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Original Article

Aging impairs induction of redox factor-1 after heat stress: a potential mechanism for heat-induced liver injury

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Abstract: Aging is associated with reduced tolerance to physiological stressors such as hyperthermia. In animal models, heat stress is associated with increased oxidative damage in the livers of old rats. In this study, we evaluated the expression of redox factor-1 (Ref-1), a DNA repair enzyme, and thioredoxin-1 (Trx-1), an antioxidant protein. We hypothesized that these proteins would be induced by heat stress in young animals, and that aging would attenuate this response. Young (6 mo) and old (24 mo) male Fischer 344 rats were exposed to a two-heat stress protocol, and livers were harvested at several time points after the second heat stress. Ref-1 and Trx-1 were evaluated by immunoblot and immunohistochemistry. In young rats, Ref-1 was induced by ~50% immediately (0 h) after heat stress, and returned to control levels at 2 h. We observed no change in Ref-1 after hyperthermia in old rats; however, aging was associated with a 2-fold increase in Ref-1 expression. At 2 h after heat stress, Trx-1 was increased in old rats, but there was no change in young rats. In tissue sections, we observed frequent ductular reactions in the old rats that were positive for both Ref-1 and Trx-1. The impairment in the induction of Ref-1 suggests a mechanism for the increased oxidative injury observed in old rats after heat stress. Furthermore, the observation of ductular reactions positive for both Ref-1 and Trx-1 demonstrates a proliferative cellular niche that develops with aging.

Keywords: Oxidative stress, ductular reaction, Kupffer cells, hyperthermia, Trx-1

Introduction

The exponential increase in the population aged 65 years and older provides strong rationale for studying age-related changes in physiology. Aged individuals are more susceptible to infections, are slower to heal than younger organisms, and exhibit altered cellular, and physiological responses to common stressors. Therefore, identifying age-related changes in cellular pathways in response to stressors could allow for therapeutic modulations that would improve health in elderly individuals.

Enhanced injury to cellular macromolecules is observed in old animals in the nonstressed condition, and this injury is augmented with physiological stressors. In the liver, heat stress is associated with augmented oxidative stress and DNA damage in old, but not in young rats [1]. Mammals have several DNA repair enzymes including p53, DNA polymerase β (β-pol), and apurinic/apyrimidinic endonuclease-1 (APE-1) or redox factor-1 (herein referred to as Ref-1). When cells are exposed to oxidative stress, Ref-1 is induced [2-4], and it translocates to the nucleus [3, 5], which facilitates its function as a base excision repair protein. Homozygous deletion of Ref-1 results in embryonic lethality [6], and when challenged with 2-nitropropane (2-NP), Ref-1 haploinsufficient mice display increased aldehydic DNA lesions [7]. Interestingly, a cytosolic function of Ref-1 has also been discovered; Ref-1 mitigates oxidative damage by inhibiting superoxide and hydrogen peroxide production by the small GTPase, Rac-1 [8, 9]. Furthermore, overexpression of Ref-1 suppresses oxidative injury and apoptosis in a model of hepatic ischemia/reperfusion [10]. Thus, Ref-1 is a protective protein under oxidative stress, and it is of interest to characterize the regulation of Ref-1 with aging and in response to physiological stressors. The observation that old animals display DNA damage

after heat stress may suggest that DNA repair mechanisms are compromised with aging. It is currently unknown whether Ref-1 responds to a physiologically relevant challenge in vivo such as heat stress or whether aging affects this potential response.

In addition to its ability to repair damaged DNA and suppress oxidative stress, Ref-1 also facilitates the activity of the transcription factor, AP-1 [11, 12], which leads to the increased expression of target genes such as metallothionein, γ-glutamylcysteine synthase, and heme oxygenase-1 [13-15]. Importantly, Ref-1 is necessary for heat-induced activation of AP-1 [16]. In this role, Ref-1 interacts with another redox-sensitive protein, thioredoxin-1 (Trx-1), and this interaction enhances AP-1 DNA binding activity [11, 12]. Like Ref-1, Trx-1 also translocates to the nucleus under conditions of stress such as UV radiation [12]. Interestingly, a previous study has observed that old rats display an attenuated AP-1 DNA binding activity after heat stress [1]. Thus, it is plausible to assume that there are impairments in Ref-1 and/or Trx-1 expression or function with aging.

Thioredoxin-1 has many other functions in the cell including facilitation of DNA synthesis, inhibition of apoptosis, and direct scavenging of reactive oxygen species (ROS) [17]. In cell culture models, Trx-1 is induced by oxidative stress, and its induction protects against apoptosis [12, 18, 19]. Studies that have characterized Trx-1 with aging have found no change in Trx-1 protein in the heart [20], and a decrease in mRNA expression in skeletal muscle [19]. However, augmenting Trx-1 expression with aging and toxic stressors improves hepatic homeostasis. For instance, in aged (26-28 month old) mice, overexpression of Trx-1 decreases hepatic protein oxidation, and serum concentrations of isoprostane [21]. Trx-1 also improved survival after administration of diquat in young (4-6 month old) mice [21]. Furthermore, overexpression of Trx-1 prevents oxidative injury as well as hepatic necrosis after thioacetamide treatment [22]. To our knowledge, the effect of aging and physiological stress on the time course of Trx-1 induction has not been investigated.

Due to the roles of Ref-1 and Trx-1 in the attenuation of oxidative damage, as well as their role in facilitating AP-1 activity, we were interested in characterizing the expression of these proteins with aging and environmental heat stress. Given the enhanced DNA damage observed in old rats [1], we hypothesized that old animals would demonstrate an impairment in Ref-1 induction after heat stress. Furthermore, since old rats display attenuated AP-1 binding activity after heat stress compared to young rats [1], we hypothesized that aging would impair the Ref-1/Trx-1 response to heat stress.

