygous thalassemias protect against severe and fatal *Plasmodium falciparum* malaria on the coast of Kenya. Blood 2005;106:368–71.
[62] Allen SJ, O'Donnell A, Alexander ND, et al. α^+-Thalassemia protects children against disease caused by other infections as well as malaria. Proc Natl Acad Sci USA 1997;94:14736–41.
[63] May J, Evans JA, Timmann C, et al. Hemoglobin variants and disease manifestations in severe falciparum malaria. JAMA 2007;297:2220–6.
[64] Mockenhaupt FP, Ehrhardt S, Gellert S, et al. α^+-Thalassemia protects African children from severe malaria. Blood 2004;104:2003–6.
[65] Wambua S, Mwangi TW, Kortok M. The effect of α^+-thalassaemia on the incidence of malaria and other diseases in children living on the coast of Kenya. PLoS Med 2006;3:e158.
[66] Williams TN. Red blood cell defects and malaria. Mol Biochem Parasitol 2006;149:121–7.
[67] Pasvol G. Does α^+-thalassemia protect against malaria? PLoS Med 2006;3:e235.
[68] Veenemans J, Andang'o PE, Mbugi EV, et al. α^+-Thalassemia protects against anemia associated with asymptomatic malaria: evidence from community-based surveys in Tanzania and Kenya. J Infect Dis 2008;198:401–8.
[69] Yamada N, Yamaya M, Okinaga S, Nakayama K, Shibahara S, Sasaki H. Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to emphysema. Am J Hum Genet 2000;66:187–95.
[70] Kimpara T, Takeda A, Watanabe K, et al. Microsatellite polymorphism in the human heme oxygenase-1 gene promoter and its application in association studies with Alzheimer and Parkinson disease. Hum Genet 1997;100:145–7.
[71] Ono K, Mannami T, Iwai N. Association of a promoter variant of the haem oxygenase-1 gene with hypertension in women. J Hypertens 2003;21:1497–503.
[72] Ono K, Goto Y, Takagi S, et al. A promoter variant of the heme oxygenase-1 gene may reduce the incidence of ischemic heart disease in Japanese. Atherosclerosis 2004;173:313–7.
[73] McGinnis RE, Spielman RS. Insulin gene 5V flanking polymorphism: length of class 1 allele in number of repeat units. Diabetes 1995;44:1296–302.
[74] Rich A, Nordheim A, Wang AHJ. The chemistry and biology of left-handed Z-DNA. Annu Rev Biochem 1984;53:791–846.
[75] Naylor LH, Clark EM. D(TG)n d(CA)n sequences upstream of the rat prolactin gene form Z-DNA and inhibit gene transcription. Nucleic Acids Res 1990;18:1595–601.
[76] Delic J, Onclecq R, Moisan-Coppey M. Inhibition and enhancement of eukaryotic gene expression by potential non-B DNA sequences. Biochem Biophys Res Commun 1991;181:818–26.
[77] Chen YH, Lin SJ, Lin MW, et al. Microsatellite polymorphism in promoter of heme oxygenase gene is associated with susceptibility to coronary artery disease in type 2 diabetic patients. Hum Genet 2002;111:1–8.
[78] Hirai H, Kubo H, Yamaya M, et al. Microsatellite polymorphism in heme oxygenase-1 gene promoter is associated with susceptibility to oxidant-induced apoptosis in lymphoblastoid cell lines. Blood 2003;102:1619–24.
[79] Epiphanio S, Mikolajczak SA, Gonçalves LA, et al. Heme oxygenase-1 is an anti-inflammatory host factor that promotes murine plasmodium liver infection. Cell Host Microbe 2008;3:331–8.
[80] Lavrovsky Y, Chatterjee B, Clark RA, Roy AK. Role of redox-regulated transcription factors in inflammation, aging and age-related diseases. Exp Gerontol 2000;35:521–32.
[81] Dick P, Schillinger M, Minar E, et al. Haem oxygenase-1 genotype and cardiovascular adverse events in patients with peripheral artery disease. Eur J Clin Invest 2005;35:731–7.
[82] Rueda B, Oliver J, Robledo G, et al. HO-1 promoter polymorphism associated with rheumatoid arthritis. Arthritis Rheum 2007;56:3953–8.
[83] Kobayashi H, Takeno M, Saito T, et al. Regulatory role of heme oxygenase 1 in inflammation of rheumatoid arthritis. Arthritis Rheum 2006;54:1132–42.
[84] Wagener FA, Toonen EJ, Wigman L, et al. HMOX1 promoter polymorphism modulates the relationship between disease activity and joint damage in rheumatoid arthritis. Arthritis Rheum 2008;58:3388–93.
[85] Lo SS, Lin SC, Wu CW, et al. Heme oxygenase-1 gene promoter polymorphism is associated with risk of gastric adenocarcinoma and lymphovascular tumor invasion. Ann Surg Oncol 2007;14:2250–6.
[86] Courtney AE, McNamee PT, Heggarty S, Middleton D, Maxwell AP. Association of functional haem oxygenase-1 gene promoter polymorphism with polycystic kidney disease and IgA nephropathy. Nephrol Dial Transplant 2008;23:608–11.
[87] Funke C, Tomiuk J, Riess O, Berg D, Soehn AS. Genetic analysis of heme oxygenase-1 (HO-1) in German Parkinson's disease patients. J Neural Transm 2009;116:853–9.

