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AGE ALTERS EXPRESSION AND INDUCIBILITY OF HEME OXYGENASE ISOZYMES IN MICE

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ABSTRACT

Heme oxygenase (HO) performs the rate limiting step in heme degradation and is induced by cell injury or stress. We wished to determine if dietary fatty acid composition, increased age and/or an induced oxidative stress would alter the expression of HO-1 (constitutive and inducible isozyme) or of HO-2 (constitutive isozyme), in mouse liver, spleen and brain. Six- and 24-month-old male B6C3F1 mice were fed AIN-76A diets containing either 5% corn oil (CO, moderately unsaturated, n=5 per age group) or 19% menhaden fish oil plus 1% corn oil (FO, highly polyunsaturated, n=20 per age group). After 2 weeks, 5 CO and 5 FO fed mice in each age group were sacrificed. The remaining FO diet mice (n=15 per age group) were then challenged with a systemic oxidative stress by intraperitoneal injection of 125 mg iron/kg body weight as iron dextran. Five stressed mice from each age group were sacrificed 1, 5, and 24 hours post injection; liver, spleen and brain were removed. Part of each tissue was fixed in formalin, and microsomal protein isolated from the remaining tissue. HO-1 and HO-2 were detected by immunoblot of microsomal protein and by immunohistochemical staining of fixed tissue in the liver and spleen, but only HO-2 was detected in the brain. There was no significant difference in HO-1 or HO-2 expression due to diet. The liver of old unstressed mice had significantly more HO-1 than young mice. However, HO-1 was significantly induced in the livers of young mice, but not of old mice, following oxidative stress. Spleen HO-1 expression was not significantly altered by age or oxidative stress. HO-2 expression was not significantly altered by age or induced oxidative stress in any tissue examined. Age-related alterations in liver HO-1 isozyme expression and inducibility may contribute to increased susceptibility to exogenous stress and disease.

KEYWORDS: Age, fatty acids, heme oxygenase, mice, oxidative stress

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INTRODUCTION

There is general consensus that aging results in a progressive loss in the ability of an organism to adapt to changes in its environment. This decrease in adaptive response may manifest itself as a reduced capacity to induce stress response genes following exposure to hormonal, nutritional, pharmacological or other environmental stressors (1). The free radical theory of aging states that the accumulation of oxidative damage to cellular macromolecules with increased age plays an integral role in the aging process as well as many of the late onset diseases associated with aging (2,3). However, questions remain as to the age-related alterations in the expression of specific enzymes which protect against oxidative damage, with the observed changes dependent upon the specific species, strain, gender, and tissue examined (1).

Heme oxygenase (HO) is one such enzyme which protects against oxidative stress (4,5). Many heme containing proteins release their heme moiety when cells are subjected to oxidative stress, providing a detrimental source of redox-active iron capable of catalyzing the generation of oxygen radicals (4). HO catalyzes the degradation of heme to biliverdin, an antioxidant, and to free iron, which can then be sequestered in a nonreactive form. Three HO isozymes have been identified to date: HO-1, HO-2 and HO-3, all of which are expressed constitutively to varying degrees in different tissues (5,6). HO-1 is the stress inducible isozyme, while the other two isozymes are noninducible (5,6).

We have recently reported that 24 month old mice showed significantly less lipid peroxidation and induced cell death in the liver than did 6 month old mice following a systemic oxidative stress induced by acute iron overload (7). In this experiment, mice were first fed a fish oil diet in order to increase the degree of unsaturation of fatty acids in cellular membranes, which in turn provided higher cellular concentrations of an oxidizable substrate to increase the susceptibility of the cells to oxidative damage (7). Oxidative damage was then induced by giving an acute iron overload (as a catalyst for oxygen free radical generation) (7). Using the same mouse model, we wished to determine if dietary fatty acid composition and/or increased age would alter the expression of HO-1 or HO-2, or alter the ability of acute iron overload to induce HO-1, in the mouse brain, liver, and spleen. Only HO-1 and HO-2 were examined in the

present study, which was performed prior to the recent discovery of HO-3 by McCoubrey et al. (6).