Methods

Animal protocols

Animal experiments were performed in the laboratory of Dr. Kevin Kregel in the department of Health and Human Development at the University of Iowa. All animal protocols were approved by the University of Iowa Institutional Animal Care and Use Committee (ACURF #0606117). Young (6 month; ~360 g) and old (24 month; ~437 g) male Fischer 344 rats were obtained from the National Institute on Aging and housed in polyethylene cages. Animal rooms were maintained on a 12 h light-dark cycle at 24 ± 2°C with a relative humidity of 50-55%. Food and water were provided ad libitum, and rats were housed in individual cages. Prior to the heat stress experiments, animals were handled by the investigators and familiarized to the equipment used for the experiments described below. Animals were exposed to a two-heat stress protocol as described in detail previously [23]. Briefly, during each heating bout, core temperature (Tc) was elevated to 41°C over 60 min, and then maintained at 41°C for an additional 30 min. The exact same heating protocol was utilized on the second day. The rationale for this protocol and the use of Fischer rats as an experimental model have been described elsewhere [24-26]. Nonheated animals served as controls and underwent sham heating protocols at similar times as the heated animals. Liver tissue was harvested either immediately after (0 h), or at 2 and 24 hours after the second heat stress in separate groups of rats (n = 7-9 animals per group). These time points were chosen to coincide with previous studies showing early increases in oxidative stress and DNA damage [1], followed by a return of these parameters to nonstressed conditions (24 h time point). Hepatic necrosis, as well as subcellular and oxidative injury resulting from aging and heat stress have been charac-

terized extensively in previous reports [1, 25, 27, 28]. At the indicated times, animals were given an overdose of pentobarbital sodium, and their livers were removed quickly and rinsed in phosphate buffered saline (PBS), then frozen in liquid nitrogen. Separate portions of liver were placed in 10% neutral buffered formalin and fixed overnight. Liver samples were subsequently dehydrated in increasing concentrations of ethanol, cleared in xylene, and then embedded in paraffin.

Immunoblotting

Frozen liver tissue was homogenized in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.25% sodium deoxycholate, 1% triton-X, 1 mM EDTA, 1 mM Na_3VO_4), and the protein concentration of each sample was determined by the Bradford protein assay (BioRad, Hercules, CA). Whole-tissue liver lysates (80 µg) were separated on 12% polyacrylamide gels, and then transferred to nitrocellulose membranes. Membranes were incubated in primary antibody overnight at 4°C (Trx-1: 1:250, Cell Signaling Technologies, #2429, in 5% bovine serum albumin in tween-tris buffered saline (TTBS)), and then incubated in secondary antibody (goat anti-rabbit HRP 1:500; Santa Cruz Biotechnologies, sc-2030) for one hour at room temperature. After secondary incubation, membranes were washed with TTBS, and then incubated in SuperSignal® West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL). Images were taken with the ChemiDoc XRS+ system (BioRad, Hercules, CA), and the brightness of bands was quantified with Image Lab software (BioRad). The same membranes were subsequently probed for Ref-1 (1:2000 Santa Cruz, sc-17774) in 5% milk, TTBS for one hour at room temperature, washed, then incubated in sheep anti-mouse HRP (1:4000; GE Healthcare; NA931V) for one hour. Membranes were developed as described above. Due to the different molecular weights, different hosts of antibodies, and strength of signal (Ref-1 >> Trx-1), this method did not result in non-specific bands. This method was reversed to ensure similar results.

To confirm equal loading and transfer, membranes were stained with Ponceau S staining solution, and protein bands within each sample lane were quantified using Image Lab software. Band densities of the protein of interest were normalized to the density of the Ponceau-stained lane for each sample. Ponceau staining has been shown by us and others to quantify protein loading better than routinely used loading controls such as β-actin, the expression of which can sometimes be affected by experimental parameters (Bloomer unpublished, and [29]). After normalization to Ponceau, the mean protein expression within the young, nonheated control group was determined and given a value of 1. The fold differences of all other groups were compared to the young, nonheated group. All immunoblots were performed in duplicate. To minimize analytic variability, every comparison (i.e., nonheated to heated, and young versus old within each time point) was run head-to-head on the same gel. For example, to compare the young and old nonheated groups, seven samples from each group were run on the same gel. This process was repeated until all comparisons were made. Values in each graph represent the values determined in this manner, and statistics were performed on these numbers.

Histological protocols

Serial 5-µm sections were prepared from paraffin blocks and affixed to Superfrost Plus microscope slides (Electron Microscopy Science, Hatfield, PA). Slides were deparaffinized in xylenes, and then rehydrated in decreasing concentrations of ethanol. Slides were rinsed with water, placed in citrate buffer (1.8 mM citric acid monohydrate, 8.2 mM trisodium citrate dehydrate, pH 6) then subjected to heat-induced epitope retrieval in a pressure cooker. The cooker was heated until it reached its maximum pressure, and then kept at maximum pressure for one minute before removing it from the heating block. Slides were cooled for 20 minutes after heating, rinsed in water, and then incubated in peroxidase blocking buffer (3% hydrogen peroxide in methanol) for 30 minutes at room temperature. Sections were rinsed in PBS, incubated in blocking solution (1% bovine serum albumin, 10% normal horse serum, and 0.1% tween in PBS) for 30 minutes at room temperature, and then incubated in primary antibody (Ref-1 at a 1:8000 dilution, or Trx-1 at a 1:800 dilution; the same antibodies as described above) in blocking solution overnight at 4°C. Sections were washed in PBS, then incubated in biotinylated secondary anti-

