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BRIEF ARTICLE

Heme status affects human hepatic messenger RNA and microRNA expression

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Abstract

AIM: To assess effects of heme on messenger RNA (mRNA) and microRNA (miRNA) profiles of liver cells derived from humans.

METHODS: We exposed human hepatoma cell line Huh-7 cells to excess iron protoporphyrin (heme) (10 μmol/L) or induced heme deficiency by addition of 4, 6-dioxoheptanoic acid (500 μmol/L), a potent inhibitor of aminolevulinic acid dehydratase, for 6 h or 24 h. We harvested total RNA from the cells and performed both mRNA and miRNA array analyses, with use of Affymetrix chips, reagents, and instruments (human genome U133 plus 2.0 and miRNA 2.0 arrays). We assessed changes and their significance and interrelationships with Target Scan, Pathway Studios, and Ingenuity software.

RESULTS: Changes in mRNA levels were most numerous and striking at 6 h after heme treatment but were similar and still numerous at 24 h. After 6 h of heme exposure, the increase in heme oxygenase 1 gene expression was 60-fold by mRNA and 88-fold by quantitative reverse transcription-polymerase chain reaction. We found striking changes, especially up-regulation by heme of nuclear erythroid-2 related factor-mediated oxidative stress responses, protein ubiquitination, glucocorticoid signaling, P53 signaling, and changes in RNAs that regulate intermediary metabolism. Fewer mRNAs were down-regulated by heme, and the fold decreases were less exuberant than were the increases. Notable decreases after 24 h of heme exposure were patatin-like phospholipase domain-containing protein 3 (-6.5-fold), neuronal PAS domain protein 2 (-1.93-fold), and protoporphyrinogen oxidase (-1.7-fold).

CONCLUSION: Heme excess exhibits several toxic effects on liver and kidney, which deserve study in humans and in animal models of the human porphyrias or other disorders.

Key words: Delta-aminolevulinic acid synthase; Heme; Heat shock proteins; Hepatotoxicity; Messenger RNA; MicroRNA

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INTRODUCTION

Iron protoporphyrin (heme) is a primordial macrocycle upon which nearly all life on earth depends. It has manifold known functions and diverse properties, but there remains much about its roles and functions that is unknown. The importance of a normal pathway and regulation of heme metabolism is underscored by the seriousness of diseases in which there are defects in heme homeostasis. For example, the porphyrias are a group of diseases in which there are defects in normal heme synthesis, due mainly to inborn errors of metabolism that produce deficient activities of the enzymes of normal porphyrin and heme synthesis[1-4]. Genes and their products of particular importance in heme metabolism are delta-aminolevulinic acid (ALA) synthase 1 (ALAS1) and heme oxygenase 1 (HMOX1), respectively, the rate-controlling enzymes of heme synthesis and catabolism. Regulation of expression of these genes and proteins is complex, but we and others have shown that heme is a major regulator of both, albeit exerting opposite effects. Specifically, heme is a negative regulator of ALAS1 by virtue of decreasing gene transcription, decreasing stability of the messenger RNA (mRNA), decreasing its uptake into mitochondria, where it carries out the synthesis of ALA, and by decreasing the half-life of the mature mitochondrial protein[2,5-7]. In contrast, heme produces rapid and profound up-regulation of the gene for HMOX1, acting to increase gene transcription by virtue of destabilizing repressive dimers of basic leucine zipper transcription factor 1 (BACH1) and small musculo-aponeurotic factor (maf) proteins and increasing nuclear erythroid-2 related factor (Nrf2)-maf dimers[8-11].

HMOX1 has emerged as an important anti-inflammatory and cytoprotective principle, and its up-regulation has been associated with several beneficial effects in diverse systems[2,12]. It is believed to function chiefly by increasing levels of carbon monoxide and biliverdin, which are important signaling and anti-oxidant molecules, respectively. In most mammals, biliverdin is rapidly reduced to the more lipophilic bilirubin, which also has potent anti-oxidant properties and which more readily passes through and dissolves in biological membranes, where it can exert its anti-oxidant and protective functions[13,14]. In addition, HMOX1 will decrease high levels of heme, which itself is a potential strong pro-oxidant, leading to formation of carbon monoxide CO, biliverdin, and iron. The importance of HMOX1 is underlined by the severe pathology of mice or humans with severe HMOX1 deficiencies[15-18].