MATERIALS AND METHODS

Six- month and 24-month-old male B6C3F1 mice (n=25 per age group) were purchased from Charles River Laboratories (Wilmington, MA) through a National Institute on Aging pilot study program two weeks before the study. Upon receipt, the mean (± SEM) weight of 6-month-old and of 24-month-old mice was 30.2 ± 1.6 g and 37.7 ± 4.3 g, respectively. All experimental procedures described below were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio (San Antonio, TX).

Materials

Dietary components for the AIN-76A formula diet included: AIN-76A vitamin mix, AIN-76A mineral mix, alphacel, casein, choline bitartrate, corn oil, corn starch, dextrose, and methionine; all of which were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Iron dextran and antioxidant free menhaden fish oil were purchased from Sigma (St. Louis, MO). Polyclonal antibodies specific for HO-1 and HO-2 as well as protein standards for each isozyme were purchased from StressGen (Victoria, B.C., Canada). Materials for western blotting included: MiniPlus SepraGels (Integrated Separation Systems, Natick, MA); Immobilon-P transfer membranes (Millipore Corp., Bedford, MA); blocking reagents, transfer reagents and molecular weight markers (BioRad, Hercules, CA); and horseradish peroxidase-linked secondary antibodies, enhanced chemiluminescence (ECL) detection reagents, and Hyperfilm for ECL (Amersham, Arlington Heights, IL). Reagents for immunohistochemical detection of bound antibodies were purchased from Dako Corp. (Carpinteria, CA). All other materials were analytical reagents of the highest grade available and were used without further purification.

Experimental Protocol

Upon receipt, all mice were fed a standard AIN-76A diet containing 5% corn oil (CO diet). After two weeks of acclimatization, 5 mice per age group continued on the CO diet and the remaining mice (n=20 per age group) were fed a modified AIN-76A diet which contained 19% menhaden fish oil plus 1% corn oil (FO diet). Diets were balanced for caloric content and made fresh weekly as described previously (8), stored in single cage portions at -20°C, and replaced in each cage daily. After 2 weeks on the different diets, young (i.e., 6-month-old) and old (i.e., 24-month-old) mice consuming the FO diet (n=15 per age group) were challenged with a systemic oxidative stress by intraperitoneal injection of 125 mg Fe/kg bw as iron dextran (9). Five iron-treated mice from each age group were sacrificed 1, 5, and 24 hours post injection. Young and old control CO and FO diet fed mice (n=5 per age and diet) received an intraperitoneal injection of normal saline and were sacrificed 1 hour later. Hence, there were five groups in each age category:

(1) 5% corn oil/normal saline injection
(2) 19% fish oil/normal saline injection
(3) 19% fish oil/sacrificed 1 hr after iron injection
(4) 19% fish oil/sacrificed 5 hr after iron injection
(5) 19% fish oil/sacrificed 24 hr after iron injection

Mice were sacrificed by cervical dislocation. The liver, spleen, and brain of each mouse was then rapidly removed, rinsed in ice cold phosphate buffered saline, weighed, a portion fixed in 10% buffered formalin, and the rest frozen in liquid nitrogen and stored at -80°C until use.