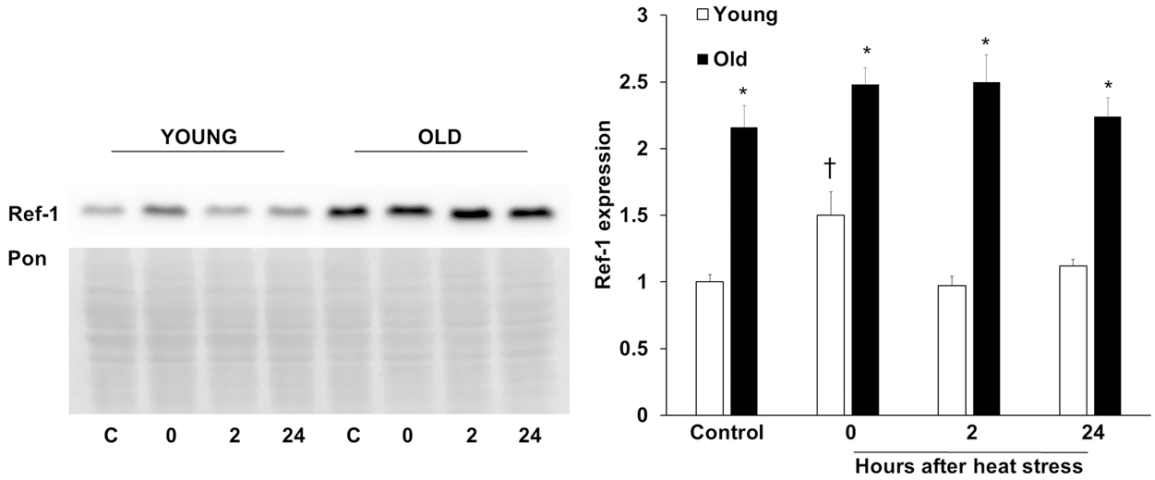

Figure 1. Heat stress stimulates Ref-1 expression in young rats. Left panel: representative immunoblot of Ref-1 in young and old animals under control conditions, and at the indicated times after heat stress (in hours). Ponceau stained membrane (Pon) demonstrates equal loading and transfer. Right panel: quantitation of Ref-1 expression, normalized to the Ponceau stain and further normalized to the young, nonheated group.*Significant effect of aging; † significant effect of heat stress (n = 7-9 young and old animals in the nonheated condition, and at each time point after heat stress).

body at a 1:350 dilution (mouse for Ref-1 and rabbit for Trx-1; Vector Labs, BA-2001, and BA-1100, respectively) for one hour at room temperature, after which they were washed in PBS, then incubated in avidin-biotin complex (Vector Labs, PK-4000). Diaminobenzidine (DAB; Vector Labs, SK-4100) was utilized for color development. In single staining experiments for Ref-1 and Trx-1, the brown DAB reaction product was used. Slides were lightly counterstained using Gills hematoxylin (Newcomer Supply, Middleton, WI), and then incubated in bluing solution (0.1% sodium bicarbonate), after which they were dehydrated, cleared in xylenes, and then coverslipped.

Double staining for Trx-1 and heme oxygenase-1 (HO-1, a Kupffer cell marker [30]) was performed by developing the Trx-1 signal first using the nickel-enhanced black reaction product of DAB. After development, slides were rinsed in PBS, incubated again in blocking solution, and then incubated overnight at 4°C in primary antibody (mouse anti-HO-1, 1:250; Enzo Life Sciences, OSA-111F). The next day, the signal was developed as described above using the brown DAB reaction product. Negative control sections were stained with the appropriate IgG (rabbit: sc-2027 or mouse: sc-2025) and/or singly for Trx-1 or HO-1. Additional double-staining experiments were performed in a similar manner for the following proteins: Ref-1 and glutamine synthetase (GS, 1:1000 dilution; BD Transduction Laboratories, # 610517); Ref-1 and β-catenin (1:25 dilution, Cell Signaling Technologies # 8480); and Ref-1 and Trx-1.

Old rats demonstrate stainable iron deposits in the nonparenchymal cell compartment of the liver [24]. Due to their role in red blood cell and transferrin turnover, the cells that display iron deposits are predominately Kupffer cells [31]. Since young rats do not demonstrate stainable iron, double staining experiments for Trx-1 and Perl's Prussian Blue for iron was performed on old rats only. Trx-1 staining was performed as described above, using the brown DAB reaction product, and then sections were incubated in a solution of 10% hydrochloric acid and 5% potassium ferrocyanide for 20 minutes. Sections were subsequently coverslipped without counter-staining.

Potential heat-induced differences in subcellular localization of Ref-1 were assessed by counting the number of positive nuclei for Ref-1 in 40 × micrographs (5-7 fields from each animal) from young, nonheated animals, and young heated animals (0 h time point) by an observer blinded to the experimental condition. The percentage of positive cells was normalized to the number of hepatocyte nuclei in each field.