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[88] Chin HJ, Cho HJ, Lee TW, et al. The heme oxygenase-1 genotype is a risk factor to renal impairment of IgA nephropathy at diagnosis, which is a strong predictor of mortality. J Korean Med Sci 2009;24:S30–7.
[89] Fu WP, Zhao ZH, Fang LZ, et al. Heme oxygenase-1 polymorphism associated with severity of chronic obstructive pulmonary disease. Chin Med J (Engl) 2007;120:12–6.
[90] Takeda M, Kikuchi M, Ubalee R, et al. Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to cerebral malaria in Myanmar. Jpn J Infect Dis 2005;58:268–71.
[91] He JQ, Ruan J, Connett JE, Anthonisen NR, Pare PD, Sandford AJ. Antioxidant gene polymorphisms and susceptibility to rapid decline in lung function in smokers. Am J Respir Crit Care Med 2002;166:323–8.
[92] Guénégou A, Leynaert B, Bénessiano J. Association of lung function decline with the heme oxygenase-1 gene promoter microsatellite polymorphism in a general population sample. Results from the European Community Respiratory Health Survey (ECRHS), France. J Med Genet 2006;43:e43.
[93] Sheu CC, Zhai R, Wang Z, et al. Heme oxygenase-1 microsatellite polymorphism and haplotypes are associated with the development of acute respiratory distress syndrome. Intensive Care Med 2009;35:1343–51.
[94] Kaneda H, Ohno M, Taguchi J, et al. Heme oxygenase-1 gene promoter polymorphism is associated with coronary artery disease in Japanese patients with coronary risk factors. Arterioscler Thromb Vasc Biol 2002;22:1680–5.
[95] Schillinger M, Exner M, Mlekusch W, et al. Heme oxygenase-1 gene promoter polymorphism is associated with abdominal aortic aneurysm. Thromb Res 2002;106:131–6.
[96] Endler G, Exner M, Schillinger M, et al. A microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with increased bilirubin and HDL levels but not with coronary artery disease. Thromb Haemost 2004;91:155–61.
[97] Lüblinghoff N, Winkler K, Winkelmann BR. Genetic variants of the promoter of the heme oxygenase-1 gene and their influence on cardiovascular disease (the Ludwigshafen Risk and Cardiovascular Health study). BMC Med Genet 2009;10:36.
[98] Chen YH, Chau LY, Chen JW, Lin SJ. Serum bilirubin and ferritin levels link heme oxygenase-1 gene promoter polymorphism and susceptibility to coronary artery disease in diabetic patients. Diabetes Care 2008;31:1615–20.
[99] Kanai M, Tanabe S, Okada M, et al. Polymorphisms of heme oxygenase-1 and bilirubin UDP-glucuronyltransferase genes are not associated with Kawasaki disease susceptibility. Tohoku J Exp Med 2003;200:155–9.
[100] Schillinger M, Exner M, Mlekusch W, et al. Heme oxygenase-1 genotype is a vascular anti-inflammatory factor following balloon angioplasty. J Endovasc Ther 2002;9:385–94.
[101] Chen YH, Chau LY, Lin MW, et al. Heme oxygenase-1 promoter microsatellite polymorphism is associated with angiographic restenosis after coronary stenting. Eur Heart J 2004;25:39–47.
[102] Tiroch K, Koch W, von Beckerath N, Kastrati A, Schömig A. Heme oxygenase-1 gene promoter polymorphism and restenosis following coronary stenting. Eur Heart J 2007;28:968–73.
[103] Exner M, Schillinger M, Minar E, et al. Heme oxygenase-1 promoter microsatellite polymorphism is associated with restenosis after percutaneous transluminal angioplasty. J Endovasc Ther 2001;8:433–40.
[104] Schillinger M, Exner M, Minar E, et al. Heme oxygenase-1 genotype and restenosis after balloon angioplasty: a novel vascular protective factor. J Am Coll Cardiol 2004;43:950–7.
[105] Chin HJ, Cho HJ, Lee TW, et al. The heme oxygenase-1 genotype is a risk factor to renal impairment of IgA nephropathy at diagnosis, which is a strong predictor of mortality. J Korean Med Sci 2009;24(Suppl.):S30–7.
[106] Exner M, Bohmig GA, Schillinger M, et al. Donor heme oxygenase-1 genotype is associated with renal allograft function. Transplantation 2004;77:538–42.
[107] Baan C, Peeters A, Lemos F, et al. Fundamental role for HO-1 in the self protection of renal allografts. Am J Transplant 2004;4:811–8.
[108] Ozaki KS, Marques GM, Nogueira E, et al. Improved renal function after kidney transplantation is associated with heme oxygenase-1 polymorphism. Clin Transplant 2008;22:609–16.
[109] Denschlag D, Marculescu R, Unfried G, et al. The size of a microsatellite polymorphism of the haemoxygenase-1 gene is associated with idiopathic recurrent miscarriage. Mol Hum Reprod 2004;10:211–4.
[110] Lo SS, Lin SC, Wu CW, et al. Heme oxygenase-1 gene promoter polymorphism is associated with risk of gastric adenocarcinoma and lymphovascular tumor invasion. Ann Surg Oncol 2007;14:2250–6.
[111] Okamoto I, Krögler J, Endler G, et al. A microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with risk for melanoma. Int J Cancer 2006;119:1312–5.
[112] Vashist YK, Uzunoglu G, Cataldegirmen G, et al. Heme oxygenase-1 promoter polymorphism is an independent prognostic marker of gastrointestinal stromal tumour. Histopathology 2009;54:303–8.
[113] Kanai M, Akaba K, Sasaki A, Sato M, Harano T, Shibahara S, et al. Neonatal hyperbilirubinemia in Japanese neonates: analysis of the heme oxygenase-1 gene and fetal hemoglobin composition in cord blood. Pediatr Res 2003;54:165–71.
[114] Arredondo M, Jorquera D, Carrasco E, Albala C, Hertrampf E. Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with iron status in persons with type 2 diabetes mellitus. Am J Clin Nutr 2007;86:1347–53.
[115] Gerbitz A, Hillemanns P, Schmid C, et al. Influence of polymorphism within the heme oxygenase-I promoter on overall survival and transplantation-related mortality after allogeneic stem cell transplantation. Biol Blood Marrow Transplant 2008;14:1180–9.
[116] Exner M, Minar E, Wagner O, Schillinger M. The role of heme oxygenase-1 promoter polymorphisms in human disease. Free Radic Biol Med 2004;37:1097–104.

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CAPÍTULO IV

Dicussão e Conclusão

Discussão e Conclusões:

A importância da enzima heme oxigenase I se mostra indiscutível a medida que observamos o grande número de publicações envolvendo a mesma. A maioria dos estudos com essa enzima estão focados na suas propriedades antioxidantes e antiapoptóticas (Willis *et al.*, 1996; Siow *et al.*, 1999; Tanaka *et al.*, 2003; Clark *et al.*, 1997; Deramaudt *et al.*, 1998; Doi *et al.*, 1999; Goodman *et al.*, 1997; Tsuji *et al.*, 1999; Deininger *et al.*, 2000; Torisu-Itakura *et al.*, 2000; Fang *et al.*, 2003). No entanto, os exatos mecanismos através dos quais a enzima HO-1 exerce suas atividades "protetoras" encontram-se longe de serem elucidados. Existem indícios na literatura que, em determinadas circunstâncias, a HO-1 possa ser mobilizada para o núcleo e assumir um papel ativo na regulação da expressão gênica (Lin *et al.*, 2007). Tal mecanismo, seria uma plausível explicação para a ampla gama de efeitos demonstrados por essa enzima em nível celular. No entanto, as evidências que suportam essa localização nuclear da HO-1, em situações de estresse, não são sólidas a ponto de indicarmos essa situação como sendo a dinâmica normal da enzima dentro da célula. A reação catalisada pela enzima HO-1 é simples, apresentando um único substrato, o grupo heme, o qual a enzima cliva gerando Fe(III), CO e bilirubina. A questão é como uma enzima que catalisa uma reação tão singular pode apresentar tamanha diversidade de efeitos quando tem sua expressão modulada. A resposta para essa pergunta não está na tentativa de atribuição de novas funções à enzima HO-1, e sim na elucidação dos diferentes efeitos promovidos pelo seu substrato, o grupo heme, em nível celular. Mesmo que presente no núcleo, como sustentado no estudo de Lin *et al.,* 2007, a enzima HO-1 provavelmente continua exercendo seus efeitos através do catabolismo do grupo "heme-livre" presente nesse compartimento celular, uma vez que já foi extensivamente mostrado na literatura o efeito que o mesmo exerce sobre, por exemplo, o fator repressor Bach1 (Ogawa *et al.*, 2001; Igarashi & Sun, 2006; Zenze-Kawasaki *et al.*, 2007). As quantidades de "heme-livre" são determinantes para a reação catalisada pela HO-1, uma vez que servirão tanto na indução da enzima, através da desrepressão de Bach1, quanto servindo de substrato da reação. Dessa forma, podemos colocar o anel pofirínico como sendo fator limitante da função fisiológica da HO-1 e de todos os efeitos decorrentes dessa atividade em nível celular.

