

Effect of Growth Hormone and Calorie Restriction on the Expression of Antioxidant Enzymes in the Liver and Kidney of Growth Hormone Receptor Knockout Mice

Khalid A Al-Regaiey

Department of Physiology, College of Medicine, King Saud University, Riyadh 11461, Kingdom of Saudi Arabia.

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Abstract

Caloric restriction (CR) can delay aging and prolong life span and these actions may be related to reduced oxidative damage. Mice with disrupted growth hormone (GH) receptor/binding protein knockout (GHRKO) live significantly longer than their normal siblings. Therefore, it is of interest to examine the effects of chronic CR on hepatic and renal antioxidant enzymes as well as lipid peroxidation (LP) as an oxidative stress marker in GHRKO mice. Female GHRKO and normal mice were either fed ad libitum (AL) or subjected to 30% CR starting at 2 months of age and examined at the age of 9 months. In the liver, catalase (CAT) activity was significantly increased in GHRKO-AL as compared to normal control -AL animals. CR reduced CAT activity in both GHRKO and normal phenotypes. Cu/Zn superoxide dismutase (SOD1) activity was also higher in GHRKO-AL as compared to normal-AL mice. However, CR reduced SOD1 activity in GHRKO mutants. Glutathione peroxidase (GPx) activity was significantly decreased in GHRKO-AL mice and further reduced in GHRKO-CR group of animals. CR significantly increased LP in GHRKOs while its activity was not altered in GHRKO-AL group of mice. In the kidney, CAT activity was lower in GHRKO-AL as compared to normal-AL, however CR did not induce any significant effect in both phenotypes. Similarly, SOD1 levels were significantly lower in GHRKO than in normal mice. GPx expression was higher in GHRKO-AL as compared to control-AL. CR reduced GPx activity in GHRKO mice but increased it in controls as compared to their AL counterparts. There was no difference in LP expression between

GHRKO-AL and normal-AL mice. However, CR significantly increased its levels in both phenotypes. Although these findings do not support the hypothesis that CR would increase the capacity of ROS defense mechanisms in GHRKO mice by increasing antioxidant enzymes levels, they do agree with some of the reported effects of CR on their expression. We suspect that GH resistance and CR may affect aging by different mechanisms and if CR delays aging in GHRKO animals it is not due to changes in the activity of antioxidant enzymes.

Introduction

It is well known that caloric restriction (CR) profoundly affects physiological and pathophysiological modifications induced by aging and markedly increases life span in a variety of species, including rats, mice, dogs, fish, flies, worms, and yeast (Weindruch and Sohal, 1997). Studies from centenarians and individuals who self-impose CR have documented beneficial effects in terms of reducing cardiovascular and diabetes-related illnesses. A research program named CALERIE (Comprehensive Assessment of the Long-term effects of Reducing Intake of Energy) had shown positive effects of CR on resting metabolic rate, reducing coronary heart disease risk factors, improves insulin sensitivity, and cognitive function (Heilbronn, et al., 2006; Fontana et al., 2007; Larson-Meyer et al., 2006; Redman et al., 2007; Martin et al., 2007).

CR also has beneficial effect on cognitive performance in

* Corresponding author: kalregai@gmail.com

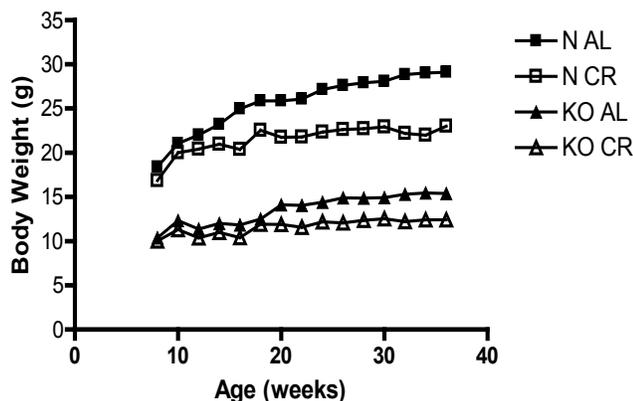
humans, mediated by increased insulin sensitivity and reduced inflammatory activity, leading to higher synaptic plasticity and stimulation of neurofacilitatory pathways in the brain (Witte et al. (2009). In animals, CR delay the occurrence of age-associated disease such as diabetes, cancer and cardiovascular disease and enhance insulin sensitivity (Colman et al., 2009; Wang et al., 2009).

However, the biological mechanism by which caloric restriction may exert its antiaging action is not yet understood. Mechanisms that are thought to contribute to CR effects include retardation of growth, reduction of body fat, reduced insulin and glucose levels, reduced reproductive capacities, delayed neuroendocrine changes, improved DNA repair capacities, reduced body temperature and metabolic rate, amelioration of oxidative stress damage, and hormesis (Sohal and Weindruch, 1996; Mattison et al., 1998; Masoro, 1998).