Gel Electrophoresis and Western Blotting

Portions of liver, spleen and brain from each mouse were thawed and homogenized using a Polytron tissue homogenizer, and microsomal fractions were isolated using differential centrifugation as previously described (10). The protein concentrations of microsomal fractions were quantitated with a protein assay kit (purchased from BioRad) prior to electrophoresis. Protein samples were boiled in a sample buffer (125 mM Tris HCl, pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate, 8 mM dithiothreitol, 0.05% bromophenol blue) for 5 minutes, then equal amounts of protein (35 µg liver, 10 µg spleen, and 35 µg brain) were separated on 12.5% polyacrylamide gels (MiniPlus SepraGels, Integrated Separation Systems, Natick, MA) according to the manufacturer's instructions and using separate gels for individual tissues. Samples were electrophoresed at 50 volts until the bromophenol blue was clear of the stacking gel, then run at 150 volts for 2 hours. The proteins were then transferred to polyvinylidene fluoride membranes (Immobilon, Millipore Corp.) for 2 hours at 100 volts and 4°C. Blots were blocked overnight at 4°C in Tris-buffered saline (TBS) containing 5% blotting grade non-fat dry milk, 20 mM Tris, pH 7.5 and 500 mM NaCl. The membranes were washed 3 times with shaking in TBS with 0.1% Tween 20 and incubated with antibodies against HO-1 or HO-2 diluted 1:1000 in the blocking medium at room temperature for 3 hours without shaking. The membranes were again washed 3 times in TBS/Tween prior to incubation with a horseradish peroxidase-linked secondary antibody diluted 1:1000 in the blocking medium. Antibody-reactive proteins were detected by the ECL chemiluminescent detection system (Amersham) according to the manufacturer's instructions. Each blot was subjected to scanning densitometry for quantitation.

Immunohistochemistry

HO-1 and HO-2 were localized immunohistochemically on four micron-thick formalin-fixed, paraffin embedded tissue cross sections mounted on coated glass slides. Slides were deparaffinized through a series of xylene and graded alcohol washes, then used in routine immunohistochemistry procedures as previously described (11), using polyclonal antibodies against either HO-1 or

HO-2 (StressGen) and a biotin-streptavidin-horseradish peroxidase detection system (Dako Corp.). Negative control slides were incubated with mouse serum in place of the primary antibody, but were otherwise treated the same. Sections of formalin-fixed mouse testis were used as a positive control for HO-1 and HO-2 immunoreactivity due to the distinct, localized expression of these two isozymes in this tissue (12).

Statistical Analyses

All statistical analyses were performed using PRISM (GraphPad Software, San Diego, CA) statistical software. Data are expressed as mean ± SEM. Two way analysis of variance (ANOVA) was performed for each isozyme and in each tissue to determine if immunoreactive blotted microsomal protein varied significantly according to age and treatment group. These tests were followed by one way ANOVAs and Student-Neuman-Keuls *a posteriori* tests to determine differences between individual treatment groups for each isozyme and in each tissue. Enzyme induction following iron treatment was also compared by linear regression analysis for each isozyme, age, and tissue examined. Significance was accepted if $p<0.05$.

RESULTS

HO-1 and HO-2 were detected by immunoblots of microsomal liver, spleen and brain protein from 6 month and 24 month old mice. There were no significant differences in the relative amounts of HO-1 or HO-2 protein due to dietary composition in any of the tissues examined; therefore data from dietary control groups were combined for presentation in Figures 2-4.

As shown in Figures 1 and 2A, there was significantly more HO-1 in the livers of old mice than in young mice prior to iron injection. However, following induction of oxidative stress by intraperitoneal injection of iron dextran, there was a significant time dependent linear increase in liver HO-1 only in the young mice, with no significant change in the old mice. Figure 2B shows that similar amounts of HO-2 protein were found in young and old livers, and that there was no change following iron treatment. Immunohistochemical staining for HO-1 and HO-2 in formalin-fixed, paraffin embedded liver sections revealed distinct patterns of cell-specific protein expression (data not shown). In the liver, both isozymes were localized to some of the endothelial cells of the sinusoids and in Kupffer cells. There were no differences in immunohistochemical staining due to differences in age, diet, or iron treatment.

As is depicted in Figure 3, HO-1 and HO-2 protein in the spleen did not significantly differ with age, fatty acid composition of the diet or following iron treatment. The increase in spleen HO-1 in both young and old mice seen in Figure 3A was not significant for either age groups when evaluated by linear regression analysis. There was also no evidence for induction of spleen HO-2 following iron treatment in either age group. Immunohistochemical staining showed that HO-1 and HO-2 co-