As células sanguíneas vermelhas (RBC) contêm grande parte do heme presente no organismo (75-80%). Os precursores dessas células sanguíneas apresentam uma taxa de síntese de heme que é de uma ordem de magnitude superior a das células do fígado, as quais correspondem ao segundo maior produtor de heme no organismo. É surpreendente saber que muito pouco é conhecido sobre o comportamento da enzima HO-1 nas células sanguíneas vermelhas, bem como nas suas precursoras presentes na medula óssea. O capítulo II dessa tese apresentou os dados relativos ao padrão de expressão e níveis proteicos da enzima HO-1 ao longo do processo de diferenciação em células fetais de fígado de camundongo (*fetal liver cells*, FL) e células eritroleucêmicas de camundongo (*murine erithroleukemia cells*, MEL). Os resultados demonstram que tanto em FL quanto em células MEL, a enzima HO-1 é regulada de forma positiva ao longo do processo de diferenciação. Essa indução da HO-1 parece estar intimamente ligada aos níveis de heme presentes nos precursores eritróides durante o processo de diferenciação, uma vez que esse aumento na expressão é prevenido pelo uso do inibidor da síntese de heme, succinil acetona (SA). O único estudo publicado até então investigando a expressão da enzima HO-1 durante o processo de diferenciação em células MEL, foi realizado em 1989, por Fujita & Sassa. As conclusões do referido estudo suportam uma redução na expressão da HO-1, durante 48h de diferenciação das células MEL com dimetil sulfóxido (DMSO). No entanto, em nossos experimentos diferenciamos as células MEL por um período mais longo de tempo (72h), sendo que nessa etapa mais tardia de diferenciação que observamos os altos níveis de HO-1. Além disso, uma vez que a expressão da HO-1 é determinada pelos níveis de heme, é esperado que o maior grau de indução da enzima ocorra após maior acumulação de heme, fato esse que ocorre após 72h de tratamento com DMSO. Por fim, semelhante ao que foi obtido nas células MEL, observamos também nas FL um aumento na expressão da enzima HO-1 ao longo do processo de diferenciação. As FL se apresentam como um modelo mais próximo do fisiológico, sendo que essas, ao contrário das células MEL, sofrem as alterações mais características do processo de diferenciação eritróide como, por exemplo, exclusão do núcleo e drástica redução no tamanho celular, além de acumularem hemoglobina (Dolznig *et al.*, 2001; Schranzhofer *et al.*, 2006). A dinâmica das alterações na expressão das células eritróides durante o processo de diferenciação, inclue a redução nas quantidades de Bach1 presentes no

núcleo concomitante com o aumento na quantidade do fator Nfr2 (Brand *et al.*, 2004). Essas alterações são importantes na indução de genes fundamentais durante o processo de síntese e acumulação de hemoglobina, como, por exemplo, os genes das α e β globinas (Tahara *et al.*, 2004a; Tahara *et al.*, 2004b). Essas alterações no padrão transcricional durante o processo de diferenciação dariam as condições necessárias para o aumento na expressão da HO-1, uma vez que o gene que codifica para essa enzima também é um dos alvos do repressor Bach1.

Na segunda parte dos resultados apresentados no capítulo II , a enzima HO-1 teve sua expressão modulada e como resultado foi observado um efeito direto sob o processo de hemoglobinização nas células MEL. Arsenato de Sódio ($NaAsO_2$) é um reagente amplamente utilizado para indução da expressão da enzima HO-1. Esse composto teve o mesmo efeito quando empregado nas células MEL. Além da esperada indução na expressão da HO-1, foi observada também uma redução na expressão das globinas, e consequente redução no processo de hemoglobinização. Tais resultados demonstram que os níveis de expressão de HO-1, atingidos empregando $NaAsO_2$, foram suficientemente altos a ponto de interferir no processo de acúmulo de heme, fato esse refletido na redução dos níveis de globina. Foram observados também que os níveis de ferritina aumentaram quando as células MEL foram expostas ao $NaAsO_2$, situação essa que indica clivagem do grupo heme pela HO-1 e consequente liberação de ferro, sendo esse metal estocado na proteína ferritina. O papel da HO-1 nos efeitos desencadeados pelo tratamento com $NaAsO_2$ foram comprovados pelo emprego do inibidor químico dessa enzima, estanho-protoporfirina (SnPP). SnPP promoveu o retorno dos níveis normais de globina e ferritina quando empregado juntamente com o $NaAsO_2$ nas células MEL tratadas com DMSO. A células eritróides possuem mecanismos que garantem que as quantidades sintetizadas de heme não excedam as quantidades de globina (Kramer *et al.*, 1976; McEwen *et al.*, 2005) durante o processo de hemoglobinização, de forma a evitar o acúmulo de "heme-livre" na célula. Nossos resultados indicam que a HO-1 tem acesso ao heme sendo sintetizado pela célula eritróide, e que esse possivelmente se encontra livre. Além de demonstrarmos, através desses resultados, a importância da enzima HO-1 no processo de diferenciação eritróide, também reforçamos perguntas até então não respondidas claramente na literatura; como, por exemplo, os caminhos percorridos pela molécula

heme até atingir o citoplasma e complexada às globinas e a posição da enzima HO-1 dentro das células eritróides. Esses questionamentos e por consequência suas respostas seriam fundamentais para determinar como a enzima HO-1 teria acesso ao heme sendo sintetizado durante o processo de hemoglobinização.

O uso de agentes químicos com o objetivo de super-expressar componentes celulares é alvo de muitas críticas. Essas críticas são muita vezes bem fudamentadas no fato de que os níveis de expressão atingidos por certo genes, como resultado do uso de agentes químicos, em pouco se comparam às variações fisiológicas normais de determinadas proteínas dentro da célula. O objetivo do emprego do $NaAsO_2$ foi baseado na indução, ainda que "descontrolada", da enzima HO-1 endógena. Obstante a esse fato, analisamos os possíveis efeitos de uma inibição na expressão da enzima HO-1 sob o processo de hemoglobinização em células MEL tratadas com DMSO. Surpreendentemente, observamos que o emprego de siRNA tendo como alvo o gene da HO-1, resultou no aumento na expressão das globinas. Esse resultado indica que em situações onde a expressão da HO-1 encontra-se reduzida, o processo de hemoglobinização ocorre de uma forma mais acentuada, sugerindo que a HO-1 possa agir de forma a co-regular o processo de diferenciação eritróide. Tanto os resultados empregando $NaAsO_2$ quanto o siRNA para HO-1, demonstraram que a HO-1 pode interferir diretamente no processo de diferenciação. Por esse motivo, os níveis dessa enzima precisam ser controlados de maneira que não venham a interferir com a síntese de heme e consequente hemoglobinização. O aumento, também observado em nossos resultados, na expressão da HO-1 durante o processo de diferenciação das células MEL e FL, apesar de inevitável, em vista do aumento na quantidade de heme, provavelmente se encontra dentro de níveis condizentes com uma hemoglobinização eficiente. Portanto, os resultados apresentados no capítulo II inciam um processo de revisão dos efeitos da enzima HO-1 na diferenciação eritróide, efeitos esses até então menosprezados.

Após o processo de diferenciação, as células vermelhas chegam à circulação para exercer sua função carregando e distribuindo o oxigênio nos diferentes tecidos do organismo. O grupo heme é parte integrante fundamental da hemoglobina, uma vez que o ferro presente nessa molécula é o grande responsável pela sua capacidade de ligação ao oxigênio. Alterações na fisiologia normal do organismo podem acarretar lise das células

vermelhas e consequente liberação da hemoglobina. Uma vez livre na circulação, a hemoglobina assume propriedades que podem ser extremamente deletérias para o organismo. Essas propriedades são fruto da molécula heme presente na hemoglobina, que pode ser liberada quando da oxidação da hemoglobina, gerando meta-hemoglobina (met-Hb) (Balla *et al.*, 1993; Jeney *et al.*, 2002). A presença de heme livre na circulação aciona uma série de mecanismos inflamatórios, resultando em trombose microvascular, vaso-oclusão e isquemia tecidual (Ferreira *et al.*, 2008). O organismo apresenta estratégias para remover os excessos de hemoglobina e heme da circulação, mecanismos esses mediados pelas moléculas haptoglobina, hemopexina e albumina (Atkinson *et al.*, 2007; Hvidberg *et al.*, 2005). Todos esses mecanismos culminam com a ação da enzima HO-1, catabolizando o heme e gerando produtos que, na sua maioria, apresentam características antioxidantes (CO e bilirrubina) (Kushida *et al.*, 2002a; Kushida *et al.*, 2002b; Stocker *et al.*, 1987a; Stocker *et al.*, 1987b; Song *et al.*, 2002; Otterbein *et al.*, 2003; Sato *et al.*, 2001). Em determinadas circunstâncias patológicas, chamadas doenças hemolíticas, como, por exemplo, anemia falciforme e talassemias, a recorrente lise das células vermelhas pode resultar na saturação dos mecanismos mediados pelas moléculas haptoglobina, hemopexina e albumina. Nesse contexto, a enzima HO-1 celular aparece como último recurso para remoção do excesso de heme.