Intravenous (IV) heme was first used as a therapeutic agent more than 40 years ago, for treatment of acute porphyrias in relapse[19], based upon the understanding that uncontrolled up-regulation of hepatic ALAS1 was a hallmark of acute porphyric attacks and that this enzyme could be dramatically and rapidly down-regulated by administration of heme intravenously. This treatment has stood the test of time, and IV heme continues to be the treatment of choice for all but mild, self-limited attacks of acute porphyria[1,3,4,20,21]. Recently, IV heme, in the form of hematin, was reported to also be of benefit in the treatment of acute pancreatitis in mice[22].

Small, non-coding RNAs, such as microRNAs (miRNAs), have emerged as important modulators of gene expression. They bind to complementary sequences (called "seed sequences") of mRNAs, especially in 3'-untranslated regions, and alter their stability and their translation into proteins. Recently, we reported important novel effects of miRNAs-122, -196, and -let 7 on expression of HMOX1 and its key repressor BACH1[7,23], and we recently discovered new and heretofore unexpected roles of proteasomal and other protease pathways that regulate levels of ALAS1, BACH1, and HMOX1[7,24].

Because of the several effects of heme on hepatic pathways and because of its role as a therapeutic agent, in this work we set out to characterize more nearly completely the comparative effects of heme excess *vs* heme deficiency in human hepatocytes. We performed detailed studies of mRNA and miRNA profiles under these conditions, and we have found evidence for increased oxidative stress and several other changes in metabolic and signaling pathways by heme.

MATERIALS AND METHODS

Chemicals and reagents

Fe protoporphyrin (heme) was purchased from Frontier Scientific (Logan, UT). 4,6-dioxoheptanoic acid (DHA) was from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Fisher Biotech (Fair Lawn, NJ). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), trypsin and TRIzol reagent were from Invitrogen Inc. (Carlsbad, CA).

Cell culture and treatments

Human hepatoma cell line, Huh-7 (Japan Health Research Resources Bank, Osaka, Japan) was cultured with DMEM supplemented with 100 units/mL penicillin, 100 mg/L streptomycin, and 10% (v/v) FBS. All cells were maintained in a humidified atmosphere of 95% room air and 50 mL/L CO_2 at 37 ℃. Freshly prepared heme (dissolved in DMSO) or DHA (dissolved in water) was added to final concentrations of 10 μmol/L or 500 μmol/L, respectively. After 6 h or 24 h at 37 ℃ in 50 mL/L CO_2/950 mL/L room air, cells were harvested and washed with ice cold phosphate buffered saline once, and lysed directly with TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was extracted according to the manufacturer's instructions and stored at -80 ℃ until mRNA and miRNA microarrays were performed.

cDNA microarray profiling

Total RNA samples were reverse transcribed, amplified and labeled using GeneChip® 3' IVT Express Kit (Affymetrix Inc., Santa Clara, CA). The resultant labeled cRNA (complementary RNA) was then purified and fragmented as per the manufacturer's instructions. The cRNA samples together with probe array controls were

Table 1 Confirmation and comparison of selected messenger RNA array results with those of quantitative real time polymerase chain reaction for selected genes

Gene	Array		qRT-PCR	
	Fold change	*P* value	Fold change	*P* value
Heme *vs* DHA-6 h time point				
HMOX1	59.9	9.97×10^{-9}	87.9	10^{-35}
IL-8	20.50	3.92×10^{-5}	13.7	0.016
ANXA1	7.18	1.87×10^{-5}	32.3	0.015
BACH1	3.04	3.26×10^{-5}	3.1	0.0019
Heme *vs* DHA-24 h time point				
HSPB8	77.1	2.66×10^{-6}	92	0.010
ANXA1	45.7	5.98×10^{-6}	138	0.015
IL-8	33.2	4.19×10^{-5}	31.7	0.016
HMOX1	25.6	2.50×10^{-5}	27.6	1.33×10^{-7}
BACH2	4.50	7.24×10^{-5}	22.6	0.011
PNPLA3	-6.48	1.74×10^{-4}	-6.65	4.59×10^{-7}