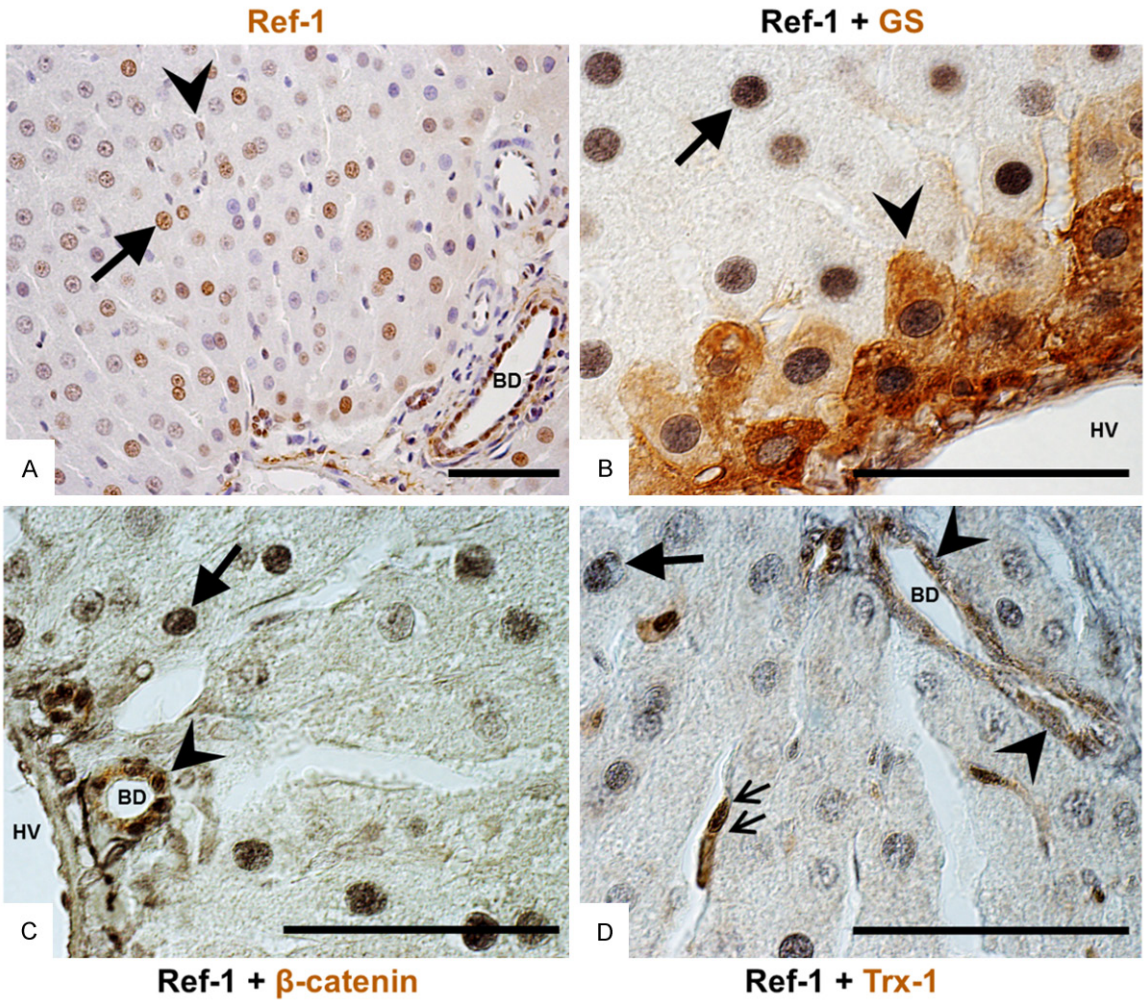

Figure 2. Immunolocalization of Ref-1 in the liver. A: Immunostaining for Ref-1 alone (brown), with hematoxylin counterstain. The arrow indicates a hepatocyte nucleus positive for Ref-1; the arrowhead indicates a sinusoidal lining cell positive for Ref-1. Positive staining was also apparent in cells lining the bile duct (BD). B: Double staining for Ref-1 (black) and glutamine synthetase (GS; brown). Colocalization (indicated by arrowhead) demonstrates Ref-1 expression in hepatocytes; the arrow indicates a hepatocyte nucleus positive for Ref-1. HV: terminal hepatic venule C: Double staining for Ref-1 (black) and β-catenin (brown). Colocalization (indicated by arrowhead) demonstrates Ref-1 expression in biliary epithelial cells; the arrow indicates a hepatocyte nucleus positive for Ref-1. D: Double staining for Ref-1 (black) and Trx-1 (brown). Arrowheads indicate colocalization in bile ducts (BD); double arrows indicate colocalization in sinusoidal lining cells (SEC; Kupffer cells). The arrow indicates a hepatocyte positive for Ref-1. Sections in panels B-D were not counterstained. All panels are images from young, control rats and are representative of 5 individual animals. The cellular localization of Ref-1 was identical in old, nonheated rats (not shown). Scale bar = 100 μm.

The ratio of normal portal tracts to ductular reactions (DR, or areas of bile duct proliferation) was assessed in old rats only since there was no evidence of DR in young rats. Portal tracts were considered normal if they displayed the characteristic rosette morphology, containing the hepatic artery, bile duct, portal vein, and lymphatic vessel. Portal tracts were considered DR if they contained several ductular structures and lacked the characteristic rosette appearance. Examples of each are presented in **Figure 5**. The number of each type of tract was counted, and the percent of abnormal tracts was determined for each animal. These percentages were averaged across the old, nonheated group.

Statistics

Immunoblotting results were analyzed by two-factor ANOVA to determine the effects of age and heat stress. Where appropriate, Tukey's