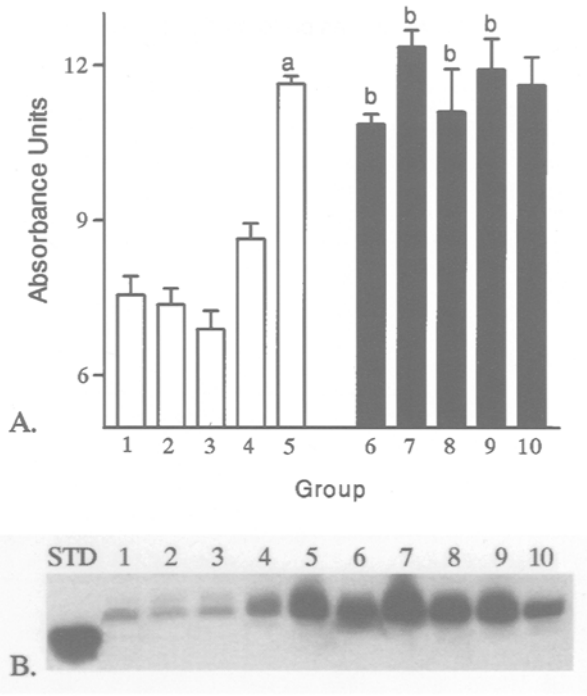

Figure 1. A. Liver heme oxygenase-1 (HO-1) protein expression (mean ± SEM) in 6 month (open bars) and 24 month (solid bars) male mice fed an AIN-76A diet containing 5% corn oil (control groups 1 and 6) or AIN-76A diet containing 19% fish oil (control groups 2 and 7). The remaining fish oil diet mice were injected intraperitoneally with iron dextran and sacrificed 1 hour (groups 3 and 8), 5 hours (groups 4 and 9) or 24 hours (groups 5 and 10) following iron injection. **a**, significantly different ($p<0.05$) from all other 6 month groups. **b**, significantly different ($p<0.05$) from comparable 6 month group. **B**. Representative immunoblot of HO-1 protein in the groups defined above.

localized in splenic macrophage, as was seen with phagocytic cells in the liver (data not shown). Immunoreactivity in the spleen was highest in macrophage in the red pulp, but the splenic chords and reticuloendothelial cells lining the venous sinuses of the red pulp were also lightly stained positive for HO-1 and HO-2. There were no differences in immunohistochemical staining due to differences in age, diet, or iron treatment.

Microsomal brain protein was also evaluated for HO-1 and HO-2 protein. HO-1 was undetectable in the brain by immunoblot. However, as pictured in Figure 4, HO-2 protein was detectable in the brain and was found in similar amounts in tissue from both age groups, with no significant differences due to diet, age or iron treatment. There was also no distinct pattern of change in HO-2 following iron treatment in either age group, nor were differences observed in the immunohistochemical staining pattern for HO-2 between the different treatment groups.

DISCUSSION

We have previously reported that maintenance of 6 month and 24 month old mice on a high polyunsaturated fatty acid diet for 2 weeks significantly increases membrane unsaturation in both age groups; and that acute

