Heavy Chain Ferritin Acts as an Anti-Apoptotic Gene That Protects Livers From Ischemia Reperfusion Injury

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ABSTRACT

Heme oxygenase-1 (HO-1) is induced under a variety of pro-oxidant conditions such as those associated with ischemia reperfusion injury (IRI) of transplanted organs. HO-1 cleaves the heme porphyrin ring releasing Fe^{2+}, which induces the expression of the Fe^{2+} sequestering protein ferritin. By limiting the ability of Fe^{2+} to participate in the generation of free radicals through the Fenton reaction, ferritin acts as an anti-oxidant. We have previously shown that HO-1 protects transplanted organs from IRI. We have linked this protective effect with the anti-apoptotic action of HO-1. Whether the iron binding properties of ferritin contributed to the protective effect of HO-1 was not clear. We now report that recombinant adenovirus mediated over-expression of the ferritin heavy chain (H-ferritin) gene protects rat livers from IRI and prevents hepatocellular damage upon transplantation into syngeneic recipients. The protective effect of H-ferritin is associated with the inhibition of endothelial cell and hepatocyte apoptosis *in vivo*. H-ferritin protects cultured endothelial cells from apoptosis induced by a variety of stimuli. These findings unveil the anti-apoptotic function of H-ferritin and suggest that H-ferritin can be used in a therapeutic manner to prevent liver IRI and thus maximize the organ donor pool used for transplantation, despite prolonged periods of cold ischemia.

INTRODUCTION

Ischemia/reperfusion injury (IRI) accounts in large measure for early dysfunction or non-function of transplanted organs and is thought to contribute to the acute and/or chronic events that can lead to graft rejection. The pathogenesis of IRI involves primarily components of the host innate immune system such as activation of the complement cascade as well as neutrophil and monocyte-macrophage activation (1, 2). A number of putative factors in the graft itself have been implicated in the pathogenesis of IRI. These include alteration of calcium concentration, ATP depletion and free radical mediated cell damage (3). In the case of transplanted livers, IRI can result in very significant levels of apoptosis, which presumably contributes to or accounts for primary graft non-function associated with prolonged IRI (2). We have recently demonstrated that over-expression of the stress responsive gene heme oxygenase-1 (HO-1) exerts potent cytoprotective effects in experimental models of hepatic (4), cardiac (5) and renal (6) IRI in rats. However, the putative mechanisms by which HO-1 leads to cytoprotection during IRI remain unclear.

Heme oxygenases (HOs) are ubiquitous enzymes that catalyze the initial and rate-limiting steps in the oxidative degradation of heme to bilirubin (7). HOs cleave a mesocarbon of the heme molecule, producing equimolar quantities of biliverdin, iron, and carbon monoxide (CO). Biliverdin is reduced to bilirubin, by bilirubin reductase, and the free iron released from heme is used in intracellular metabolism and induces the expression of the iron chelator ferritin, by which it is sequestered (8). Three HO isoforms have been identified: HO-1 (an inducible heat shock protein, i.e. hsp32), HO-2, (a constitutive form expressed primarily in brain and testis), and a less well-characterized HO-3. HO-1 is the only inducible form of the HOs and confers potent cytoprotective effects in the context of organ transplantation (*reviewed in* (9)). At a cellular level the mechanism(s) by which HO-1 confers cytoprotection against oxidative stress was linked

initially to its ability to induce the expression of ferritin (10).

Ferritin is a ubiquitous protein forming a shell consisting of 24 symmetrically related subunits. Two different subunits are known: a heavy (H) and a light (L) chain. Ferritin has a high capacity to store free iron by oxidizing Fe^{2+} to Fe^{3+}, and thus acts as an effective anti-oxidant molecule by reducing the availability of intracellular free Fe^{2+} that can participate in the generation of free radicals through the Fenton reaction (11). This property of ferritin is attributed primarily to the heavy chain subunit (H-ferritin), the only one that has ferroxidase activity (12). Expression of HO-1 is associated with up-regulation of H-ferritin, which is thought to contribute to the ability of HO-1 to protect endothelial cells (EC) from the cytotoxic effects of activated neutrophils and H_2O_2 (10) as well as from oxidized low-density lipoproteins (13). Other molecules that chelate free iron (e.g., deferoxamine mesylate) have also been shown to prevent EC from undergoing apoptosis (14), suggesting that H-ferritin may have similar effects.

In the present study we tested a hypothesis that expression of H-ferritin contributes in a critical manner to the effect of HO-1 in terms of protecting cells from apoptosis and transplanted organs from IRI. We have obtained several independent lines of evidence suggesting that this indeed is the case.

METHODS

Animals. Inbred male Sprague Dawley (SD) rats weighing 200 to 250 g (Harlan Sprague Dawley, Indianapolis, IN) were used throughout this study. Rats were fed standard rodent chow and water *ad libitum*, and cared for according to guidelines approved by the American Association of Laboratory Animal Care.

Cell culture. The murine 2F-2B EC line (American Type Culture Collection, ATCC # CRL-2168, Rockville, MD) and primary Bovine Aortic ECs (BAEC) were cultured as described before (15, 16).

Cell treatment and reagents. Actinomycin D (Act.D; Sigma Chemical CO., Saint Louis, MO) was dissolved in sterile PBS and added to the culture medium, 24 hours after transfection. Act.D concentration used corresponded to the optimal concentration necessary to sensitize EC to TNF-α mediated apoptosis, e.g. 10 μg/ml for 2F-2B EC and 0.1 μg/ml for BAEC. When indicated, EC apoptosis was induced by etoposide (200 μM, 8 hours) (Sigma Chemical CO) or by serum deprivation (0.1% FCS for 24 hours). The iron chelator deferoxamine mesylate (DFO) (Sigma Chemical CO) was dissolved in water and added to culture medium (1-100 μM). Iron protoporphyrin (FePP/heme) and tin protoporphyrin (SnPPIX) (Porphyrin Products Inc., Logan, UT) were dissolved (10 mM) in 100 mM NaOH and stored at -20^{o} C until used. Metalloporphyrins were added to the culture medium (50 μM), 6 hours after transfection. Human recombinant TNF-α (R&D, Systems, Minneapolis, MN) was dissolved in PBS, 0.1% bovine serum albumin and added to the culture medium (10-100 ng/ml), 24 hours after transfection.

CO exposure. Cells were exposed to varying concentration of CO (250 and 10,000 ppm), as described elsewhere (14).

Expression plasmids. β-galactosidase cDNA (Clontech Laboratories, Palo Alto, CA) was cloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA), as described before (15). A 1.0-kbp X*ho*I-H*ind*III fragment encoding the rat full length HO-1 cDNA was cut from the prHO-1 vector (17) and sub-cloned into the pcDNA3 vector in order to achieve expression of the HO-1 cDNA under the control of the cytomegalovirus (CMV) enhancer/promoter (pcDNA3/HO-1). A 0.6-kbp E*co*RI-B*am*HI fragment encoding the full-length human H-ferritin cDNA was cut from the pSG5-H Ferritin vector (kind gift from Dr. P. Arosio, University of Brescia, Brescia, Italy), and sub-cloned into pcDNA3.