A malária é uma doença causada pela infecção do indivíduo com o protozoário *Plasmodium*. O segundo estágio da infecção com esse parasita, resulta na contaminação dos eritrócitos com o *Plasmodium*. Como consequência desse processo, as células vermelhas contaminadas acabam lisando e liberando a hemoglobina na circulação. Tal situação faz com que a malária seja caracterizada por ciclos de elevada hemólise. Muitos dos sintomas apresentados pelos indíviduos infectados são resultado desse aumento de hemoglobina e heme na circulação, culminando com dano vascular e sinalização pro-inflamatória. Dessa forma, a enzima HO-1 mais uma vez se coloca como peça fundamental na defesa do organismo, removendo o excesso de heme e reduzindo o quadro pró-inflamatório resultante da infecção com esse parasita. A importância da HO-1 na malária já está descrita na literatura, inclusive promovendo, quando em níveis elevados, proteção contra malária cerebral, principal responsável pelas mortes

relacionadas à infecção com Plasmodium (Pamplona *et al.*, 2007; Schluesener *et al.*, 2001; Medana *et al.*, 2001; Clark *et al.*, 2003).

O capítulo III sustenta a hipótese de que genótipos específicos do gene *HMOX1* estariam sendo selecionados em regiões endêmicas para malária, anemia falciforme e talassemia. Os genótipos de *HMOX1* que concedem alta expressão da enzima seriam o alvo dessa seleção positiva, concedendo, como resultado, proteção contra malária.. A região promotora do gene da *HMOX1* apresenta uma série de repetições dinucleotídicas, purina-pirimidina (GT), com alta variação no número de repetições, sendo, dessa forma, muito útil em estudos genéticos (McGinnis & Spielman, 1995). A influência das repetições GT na atividade do promotor da *HMOX1* já foi experimentalmente demonstrada, onde construtos de promotores com <25 repetições demonstraram uma maior atividade (Delic *et al.*, 2001). Estudos *in vivo* também demonstraram maior atividade dos promotores que carregam <25 repetições (Hirai *et al.*, 2003). O polimorfismo no promotor do gene da *HMOX1* apresenta grande variabilidade dentro das diferentes populações humanas, o que, portanto, faz com que esse componente genético possa ser alvo de seleção. Como já descrito na literatura, tanto anemia falciforme quanto talassemias conferem proteção contra malária (Williams *et al.*, 2005; Allen *et al.*, 1997; May *et al.*, 2007; Mockenhaupt *et al.*, 2004). A característica endêmica da malária e a pressão de seleção exercida por essa doença, resulta numa alta frequência dos alelos falciforme e talassêmicos nessa região de endemia para malária quando comparada com outras regiões livres dessa pressão de seleção. A HO-1 aparece como elemento principal na proteção do tecido endotelial em modelos animais de anemia falciforme (Belcher *et al.*, 2006), removendo o excesso de heme e limitando a toxicidade promovida por essa molécula. Além disso, foi demonstrado que pacientes com anemia falciforme apresentam níveis elevados da enzima HO-1 (Lanaro *et al.*, 2009), fato esse que indica um papel ativo dessa enzima no combate ao heme liberado na hemólise das células vermelhas. Considerando as semelhanças entre talassemias e anemia falciforme, na medida em que ambas tem na presença do heme livre na circulação um potente indutor da ativação endotelial, não seria exagerado considerar uma importância da HO-1 na proteção do dano mediado por heme em indivíduos talassêmicos (situação essa até então não descrita na literatura). Portanto, a pressão seletiva excercida pela malária estaria promovendo o

aumento da frequência não só dos alelos falciforme e talassêmicos mas também dos alelos de *HMOX1* que conferem maior expressão desse gene. Os indivíduos portadores dos alelos faciforme e talassêmicos que possuissem alelos curtos (<25) da *HMOX1*, o que, por sua vez, permite um eficiente catabolismo do heme livre, teriam também uma vantagem quando da contaminação com a *Plasmodium*, uma vez que, como já demonstrado na literatura, maior expressão da enzima HO-1 reduz as possibilidades de desenvolvimento da malária cerebral (principal causa das mortes relacionadas a essa doença) e ameniza os sintomas característicos da malária (Epiphanio *et al.*, 2008). Dessa forma, o capítulo III sustenta a hipótese de que os alelos sendo selecionados de forma positiva pela malária seriam os alelos curtos do gene da *HMOX1*, sendo esses, por sua vez, presentes em maior frequência em indivíduos talassêmicos e falciformes.

A reação singular catalisada pela enzima HO-1 coloca a mesma como peça fundamental em todos os mecanismos de proteção celular contra toxicidade mediada por heme. As inúmeras funções desempenhadas por essa molécula heme, composta de um anel porfirínico ligado a ferro, fazem com que a sua rota de degradação enzimática assuma uma grande importância. Todos os dias encontramos novas evidências na literatura que indicam a importância da HO-1 tanto em diferentes situações fisiológicas normais bem como situações patológicas. Essa tese aponta a HO-1 como um importante fator durante o processo de diferenciação eritróide, fato esse até então sem precedentes na literatura. Além disso, elaboramos uma hípotese bem embasada que coloca variantes polimórficas do gene da *HMOX1* como importante fator genético dentro de áreas de endemia para malária. Acima de tudo, nosso trabalho levanta uma série de novas perguntas que precisam ser esclarecidas para o melhor entendimento dos reflexos da atividade da HO-1 dentro dos organismos vivos.

Bibliografia

Abraham NG, Kappas A (2008) Pharmacological and clinical aspects of heme oxygenase. Pharmacol Rev 60:79 –127.

Abraham NG, Friedland ML, Levere RD (1983) Heme metabolism in hepatic and erythroid cells. In: Brown E, ed. Progress in Hematology. Vol XIII ed. New York, NY: Grune and Stratton 75–130.

Alam J, Wicks C, Stewart D, Gong P, Touchard C, Otterbein S, Choi AMK, Burow ME, Tou T (2000) Mechanism of heme oxygenase-1 gene activation by cadmium in MCF-7 mammary epithelial Cells. Role of p38 kinase and Nrf2 transcription factor. J Biol Chem 275: 27694–27702.

Alam J, Stewart D, Touchard C, Boinapally S, Choi AM,Cook JL (1999) Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. J Biol Chem 274: 26071–26078.

Alam J, Camhi S, Choi AMK (1995) Identification of a second region upstream of the mouse heme oxygenase-1 gene that functions as a basal level and inducer-dependent transcription enhancer. J Biol Chem 270: 11977–11984.

Alam J, Cai J, Smith A (1994) Isolation and characterization of the mouse heme oxygenase-1 gene. Distal 5' sequences are required for induction by heme or heavy metals. J Biol Chem 269: 1001–1009.

Alam S. Shibahara S, Smith A (1989) Transcriptional activation of the heme oxygenase gene by heme and cadmium in mouse hepatoma cells. J Biol Chem 264: 6371–6375.

