Induction after Administering Paraquat of Heme Oxygenase-1 and Heat Shock Protein 70 in the Liver of Senescence-accelerated Mice

Yukiko NAKANISHI and Kyoden YASUMOTO†

Research Institute for Food Science, Kyoto University, Gokasho, Uji, Kyoto 611, Japan

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Age-associated changes in the induction of heme oxygenase (HO-1) and heat shock protein 70 (HSP70) after the administration of paraquat were investigated in the liver of senescence-accelerated mice (SAMs). The extent of HO-1 and HSP70 induced in response to paraquat decreased significantly with age, and the level of oxidized proteins increased with age. Moreover, the extent of induced HSP70 was lower in mice that were prone to accelerated senescence (SAMP1/Fky) than in mice that were resistant to accelerated senescence (SAMR1/Fky) of the same age. These results suggest that an age-associated decline in the levels of HO-1 and HSP70 enhanced oxidative damage during the aging process. Age-dependent changes in HO-1 and in the levels of oxidized proteins were examined in SAMP1/Fky. The accumulation of oxidized proteins was suppressed when HO-1 was induced, but increased markedly after the induction of HO-1 decreased. Free iron, the residuum from heme degradation, might mediate free radical production. The role of HO-1 is discussed in relation to the oxidative damage associated with age.

Key words: age; senescence-accelerated mouse; heat shock protein 70; heme oxygenase; stress

Aging is an intrinsic and inevitable property of most living mammals. Johnson has recently revealed that mutations in any one of several genes in Caenorhabditis elegans resulted in a significant extension of the mean and maximum life span.12−14 However, extrinsic or environmental factors can influence aging. Of these factors, an accumulation of molecular oxidative damage is attributed to the senescence-associated loss of functional capacity.15 In our laboratory, an age-associated decline in the functional defense capacity against oxidants was shown with senescence-accelerated mice (SAMs)5−7 which has been widely employed for investigating the mechanism of the aging process: 1) the activity of such antioxidative enzyme activities as glutathione peroxidase, glutathione-S-transferase and superoxide dismutase in erythrocytes and liver decreased with age.8 2) the ratio of DNA single-strand breaks and the level of oxidized protein in the liver increased with age,9−11 and 3) these changes in mice that were prone to accelerated senescence (SAMP1/Fky) were more marked than in mice that were resistant to accelerated senescence (SAMR1/Fky).8−11

The ability to withstand stressors such as oxidizing molecules may be the key requirement for a longer life span. The HSP70 family of proteins are expressed in most organisms with environmental conservation.12,13 The HSP70 family including hsp70 and hsc70. The level of hsp70 is very low in nonstressed cells, and its level increases drastically with hyperthermia; hsc70 is classified as a constitutive type of protein. Changes in the induction of HSPs in various tissues might be an important factor in the aging process. The induction of hsp70 in isolated hepatocytes from rats,14,15 in cultures of lung- and skin-derived fibroblasts from rats,16 and in human diploid fibroblasts17 significantly decreased with the age of the animals. Holbrook et al. have reported that the induction of restraint-induced HSP70 was attenuated by age.18,19 The most characteristic feature of senescent organisms is that they show diminished responsiveness to stimuli or stresses in maintaining homeostasis. Very little is currently known about the in vivo effect of aging on the induction of HSPs in animals. Most investigations of HSPs have been performed with isolated cells from animals, and there have only been a few investigations about the induction of HSP70 in animal experiments.18,19,23−28

Heme oxygenase, the rate-limiting enzyme in the heme-degradative pathway, catalyzes the degradation of heme to biliverdin, carbon monoxide, and the ferric ion.29 Biliverdin is rapidly converted to bilirubin by biliverdin reductase.30 Bilirubin has recently been shown to scavenge the reactive oxygen species that are produced in vivo by oxidative stress.31 Two isozymes of heme oxygenase, known as heme oxygenase-1 (HO-1) and heme oxygenase-2 (HO-2), have been characterized to date. HO-1 was initially identified as a metal- and oxidative stress-inducible isozyme,32−36 and is considered to function in the cellular defense system against oxidative stress.37 In contrast, Nutter et al. have shown that the induction of heme oxygenase did not protect human cells against oxidant stress.38 The more recently identified isozyme of heme oxygenase, HO-2, is a constitutive and noninducible in rats, rabbits, and humans.39−41 In this respect, heme oxygenase resemble members of the HSP70 family, although a great deal remains to be studied about their functions.