The free radical theory of aging proposes that oxidative stress is a causal factor in aging process. The imbalance between free radical production and cellular antioxidant defense leads to the accumulation of oxidative damage to the cellular macromolecules. Cellular endogenous antioxidant defense includes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and others. The importance of antioxidant enzymes in defying oxidative stress is evidenced by the observation that overexpression of antioxidant enzymes extends lifespan in *Drosophila* (Orr and Sohal, 1994; Parks et al., 1998) and that treatment of *C. elegans* with synthetic SOD/CAT mimetics extended its lifespan (Melov et al., 2006).

Studies in *Drosophila*, *C. elegans*, and mutant mice provide evidence that insulin-like signaling contributes to the aging process. Mutations in the *daf-2* insulin receptor-like gene or the downstream *age-1* gene extend the life span of *C. elegans* two- to threefold (Kenyon et al. 1993). Ames and Snell dwarf mice, deficient in GH (growth hormone), PRL (prolactin) and TSH (thyroid stimulating hormone), live much longer than their normal siblings (Brown-Borg et al., 1996; Flurkey et al., 2001). CR was shown to cause significant extension of lifespan in long-lived Ames dwarf mice (Bartke et al., 1999) and in long-lived *daf-2* mutant *C. elegans* (Lakowski and Hekimi, 1998). Moreover, The

Figure 1. Weekly body weight changes for female normal and GHR-KO mice that were fed AL or subjected to 30% CR for 9 months starting at 2 months of age.



activity of two important antioxidant enzymes, CAT and Cu/ZnSOD in the liver, kidney, and hypothalamus of Ames dwarf mice is higher than that in their normal siblings (Brown-Borg et al., 1999; Hauck and Bartke, 2000).

GH receptor/GH binding protein knockout mice (GHRKO) are GH-resistant and IGF-1 deficient and were also reported to live significantly longer than their normal counterparts (Tatar et al., 2003). Studies conducted in our lab had shown that in GHRKO mice, CR did not increase the overall, median or average life span of animals and it was suggested that somatotrophic signaling played a key role in mediating the effects of CR to enhance longevity (Bonkowski et al., 2006). In the current study we examined the effects of CR on antioxidant enzymes, namely catalase (CAT), superoxide dismutase (SOD1) and glutathione peroxidase (GPx1) activity and on lipid peroxidation (LP) as an oxidative damage marker in livers and kidneys of these GHRKO mice.

Materials and Methods

Animals

GHRKO female mice and normal (N) littermate controls were produced in a closed colony derived from animals provided by Dr. J. Kopchick and maintained at Southern Illinois University by mating knockout (-/-) males with heterozygous (+/-) female carriers of the disrupted GHR/GH-binding protein gene. Animals were housed on a 12-h light, 12-h dark cycle at 22 ± 2 °C and were fed (Lab Diet Formula 5008, Ralston Purina Corp., St. Louis, MO) and watered ad libitum, except as noted below. All animal procedures were approved by the laboratory animal care and user committee at Southern Illinois University School of Medicine. GHRKO mice and N siblings were fed either ad libitum (AL) or submitted to 30% CR protocol (eight animals per phenotype per diet) as described previously (Bonkowski et al., 2006).

Samples

At 9 months of age, animals were anesthetized by isoflurane, bled by cardiac puncture, and decapitated. To avoid stressing animals, the time between taking the animal from the cage, anesthetizing, bleeding, and decapitation was minimized (≈ 1 min). Livers and kidneys were rapidly removed, quickly frozen on dry ice, and stored at -80°C until processed.

Trunk blood was collected in tubes containing EDTA. After collection, blood was centrifuged at $6000 \times g$ for 15 min at 4°C , the plasma was collected and stored at -80°C .

Total proteins were obtained from whole tissue homogenates. Approximately 100 mg liver/kidney samples were homogenized in 500 μl ice-cold homogenizing buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton 100, with protease inhibitors cocktail and phosphatase inhibitors cocktails (Sigma-Aldrich Corp.)] and spun at $14,000 \times g$ for 45 min. The supernatant was removed and stored at -80°C . Protein concentrations were determined using the bicinchoninic acid assay (Pierce Corp., Rockford, IL) according to the manufacturer's instructions.

Glucose concentrations were determined using a OneTouch Ultra glucose meter (LifeScan, Milpitas, CA). Insulin was assessed by ELISA (Linco Research, Inc. St. Charles, MO), and corticosterone was determined using RIA kits from ICN.