Transient transfections. BAEC and 2F-2B EC were transiently transfected, as described elsewhere (15, 16, 18). $3x10^5$ cells were culterd in 35-mm wells and transfected 24h later using Lipofectamine (Life Technologies, Galthersburg, MD) according to the manufacturer's suggestion (1.5–2 mg DNA/ 4-6 ml Lipofectamine, 5-6h). All experiments were carried out 24 or 48h after transfection. Percentage of viable cells was assessed by evaluating the number of β-galactosidase expressing cells that retained normal morphology (19-21). The number of random fields counted was determined to have a minimal of 200 viable transfected cells per control well. The percentage of viable cells was normalized, for each DNA preparation, to the number of transfected cells counted in the absence of the apoptosis inducing agent (100% viability). All experiments were performed at least three times in duplicate.

Adenovirus. The recombinant β-galactosidase adenovirus was a gift of Dr. Robert Gerard (University of Texas Southwestern Medical Center, Dallas, Texas). The replication defective adenovirus type V (a kind gift of Dr. Robert Gerard, University of Texas Southwestern Medical Center, Dallas, Texas) was used to generate recombinant vectors. The recombinant adenovirus bearing the gene for human H-chain of ferritin was constructed by a homologous recombination

in 293 cells as described earlier (22). Briefly, cDNA coding for H-chain of ferritin (kind gift from Dr. P. Arosio, University of Brescia, Brescia, Italy) was cloned into the pAC.CMV-pLpASR^{+} vector (23, 24) and cotransfected into 293 cells with the pJM17 recombinant plasmid containing the full length adenoviral genome with a deletion of the E1 region (25). Adenoviruses were produced, extracted, purified, and titrated, as described previously (25). Confluent BAECs were infected with a multiplicity of infection of 200 plaque forming units (pfu) per cell (pfu/cell), as described elsewhere (16).

Ex-vivo liver perfusion. Weight matched SD rats received either no treatment, H-Ferritin adenovirus vector ($2.5x10^9$ pfu iv), or β-galactosidase adenovirus vector ($2.5x10^9$ pfu iv) (n=6 rats/group.). Livers were harvested 48h later, stored for 24h at 4°C in University of Wisconsin (UW) solution, and then perfused by the rat blood for 2h on an isolated perfusion rat liver apparatus under stable temperature (37° C), flow pressure (13 cm H_2O), and pH (7.3-7.4). Portal venous blood flow, bile production, and serum AST and ALT levels were assessed serially according to a previously described method (4). At the conclusion of experiment, liver samples were collected for histological evaluation and myeloperoxidase (MPO) activity according to a previously described method (26).

Rat orthotopic liver transplantation. Donor SD rats were either untreated, pretreated with H-Ferritin or β-galactosidase adenoviruses ($2.5x10^9$ pfu iv). Livers were harvested 48h later, stored for 24 h at 4^0 C in UW solution, and grafted orthotopically into SD rat recipients (n=6 rats/group). Orthotopic liver grafting was carried out by utilizing Kamada's method with modifications (27). Liver recipients were monitored daily up to 2 weeks post-transplantation, and their survival was assessed.

Histology. Liver samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with Hematoxylin and Eosin. The Suzuki's criteria were used to evaluate the histologic severity of hepatic IRI (28). In this classification, sinusoidal congestion, hepatocyte necrosis and ballooning degeneration are graded from 0 to 4. No necrosis, congestion or centrilobular

ballooning is given a score of 0, while severe congestion and ballooning degeneration as well as > 60% lobular necrosis is given a value of 4 (28).

Detection of apoptosis. Liver specimens were processed for immunostaining using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) technique, as described (29). Accumulated internucleosomal DNA fragments (apoptosis) were detected serially on cardiac sections (4 μm in thickness) using an TACS 2 TdT in situ apoptosis detection kit (Trevigen™, Inc. Gaithersburg, MD).

Cell extracts and Western blot analysis. Cell extracts were prepared, electrophoresed under denaturing conditions (10-12.5% polyacrylamide gels) and transferred on polyvinyldifluoridine (PVDF) membranes (Immobilon P, Millipore, Bedford, MA), as described elsewhere (14). HO-1 was detected using a rabbit anti-human HO-1 polyclonal antibody (StressGen, Biotechnologies Corp., Victoria, CA). H-ferritin was detected using a rabbit anti-human ferritin polyclonal antibody (Dako Corp., Carpinteria, CA). β-tubulin was detected using a murine anti-human β-tubulin monoclonal antibody (Boehringer Mannheim, Indianapolis, IN). Primary antibodies were detected using horseradish peroxidase conjugated donkey anti-rabbit or goat anti-mouse IgG secondary antibodies (Pierce, Rockford, IL). Peroxidase activity was visualized using the Enhanced ChemiLuminescence Assay (Amersham Life Science Inc., Arlington Heights, IL), according to manufacturer's instructions and stored in the form of photoradiographs (Biomax™MS, Eastman Kodak, Rochester, NY). Digital images were obtained using an image scanner (Arcus II, Agfa, Ridgefield Park, NJ) equipped with FotoLook and Photoshop software. The amount of H-ferritin expression was quantified using ImageQuant software (Molecular Dynamics, Sunny Vale, CA).

Statistical analysis. All results were calculated as the mean ± standard deviation (mean ± SD). The unpaired student's t-test was applied to analyze differences between the groups. For the comparison of the graft survival, the Mann-Whitney U test was used. All differences were considered statistically significant at the p-value of <0.05.

RESULTS

H-Ferritin is a product of endogenous heme catabolism by HO-1.

We have confirmed that up-regulation of endogenous HO-1 in EC by iron protoporphyrin (Hemin/FePP) is associated with a concomitant up-regulation of ferritin expression (Fig. 1A and B)(10). The murine EC line (2F-2B) or primary bovine aortic EC (BAEC) were exposed for 24h to increasing concentrations of FePP; HO-1 and ferritin protein expression was analyzed by Western blot. Untreated EC were used as controls. Increasing concentrations of FePP resulted in higher levels of HO-1 and ferritin expression (Fig. 1). Similar results were obtained in BAEC (Fig. 1A) and murine 2F-2B cells (Fig. 1B). Hemin-stimulated ferritin up-regulation in EC requires the release of free iron from heme as illustrated by the observation that iron chelation by DFO prevented heme stimulated ferritin expression (Fig. 1B).

H-ferritin protects endothelial cells from undergoing apoptosis.

Control EC (pcDNA3 transfected) showed 60-70% apoptotic cells after exposure to TNF-α in the presence of the transcription inhibitor Act. D. (Fig. 2A). Expression of pcDNA3/H-ferritin suppressed TNF-α mediated EC apoptosis (10% apoptotic EC) (Fig. 2A). This protective effect was observed in the EC line 2F-2B as well in primary BAEC (Fig. 2A). The anti-apoptotic effect of H-ferritin was also observed with other pro-apoptotic stimuli such as etoposide or serum deprivation (Fig. 2B). This effect was dose-dependent, showing protection from 1 ng to 100 ng of the pcDNA3/H-ferritin expression vector per 3 x 10^5 cells. A level of pcDNA3/H-ferritin over 100 ng led to the loss of its protective effect, as evidenced by an increasing number of apoptotic cells in TNF-α/Act.D stimulated cells, as well as in control cells (H-ferritin plus Act.D only)

(Fig. 2C). Therefore all subsequent experiments were carried out using H-ferritin in the protective range (1-100 ng per 3 x 10^5 cells); i.e. at doses lower than any noted to cause toxicity.

In the absence of HO-1 activity H-Ferritin protects EC from undergoing apoptosis.