P values are for comparison of results following treatment with iron protoporphyrin (heme) *vs* 4, 6-dioxoheptanoic acid (DHA). Listing of all messenger RNAs with ≥ 1.5 fold differences in expression at 6 h, 24 h after treatment of Huh-7 cells with heme (10 μmol/L) *vs* DHA (500 μmol/L). Cells were cultured, treated, harvested and total RNA prepared and assays as described in Materials and Methods. qRT-PCR: Quantitative real time polymerase chain reaction; HMOX1: Heme oxygenase 1; IL-8: Interleukin-8; ANXA1: Annexin A1; BACH: Basic leucine zipper transcription factor; HSPB8: Heat shock 22kDa protein 8; PNPLA3: Patatin-like phospholipase domain-containing protein 3.

hybridized onto Affymetrix GeneChip® Human Genome U133 Plus 2.0 arrays. Hybridization controls were spiked into the cRNA samples in order to monitor and troubleshoot the hybridization process. Probes for housekeeping genes were used to assess sample integrity. Hybridization, washing, staining and scanning were performed using Affymetrix GeneChip® system instruments and protocols.

miRNA microarray profiling

The total RNA was Poly (A) tailed and ligated to biotinylated signal molecules using the FlashTag™ Biotin RNA labeling Kit (Genisphere, Llc in Hatfield, PA, United States). An enzyme linked oligosorbent assay quantitative-competitive assay was performed to verify labeling prior to array hybridization to GeneChip® miRNA 2.0 microarrays (Affymetrix, Santa Clara, CA, United States). Hybridization, washing, staining and scanning were performed using Affymetrix GeneChip® system instruments and protocols.

Real-time fluorescent reverse transcription-polymerase chain reaction for quantification of mRNAs

First-strand complementary DNA was synthesized using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, United States). The reverse transcription reaction was incubated at 42 ℃ for 30 min and stopped by heating to 85 ℃ for 5 min. 50 ng of final product was used as template for polymerase chain reaction (PCR). Quantitative reverse transcriptase (qRT)-PCR was performed using TaqMan® Probe-Based Detection (Applied Biosystems, Foster City, CA, United States) per manufacturer's instructions with an ABI Prism 7500 Fast Real-Time PCR System, using Taqman® gene expression assays and Taqman® Gene expression master mix (Applied Biosystems). Template was amplified by 40 cycles of denaturation at 95 ℃ for 15 s, annealing of primers and probe together with extension at 60 ℃ for 1 min in triplicate reactions. Fluorescence data were acquired during a combined anneal/extension step. RT negative reactions were run on each plate to confirm the absence of DNA contamination. Fold change values were calculated using comparative Ct analysis and normalized to those of glyceraldehyde phosphate dehydrogenase, which was an invariant control[25].

Statistical analysis

Affymetrix GeneChip® Command Console Software version 3.0.1 was used to analyze microarray image data and to compute intensity values. Affymetrix. files containing raw, probe-level signal intensities were analyzed using Partek Genomics Suite (Partek, St. Louis, MO, United States). Robust multichip averaging was used for background correction, quantile normalization, and probe set summarization with median polish[26]. Statistical differences were assessed by two-way analysis of variance analysis with false discovery rate. Partek miRNA workflow was used to access TargetScan[27] target prediction databases to perform miRNA-mRNA target integration. Pathway analysis was performed using Ariadne Pathway Studios (Ariadne Genomics, Rockville, MD, United States). The core analysis function in Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, United States) was used for canonical and toxicity pathway analyses. Cluster and Treeview software were used to perform miRNA hierarchical cluster analysis and visualization.

RESULTS

Heme excess, compared to control or to heme deficiency, produced by DHA, produced a number of striking changes, particularly up-regulation of mRNAs in Huh-7 cells, as shown in Figure 1A and B. The changes were most numerous and striking at 6 h after heme treatment (Figure 1A), but also were similar and numerous at 24 h (Figure 1C). After 6 h of heme exposure, the increase in *HMOX1* gene expression was 60-fold ($P = 9.97 \times 10^{-9}$) by mRNA array and 88-fold by qRT-PCR (Table 1). HMOX1 is also known as heat shock protein (HSP)-32. Several other stress-response/heat shock-responsive genes also were markedly up-regulated, including interleukin-8 (*IL-8*), sestrin 2 (*SESN2*), heat shock 22kDa protein 8 (*HSPB8*), stanniocalcin 2, *MAFF*, and annexin A1 (*ANXA1*). mRNAs for lipoprotein receptor and BACH1, the latter a major repressor of HMOX1, were increased 3.9 and 3.0-fold, respectively Table 1 provides a summary list of some of the more striking changes in mRNA levels at 6 h. It also shows results for the more quantitative method of qRT-PCR. Note that, for the genes studied, the fold