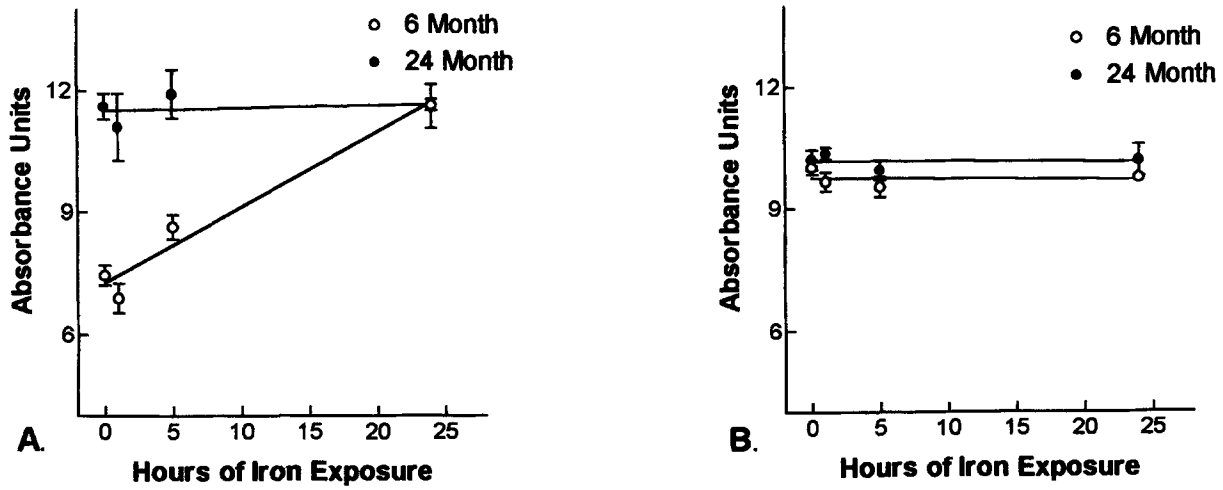

Figure 2. A. Liver heme oxygenase-1 (HO-1) protein expression (mean ±SEM) in 6 month old (open symbols) and 24 month old (solid symbols) male mice. Linear regression analysis showed a significant increase with time following iron injection in HO-1 in the young but not the old mice. **B.** Liver heme oxygenase-2 (HO-2) protein expression in the groups defined above. There were no significant differences in HO-2 protein expression between treatment groups.

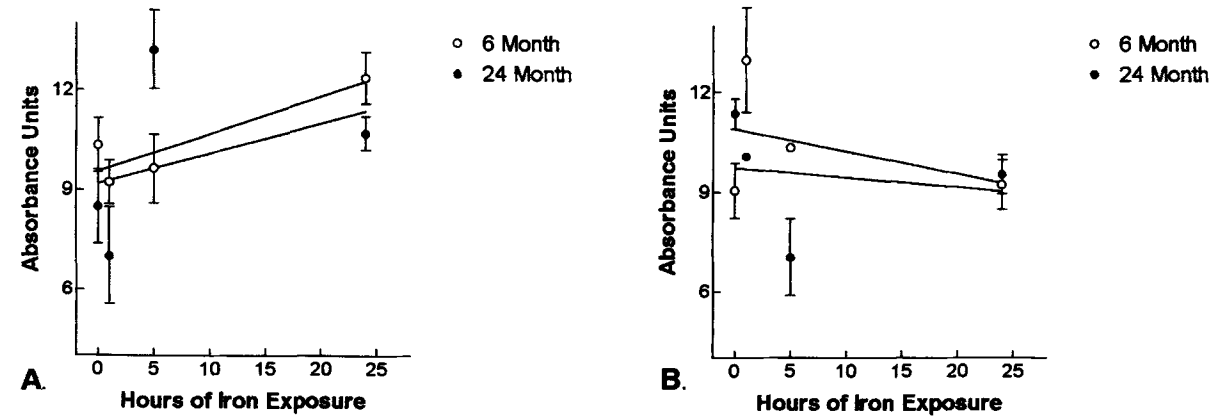

Figure 3. A. Spleen heme oxygenase-1 (HO-1) protein expression (mean ±SEM) in 6 month old (open symbols) and 24 month old (solid symbols) male mice. Linear regression analysis showed no significant increase with time following iron exposure in HO-1 protein expression in either age group. **B.** Spleen heme oxygenase-2 (HO-2) protein expression in the groups defined above. Linear regression analysis showed no significant increase with time in HO-2 protein expression in either age group.

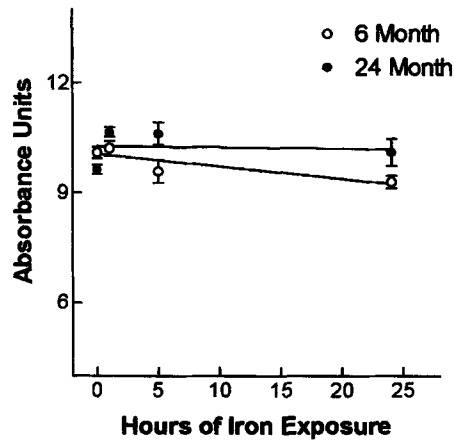

Figure 4. Brain heme oxygenase-2 (HO-2) protein expression (mean ±SEM) in 6 month old (open symbols) and 24 month old (solid symbols) male mice. Linear regression analysis showed no significant increase with time of iron exposure in HO-2 protein expression in either age group.