Blocking HO-1 activity with SnPPIX suppressed HO-1-derived ferritin expression (*data not shown*). The absence of H-ferritin under such conditions also led to the loss of the protective effect of HO-1 (Fig. 3A). In contrast, SnPPIX did not impair the anti-apoptotic effect of H-ferritin expression secondary to transient transfection (Fig. 3A) or exogenously administered CO (10,000 ppm) (Fig. 3B).

H-Ferritin and CO have additive protective activity in EC.

As shown above, H-ferritin makes a significant contribution to the anti-apoptotic function of HO-1. We have previously shown that CO also has anti-apoptotic properties (14, 30). Therefore we asked whether these two products of HO-1 activity would complement each other in their protective function. EC were transfected with various amounts of H-ferritin ranging from sub-optimal (0.1ng per 3 x 10^5 cells) to optimal (1ng per 3 x 10^5 cells) doses. Cells were separately exposed to low level of exogenous CO (250 ppm), which protects EC from apoptosis to only a minor extent (30-40% apoptotic cells)(Fig. 4). The combination of exogenous CO plus low dose H-ferritin expression protected EC to almost 100%, even though each component was used at a sub-optimal dose (Fig. 4). This is especially apparent at 0.1 ng/ per 3 x 10^5 cells of pcDNA3/H-ferritin (which alone showed no significant protective effect) plus CO, with the combination showing protection significantly above CO alone.

Recombinant adenovirus mediated H-ferritin expression protects livers from ex-vivo reperfusion injury.

Rat livers exposed to prolonged cold ischemia (UW solution, 4^{o}C, 24h) showed severe signs of injury once re-perfused ex-vivo with whole syngeneic blood (4). Liver injury was evidenced by the relative low increase in portal blood flow (from 0.63 ± 0.076 ml/min/g at time 0 to 1.13±0.23 at ml/min/g at 120 min)(Fig. 5A) and bile production (from 0.00338±0.0078 ml/g at time 0 to 0.025±0.01 ml/g at 120 min)(Fig. 5B) following reperfusion as well as by a significant increase in ALT release (from 7.2 ± 4.9 IU/l at time 0 to 173±71 IU/l at 120 min)(Fig. 5C). Similar results were obtained in β-galactosidase recombinant adenovirus transduced livers in that there was a similar level of portal blood flow (from 0.626±0.079 ml/min/g at time 0 to 1.035±0.105 ml/min/g at 120 min) and relative low level of bile production (from 0.0005±0.0002 ml/g at time 0 to 0.03±0.0085 ml/g at 120 min) as well as significant ALT release (from 12.5 ± 10.5 IU/l at time 0 to 148 ± 92 IU/l at 120 min) (Fig. 5). Unlike untreated or β-galactosidase transduced groups, H-ferritin transduced livers showed a significant ($p<0.01$) increase in portal blood flow (from 0.62±0.099 ml/min/g at time 0 to 1.372±0.133 ml/min/g at 120 min)(Fig. 5A) and bile production (from 0.00621±0.0029 ml/g at time 0 to 0.043±0.0088 at 120 min)(Fig. 5B) while ALT release (from 9.3±4.5IU/l at time 0 to 68.6±14.8 IU/l at 120 minutes) (Fig. 5C) remained at relatively low levels. At 2 hours of reperfusion, MPO activity, a marker of neutrophil mediated oxidative stress injury was significantly inhibited (p,0.05) in H-ferritin transduced livers (0.736±0.58 units/g), as compared to untreated (1.35±0.227 units/g) or β-galactosidase transduced (3.12±0.9 units/g) livers (Fig. 5D). Taken together, these results support the notion that over-expression of H-ferritin protects livers from IRI as evidenced by amelioration of hepatocellular damage upon ex-vivo reperfusion, despite prolonged periods of cold ischemia.

Livers transduced with the H-ferritin gene also had a significantly better preservation of their histological detail, as compared to non-transduced ($p<0.05$) or β-galactosidase ($p<0.05$) transduced livers, as assessed by standard Suzuki's pathological scoring (Fig. 6). H-ferritin transduced livers had relatively lower levels of congestion, centrilobular ballooning, and necrosis as compared to non-tranduced or β-galactosidase transduced livers (Fig. 6).

Recombinant adenovirus mediated H-ferritin expression prevents IRI following orthotopic liver transplantation.

Livers from SD rats exposed to prolonged cold ischemia (U.W. solution, 4^{o}C, 24 hours) showed severe signs of hepatocellular damage following transplantation into syngeneic SD recipients (Fig. 7). This was evidenced by the high serum levels of AST 24 hours post-transplant (3928±1455 IU/L)(Fig. 7). Similar results were obtained in liver transplant recipients transduced with a β-galactosidase recombinant adenovirus (4887±500 IU/L)(Fig. 7). In marked contrast, in animals bearing liver transplants transduced with the H-ferritin recombinant adenovirus AST release (1368±550.8 IU/L) was significantly inhibited as compared to β-galactosidase/untreated groups ($p< 0.05$) (Fig. 7).

That H-ferritin transduced livers were protected from IRI is strongly supported by the demonstration that up to 90% of livers transduced with H-ferritin survived long-term (>14days) when transplanted into syngenic SD recipients. In marked contrast, only 40-50% of non-transduced or β-galactosidase transduced livers survived >14 days when transplanted into syngenic SD recipients (Fig. 8). The relative number of cells undergoing apoptosis in H-ferritin transduced livers transplanted into syngeneic recipients was significantly reduced as compared to non-transduced or β-galactosidase transduced livers transplanted under the same conditions (Fig.

9). This finding is consistent with the hypothesis that the anti-apoptotic effect of H-ferritin may account for its overall cytoprotective function in transplanted livers.

DISCUSSION

It has been well established that the expression of the cytoprotective gene HO-1 protects a variety of cell types from undergoing apoptosis (21, 31). The enzymatic action of HO-1 on heme engenders the release of the gas CO (32), which can account for at least some of the anti-apoptotic effect of HO-1 (14). Several other studies have suggested the importance of the regulation of the free intracellular iron (Fe^{2+}) pool for the anti-apoptotic effect of HO-1 (31, 33, 34).

Free intracellular Fe^{2+} can be cytotoxic by its capacity to promote the generation of $OH^{\cdot}$ and OH^{-} species through the Fenton reaction (11, 35). The action of HO-1 on heme releases Fe^{2+} that could act in such a way. However, heme derived Fe^{2+} activates an ATPase iron pump that increases Fe^{2+} efflux from cells and thus decreases the intra-cellular pool of free Fe^{2+} (31). Activation of this Fe^{2+} pump is thought to contribute to the anti-apoptotic action of HO-1 (31). In addition, free Fe^{2+} released through heme degradation also leads to the rapid induction of ferritin expression (8, 10) (Fig. 1). Presumably, sequestration of Fe^{2+} by ferritin devoid the Fe^{2+} from participating in the Fenton reaction and thus inhibits the pro-oxidant capacity of intracellular Fe^{2+}. In different cell types, over-expression of ferritin protects from stimuli such as H_2O_2 (10) ultraviolet irradiation and oxidized low-density lipoproteins (13).