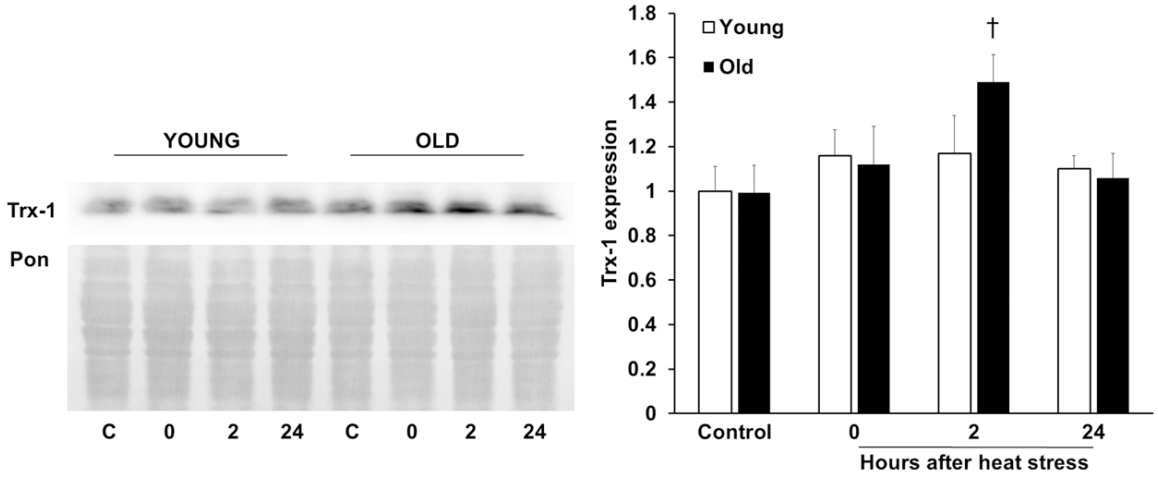

Figure 3. Increase in Trx-1 abundance in old animals after hyperthermia. Left panel: representative immunoblot of Trx-1 in young and old animals under control conditions, and at the indicated times after heat stress (in hours). Ponceau stained membrane (Pon) demonstrates equal loading and transfer. Right panel: quantitation of Trx-1 expression, normalized to the Ponceau stain and further normalized to the young, nonheated group. †Significant effect of heat stress (n = 7-9 young and old animals in the nonheated condition, and at each time point after heat stress).

post hoc test was used to determine significant differences between groups. A *p*-value of less than 0.05 was considered statistically significant. The SPSS program (IBM) was utilized for statistical purposes.

Results

In the nonheated condition, expression of Ref-1 was more than 2-fold greater in old rats, compared to young rats ($p < 0.05$); this age-related difference was also observed at all time points after heat stress. While young rats demonstrated an increase (~50%) in Ref-1 protein abundance immediately (0 h) after heat stress, old rats did not experience changes in Ref-1 after hyperthermia. This increase in Ref-1 in young rats was transient, and returned to control values by 2 h after heat stress (**Figure 1**).

To determine the cellular localization of Ref-1 in the liver, we utilized immunohistochemistry on paraffin-embedded sections. Single-staining experiments suggested a localization of Ref-1 in hepatocytes and biliary epithelial cells, as well as occasional staining in sinusoidal-lining cells (**Figure 2A**). Immunohistochemistry for Ref-1 and glutamine synthetase (GS; an enzyme expressed in hepatocytes adjacent to terminal hepatic venules [32]) confirmed hepatocyte localization of Ref-1 (**Figure 2B**). Additional double-staining experiments with β-catenin (which is expressed in biliary epithelial cells [33]) and Trx-1 (expressed in Kupffer cells; **Figure 4**) demonstrated that Ref-1 is also expressed in both of these cell types (**Figure 2C** and **2D**). With the exception of DR (as described below), identical cellular staining patterns of Ref-1 were observed in young and old nonheated rats. Therefore, images from only young control rats are shown in **Figure 2**.

Due to the heat-induced increase in Ref-1 in the young rats, we assessed potential nuclear translocation by counting nuclei positive for Ref-1 in the young control, and young heated group (0 h). We did not observe a difference in the percentage of positive nuclei with hyperthermia, nor were there heat-induced differences in cytoplasmic staining (not shown). In all groups, the percent of hepatocyte nuclei positive for Ref-1 was ~88% (not shown), which is consistent with a previous investigation [34].

Hepatic expression of Trx-1 was not affected by aging; however, we observed a significant increase in Trx-1 at 2 h after heat stress in the old, but not in the young group (**Figure 3**). To obtain consistent results for Trx-1 via immunoblot, it was necessary to load large amounts of lysate, and to use relatively high antibody concentrations (see Methods). It is also worth noting that immunostaining for Trx-1 required a more concentrated antibody dilution than for Ref-1. Therefore, it appears that Trx-1 expression in the liver is much lower than that of Ref-

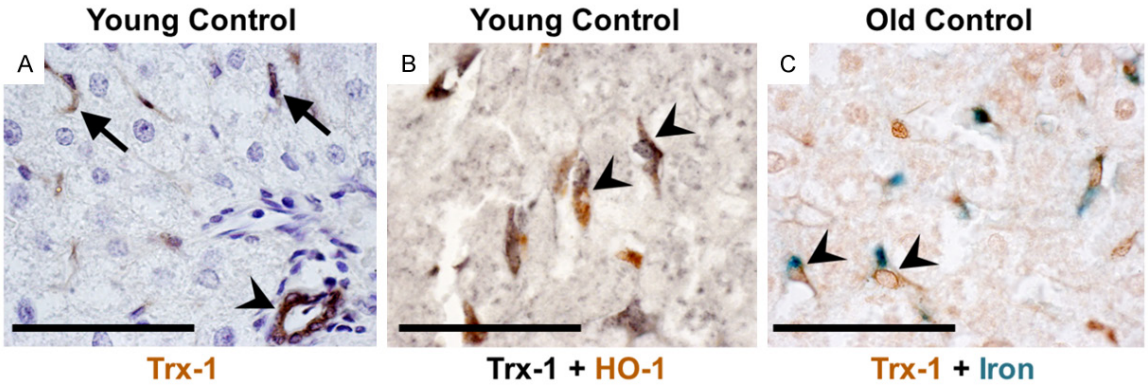

Figure 4. Localization of hepatic Trx-1. A: Trx-1 staining in a young, control liver. An identical pattern of cellular localization was observed in the old control rats (not shown). The arrowhead indicates positive staining in a bile duct, arrows indicate positive staining in nonparenchymal cells. B: Colocalization of Trx-1 (black) and HO-1 (brown) in a young control rat; double-positive cells are indicated by arrowheads (results are representative of 3 separate young and old control animals, with the experiment run in triplicate). C: Colocalization of Trx-1 (brown) and iron deposits (cyan) in an old, control rat, double positive cells are indicated by arrowheads (results are representative of 3 separate old animals). Scale bar = 100 μm.