The reduced ability of senescent organisms to respond to stress and to maintain homeostasis can be observed in many mammal species. In this study, we investigated the age-associated changes in response of heme oxygenase and of HSP70 to paraquat (methyl viologen; a potent herbicide)

† To whom all correspondence should be addressed.

Abbreviations: ANOVA, analysis of variance; DNPH, 2,4-dinitrophenylhydrazine; Fisher’s LSD test, Fisher’s least significant difference test; HO, heme oxygenase; HSPs, heat shock proteins; hsp70, inducible heat shock protein 70; hsc70, constitutive heat shock protein 70; HSP70, hsp70 and hsc70; SAM, senescence-accelerated mice.
in the liver of SAMR1/Fky mice and SAMPI/Fky mice. We also examined the physiological role of HO-1 induced by the administration of paraquat.

**Materials and Methods**

*Animals and treatment.* Mice were bred in our laboratory and maintained at 23°C with a 12-h light-dark cycle and 60% humidity. A commercial diet (CE-2; Nihon CLEA, Tokyo, Japan) and deionized water were supplied ad libitum. In experiment 1, male mice of different ages (2, 7, and 12 months for SAMPI/Fky, and 2, 7, 12, and 17 months for SAMR1/Fky) were each divided into two groups. One group of mice of each strain was given a subcutaneous injection of paraquat (0.3 mmol/kg of body wt), and the other group of mice was used as the control. Three hours after the injection, the mice were anesthetized with ether and killed by decapitation. In experiment 2, the 2- and 7-month-old male SAMPI/Fky mice were given a subcutaneous injection of paraquat (0.3 mmol/kg of body wt). When 0, 3, 6, 9, 12, 18, and 24 h had elapsed after the paraquat administration, the mice were anesthetized with ether and killed by decapitation.

*Preparation of the liver homogenates.* After their perfusion with a 0.9% NaCl saline solution, the livers were removed and stored at -80°C. The frozen livers were homogenized in a glass-Teflon homogenizer with an ice-cooled 20 mM Tris-HCl buffer (pH 6.8). Each homogenate was centrifuged (10,000 × g, 10 min), and the resulting supernatant was used for the subsequent analyses.

*Quantification of expressed HO-1, HO-2, and HSP70.* The concentration of proteins in the supernatant was determined by the modified method of Lowry et al., with bovine serum albumin as a standard. The proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 13.5% polyacrylamide gel for HO-1 and 7% polyacrylamide gel for HSP70, as described by Laemmli. The separated proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane, and HO-1, HO-2, and HSP70 were detected by Western blotting with antibodies raised against each protein (StressGen Corp., Victoria, BC, Canada). Band intensity was determined with a CS-9000 scanning densitometer (Shimadzu, Kyoto, Japan). Calibration curves were prepared by using purified HSP70 (StressGen Corp.) or HO-1 (StressGen Corp.), as a standard at a concentration from 2.0 ng to 200 ng of protein per lane. Good correlation was obtained: \( r^2 = 0.978 \) for HO-1 and \( r^2 = 0.966 \) for HSP70. To compare the levels of HO-2, samples were used from each group of 2-month-old mice, because purified HO-2 was not available.

*Measurement of oxidized protein levels.* The carbonyl content of the proteins was spectrophotometrically determined after a reaction with 2,4-dinitrophenylhydrazine (DNPH).

*Statistical analyses.* Each result is expressed as the mean ± SEM for five animals in experiment 1 and for three animals in experiment 2. One-way and two-way analyses of variance (ANOVA) were performed as appropriate in each experiment. When a difference was significant (\( p < 0.05 \)), Fisher’s least significant difference test (Fisher’s LSD test) was applied to examine differences between individual groups with the StatView statistical software (1992 version; Abacus Concepts, Berkeley, CA, U.S.A.).

**Results**

*Induction of HSP70 in the liver in response to the administration of paraquat.* We first examined whether or not paraquat, a generator of oxygen-derived free radicals in vitro, would induce the expression of HSP70.