The mechanism underlying the protective effect of ferritin is not clear. We demonstrate here that H-ferritin protects EC from undergoing apoptosis (Fig. 2). This protective effect is dose-dependent and is observed for several pro-apoptotic agonists such as TNF-α, etoposide and serum deprivation (Fig. 2). We have tested specifically the anti-apoptotic action of the H-chain of ferritin, as only the H-chain manifests ferroxidase activity and the L-chain is not able to counteract iron oxidation (12, 36). By suppressing endogenous HO-1 activity with SnPPIX, we

showed that the anti-apoptotic effect of H-ferritin does not require endogenous HO-1 activity (Fig. 3).

Similarly to HO-1(37), H-ferritin shows toxic effects at high concentrations (Fig. 2). This is consistent with studies showing the potential pro-oxidant nature of ferritin caused by released reactive iron (38, 39). However, our data suggest that H-ferritin has protective effects in a very wide range (approximately 100 fold) before becoming toxic whereas HO-1 has a much narrower therapeutic range (approximately four-fold, *data not shown*).

At low doses, at which H-ferritin showed no significant anti-apoptotic effect and CO only a minor one, the combination of the two protected EC to nearly 100% (Fig. 4). This suggests that these two end products of HO-1 action on heme can act together in an additive, perhaps synergistic manner. Given that HO-1 and ferritin are toxic at high levels, and given the known harmful effects of CO at high concentrations (formation of carboxyhemoglobin and inhibition of mitochondrial respiration), the use of these two molecules, at low non-toxic doses, might be better for *in vivo* therapeutic applications.

Based on these findings we asked whether the anti-apoptotic action of H-ferritin could be used in a therapeutic manner to prevent the deleterious effects resulting from acute inflammatory conditions. We have tested this hypothesis in an experimental model of liver IRI because of the key role of apoptosis in this type of injury. We found that in recombinant H-ferritin adenovirus transduced livers exposed to prolonged cold ischemia there was a significant protection from injury caused by ex-vivo reperfusion with whole blood, as compared to non-transduced or β-galactosidase recombinant adenovirus transduced livers (Fig. 5). This notion is supported by the observation that H-ferritin transduced livers had higher levels of portal blood flow and bile production (two markers of liver function in this experimental system), as compared to non-

transduced or β-galactosidase transduced livers (Fig. 5). In addition, H-ferritin transduced livers also had significant lower levels of ALT release (a marker of hepatocellular injury) and MPO accumulation (a marker of neutrophil activity) as well as a significantly better preservation of morphological structure as compared to non-transduced or β-galactosidase transduced livers (Fig. 5 and 6).

We found that the protection of livers from IRI, conferred by the expression of the H-ferritin gene, was still observed upon liver transplantation (Fig. 7, 8 and 9). Indeed, the survival rate of syngeneic recipients transplanted with H-ferritin transduced livers was 90% while that of syngeneic recipients transplanted with non-transduced or β-galactosidase transduced livers was only 40-50% (Fig. 8). The higher rate of survival in recipients transplanted with H-ferritin transduced livers was consistent with the significantly improved hepatocyte function (Fig. 7) as well by a lower relative number of cells undergoing apoptosis (Fig. 9), as compared to non-transduced or b-galactosidase transduced livers.

The molecular mechanism by which the expression of H-ferritin prevents apoptosis and protects livers from IRI is not clear. A number of studies have suggested that accumulation of free intracellular Fe^{2+} can exacerbate the deleterious effects of oxidative stress such it occurs during liver IRI (40, 41). For example intracellular free Fe^{2+} primes the activation of the transcription factor nuclear factor kappa B (NF-κB) in Kupffer cells (40) and probably in EC as well. Activation of NF-κB contributes to the expression of pro-inflammatory genes such the cytokine TNF-α in monocyte macrophages (42) and E-selectin, P-selectin ICAM-1 and VCAM-1 in EC (22). Expression of these genes promotes the recruitment and activation of circulating leukocytes, which exacerbates the deleterious effects of IRI. Given the above, Fe^{2+} chelation and/or specific inhibition of the expression of these pro-inflammatory genes should prove

beneficial in terms of preventing liver IRI. This notion is strongly supported by our recent demonstration that inhibition of P-selectin function by administration of recombinant P-selectin glycoprotein ligand immunoglobulin, a glycoprotein that binds to P-selectin and inhibits neutrophil adhesion, confers partial protection against liver IRI (2, 43). Moreover, the combination of P-selectin inhibition with Fe^{2+} chelation confers a stronger level of protection in these livers against IRI. Presumably expression of H-ferritin chelates free Fe^{2+} in a manner that suppresses the oxidative burst required for the activation of NF-κB and thus inhibits at least to some extent the expression of NF-κB dependent pro-inflammatory genes. It is not clear from our studies whether the Fe^{2+}chelating effect of H-ferritin is sufficient to act in such a manner. There are, however, observations supporting such a role. Fe^{2+} chelation by deferoxamine mesylate suppresses NF-κB activation (*data not shown*)(44) as well as the expression of NF-κB dependent genes in EC (*data not shown*). Based on its ability to suppress the activation of the transcription factor NF-κB, H-ferritin would be protective in livers exposed to IRI. This is supported by the observation that specific inhibition of NF-κB activation by recombinant adenovirus mediated over- expression of IκBα, the natural inhibitor of NF-κB, protects livers from IRI (*data not shown*).

If there were no additional effects of H-ferritin beside its ability to modulate NF-κB activation, then H-ferritin would sensitize liver heptocytes to TNF-α mediated apoptosis, since NF-κB activation is required for the expression of anti-apoptotic genes that protect these cells from TNF-α mediated apoptosis (45). Based on this reasoning, we hypothesized that H-ferritin expression in the liver must have an anti-apoptotic effect as well. Our data supports this hypothesis, both *in vitro* (Fig. 2) and *in vivo* (Fig. 9). Presumably this anti-apoptotic effect of H-ferritin contributes to the overall protection of livers against IRI, consistent with our most recent

data using IDN-6556, an irreversible caspase-3 inhibitor which also protects livers from IRI in the same experimental model (*unpublished observation*).

In conclusion, our present studies provide evidence that H-ferritin has a potent anti-apoptotic function. In addition, we show that H-ferritin can be used therapeutically to protect livers from IRI.

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FIGURE LEGENDS

Figure 1. Up-regulation of HO-1 by hemin leads to H-ferritin expression. (A) BAEC were incubated with increasing concentrations of FePP (Hemin; 3-100 μM) for 24h. Ferritin, HO-1 and α-tubulin were detected by western blot. The same membrane was used for all immunochemical detections. NT, non-treated. **(B)** Corresponding western blot with 2F-2B. **(C)** 2F-2B cells were incubated with FePP (Hemin; 50 μM) and DFO (10 mM) for 24h. Ferritin, HO-1 and α-tubulin were detected by western blot. the membrane was used for all immunochemical detections.

Figure 2. H-Ferritin protects EC from apoptosis. (**A**) Two different EC systems were used: murine 2F-2B EC line and primary bovine aortic EC (BAEC). EC were cotransfected with β-galactosidase plus control (pcDNA3 or pcDNA3/HO-1) or pcDNA3/H-Ferritin. EC apoptosis was induced by TNF-α plus Act.D and apoptosis was quantified as described in Methods. Gray bars represent EC treated with Act.D and black bars represent EC treated with TNF-a and Act.D. One representative experiment out of six are shown. (**B**) 2F-2B were cotransfected with β-galactosidase plus control (pcDNA3) or pcDNA3/H-ferritin. Gray bars represent untreated EC and black bars represent EC treated with etoposide (200 mM, 8h) or subjected to serum deprivation (0.1% FCS, 24h). One representative experiment out of three is shown. (**C**) 2F-2B were cotransfected with β-galactosidase plus increasing amounts of pcDNA/H-ferritin. As controls pcDNA3 and pcDNA3/HO-1 was used. EC apoptosis was induced by TNF-α plus Act.D and the apoptosis of β-galactosidase transfected EC was quantified. One representative experiment out of three is shown. All results shown are the mean ± SD from duplicate wells.