Table 2 Summary of data analysis with use of ingenuity pathway

Name	6 h		24 h	
	P value	Ratio	*P* value	Ratio
Top canonical pathways				
Nrf2-mediated oxidative stress response	2.64×10^{-11}	28/193 (0.145)	1.58×10^{-9}	61/193 (0.316)
Protein ubiquitination pathway	1.44×10^{-9}	31/274 (0.133)	3.26×10^{-8}	75/274 (0.274)
Aldosterone signaling in epithelial cells	8.31×10^{-8}	21/170 (0.124)	-	-
Glucocorticoid receptor signaling	1.6×10^{-5}	24/295 (0.081)	-	-
P53 signaling	1.75×10^{-3}	10/96 (0.104)	-	-
Biosynthesis of steroids	-	-	3.22×10^{-7}	14/121 (0.116)
Propanoate metabolism	-	-	1.45×10^{-6}	24/121 (0.198)
Urea cycle and metabolism of amino groups	-	-	5.8×10^{-6}	16/78 (0.205)
Top toxicity lists				
Nrf2-mediated oxidative stress response	3.03×10^{-11}	30/237 (0.127)	5.41×10^{-9}	66/237 (0.278)
Renal necrosis/cell death	1.05×10^{-4}	25/314 (0.08)	1.06×10^{-5}	79/314 (0.252)
Liver necrosis/cell death	1.79×10^{-3}	14/166 (0.084)	-	-
P53 signaling	2.06×10^{-3}	10/95 (0.105)	$1.54E \times 10^{-4}$	30/95 (0.316)
Liver proliferation	2.54×10^{-3}	12/133 (0.09)	-	-
Cholesterol biosynthesis	-	-	2.26×10^{-13}	16/16 (1)
FXR/RXR activation	-	-	5.84×10^{-4}	26/86 (0.302)

P values are for comparison of results following treatment with iron protoporphyrin (heme) *vs* 4, 6-dioxoheptanoic acid (DHA). Listing of all microRNAs with ⩾ 1.5 fold differences in expression at 6 h after treatment of Huh-7 cells with heme (10 μmol/L) *vs* DHA (500 μmol/L). Cells were cultured, treated, harvested and total RNA prepared and assays as described in Materials and Methods. FXR/RXR: Farnesoid X receptor/retinoic X receptor; Nrf2: Nuclear erythroid-2 related factor.

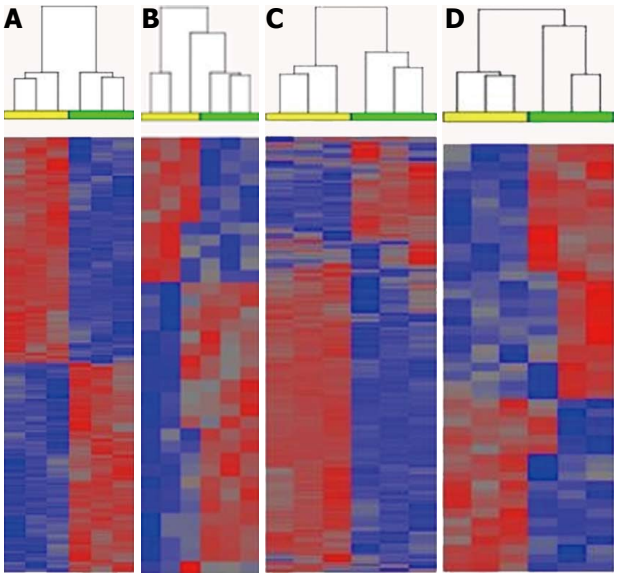

Figure 1 Heat maps with hierarchical clustering of mRNAs (A, C) and microRNAs (B, D) in Huh-7 cells exposed to heme, 10 μmol/L (yellow bars, left side of each panel) or 4, 6-dioxoheptanoic acid, 500 μmol/L (green bars, right side of each panel). A and B: The duration of exposure to heme or DHA was 6 h; C and D: The duration of exposure was 24 h. Cells were cultured, treated, harvested, and RNA arrays performed and analyzed as described in Materials and Methods. Blue color indicates decreased expression; red color indicates increased expression.

differences in expression of mRNAs, comparing heme to DHA treatment, were generally similar by the two methods. The directions of the differences (+ or -) produced by heme *vs* DHA were always the same (because of limited funds and limited amounts of RNA we were able to directly compare results of mRNA arrays and qRT-PCR for only 4-5 genes).