Allen SJ, O'Donnell A, Alexander ND, Alpers MP, Peto TE, Clegg JB, Weatherall DJ (1997) alpha+-Thalassemia protects children against disease caused by other infections as well as malaria. Proc Natl Acad Sci USA 94: 14736–14741.

Andrews NC, Erdjument-Bromage H, Davidson MB, Tempst P, Orkin SH (1993) Erythroid transcription factor NF-E2 is a haematopoietic-specific basic-leucine zipper protein. Nature 362: 722–728.

Atkinson SH, Mwangi TW, Uyoga SM, Ogada E, Macharia AW, Marsh K, Prentice AM, Williams TN (2007) The haptoglobin 2–2 genotype is associated with a reduced incidence of Plasmodium falciparum malaria in children on the coast of Kenya. Clin Infect Dis 44: 802–809.

Balla G, Jacob HS, Eaton JW, Belcher JD and Vercellotti GM (1991) Hemin: a possible physiological mediator of low density lipoprotein oxidation and endothelial cell injury. Arterioscler Thromb 11: 1700-1711.

Balla J, Jacob HS, Balla G, Nath K, Eaton JW, Vercellotti GM (1993) Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage. Proc Natl Acad Sci USA 90:9285–9289.

Belcher JD, Mahaseth H, Welch TE, Otterbein LE, Hebbel RP, Vercellotti GM (2006) Heme oxygenase-1 is a modulator of inflammation and vaso-occlusion in transgenic sickle mice. J Clin Invest 116: 808–816.

Brand M, Ranish JA, Kummer NT, Hamilton J, Igarashi K, Francastel C, Chi TH, Crabtree GR, Aebersold R, Groudine M (2004) Dynamic changes in transcription factor complexes during erythroid differentiation revealed by quantitative proteomics. Nat Struct Mol Biol 11:73-80.

Bratosin D, Estaquier J, Petit F, Arnoult D, Quatannens B, Tissier JP, Slomianny C, Sartiaux C, Alonso C, Huart JJ, Montreuil J, Ameisen JC (2001) Programmed cell death in mature erythrocytes: a model for investigating death effector pathways operating in the absence of mitochondria. Cell Death Differ 8: 1143-56.

Camejo G, Halberg C, Manschik-Lundin A, Hurt-Camejo E, Rosengren B, Olsson H, Hansson GI, Forsberg GB and Ylhen B (1998) Hemin binding and oxidation of lipoproteins in serum: mechanisms and effect on the interaction of LDL with human macrophages. J Lipid Res 39: 755-766.

Canonne-Hergaux F, Zhang AS, Ponka P, Gros P (2001) Characterization of the iron transporter DMT1 (NRAMP2/DCT1) in red blood cells of normal and anemic mk/mk mice. Blood 98: 3823-3830.

Civil A, van Genesen S, Lubsen N (2002) c-Maf, the gammaD-crystallin Maf-responsive element and growth factor regulation. Nucleic Acids Res 30: 975–982.

Chen YH, Lin SJ, Lin MW, Tsai HL, Kuo SS, Chen JW, Charng MJ, Wu TC, Chen LC, Ding YA, Pan WH, Jou YS, Chau LY (2002) Microsatellite polymorphism in promoter of heme oxygenase gene is associated with susceptibility to coronary artery disease in type 2 diabetic patients. Hum Genet 111: 1–8.

Clark IA, Awburn MM, Harper CG, Liomba NG, Molyneux ME (2003) Induction of HO-1 in tissue macrophages and monocytes in fatal falciparum malaria and sepsis. Malar J 2: 41.

Clark JE, Green CJ and Motterlini R (1997) Involvement of the heme oxygenase-carbon monoxide pathway in keratinocyte proliferation. Biochem Biophys Res Commun 241: 215–220.

Conner SD, Schmid SL (2003) Differential requirements for AP-2 in clathrinmediated endocytosis. J Cell Biol 162: 773-779.

Creusot F, Verdiere J, Gaisne M, Slonimski PP (1988) CYP1 (HAP1) regulator of oxygen-dependent gene expression in yeast. I. Overall organization of the protein sequence
displays several novel structural domains. J Mol Biol 204: 263–276.

Davies KJA and Delsignore M (1986) Protein damage and degradation by oxygen radicals III. Modification of secondary and tertiary structure. J. Biol. Chem. 262: 9908–9913.

De Domenico I, McVey Ward D, Kaplan J (2008) Regulation of iron acquisition and storage: consequences for iron-linked disorders. Nat Rev Mol Cell Biol9: 72-81.

Deininger MH, Meyermann R, Trautmann K, Duffner F, Grote EH, Wickboldt J and Schluesener HJ (2000) Hemeoxygenase (HO)-1 expressing macrophages/microglial cellsaccumulate during oligodendroglioma progression. Brain Res 882: 1–8.

Delaby C, Pilard N, Puy H, Canonne-Hergaux F (2008) Sequential regulation of ferroportin expression after erythrophagocytosis in murine macrophages: early mRNA induction by
haem, followed by iron-dependent protein expression. Biochem J 411: 123-131.

Delaby C, Pilard N, Goncalves AS, Beaumont C, Canonne-Hergaux F (2005) Presence of the iron exporter ferroportin at the plasma membrane of macrophages is enhanced by iron loading and down-regulated by hepcidin. Blood 106: 3979-3984.

Deramaudt BM, Braunstein S, Remy P and Abraham NG (1998) Gene transfer of human heme oxygenase into coronary endothelial cells potentially promotes angiogenesis. J Cell Biochem 68: 121–127.

Desjardins M (2003) ER-mediated phagocytosis: a new membrane for new functions. Nat Rev Immunol 3: 280-91.

Doi K, Akaike T, Fujii S, Tanaka S, Ikebe N, Beppu T, Shibahara S, Ogawa M and Maeda H (1999) Induction of haem oxygenase-1 by nitric oxide and ischaemia in experimental solid tumours and implications for tumour growth. Br J Cancer 80:1945–1954.

Dolznig H, Boulmé F, Stangl K, Deiner EM, Mikulits W, Beug H, Müllner EW (2001) Establishment of normal, terminally differentiating mouse erythroid progenitors: molecular characterization by cDNA arrays. FASEB J 15(8): 1442-1444.

Eisenstein RS, Munro H (1990a) Translational regulation of ferritin synthesis by iron. Enzyme 44: 42–58.

Epiphanio S, Mikolajczak SA, Gonçalves LA, Pamplona A, Portugal S, Albuquerque S, Goldberg M, Rebelo S, Anderson DG, Akinc A, Vornlocher HP, Kappe SH, Soares MP, Mota MM (2008) Heme oxygenase-1 is an anti-inflammatory host factor that promotes murine plasmodium liver infection. Cell Host Microbe 3:331–338.

Fang J, Akaike T and Maeda H (2003) Antiapoptotic role of heme oxygenase (HO) and the potential of HO as a target in anticancer treatment. Apoptosis 9: 27–35.

Ferreira A, Balla J, Jeney V, Balla G, Soares MP (2008) A central role for free heme in the pathogenesis of severe malaria: the missing link? J Mol Med 86: 1097–1111.

Fujita H, Yamamoto M, Yamagami T, Hayashi N, Bishop TR, De Verneuil H, Yoshinaga T, Shibahara S, Morimoto R, Sassa S (1991) Sequential activation of genes for heme pathway enzymes during erythroid differentiation of mouse Friend virus-transformed erythroleukemia cells. Biochim Biophys Acta 1090: 311-316.

Fujita H, Sassa S (1989) The rapid and decremental change in haem oxygenase mRNA during erythroid differentiation of murine erythroleukaemia cells. Br J Haematol 73: 557-560.