Figure 3. H-Ferritin and CO protect EC in absence of HO-1 activity. (A) 2F-2B were cotransfected with β-galactosidase plus control (pcDNA3 or pcDNA3/HO-1) or pcDNA/H-ferritin) expression vectors. Gray bars represent EC treated with Act.D alone and black bars represent EC treated with TNF-α and Act.D. When indicated (+) HO-1 enzymatic activity was inhibited by SnPPIX. **(B)** When indicated (+) 2F-2B were exposed to exogenous CO (10,000 ppm). All results shown are the mean ± SD from duplicate wells taken from one representative experiment out of three.

Figure 4. H-Ferritin and CO have additive effects in suppressing EC apoptosis. 2F-2B cells were co-transfected with β-galactosidase plus control (pcDNA) or pcDNA/H-ferritin. When indicated EC were exposed to 250 ppm CO. Light gray bars represent EC treated with Act.D and dark gray and black bars represent EC treated with TNF-α and Act.D. The dark gray bars represent EC only treated with increasing doses of H-ferritin. The black bars represent the EC treated additionally with 250 ppm exogenous CO. All results shown are the mean ± SD from duplicate wells taken from one representative experiment out of three.

Figure 5. Recombinant adenovirus mediated expression of H-ferritin prevents the cytotoxic effects of IRI and promotes liver function upon IRI. Livers were harvested from SD rats and exposed to a prolonged period of ischemia (24h, 4^{o}C, UW solution), as described in Methods. When indicated livers were transduced with β-galactosidase or H-ferritin adenoviruses, as described in Methods. Different liver parameters that correlate with liver metabolic function and cytotoxicity were analyzed in a ex-vivo reperfusion system using whole syngenic blood, as described in Methods. (**A)** Portal blood flow was analyzed as described in Material Methods.

Results shown are the mean ± standard deviation from n=4. Notice that at 120 minuets portal blood flow was significantly higher in livers transduced with H-ferritin versus non-transduced ($p<0.001$) or β-galactosidase transduced livers ($p<0.001$). (**B)** Bile production was analyzed as described in Material Methods. Results shown are the mean ± standard deviation from n=4. Notice that bile production at 120 minutes was significantly higher in livers transduced with H-ferritn versus non-transduced ($p<0.001$) or β-galactosidase transduced livers ($p<0.02$). **(C)** ALT release was analyzed as described in Material Methods. Results shown are the mean ± standard deviation from n=4. Notice that at 90 and 120 minutes ALT release was significantly lower in livers transduced with H-ferritn versus non-transduced ($p<0.05$)or b-galactosidase transduced livers ($p<0.05$). (**D)** MPO activity was analyzed at 120 hours after reperfusion as described in Material Methods. Results shown are the mean ± standard deviation from n=4. Notice that MPO activity was significantly lower in livers transduced with H-ferritn versus non-transduced ($p<0.05$) or β-galactosidase transduced livers ($p<0.05$).

Figure 6. Recombinant adenovirus mediated expression of H-ferritin preserves liver morphology upon IRI. Livers were harvested from SD rats and exposed to a prolonged period of ischemia (24h, 4^{o}C, UW solution), as described in Methods. When indicated the livers were transduced with control β-galactosidase or H-ferritin adenoviruses, as described in Methods. (**A**) Samples were collected 120 hours after reperfusion and stained with Eosin Hematoxilin. All magnifications are 200 x. (**B**) Morphological changes associated with IRI were quantified using Susuki's, criteria, as described in Methods. Results shown are the mean ± standard deviation from n=4. Notice that there is significantly less injury in livers transduced with H-ferritn versus non-transduced ($p<0.05$) or β-galactosidase transduced livers ($p<0.05$).

Figure 7. Recombinant adenovirus mediated expression of H-ferritin prevents liver cytotoxicity following transplantation. Livers were harvested from SD rats, exposed to ischemia (24h, 4^{o}C, UW solution) and tranplanted into syngenic recipients as described in Methods. When indicated the livers were transduced with control b-galactosidase or H-ferritin adenoviruses, as described in Methods. AST release was measured 24 hours after transplanatation, as described in Methods. Decreased ALT recipients receiving H-ferritin transduced livers (n=4) was signficantly enhanced as compared to recipients transplanated with non transduced ($p<0.05$; n=4) or β-galactosidase transduced livers ($p<0.05$; n.=4).

Figure 8. Recombinant adenovirus mediated expression of H-ferritin promotes graft survival upon liver transplantation. Livers were harvested from SD rats, exposed to ischemia (24h, 4^{o}C, UW solution) and transplanted into syngenic recipients as described in Methods. When indicated the livers were transduced with control β-galactosidase or H-ferritin

adenoviruses, as described in Methods. Recipient survival is illustrated in a Kaplan Mayer format. Eight to ten animlas were analyzed per group. Prologed survival in recipients receiving H-ferritin recombinant adenovirus transduced livers was signficantly enhanced as compared to recipients transplanated with non transduced or β-galactosidase recombinant adenovirus transduced livers ($p<0.001$).

Figure 9. Recombinant adenovirus mediated expression of H-ferritin prevents liver apoptosi following transplantation. Livers were harvested from SD rats, exposed to ischemia (24h, 4^oC, UW solution) and transplanted into syngenic recipients, as described in Methods. When indicated livers were untreated (a-c), transduced with control β-galactosidase (d-f) or H-ferritin (g-i) adenoviruses, as described in Methods. Apoptosis was detected 24 hours after transplanatation, as described in Methods. Apoptotic cells are signaled with black arrows. Magnifications are 100 x (a, c, d, f, g, i) or 400 x (b, e, h).

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Abbreviations used.

Act.D.: Actinomycin D; BAEC: bovine aortic endothelial cells; CO: carbon monoxide; DFO: deferoxamine mesylate; EC: endothelial cell; HO-1: heme oxygenase-1; LPS: lipopolysaccharide; MPO: myeloperoxidase; MAPK: mitogen-activated protein kinases; ROS: reactive oxygen species; SD: Sprague Dawley; SnPPIX: tin protoporphyrin; TNF-α: tumor necrosis factor-α;