Fewer mRNA levels were lower with heme excess, and the fold decreases were much less exuberant than were the fold increases. After 24 h of exposure, the greatest fold increase by mRNA array was that of *HSPB8* (77-fold), followed by *ANSA1, IL-8, HMOX1, HSPA1A,* and *SESN2* (46 to 11-fold). At 24 h, there was a notable increase in expression of the *BACH1* gene both by mRNA array (4.5-fold increase by heme) and by qRT-PCT (22.6-fold increase by heme). Notable decreases after 24 h of heme exposure were observed for patatin-like phospholipase domain-containing protein 3 (-6.5-fold), neuronal PAS domain protein 2 (-1.93-fold), and protoporphyrinogen oxidase, the penultimate enzyme of the heme biosynthetic pathway (-1.7-fold). Results of some of the striking differences after 24 h of heme excess *vs* heme deficiency are summarized in Table 1, which also again shows generally good agreement between results by mRNA array *vs* qRT-PCR. There also were several changes in miRNA profiles, as shown in Figure 1B and D.

Next, we subjected the mRNA array profiles to analysis by Ingenuity Pathway algorithms. We found striking and highly statistically significant effects at 6 h in Nrf2-mediated oxidative stress response genes, in protein ubiquitination pathway genes, in aldosterone signaling genes in epithelial cells, and in glucocorticoid receptor signaling genes (Table 1). At 24 h, the NRF2-mediated oxidative stress response and the protein ubiquitin pathways continued to show highly significant differences ($P = 1.6 \times 10^{-9}$ and 3.3×10^{-8}, respectively), and pathways involved in biosynthesis of steroids and in propanoate and urea and amino acid metabolism were also significantly affected. Other notable and major effects of heme excess occurred in pathways involved in toxicity, including those that mediate renal necrosis, hepatic necrosis, and

Table 3 Summary of analysis with ingenuity pathway analytical tools

Name	6 h		24 h	
	P value	No. molecules	*P* value	No. molecules
Hepatotoxicity				
Liver proliferation	6.71×10^{-4} - 6.26×10^{-1}	12	4.24×10^{-3} - 1.63×10^{-1}	36
Liver necrosis/cell death	2.08×10^{-3} - 3.77×10^{-1}	15	-	-
Liver damage	3.31×10^{-3} - 1.25×10^{-1}	12	-	-
Liver hemorrhaging	7.28×10^{-3} - 7.28×10^{-3}	2	-	-
Hepatocellular peroxisome proliferation	3.57×10^{-2} - 3.57×10^{-2}	1	-	-
Liver cholestasis	-	-	1.11×10^{-5} - 6.55×10^{-1}	32
Hepatocellular carcinoma	-	-	2.46×10^{-4} -2.46×10^{-4}	54
Liver steatosis	-	-	2.59×10^{-3} - 1.00×10^{-0}	34
Liver fibrosis	-	-	1.51×10^{-2} - 4.43×10^{-1}	19
Nephrotoxicity				
Renal necrosis/cell death	7.22×10^{-5} - 4.21×10^{-1}	25	7.44×10^{-7} - $1.00E \times 10^{-0}$	79
Kidney failure	1.27×10^{-3} - 2.24×10^{-1}	10	2.64×10^{-2} - 6.55×10^{-1}	26
Renal enlargement	1.27×10^{-3} - 1.03×10^{-1}	2	2.64×10^{-2} - 4.13×10^{-1}	2
Renal damage	3.09×10^{-2} - 5.83×10^{-1}	4		
Glomerular	3.57×10^{-2} - 5.17×10^{-1}	2		
Renal tubule injury			4.27×10^{-5} - 1.63×10^{-1}	30
Renal proliferation			2.17×10^{-3} - 4.83×10^{-1}	24

P values for the comparison of iron protoporphyrin (heme) *vs* 4, 6-dioxoheptanoic acid (DHA) are shown. Listing of all microRNAs with ⩾ 1.5 fold differences in expression at 24 h after treatment of Huh-7 cells with heme (10 μmol/L) *vs* DHA (500 μmol/L). Cells were cultured, treated, harvested and total RNA prepared and assays as described in Materials and Methods.

those involved in P53 signaling, liver proliferation, cholesterol biosynthesis, and nuclear receptor (farnesoid X receptor, retinoic X receptor) dependent activation (Table 2).