Furuyama K, Kaneko K, Vargas PD (2007) Heme as a magnificent molecule with multiple missions: heme determines its own fate and governs cellular homeostasis. Tohoku J Exp Med
213: 1-16.

Goodman AI, Choudhury M, da Silva JL, Schwartzman ML and Abraham NG (1997) Overexpression of the heme oxygenase gene in renal cell carcinoma. Proc Soc Exp Biol Med 214: 54–61.

Granick S, Sincalir P, Sassa S and Grieninger G (1975) Effects of heme, insulin, and serum albumin on heme and protein synthesis in chick embryo liver cells cultured in a chemically defined medium, and a spectrofluorometric assay for porphyrin composition.
J Biol Chem 250: 9215–9225.

Grosveld F, Blom van Assendelft G, Greaves DR, Kollias G (1987) Position-independent, high-level expression of the b-globin gene in transgenic mice. Cell 51: 975–985.

Hara E, Takahashi K, Takeda K, Nakayama M, Yoshizawa M, Fujita H, Shirato K and Shibahara S (1999) Induction of heme oxygenase-1 as a response in sensing the signals evoked by distinct nitric oxide donors. Biochem Pharmacol 58: 227–236.

Hartsfield SL, Alam J, Cook JL and Choi AMK (1997) Regulation of heme oxygenase-1 gene expression in vascular smooth muscle cells by nitric oxide. Am J Physiol 273: 980–988.

Hirai H, Kubo H, Yamaya M, Nakayama K, Numasaki M, Kobayashi S, Suzuki S, Shibahara S, Sasaki H (2003) Microsatellite polymorphism in heme oxygenase-1 gene

promoter is associated with susceptibility to oxidantinduced apoptosis in lymphoblastoid cell lines. Blood 102: 1619–1624.

Hoshino H, Igarashi K (2002) Expression of the oxidative stress-regulated transcription factor bach2 in differentiating neuronal cells. J Biochem (Tokyo) 132: 427–431.

Hunter GC, Dubick MA, Keen CL and Eskelson CD (1991) Effects of hypertension on aortic antioxidant status in human abdominal aneurysmal and occlusive disease. Proc Soc Exp Biol Med 196: 273-279.

Hvidberg V, Maniecki MB, Jacobsen C, Hojrup P, Moller HJ, Moestrup SK (2005) Identification of the receptor scavenging hemopexin–heme complexes. Blood 106: 2572–2579.

Igarashi K, Sun J (2006) The heme-Bach1 pathway in the regulation of oxidative stress response and erythroid differentiation. Antioxid Redox Signal 8: 107-118.

Inamdar NM, Ahn YI, Alam J (1996) The heme-responsive element of the mouse heme oxygenase-1 gene is an extended AP-1 binding site that resembles the recognition sequences for MAF and NF-E2 transcription factors. Biochem Biophys Res Commun 221: 570–576.

Iolascon A, De Falco L, Beaumont C (2009) Molecular basis of inherited microcytic anemia due to defects in iron acquisition or heme synthesis. Haematologica 94: 395-408.

Ishii T, Itoh K, Ruiz E, Leake DS, Unoki H, Yamamoto M, Mann GE (2004) Role of Nrf2 in the regulation of CD36 and stress protein expression in murine macrophages: activation
by oxidatively modified LDL and 4-hydroxynonenal. Circ Res 94: 609–616.

Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa T, Katoh Y, Bannai S, Yamamoto M (2000) Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J Biol Chem 275: 16023–16029.

Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y (1997) An Nrf2/small Maf heterodimer mediates
the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 236: 313–322.

Jeney V, Balla J, Yachie A, Varga Z, Vercellotti GM, Eaton JW and Balla G (2002) Pro-oxidant and cytotoxic effects of circulating heme. Blood 100: 879-887.

Kanezaki R, Toki T, Yokoyama M, Yomogida K, Sugiyama, Yamamoto M, Igarashi K, Ito E (2001) Transcription factor BACH1 is recruited to the nucleus by its novel alternative spliced isoform. J Biol Chem 276: 7278–7884.

Kartikasari AE, Wagener FA, Yachie A, Wiegerinck ET, Kemna EH, Swinkels DW (2008) Hepcidin suppression and defective iron recycling account for dysregulation of iron homeostasis in heme oxygenase-1 deficiency. J Cell Mol Med 13: 3091-3102.

Katz JH (1961) Iron and protein kinetics studied by means of doubly labeled human crystalline transferrin. J Clin Invest 61: 2143-2152.

Kawashima A, Oda Y, Yachie A, Koizumi S, Nakanishi I (2002) Heme oxygenase-1 deficiency: the first autopsy case. Hum Pathol 33: 125-130.

Keyse SM and Tyrrell RM (1989) Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. Proc Natl Acad Aci USA 86: 99–103.

Kikuchi G, Shemin D, Bachmann BJ (1958) The enzymic synthesis of delta-aminolevulinic acid. J Biol Chem 233:1214-1219.

Knutson M, Wessling-Resnick M (2003) Iron metabolism in the reticuloendothelial system. Crit Rev Biochem Mol Biol 38: 61-88.

Kramer G, Cimadevilla JM, Hardesty B (1976) Specificity of the protein kinase activity associated with the hemincontrolled repressor of rabbit reticulocyte. Proc Natl Acad Sci USA
73: 3078-3082.

Kushida T, LiVolti G, Goodman AI, Abraham NG (2002a) TNF-alpha-mediated cell death is attenuated by retrovirus delivery of human heme oxygenase-1 gene into human microvessel endothelial cells. Transplant Proc 34:2973–2978.

Kushida T, Li Volti G, Quan S, Goodman A, Abraham NG (2002b) Role of human heme oxygenase-1 in attenuating TNF-alpha-mediated inflammation injury in endothelial cells. J Cell Biochem 87: 377–385.

Kwak M, Itoh K, Yamamoto M, Sutter T, Kensler T (2001) Role of transcription factor Nrf2 in the induction of hepatic phase 2 and antioxidative enzymes in vivo by the cancer chemoprotective agent, 3H-1, 2-dimethiole-3-thione. Mol Med 7: 135–145.

Lanaro C, Franco-Penteado CF, Albuqueque DM, Saad ST, Conran N, Costa FF (2009) Altered levels of cytokines and inflammatory mediators in plasma and leukocytes of sickle cell anemia patients and effects of hydroxyurea therapy. J Leukoc Biol 85: 235–242.

Lee HC, Hon T, Zhang L (2002) The molecular chaperone Hsp90 mediates heme activation of the yeast transcriptional activator Hap1. J Biol Chem 277: 7430–7437.

Lee TS, Tsai HL and Chau LY (2003) Induction of Heme Oxygenase-1 Expression in Murine Macrophages is Essential for the Anti-inflammatory Effect of Low Dose 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2. J Biol Chem 278: 19325-19330.

Lee TS and Chau LY (2002) Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. Nat Med 8: 240–246.

Lin Q, Weis S, Yang G, Weng YH, Helston R, Rish K, Smith A, Bordner J, Polte T, Gaunitz F, Dennery PA (2007) Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress. J Biol Chem 282: 20621-20633.

Lutz HU, Fasler S, Stammler P, Bussolino F, Arese P (1988) Naturally occurring anti-band 3 antibodies and complement in phagocytosis of oxidatively- stressed and in clearance of senescent
red cells. Blood Cells 14: 175-203.

Maines MD (1988) Heme oxygenase: Function, multiplicity, regulatory mechanisms, and clinical applications. FASEB J 2: 2557–2568.

Martin FM, Bydlon G, Friedman JS (2006) SOD2-deficiency sideroblastic anemia and red blood cell oxidative stress. Antioxid Redox Signal 8: 1217-1225.