Figure 1

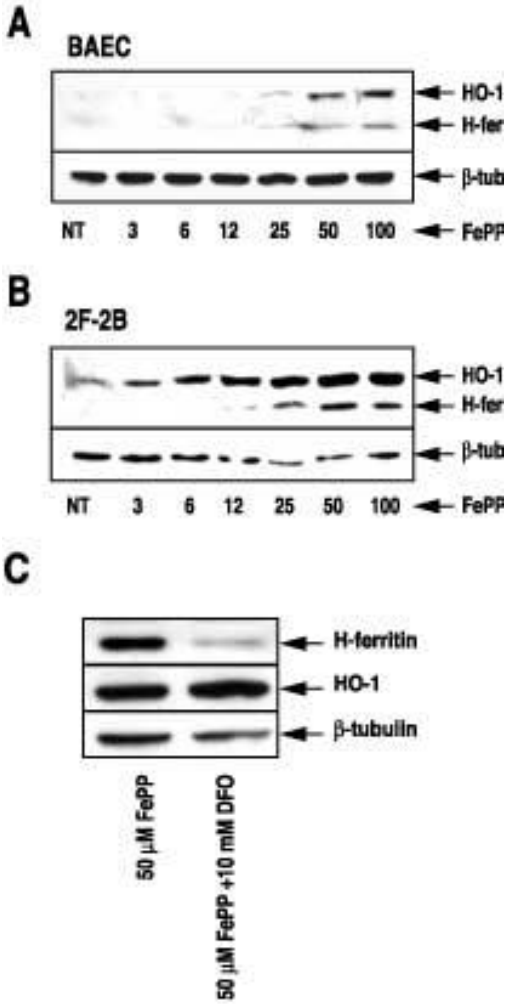

A
BAEC
HO-1
H-fer
β-tub
NT 3 6 12 25 50 100
FePP
B
2F-2B
HO-1
H-fer
β-tub
NT 3 6 12 25 50 100
FePP
C
H-ferritin
HO-1
β-tubulin
50 μM FePP
50 μM FePP +10 mM DFO

Figure 2

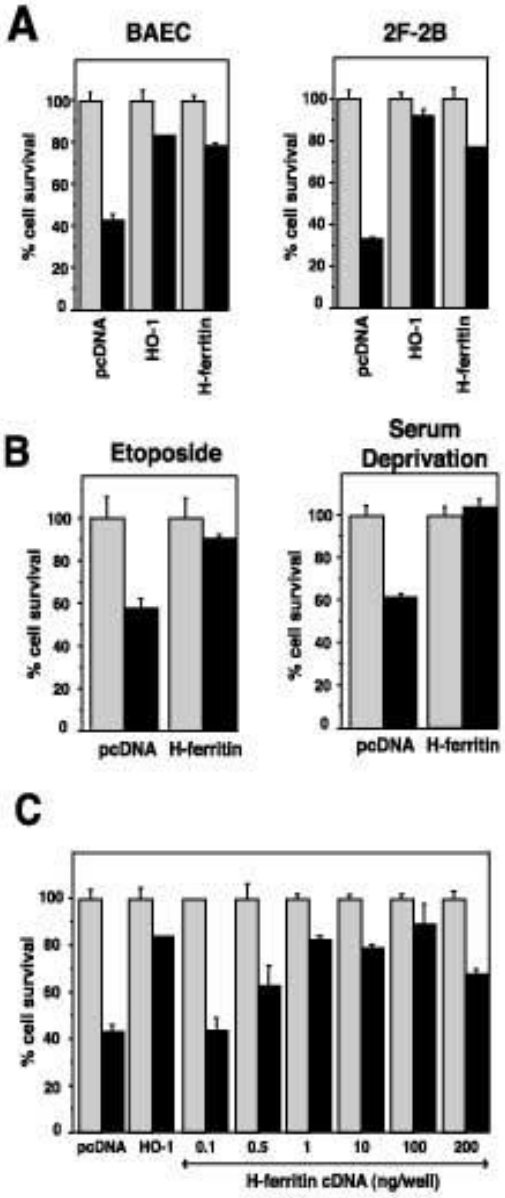

A
BAEC
2F-2B
% cell survival
100
80
60
40
20
0
pcDNA
HO-1
H-ferritin
B
Etoposide
Serum Deprivation
pcDNA
H-ferritin
C
pcDNA
HO-1
0.1
0.5
1
10
100
200
H-ferritin cDNA (ng/well)

Figure 3

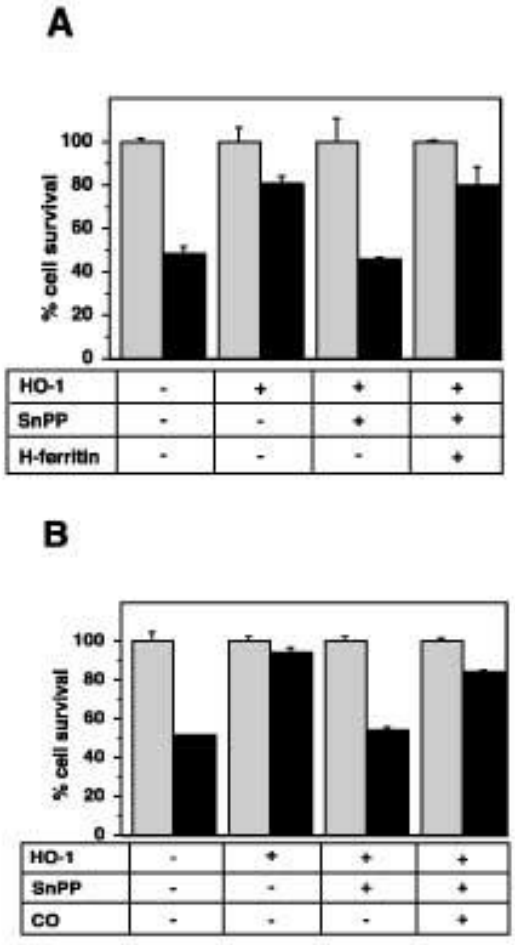

A
% cell survival
100
80
60
40
20
0
HO-1 - + + +
SnPP - - + +
H-ferritin - - - +
B
% cell survival
100
80
60
40
20
0
HO-1 - + + +
SnPP - - + +
CO - - - +

Figure 4

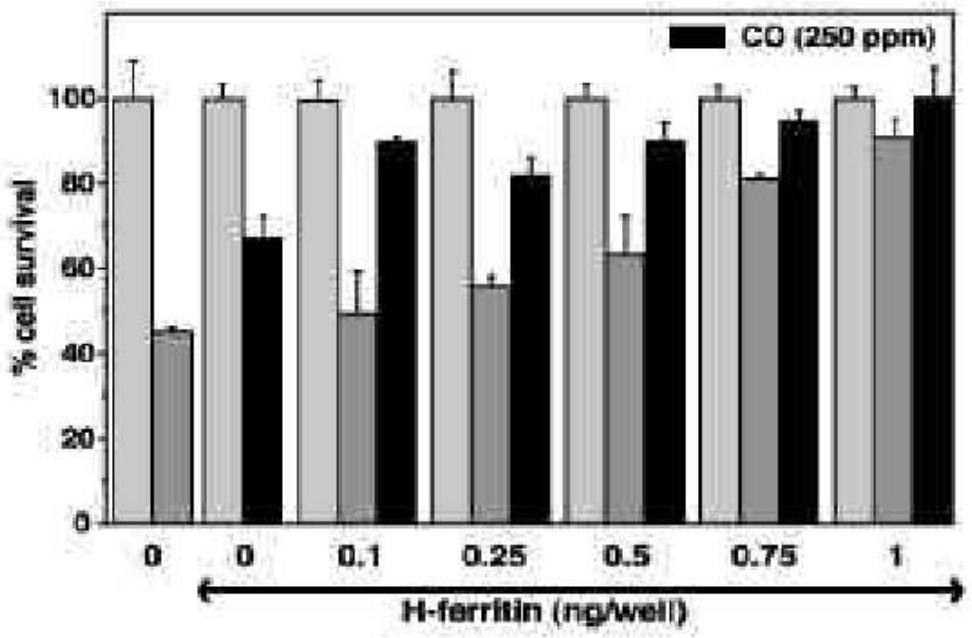

CO (250 ppm)
% cell survival
100
80
60
40
20
0
0
0
0.1
0.25
0.5
0.75
1
H-ferritin (ng/well)