Catalase, Cu/Zn SOD, and GPx Activity

Catalase activity was determined by UV method (Spectronic Genesys 5 spectrophotometer: Milton Roy, Rochester, NY, USA), as previously described (Aebi, 1974). One unit of the enzyme was defined as $\mu\text{mol H}_2\text{O}_2$ disproportionated/min/mg protein. Total Cu/Zn SOD activity was determined by adaptation of the method described by Sun et al., (1994). One arbitrary unit (au) of SOD activity was defined as the amount of cellular protein causing 50% inhibition of the rate of nitroblue tetrazolium reduction. Catalytic activity of the samples was determined by comparison to a standard curve of commercial Cu/Zn SOD (Sigma, St. Louis, MO, USA).

GPx1 activity was determined as described by (Tappel, 1978). One unit of activity was defined as the amount of enzyme required to oxidize 1 μmol of reduced nicotinamide adenine dinucleotide phosphate to NADP per minute per milligram

of protein.

Cellular Thiobarbituric acid-reactive substances (TBARS) were measured (as reaction equivalent to malondialdehyde) as described by Hashimoto et al. (1999). TBARS formation was expressed as nanomoles of MDA per milligram of protein.

Statistical Analysis

Data are expressed as the mean \pm SE. The statistical evaluation was performed using two-factor ANOVA (phenotype and diet), followed by Fisher's protected least significant difference test as a post hoc test. A *t* test was also used to evaluate the effect of diet within phenotypes and phenotype within diet. $P < 0.05$ was considered significant. All statistical analyses were performed using StatView 5.0 software (SAS Institute, Inc., Cary, NC).

Results and Discussion

Despite the fact that the effects of CR and insulin/insulin-like signaling disruption share many similarities, there is evidence that they have their distinct actions. It was previously reported