We examined the pathways of hepatotoxicity and nephrotoxicity in greater detail (Table 3). At 6 h after heme or DHA treatment, 12 molecules involved in liver proliferation, 15 in liver cell damage/necrosis, and 12 in liver damage pathways were markedly affected, whereas, at 24 h, 32 involved in cholestasis, 54 in hepatocellular carcinoma, 34 in hepatic steatosis, and 19 in hepatic fibrosis were increased by heme (Table 3). With respect to molecules involved in pathways of renal damage, many involved in renal cell damage, renal failure, tubular injury and proliferation were up-regulated following heme exposure (Table 2).

Changes in miRNA levels are shown as heat maps in Figure 1B and D. Note that miR-181c, -296-5p, -513a-5p, and-637, were significantly affected by heme, compared with DHA. All were up-regulated by heme, with the exception of miR-513a-5p, which was down-regulated -3.84 fold ($P = 0.034$) at 24 h post-treatment.

Figure 1C and D provides a summary of the major pathways affected by heme excess (*vs* heme deficiency, induced by DHA) in human Huh-7 cells. Note the striking effects on HSPs, on *HMOX1*, on *BACH1* and *BACH2*, and on *FOXP3, CEBP, JUN, MYC, ATF3, GCLO, GDF, IRS2, EGR1*, and lesser but wide-spread up-regulation of expression of *CYP* genes, including *CYP2E1, CY1, 3A4, CYP2B6, CYP2C9, CYP1A2*.

DISCUSSION

The major findings of this work are as follows: (1) heme excess *vs* heme deficiency, produced by exposure of cells to DHA for 6 or 24 h, leads to marked changes in global gene expression in the Huh-7 line of cells derived from a human hepatocellular carcinoma; (2) a large number of genes that are activated by stressful conditions (stress response genes), including several HSPs, Nrf2 and NQ01 are markedly up-regulated by heme; (3) several important transcription factors, including FOS, JUN, MYC, ATF, BACH1, BACH2, SMADs, CEBP, are up-regulated by heme excess; (4) marked up-regulation of NRF2-mediated, protein ubiquitination, steroid signaling, P53 signaling, and renal and liver cell necrosis pathways is produced by heme excess; and (5) heme excess also produces changes in miRNA profiles, which likely contribute to the modulation of mRNA expressions observed (Figure 1).

A deficiency of hepatic heme is well-known to be associated with disease phenotypes, especially the acute porphyrias in relapse, in which there are partial defects in genes and enzymes of heme synthesis, which, with other genetic and environmental factors, lead to a critical deficiency in the regulatory heme pool of hepatocytes and hence to uncontrolled and marked up-regulation of hepatic ALS synthase-1, which normally is the rate-controlling enzyme of heme synthesis[1-4]. Acute attacks of porphyria cause much morbidity loss of productivity, hospitalization, and occasionally acute mortality.

Realization of this cascade of effects led one of us (Bonkovsky HL) to develop heme as a potential therapy for acute porphyria in relapse[19]. This therapy has withstood the test of time and still today. The prompt administration of heme intravenously is the treatment of choice for acute porphyria attacks[1,20,21]. It also has benefits in other diseases in which there are defects in normal heme synthesis or excesses of ALA production,