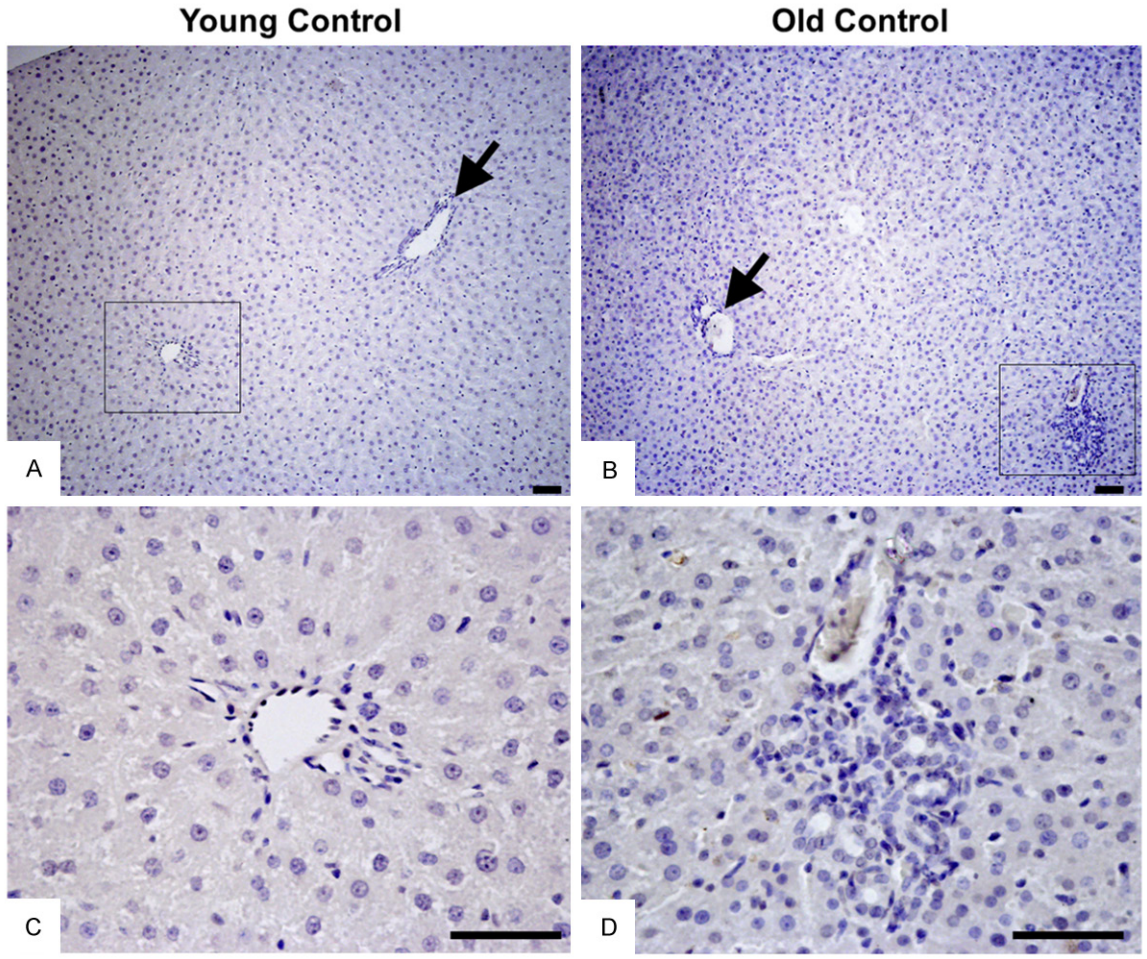

Figure 5. Aging is associated with ductular reactions in the liver. A: Low magnification micrograph of a young control liver. The arrow indicates a normal portal tract. The area within the box is shown in Panel C at a higher magnification. B: Low magnification view of an old control liver. The arrow points to a normal portal tract, and the area within the box is shown in Panel D at a higher magnification. Scale bar = 100 μm.