The steady-state level of HSP70 gradually increased with age in the liver of SAMs. The levels of HSP70 in the livers of 17-month-old SAMR1/Fky mice and 12-month-old SAMPI/Fky mice were 9-fold and 3-fold greater, respectively, than that of 2-month-old mice of either strain (Fig. 1A). To evaluate the relative change of stress response with age, the relative ratio of HSP70 level to that in the control mice was calculated as the same age for each strain. The HSP70 level increased markedly in the liver of mice 3 h after the administration of paraquat. The induction of HSP70 after paraquat administration in 2-month-old SAMR1/Fky mice was 30-fold greater than that in control mice. In the case of SAMPI/Fky mice, the induction of HSP70 was very low (less than 6-fold) and decreased with age (Fig. 1B).

*Induction of HO-1 in the liver in response to the administration of paraquat.* No steady-state level of HO-1 was detectable (less than 20 ng/mg of protein), but the levels of HO-1 increased markedly in the liver of mice 3 h after administration of paraquat. There was no significant difference between the SAMR1/Fky and SAMPI/Fky mice at the same month of age, and the induction of HO-1 decreased with the age of both types (Fig. 2).

*Level of oxidized protein in the liver after administering paraquat.* The steady-state level of oxidized protein increased with age, this change being more marked in SAMPI/Fky than in SAMR1/Fky mice. Paraquat administration significantly increased the level of oxidized protein in both types (Fig. 3).

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**Fig. 1. Age-associated Change in the Induction of HSP70 in the Liver in Response to Administering Paraquat.**

Each value in the mean ± SEM (n = 5 for both strains of mice). The level of HSP70 (A) and calculated relative level (B) in the liver of SAMPI/Fky (filled circles) and SAMR1/Fky (open circles) mice determined as described in Materials and Methods. A) Solid lines show the PO-treated group and dashed lines show the control group. B) Stress response is given by the relative concentration of HSP70 to that of the control group. Values not sharing the same superscript are significantly different (\( p < 0.05 \)) within each strain of mouse as determined by Fisher’s LSD test. Statistical differences are shown between SAMPI/Fky mice and SAMR1/Fky mice of the same age: *p < 0.05 as determined by Student’s t-test.
Fig. 2. Age-associated Change in the Level of HO-1 in the Liver in Response to Administering Paraquat.

Each value is the mean ± SEM (n = 5 for both strains of mice). The level of HO-1 in the liver of SAMPl/Fky (filled circles) and SAMR1/Fky (open circles) mice were determined as described in Materials and Methods. No HO-1 was detected in the control group. Superscript letters and asterisks are defined in Fig. 1.

Fig. 3. Age-associated Change in the Level of Oxidized Proteins in the Liver in Response to Administering Paraquat.

Each value is the mean ± SEM (n = 5 for both strains of mice). The levels of HO-1 and oxidized proteins in the liver of SAMPl/Fky (filled circles) and SAMR1/Fky (open circles) mice were determined as described in Materials and Methods. Superscript letters and asterisks are defined in Fig. 1.

Time-course plots for the induction of HSP70 and HO-1, and for the levels of oxidized protein in response to paraquat administration in the liver of SAMPl/Fky mice

The plots for the induction of HSP70 and HO-1, and for the levels of oxidized protein in 2-month-old were different from those in the 7-month-old SAMPl/Fky mice. An increases in the level of oxidized protein followed the decreased induction of HO-1 after administering paraquat (Figs. 4 and 5).

Discussion

The induction of HO-1 and HSP70 was maximal in 2-month-old mice and then decreased with age (Figs. 1 and 2). In particular, the extent of HSP70 induction was higher in SAMR1/Fky mice than in SAMPl/Fky mice of the same age. HSP70 has an important role in cells as a molecular chaperone and helper along the pathway to protein folding. Therefore, the decreased induction of HSP70 in response to oxidative stress suggests the in vivo accumulation of denatured proteins. The extent of HO-1 and HSP70 induction was significantly affected by age (p < 0.001 and p < 0.001, respectively) and by the administration of paraquat (p < 0.005 and p < 0.05, respectively). Two-way ANOVA for the induction of HO-1 and HSP70 revealed significant interaction (p < 0.005 and p < 0.001) between the administration of paraquat and age. These results indicate an age-dependent decrease in the intensity of stress response. The administration of paraquat did not induce HO-2 in the liver of mice at any age (data not shown).