Figure 5

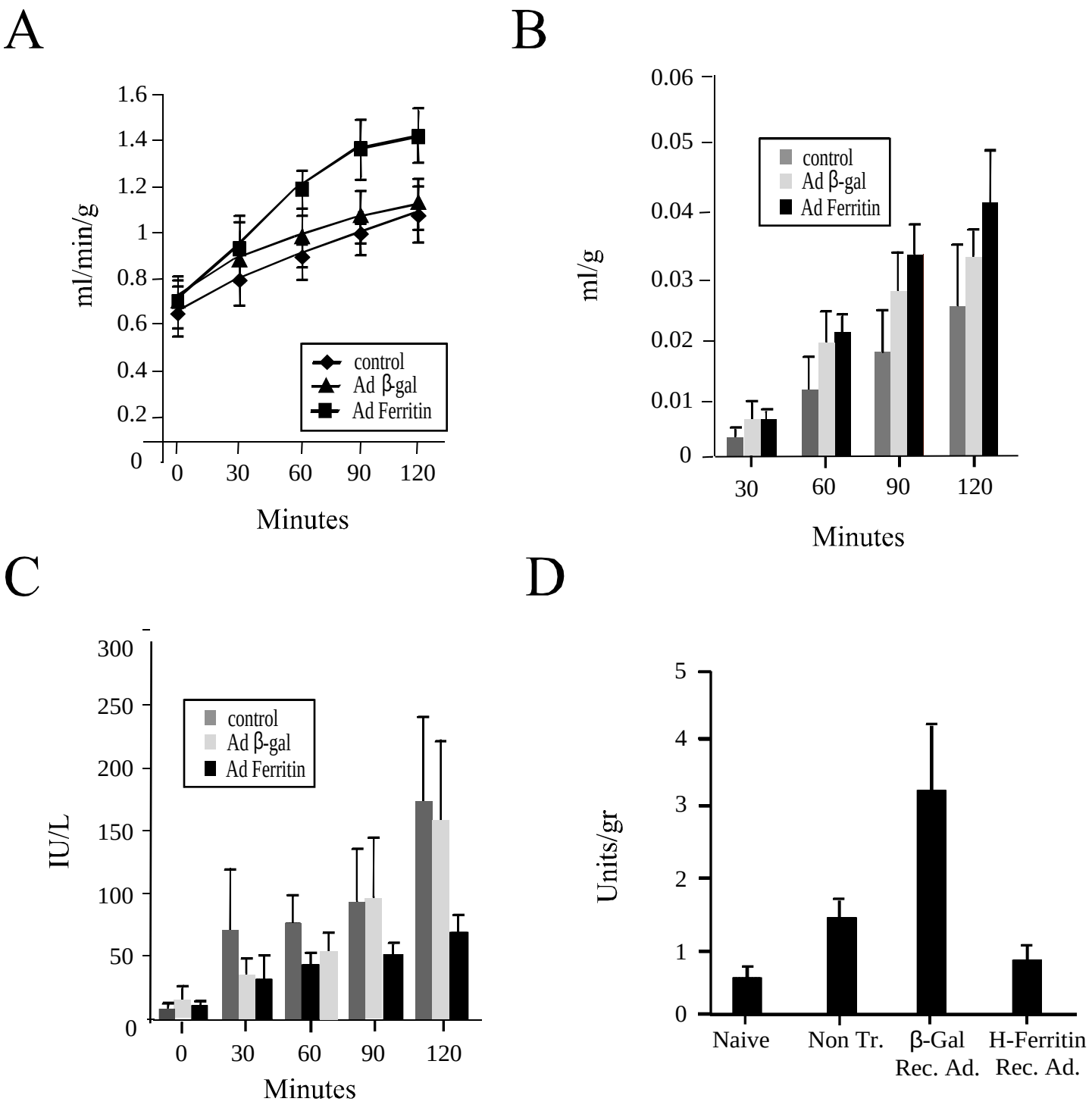
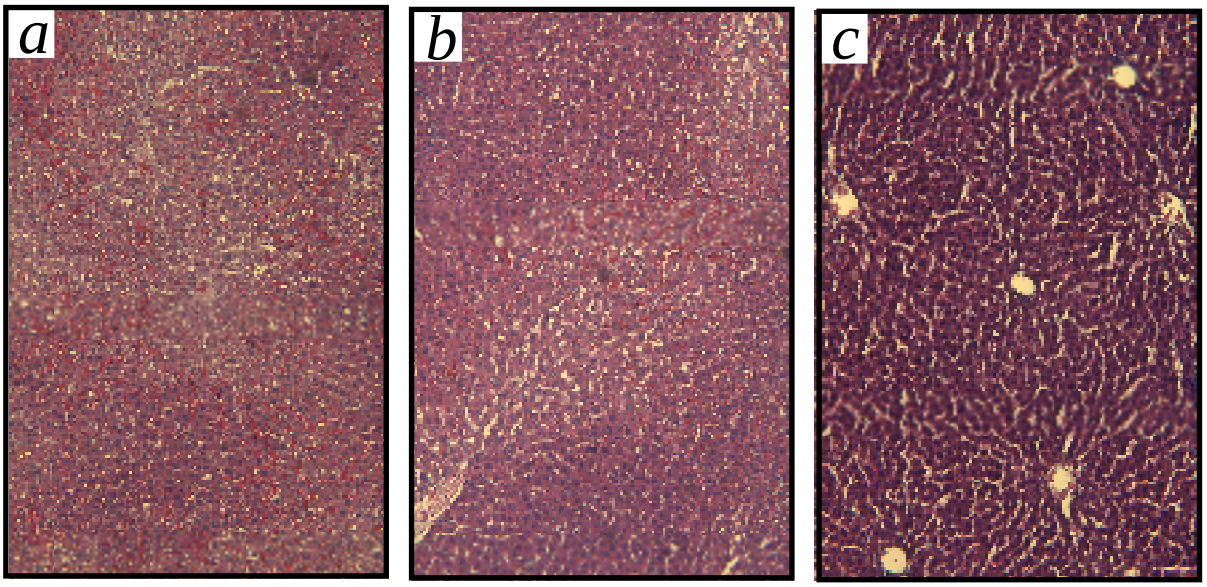

A
ml/min/g
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
30
60
90
120
Minutes
control
Ad β-gal
Ad Ferritin
B
ml/g
0.06
0.05
0.04
0.03
0.02
0.01
0
30
60
90
120
Minutes
control
Ad β-gal
Ad Ferritin
C
IU/L
300
250
200
150
100
50
0
0
30
60
90
120
Minutes
control
Ad β-gal
Ad Ferritin
D
Units/gr
5
4
3
2
1
0
Naive
Non Tr.
β-Gal Rec. Ad.
H-Ferritin Rec. Ad.