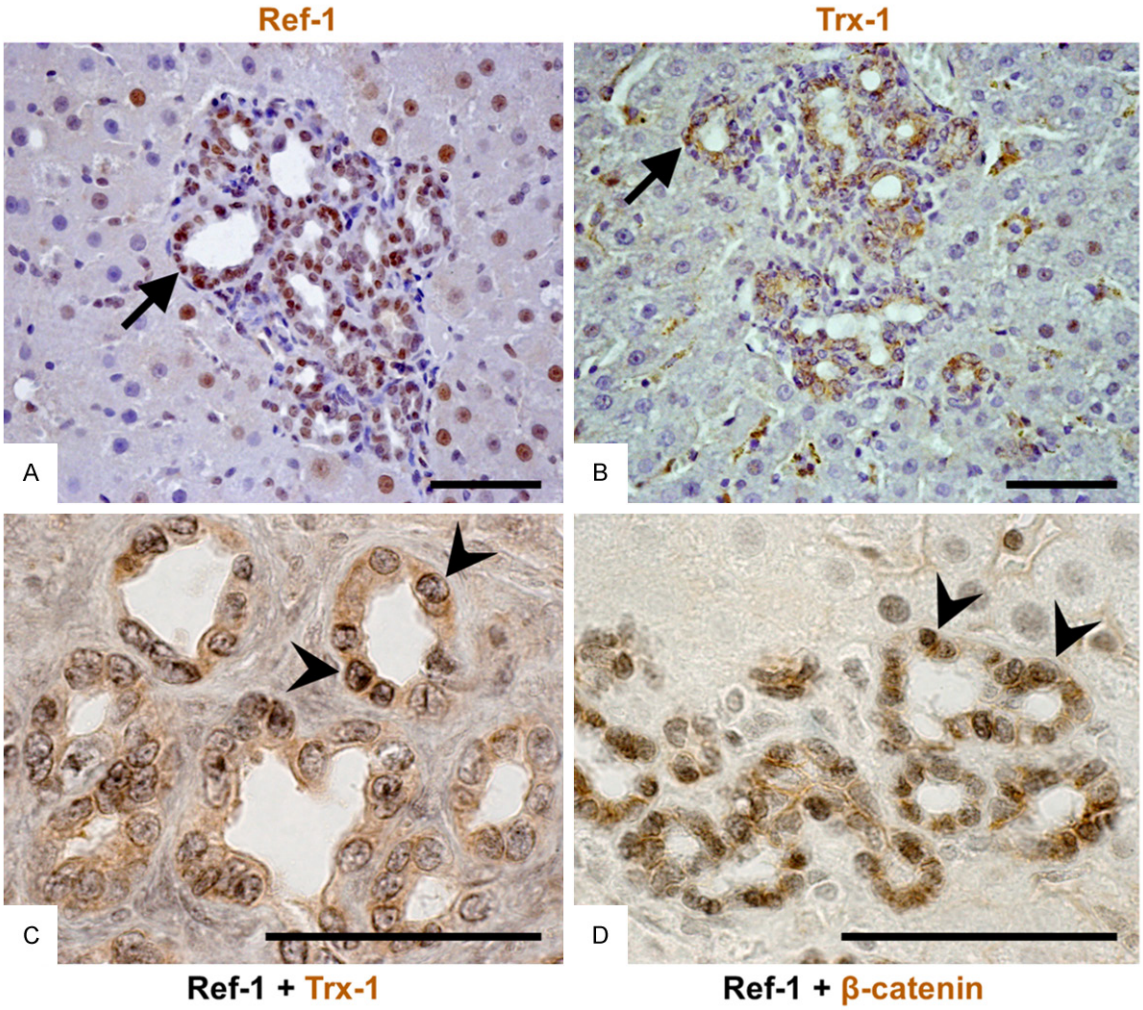

Figure 6. Colocalization of Ref-1 and Trx-1 within ductular reactions of aged animals. A: Ductular reaction (DR) in an old rat showing positive staining for Ref-1. The arrow indicates a cell positive for Ref-1. Multiple nuclei within bile ducts cells are positive for Ref-1. B: Ductular reaction in an old rat showing positive staining for Trx-1. The arrow indicates a cell positive for Trx-1. Frequent positive staining for Trx-1 was observed in cells within DR. Sections in Panels A and B were counterstained with hematoxylin. C: Colocalization of Ref-1 (black) and Trx-1 (brown) in cells of a DR. Arrowheads indicate cells positive for both Ref-1 and Trx-1. D: Colocalization of Ref-1 (black) and β-catenin (brown) in biliary epithelial cells of a DR. Arrowheads indicate cells positive for both Ref-1 and β-catenin. Sections in Panels C and D were not counterstained. Scale bar = 100 μm.

1. Immunohistochemistry for Trx-1 confirmed these results, showing punctate staining in bile ducts and nonparenchymal cells, with weak to negative staining in hepatocytes (**Figures 2D**, **4**, **6B** and **6C**). We did not observe clear changes in Trx-1 immunoreactivity after heat stress in either age group (not shown).

Due to the nonparenchymal appearance of the Trx-1-positive cells, we suspected that they were Kupffer cells (hepatic macrophages). To better identify the cell type(s) expressing Trx-1, we performed two double-staining experiments. First, we stained for both Trx-1, and heme oxygenase-1 (HO-1), a specific marker of Kupffer cells in quiescent liver [30]. We observed colocalization of Trx-1 and HO-1 in both young and old control animals (**Figure 4**). Additionally, we stained for Trx-1 and iron deposits in old rats only (since young rats do not display stainable iron), and observed colocalization of Trx-1 and iron in nonparenchymal cells (**Figure 4C**).

While assessing the staining patterns of Ref-1 and Trx-1, we observed that old rats displayed frequent ductular reactions (DR; **Figures 5** and **6**), which are areas of bile duct proliferation. DR

comprised 43% ± 8 (S.D.) of all portal tracts in the old, nonheated group. While the sizes of the DR were highly variable, they were present in all (8 of 8) of the old control rats examined, and none of the young rats (**Figure 5**). Recently, mild heat stress has been shown to increase the proliferative ability of hepatocellular carcinoma cells [35]; therefore, it was of interest to characterize the effects of heat stress on the number of DR in old rats. Heat stress did not alter the number of DR in old rats (not shown). DR were positive for Ref-1 and Trx-1, and double-staining experiments using β-catenin demonstrated colocalization of these proteins within biliary epithelial cells (**Figure 6**).

Discussion

After physiological heat stress, old animals experience hepatic necrosis [28] and oxidative injury [1]. In this investigation, we have identified an upstream mechanism for these observations - an inability to induce Ref-1 protein. Although old rats had 2-fold higher levels of Ref-1 compared to young rats, aging resulted in a blunted induction of Ref-1 after hyperthermia. Despite greater expression of this protective protein, steady-state levels of ROS and oxidative injury are elevated in old rats [1], which suggests that Ref-1 dysfunction occurs with aging. The mechanism for the age-related increase in steady-state levels of Ref-1 could involve both transcriptional and translational mechanisms. For example, since Ref-1 mRNA and protein both increase in response to ROS [2-4], it is possible that a chronic state of oxidative stress, such as that which occurs with aging, stimulates the expression of Ref-1 protein in this age group. It is also possible that the accumulation of Ref-1 is due to impaired degradation of the protein, likely due to decreases in both proteasomal function and chaperone-mediated autophagy with aging [36, 37].