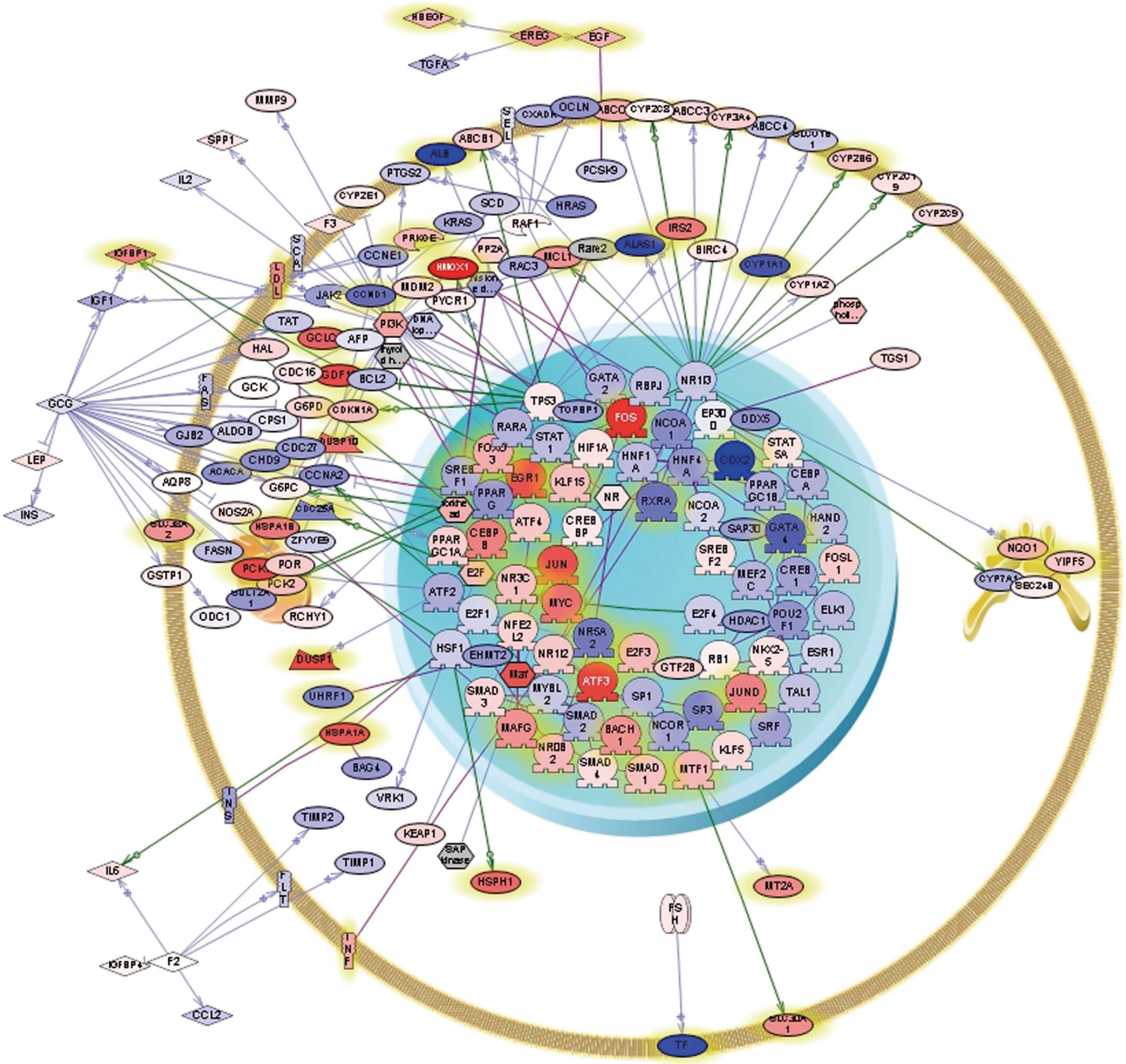

Figure 2 Pathway analysis illustrating differential gene expression in iron protoporphyrin- *vs* 4, 6-dioxoheptanoic acid- treated Huh-7 cells. Cells were cultured and exposed to iron protoporphyrin (10 μmol/L) or 4, 6-dioxoheptanoic acid (500 μmol/L) for 6 h, after which they were harvested and total RNA isolated using TRIzol. Pathway analysis was performed using Ariadne Pathway Studio by defining sub-networks of genes selected using gene set enrichment analysis ($P \leq 0.01$). The resulting sub-networks were combined. Entities satisfying the filtering criteria are depicted. The intensity of red or blue of the entities themselves indicates the corresponding degree of up- or down-regulation, respectively. Differing shapes are used to represent the entity types and the relationships among them.

which likely is neurotoxic[28]. In addition, heme has been of benefit in managing other forms of porphyria, especially congenital erythropoietic (uro)porphyria[29-31] and erythropoietic protoporphyria[32-34].

In addition, as shown previously by us[5,9,12,35,36] and others[37], and as confirmed in this work (Figure 1A and B; Table 1), heme is a potent inducer of the *HMOX1* gene. *HMOX1* is a key cytoprotective and anti-oxidant gene, exerting a myriad of beneficial effects on diverse tissues and in diverse conditions, especially oxidative stress (for reviews see[2,12,38-40]). Indeed, heme has recently been shown to ameliorate experimental pancreatitis in mice[22,41], and similar effects seem likely for other inflammatory diseases, based upon the emerging anti-inflammatory and immunosuppressive effects of carbon monoxide, biliverdin, and bilirubin, the products of the HMOX-catalyzed breakdown of heme[21,42]. Heme has also shown promise for blocking the replication of the hepatitis C virus[23,24,36], at least in part by virtue of its effects BACH1 and HMOX1. The results of this work introduce a note of caution into the therapeutic uses of heme. It may be that heme itself is acting as a pro-oxidant and is increasing oxidative stress and reactive oxygen species, leading to up-regulation of several cytoprotective genes. Thus, heme itself may also be toxic, especially if the doses are too high or if HMOX is deficient[15-17].