Figure 6

A



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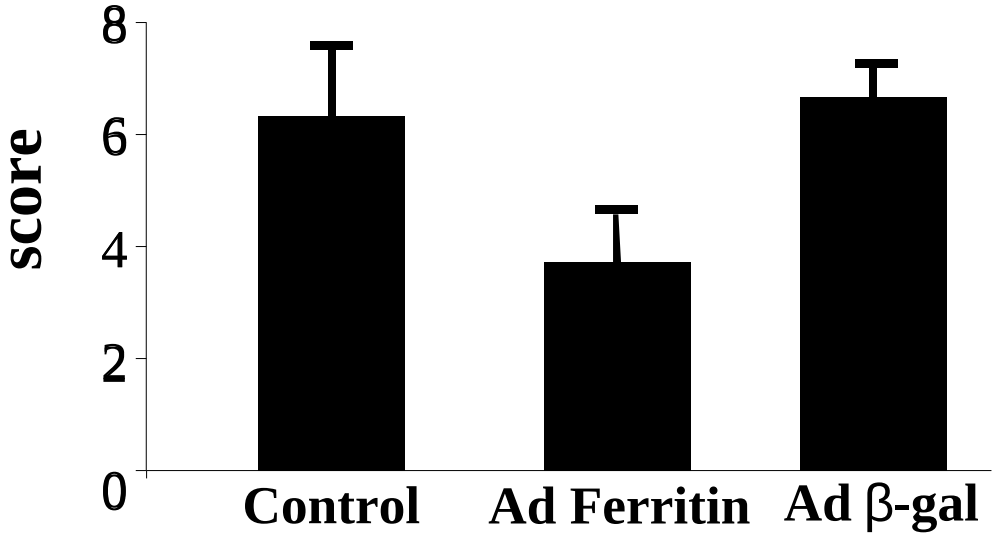

Figure 7

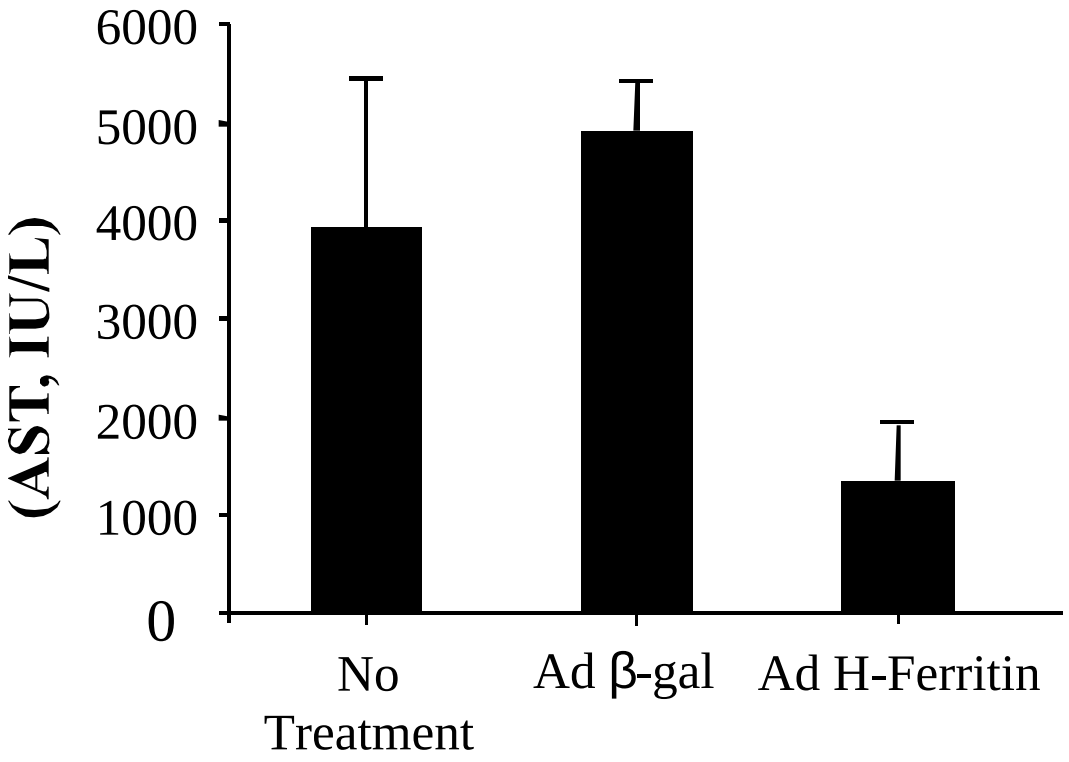
6000
5000
4000
3000
2000
1000
0
(AST, IU/L)
No Treatment
Ad β-gal
Ad H-Ferritin

Figure 8

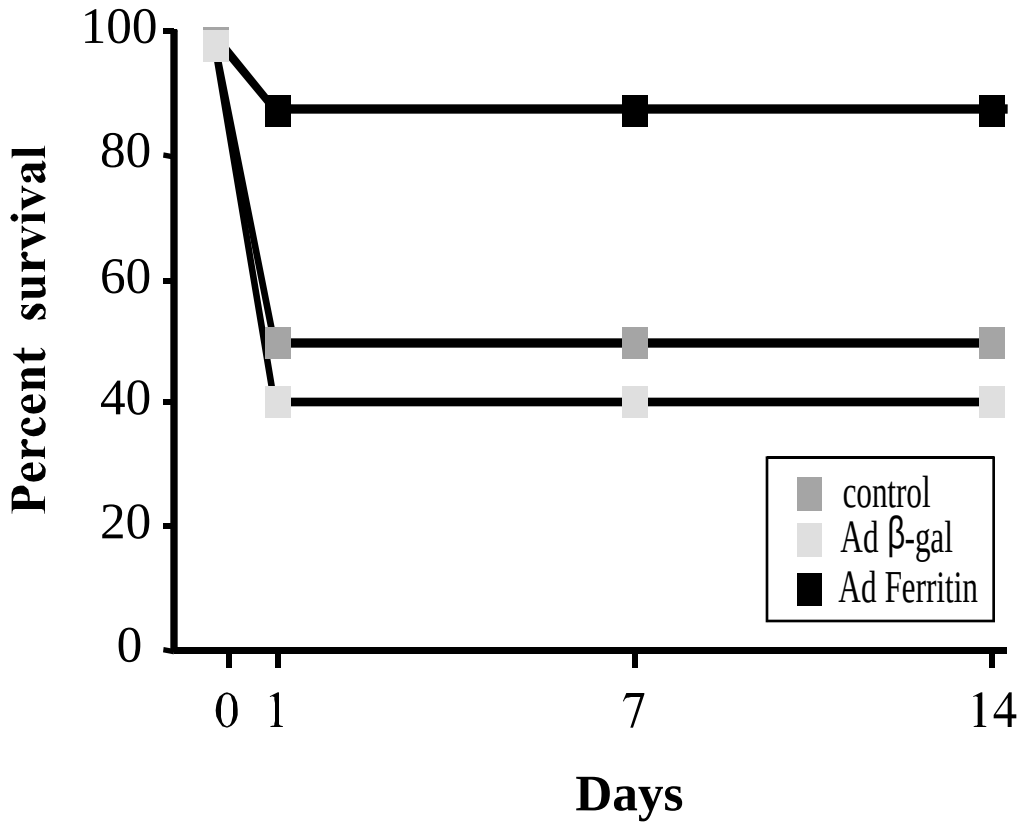
100
80
60
40
20
0
Percent survival
0
1
7
14
Days
control
Ad β-gal
Ad Ferritin

Figure 9