In contrast to old rats, young rats induced Ref-1 by approximately 50% immediately (0 h) following heat stress. In cell culture models, hydrogen peroxide treatment stimulates Ref-1 protein expression and activity, which confers protection against oxidative stress and DNA damage [8-10]. The protective activity of Ref-1 is abolished with cycloheximide (CHX; a protein synthesis inhibitor) [4], suggesting that new protein synthesis is necessary for maintenance of homeostasis after an oxidative challenge. Combined with our present results, we suggest that the increase in Ref-1 protein in young rats plays a protective role after heat stress since young rats do not develop oxidative injury in this model [1]. The lack of induction of Ref-1 in old rats coincided with an increase in DNA damage [1], further suggesting that the repair function of Ref-1 is diminished with aging. Our observations are similar to previous reports utilizing toxic stressors. For example, in response to carcinogen exposure (2-nitropropane), young animals exhibited a robust increase in Ref-1, while old animals demonstrated a decrease in protein expression and activity [38].

While the interaction of Ref-1 and Trx-1 enhances AP-1 activation [11, 12], increasing Ref-1 protein levels alone can stimulate AP-1 activity [39]. Aging attenuates the activation of AP-1 after heat stress [1], and in this study, we observed a blunted induction of Ref-1 protein after heat stress in old animals. Therefore, our results suggest that the inability to increase protein levels of Ref-1 may contribute to the blunted activation of AP-1 in old rats after heat stress. Supporting this finding, in previous studies, we have observed that old rats demonstrate an impairment in HO-1 induction after hyperthermia [40], which is an AP-1 target protein [15]. Since we did not observe nuclear staining of Trx-1 in hepatocytes under any condition (which is consistent with another study [41]), this suggests that these two proteins do not interact to facilitate AP-1 activity in this model. However, it is possible that trace amounts of Trx-1 exist in hepatocyte nuclei that were not detected by our methods.

Trx-1 is induced by oxidative stress, and protects against cellular injury in several model systems [12, 18, 19, 21, 22]. In our model, old animals demonstrate exaggerated oxidative stress after hyperthermia compared to young rats [1]; therefore, the augmented concentrations of ROS might stimulate Trx-1 protein expression. Since it has been reported previously that old rats also have increased oxidative injury at similar times as the increase in Trx-1 [1], our results suggest that Trx-1 does not mitigate heat-induced ROS. Manipulation of Trx-1 expression will be necessary to more fully delineate its role after heat stress in the old cohort.

Using two double-staining methods, we have demonstrated that Trx-1 is expressed in Kupffer cells in both young and old animals. This non-parenchymal localization has been observed previously [22], but the cell type expressing Trx-1 has not been thoroughly investigated. The predominately nonparenchymal expression of Trx-1 in the liver helps to explain the low levels detected via immunoblot; since hepatocytes are the most numerous cell type in the liver, immunoblotting on whole-tissue lysates would tend to detect proteins in hepatocytes primarily. It is tempting to speculate that Trx-1 in Kupffer cells has a strong protective role, considering that these cells continuously turn over iron [31], and are thus potentially exposed to labile iron, which can facilitate oxidative injury.

Nonparenchymal staining of Trx-1 was also observed in bile ducts, and in DR in old rats. To our knowledge, this cellular localization of Trx-1 has not yet been reported. The incidence of ductular reactions increases with aging in rats [42]. Our investigation has furthered this observation by showing that biliary epithelial cells (which express β-catenin) within these structures are positive for both Ref-1 and Trx-1. Given that these proteins play anti-apoptotic, and cell cycle-stimulatory roles, it is likely that they contribute to the proliferative phenotype in this cellular niche [17, 43]. It is also interesting to note that the expression of Ref-1 and Trx-1 in hepatocellular carcinoma (HCC) is predictive of poor prognosis [44, 45]. While the cause of the development of DR with aging remains to be elucidated, it is possible that inflammation-, or ROS-induced cell-cycle stimulation could play a role in this process [46]. Kupffer cell numbers are greater in the periportal, compared to the perivenous region of the liver [40], and their numbers increase with age [40, 47]. Since Kupffer cells stimulate both inflammation and oxidative stress, the monocyte-macrophage lineage of cells could be contributing to the increase in DR with aging. If bile ducts are a source of proliferative cells that do contribute to HCC, it would suggest that old animals are predisposed to liver disease. Indeed, old donor livers are more susceptible to fibrosis [48], and aged animals experience an exaggerated fibrotic response to carbon tetrachloride-induced hepatic fibrosis [49]. Moreover, the frequency of DR increases with age in the context of liver disease in humans [50-54], and the presence of DR is associated with poor prognosis in HCC [55]. Here we show that aging is associated with DR; therefore the Fischer rat might serve as a useful model to study the development and significance of DR with aging and hepatic disease.

Conclusion

Overall, we have shown that aging is associated with an inability to induce Ref-1 after a physiologically relevant challenge, which identifies a mechanism for the hepatic cell death [28] and oxidative injury previously observed after heat stress [1, 27]. Furthermore, the inability of old rats to induce Ref-1 after heat stress likely contributes to reduced cellular protection after heat stress via decreased activation of AP-1, leading to decreased transcription of target genes such as HO-1. Thus, potential therapeutic modalities that improve Ref-1 activity will likely prove useful to treat heat-related illness in elderly populations.

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Disclosure of conflict of interest

None.

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