The current findings also lend greater weight to the possible usefulness of other means of inducing HMOX1,

such as by cobalt protoporphyrin, which we[9,35] and others[43-45] have shown is a potent and long-acting inducer and which, unlike heme, does not increase oxidative stress nor undergo catabolism by HMOX. Thus, its effects may be achieved with lower doses and for longer times than for heme.

We recognize that our results have limitations: We have performed arrays only of mRNA's and miRNAs, and we have not yet confirmed all major changes and results with qRT-PCR. However, it is reassuring that the qRT-PCR data generally are well correlated with the array data (Table 1). In addition, we have not yet performed detailed proteomic analyses or heme-excess *vs* heme-deficient hepatocytes. However, in a preliminary proteomics study, we have found several changes consistent with our findings. Specifically, at 18 h of heme treatment (10 μmol/L) *vs* the vehicle, DMSO, we found sixty-six proteins differentially expressed to a highly significant degree. Among these, a total of 24 were decreased, whereas 42 were increased in expression, in keeping with the greater up-regulation of mRNAs by heme, noted above. Among those most strongly increased in expression were HMOX1, ubiquitin and ribosome protein S27a precursor, retinal dehydrogenase, HSP70, ferritin light chain, and ferritin heavy chain. In a similar comparison of DHA (500 μmol/L *vs* DMSO), 5 proteins were significantly decreased in expression, namely, ATIC, CCT8, GANAB, EN01, and XP07. Thus, there are similarities in the mRNA and protein results, notably HMOX1 and other HSPs. The increases in ferritin light and heavy chain peptides, but not mRNAs, are in keeping with the known post-transcriptional up-regulatory effects of iron on ferritin chain synthesis[46,47]. One expects that heme treatment will lead to increases in free iron in hepatocytes, as a result of the action of HMOX[1,12].This will be the subject of a later manuscript in this series (Figure 2).

In summary, heme excess in a human hepatocyte line produces manifold changes in mRNA and miRNA profiling, including especially effects on oxidative/stress response, protein ubiquitination, steroid signaling (aldosterone, glucocorticoids), cholesterol, propanoate, urea, and amino acid metabolism. These findings emphasize that additional and more in-depth studies of effects of heme on the transcriptome and proteome are needed and that heme should be used with due caution as a treatment of human disease.

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COMMENTS

Background

Heme is used as therapy for acute porphyria in relapse and for other conditions of iron protoporphyrin (heme) deficiency. Because it is a potent inducer of heme oxygenase-1, a major cytoprotective enzyme, heme is proposed as a drug that may benefit a variety of acute or chronic inflammatory conditions. Heme has also been found recently to bind to and influence levels and activities of several proteins involved in circadian rhythms and intermediary metabolism of carbohydrates and lipids.

Research frontiers

Although heme is a primordial molecule upon which aerobic life as the authors know it depends, the myriad effects of heme on gene and protein expression and metabolic and circadian pathways remain imperfectly understood.

Innovations and breakthroughs

In this work, authors have carried out detailed analyses of alterations caused by heme excess vs heme deficiency on messenger RNA and microRNA profiles in the Huh-7 liver cell line derived from a human subject. They also have performed exploratory measures of effects of heme on the proteome of these cells.

Applications

Their results have important implications especially for cautions regarding the use of heme as a therapeutic agent in humans.

Terminology

Heme is one of the class of metalloporphyrins, which include cobalt protoporphyrin, tin- and zinc porphyrins, an others. The circadian rhythm proteins are proteins that influence the normal daily sleep-wake and other 24 h cycles upon which life on earth depends.

Peer review

The data highlight significant heme-dependent alterations in biochemical pathways, which could contribute in understanding aspects of pharmacological heme toxicity. The findings are appropriately discussed and potential limitations are acknowledged. The study is relatively well-done and the results of the microarray approach are convincing.

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