The administration of paraquat did, however, increase the level of oxidized proteins (Fig. 3). The level of oxidized proteins was significantly increased as a function of the age of the animals (p < 0.005) and the administration of paraquat (p < 0.001). Thus, our results suggest that aging decreased the extent of HSPs induction in response to stress. Therefore, the older animals appear to have been more susceptible to the potentially deterious reactions induced by...
oxidative stress than the younger animals. In SAMPI/Fky mice, a more profound decrease in the extent of HSPs induction was noted in response to paraquat than that in SAMR1/Fky mice of the same age.

The effects of aging on the time-course for the induction of HO-1 and HSP70 and for the level of oxidized protein in response to the administration of paraquat were then examined. Paraquat administration caused a rapid and transient increase in the hepatic levels of HO-1 and HSP70 within 3 h. However, the level of HO-1 returned to the basal value earlier than that of HSP70 in the 2-month-old SAMPI/Fky mice (Figs. 4 and 5). Mitani et al. have reported that metalloporphyrins strongly induced mRNA for HO-1 without any effect on the level of mRNA for HSP70.43 In considering these findings, our results also suggest the existence of a different signaling pathway for the induction of HO-1 from that of HSP70, even though the regulatory elements for gene expression are shared by these stress proteins.

The level of HSP70 decreased earlier in the 2-month-old SAMPI/Fky mice than in the 7-month-old SAMPI/Fky mice (Fig. 4). Heydari et al. have shown that the half-life of mRNA for HSP70 was longer in hepatocytes isolated from old rats than that of HSP70 mRNA in hepatocytes isolated from young adult rats.15 Thus, it seems likely that HSP70 is degraded more slowly with increasing age.

The level of HO-1 transiently increased before the level of oxidized protein increased (Fig. 5). Llesuy et al. have reported as increase in heme oxygenase activity that followed a transient decrease in the intracellular glutathione pool and an increase in the level of intracellular hydrogen peroxide after administering cobalt chloride.37 The induction of heme oxygenase leads to the production of bilirubin.29,30 Bilirubin has recently been shown to scavenge reactive oxygen species that are produced in vivo by oxidative stress.31 Our results support the hypothesis that HO-1 plays an important role in the antioxidative system.

The level of oxidized proteins increased significantly after a decrease in the extent of HO-1 induced. Nine hours after administering paraquat, the level of oxidized proteins in 2-month-old SAMPI/Fky mice was higher than that in 7-month-old SAMPI/Fky mice (Fig. 5). This result supports the hypothesis of Nutter et al. that heme oxygenase does not protect human cells against oxidative stress.38 Heme oxygenase, the rate-limiting enzyme in the heme-degradative pathway, catalyzes the degradation of heme to biliverdin, carbon monoxide, and the ferric ion.28 An increased level of the free ferric ion in tissues accelerates the production of reactive oxygen species. Thus, subsequent oxidative damage might be a result of the increased level of the ferric ion. Consequently, the increased level of oxidized protein suggests that induced HO-1 acts as a pro-oxidant. On the other hand, He et al. have shown that a dietary paraquat administration diminished the activities of antioxidative enzymes, and increased the level of oxidized proteins and the values of thiobarbituric acid-reactive substances (TBARS) in the blood and liver.5 The increase in oxidized protein level was also attributed to the addition of malondialdehyde derived from peroxide lipids.

That the SAMPI/Fky mouse was prone to senescence is the most prominent biological feature of this strain of mouse, which is deemed suitable as a model for a variety of studies involving stress treatment. As the molecular events governing the mechanism for accelerated senescence in the SAMPI/Fky strain have not yet been elucidated, the metabolic defects responsible for the observed induction of stress proteins caused by paraquat-induced oxidative stress remain to be elucidated. However, we examined in this study the effect of aging on the induction of HO-1 and HSP70 after administering paraquat to mice that were resistant to and prone to accelerated senescence. Our results suggest that an age-associated decline in the extent of induced HO-1 and HSP70 would enhance oxidative injury. Furthermore, we found that the level of oxidized protein markedly increased after the induction of HO-1 decreased under the state of oxidative stress.

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