Marro S, Chiabrando D, Messana E, Stolte J, Turco E, Tolosano E, Muckenthaler MU (2010) Heme controls ferroportin1 (FPN1) transcription involving Bach1, Nrf2 and a MARE/ARE sequence motif at position -7007 of the FPN1 promoter. Haematologica Feb 23. [Epub ahead of print].

May J, Evans JA, Timmann C, Ehmen C, Busch W, Thye T, Agbenyega T, Horstmann RD (2007) Hemoglobin variants and disease manifestations in severe falciparum malaria. JAMA 297: 2220–2226.

McCoubrey WK Jr, Huang TJ and Maines MD (1997) Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. Eur J Biochem 247: 725–732.

McEwen E, Kedersha N, Song B, Scheuner D, Gilks N, Han A, Chen JJ, Anderson P, Kaufman RJ (2005) Heme-regulated inhibitor kinase- mediated phosphorylation of eukaryotic translation initiation factor 2 inhibits translation, induces stress granule formation, and mediates survival upon arsenite exposure. J Biol Chem 280: 16925-16933.

McGinnis RE, Spielman RS (1995) Insulin gene 5V flanking polymorphism: length of class 1 allele in number of repeat units. Diabetes 44: 1296–1302.

Medana IM, Mai NT, Day NP, Hien TT, Bethell D, Phu NH, Farrar J, White NJ, Turner GD (2001) Cellular stress and injury responses in the brains of adult Vietnamese patients with fatal Plasmodium falciparum malaria. Neuropathol Appl Neurobiol 27:421–433.

Mignotte V, Wall L, deBohr E, Grosveld F, Romeo P-H (1989a) Two tissue-specific factors bind the erythroid promoter of human porphobilinogen deaminase gene. Nucleic Acids Res 17: 37–54.

Mignotte V, Eleouet JF, Raich N, Romeo P-H (1989b) Cisand trans-acting elements involved in the regulation of the erythroid promoter of the human porphobilinogen deaminase
gene. Proc Natl Acad Sci USA 86: 6548–6552.

Miller YI and Shaklai N (1994) Oxidative crosslinking of LDL protein induced by hemin: involvement of tyrosines. Biochem Mol Biol Int 34: 1121-1129.

Milot E, Strouboulis J, Trimborn T, Wijgerde M, de Boer E, Langeveld A, Tan-Un K, Vergeer W, Yannoutsos N, Grosveld F, Fraser P (1996) Heterochromatin effects on the frequency and duration of LCR-mediated gene transcription. Cell 87: 105–114.

Mitani K, Fujita H, Fukuda Y, Kappas A and Sassa S (1993) The role of inorganic metals and metalloporphrins in the induction of heme oxygenase and heat-shock protein 70 in human hepatoma cells. Biochem J 290: 819–825.

Mockenhaupt FP, Ehrhardt S, Gellert S, Otchwemah RN, Dietz E, Anemana SD, Bienzle U (2004) alpha+-Thalassemia protects African children from severe malaria. Blood 104: 2003–2006.

Monson EK, Weinstein M, Ditta GS, Helinski DR (1992) The FixL protein of Rhizobium meliloti can be separated into a heme-binding oxygen-sensing domain and a functional C-terminal kinase domain. Proc Natl Acad Sci USA 89: 4280–4284.

Morgan EH (1981) Transferrin biochemistry, physiology and clinical significance. Mol Aspects Med 4:1-123.

Morita T, Mitsialis S, Koike H, Liu Y, Kourembanas S (1997) Carbon monoxide controls the proliferation of hypoxic vascular smooth muscle cells. J Biol Chem 272: 32804–32809.

Motterlini R, Foresti R, Bassi R, Calabrese V, Clark JE and Green CJ (2000) Endothelial heme oxygenase-1 induction by hypoxia. Modulation by inducible nitric oxide synthase and S-nitrosothiols. J Biol Chem 275: 13613–13620.

Motterlini R, Foresti R, Intaglietta M, Winslow RM (1996) NO-mediated activation of heme oxgenase: endogenous cytoprotection against oxidative stress to endothelium. Am J Physiol 270: H107–H114.

Muckenthaler MU, Galy B, Hentze MW (2008) Systemic iron homeostasis and the Iron-responsive Element/iron-Regulatory Protein (IRE/IRP) Regulatory Network. Annu Rev Nutr 28: 197-213.

Muhlenhoff U, Stadler JA, Richhardt N, Seubert A, Eickhorst T, Schweyen RJ, Lill R, Wiesenberger G (2003) A specific role of the yeast mitochondrial carriers MRS3/4p in mitochondrial iron acquisition under iron-limiting conditions. J Biol Chem 278: 40612-40620.

Napier I, Ponka P, Richardson DR (2004) Iron trafficking in the mitochondrion: novel pathways revealed by disease. Blood 105:1867-1874.

Nemeth E, Roetto A, Garozzo G, Ganz T, Camaschella C (2005) Hepcidin is decreased in TFR2 hemochromatosis. Blood 105: 1803-1806.

Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, Kaplan J (2004) Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 306: 2090-2093.

Ndisang JF, Tabien HE, Wang R (2004) Carbon monoxide and hypertension. J Hypertens 22: 1057–1074.

Neuzil J and Stocker R (1993) Bilirubin attenuates radical-mediated damage to serum albumin. FEBS Lett 331: 281–284.

Nishizawa M, Kataoka K, Goto N, Fujiwara KT, Kawai S (1989) v-maf, a viral oncogene that encodes a leucine zipper motif. Proc Natl Acad Sci USA 86: 7711–7715.

Ogawa K, Sun J, Taketani S, Nakajima O, Nishitani C, Sassa S, Hayashi N, Yamamoto M, Shibahara S, Fujita H, Igarashi K (2001) Heme mediates derepression of Maf

recognition element through direct binding to transcription repressor Bach1. EMBO J 20: 2835–2843.

Otterbein LE, Zuckerbraun BS, Haga M, Liu F, Song R, Usheva A, Stachulak C, Bodyak N, Smith RN, Csizmadia E, Tyagi S, Akamatsu Y, Flavell RJ, Billiar TR, Tzeng E, Bach FH, Choi AM, Soares MP (2003) Carbon monoxide suppresses arteriosclerotic lesions associated with chronic graft rejection and with balloon injury. Nat Med 9: 183-190.

Pamplona A, Ferreira A, Balla J, Jeney V, Balla G, Epiphanio S, Chora A, Rodrigues CD, Gregoire IP, Cunha-Rodrigues M, Portugal S, Soares MP, Mota MM (2007) Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. Nat Med 13: 703–710.

Ponka P, Beaumont C, Richardson DR (1998) Function and regulation of transferring and ferritin. Semin Hematol 35: 35-54.

Ponka P. Tissue-specific regulation of iron metabolism and heme synthesis: distinct control mechanisms in erythroid cells. Blood. 89: 1-25, 1997.

Ponka P. Cell biology of heme. Am J Med Sci. 318: 241-256, 1999.

Poss KD, Tonegawa S (1997) Heme oxygenase 1 is required for mammalian iron reutilization. Proc Natl Acad Sci USA 94: 10919–10924.

Ryter SW And Tyrrell RM (2000) The Heme Synthesis And Degradation Pathways: Role In Oxidant Sensitivity. Free Radic Biol Med 28: 289-309.

Sato K, Balla J, Otterbein L, Smith RN, Brouard S, Lin Y, Csizmadia E, Sevigny J, Robson SC, Vercellotti G, Choi AM, Bach FH and Soares MP (2001) Carbon monoxide generated by heme oxygenase-1 suppresses the rejection of mouse-to-rat cardiac transplants. J Immunol 166: 4185–4196.