iron overload results in similar temporal patterns of increased serum iron and tissue iron accumulation (7). However, significantly more liver oxidative damage (as indicated by malondialdehyde accumulation) and induced cell death (as indicated by an *in situ* apoptosis assay) were present in the young mice when compared with the old mice (7). As HO-1 is a stress inducible protein involved in iron homeostasis, we chose to investigate in the present study whether there was altered expression or induction of this enzyme with increased age in this model of acute systemic oxidative stress.

Liver HO-1 protein, but not HO-2 protein, was significantly increased in the 24 month old mice. Elevated total HO activity with increased age has been previously reported in rat liver (13-15), but to our knowledge this is the first report of age-related changes in specific HO isozymes in the mouse liver. As elevated HO-1 in the old livers correlates with tissue protection against cell damage and cell death in the same model of systemic oxidative stress (7), it is possible that HO-1 is protective against this type of stress. It remains to be established

whether conditions in the old mouse liver failed to cross the threshold for HO-1 induction, or whether the enzyme was simply unable to be induced to a higher amount. Previous reports show that HO activity in the livers of old rats is inducible following exposure to heavy metals (13,16), but we are unaware of existing data on old mouse tissue. We recognize that this study is limited by the investigation of changes at the protein level only; future studies evaluating changes in HO gene transcription, mRNA levels, as well as enzyme activity will provide further explanation of the mechanism of altered mouse liver HO-1 protein with age. Finally, this study was performed prior to the recent discovery of HO-3 by McCoubrey et al. (6). It would be of interest to investigate the role of this novel HO isozyme in age-related alteration in stress response.

Immunohistochemical localization of HO isozymes in specific cell types of the liver, spleen and brain were not noticeably different with increased age or following iron induction. Also, specific cell types expressing the different isozymes were essentially the same as has been previously reported (5 and references therein; data not shown).

Spleen HO-1 was not significantly induced in the present experiment, although there was a significant increase in spleen tissue iron deposition with time following iron injection (7). The lack of spleen HO-1 induction under conditions of liver HO-1 induction could be due to tissue heterogeneity in HO inducibility and inhibition. It has been previously reported that splenic HO-1 activity could not be induced by hematin, whereas liver HO-1 was significantly increased (17). Likewise, spleen and liver HO activity are differentially regulated by metalloporphyrin HO inhibitors (18). Alternatively, it has been suggested that spleen HO is maintained in the induced state as a result of constant exposure to hemoglobin released from senescent erythrocytes (17).

In the present study, HO-1 protein was not detected in the mouse brain, whereas HO-2 was present in the brain in similar amounts regardless of treatment group. HO-2 is by far the predominant HO isozyme in neuronal tissue (5). In fact, initial studies of rodent brain could not detect HO-1 protein under normal conditions (19); but with hyperthermia, brain HO-1 was rapidly induced (20,21). We have found in a recent study that following acute iron overload, brain tissue sections stained positive for Prussian blue (indicative of iron deposition) in the choroid plexus (which maintains the blood-brain barrier), but that there was no evidence for iron accumulation in other regions of the brain (unpublished data from our group). Thus acute iron overload carried out under the current conditions does not appear to induce brain HO-1, probably due to a lack of iron penetration and subsequent oxidative stress in this tissue.

In conclusion, the constitutive expression and inducibility of mouse liver HO-1 protein is significantly altered with increased age. Increased HO-1 in the old livers may partially explain a protection with increased age against oxidative stress (7). However, because of an apparent lack of inducibility and thus ability to mount a stress response, any benefit due to upregulated HO-1 against an extended stress or stressors is questionable. Investigations into the mechanism of the upregulation of liver HO-1 and changes in HO activity with increased age are warranted.

ABBREVIATIONS: ANOVA, analysis of variance; HO, heme oxygenase; ECL, enhanced chemiluminescence; TBS, Tris buffered saline; CO, corn oil; FO, menhaden fish oil.

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