Schacter BA (1988) Heme catabolism by heme oxygenase: physiology, regulation, and mechanism of action. Semin Hematol 25:349 –369.

Schluesener HJ, Kremsner PG, Meyermann R (2001) Heme oxygenase-1 in lesions of human cerebral malaria. Acta Neuropathol (Berl) 101: 65–68.

Schranzhofer M, Schifrer M, Cabrera JA, Kopp S, Chiba P, Beug H, Müllner EW (2006) Remodeling the regulation of iron metabolism during erythroid differentiation to ensure efficient heme biosynthesis. Blood 107: 4159-4167.

Shaw GC, Cope JJ, Li L, Corson K, Hersey C, Ackermann GE, Gwynn B, Lambert AJ, Wingert RA, Traver D, Trede NS, Barut BA, Zhou Y, Minet E, Donovan A, Brownlie A, Balzan R, Weiss MJ, Peters LL, Kaplan J, Zon LI, Paw BH (2006) Mitoferrin is essential for erythroid iron assimilation. Nature 440: 96-100.

Sheftel AD, Zhang AS, Brown C, Shirihai OS, Ponka P (2007) Direct interorganellar transfer of iron from endosome to mitochondrion. Blood 110:125-132.

Shibahara S (1988) Regulation of heme oxygenase gene expression. Semin Hematol 25: 370–376.

Siow RC, Sato H and Mann GE (1999) Heme oxygenase-carbon monoxide signalling pathway in atherosclerosis: Anti-atherogenicactions of bilirubin and carbon monoxide? Cardiovasc Res 41: 385–394.

Snyder SH, Baranano DE (2001) Heme oxygenase: a font of multiple messengers. Neuropsychopharmacology 25: 294 –298.

Solari V, Piotrowska AP, Puri P (2003) Expression of heme oxygenase-1 and endothelial nitric oxide synthase in the lung of newborns with congenital diaphragmatic hernia and persistent pulmonary hypertension. J Pediatr Surg 38: 808–813.

Song R, Mahidhara RS, Zhou Z, Hoffman RA, Seol DW, Flavell RA, Billiar TR, Otterbein LE and Choi AM (2004) Carbon monoxide inhibits T lymphocyte proliferation via caspase-dependent pathway. J Immunol 172: 1220-1226.

Song R, Mahidhara RS, Liu F, Ning W, Otterbein LE and Choi AM (2002) Carbon monoxide inhibits human airway smooth muscle cell proliferation via mitogen-activated protein kinase pathway. Am J Respir Cell Mol Biol 27: 603-610.

Stanford SJ, Walters MJ, Hislop AA, Haworth SG, Evans TW, Mann BE, Motterlini R, Mitchell JA (2003) Heme oxygenase is expressed in human pulmonary artery smooth muscle where carbon monoxide has an anti-proliferative role. Eur J Pharmacol 473: 135–141.

Steiner H, Kispal G, Zollner A, Haid A, Neupert W, Lill R (1996) Heme binding to a conserved Cys-Pro-Val motif is crucial for the catalytic function of mitochondrial heme lyases. J Biol Chem 271: 32605–32611.

Stocker R, Peterhans E (1989a) Synergistic interaction between vitamin E and the bile pigments bilirubin and biliverdin. Biochim Biophys Acta 1002: 238–244.

Stocker R, Peterhans E (1989b) Antioxidant properties of conjugated bilirubin and biliverdin: biologically relevant scavenging of hypochlorous acid. Free Radic Res Commun 6: 57–66.

Stocker R, Glazer AN, Ames BN (1987a) Antioxidant activity of albumin-bound bilirubin. Proc Natl Acad Sci USA 84: 5918 –5922.

Stocker R, Yamamoto Y, McDonagh A, Glazer A and Ames BN (1987b) Bilirubin is an antioxidant of possible physiological importance. Science 235: 1043–1045.

Stocker R, Ames B (1987c) Potential role of conjugated bilirubin and copper in the metabolism of lipid peroxides in the bile. Proc Natl Acad Sci USA 84: 8130–8134.

Sun J, Hoshino H, Takaku K, Nakajima O, Muto A, Suzuki H, Tashiro S, Takahashi S, Shibahara S, Alam J, Taketo MM, Yamamoto M, Igarashi K (2002) Hemoprotein Bach1 regulates enhancer availability of heme oxygenase-1 gene. EMBO J 21: 5216–5224.

Sun J, Brand M, Zenke Y, Tashiro S, Groudine M, Igarashi K (2004) Heme regulates the dynamic exchange of Bach1 and NF-E2-related factors in the Maf transcription factor network. Proc Natl Acad Sci USA 101: 1461–1466.

Tanaka S, Akaike T, Fang J, Beppu T, Ogawa M, Tamura F, Miyamoto Y and Maeda H (2003) Antiapoptotic effect of heme oxygenase-1 induced by nitric oxide in experimental solid tumor. Br J Cancer 88: 902–909.

Tahara T, Sun J, Igarashi K, Taketani S (2004a) Heme-dependent up-regulation of the alpha-globin gene expression by transcriptional repressor Bach1 in erythroid cells. Biochem Biophys Res Commun 324: 77-85.

Tahara T, Sun J, Nakanishi K, Yamamoto M, Mori H, Saito T, Fujita H, Igarashi K, Taketani S (2004b) Heme positively regulates the expression of beta-globin at the locus control region via the transcriptional factor Bach1 in erythroid cells. J Biol Chem 279: 5480-5487.

Torisu-Itakura H, Furue M, Kuwano M and Ono M (2000) Coexpression of thymidine phosphorylase and heme oxygenase-1 in macrophages in human malignant vertical growth melanomas. Jpn J Cancer Res 91: 906–910.

Trimarchi JM, Lees JA (2002) Sibling rivalry in the E2F family. Nat Rev Mol Cell Biol 3: 11–20.

Tsuji MH, Yanagawa T, Iwasa S, Tabuchi K, Onizawa K, Bannai S, Toyooka H and Yoshida H (1999) Heme oxygenase-1 expression in oral squamous cell carcinoma as involved in lymph node metastasis. Cancer Lett 138: 53–59.

Weiss G, Goodnough LT (2005) Anemia of chronic disease. N Engl J Med 352: 1011-1023.

Willis D, Moore AR, Frederick R and Willoughby DA (1996) Heme oxygenase: A novel target for the modulation of the inflammatory response. Nat Med 2: 87–90.

Williams TN, Wambua S, Uyoga S, Macharia A, Mwacharo JK, Newton CR, Maitland K (2005) Both heterozygous and homozygous thalassemias protect against severe and fatal Plasmodium falciparum malaria on the coast of Kenya. Blood 106: 368–371.

Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y, Koizumi S (1999) Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. J Clin Invest 103: 129-135.

Zamora R, Vodovotz Y, Aulak KS, Kim PK, Kane JM 3rd, Alarcon L, Stuehr DJ and Billiar TR (2002) A DNA microarray study of nitric oxide-induced genes in mouse hepatocytes: Implications for hepatic hemeoxygenase-1 expression in ischemia/reperfusion. Nitric Oxide 7: 165–186.

Zenke-Kawasaki Y, Dohi Y, Katoh Y, Ikura T, Ikura M, Asahara T, Tokunaga F, Iwai K, Igarashi K (2007) Heme induces ubiquitination and degradation of the transcription factor Bach1. Mol Cell Biol 27:6962-6971.

Zhang X, Shan P, Jiang D, Noble P, Abraham N, Kappas A, Lee P (2004) Small interfering RNA targeting heme oxygenase-1 enhances ischemia-reperfusion-induced lung apoptosis. J Biol Chem 279: 10677–10684.