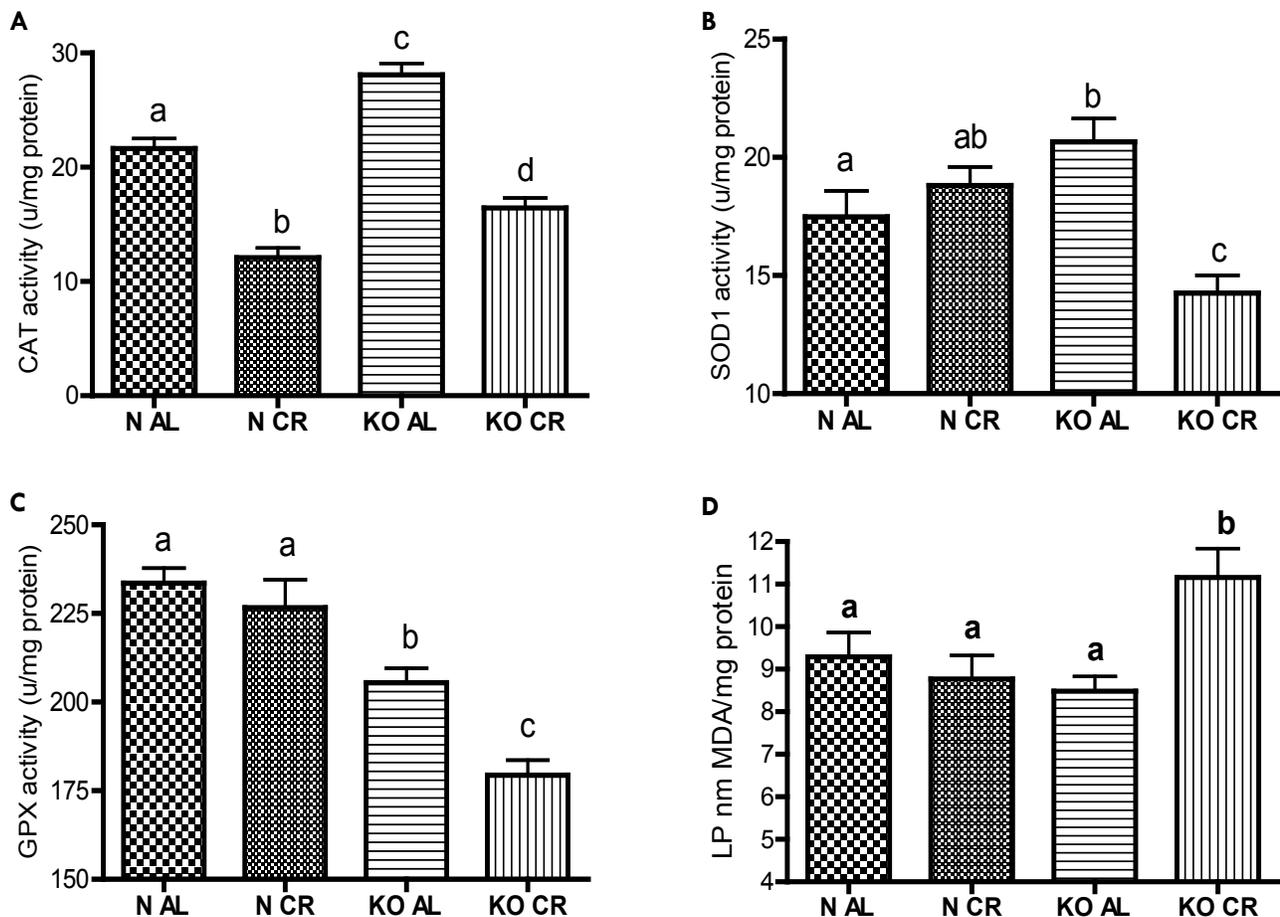


Figure 2. Hepatic antioxidant enzyme activity (A) catalase, (B) Cu/ZnSOD, (C) Gpx1, and (D) lipid peroxidation (LP) in female GHR-KO (KO) and normal (N) mice subjected to caloric restriction (CR) or fed ad libitum (AL). Data reported as mean \pm SE. Groups that do not share a common superscript are significantly different ($p < 0.05$).

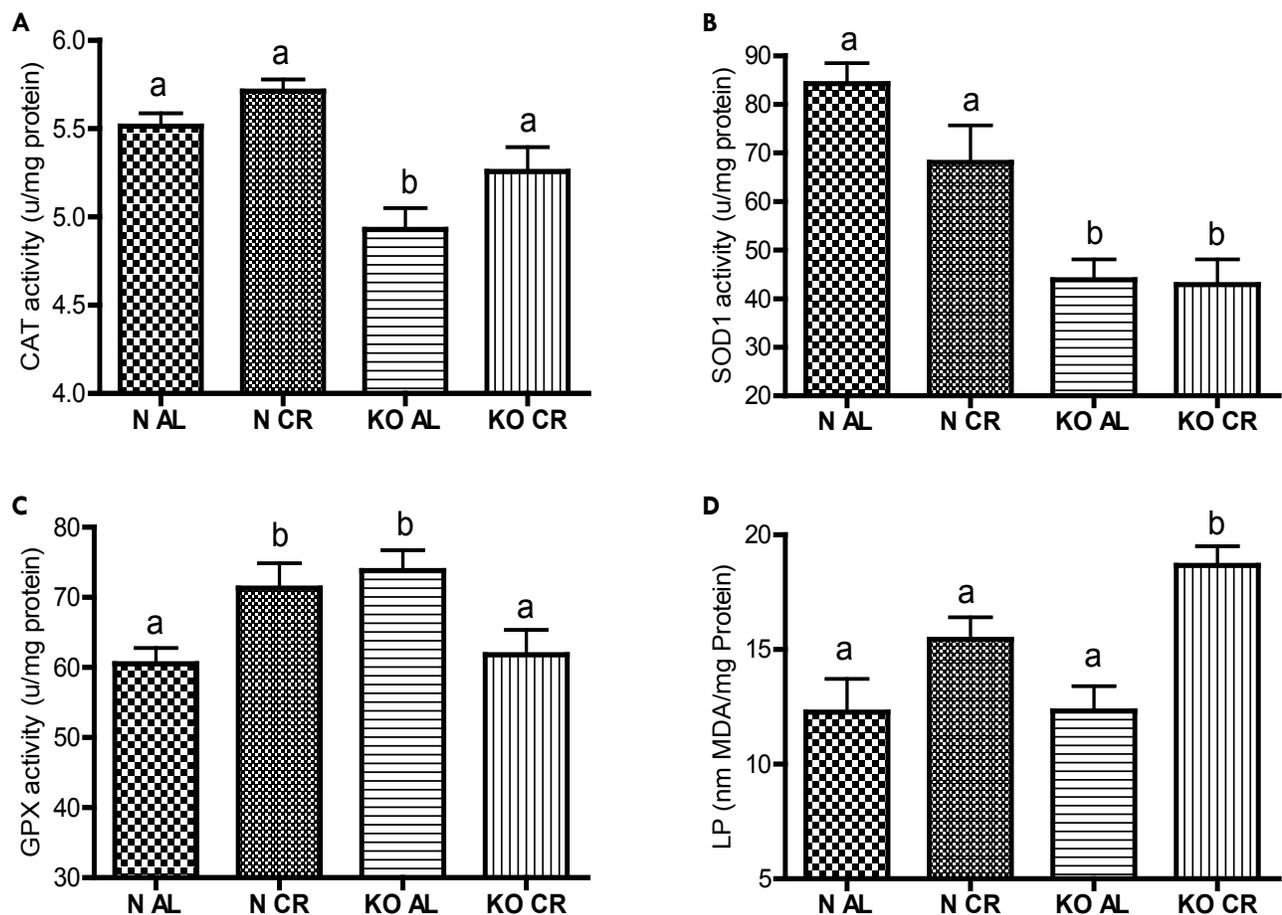


Figure 3. Renal antioxidant enzyme activity (a) catalase, (b) Cu/ZnSOD, (c) Gpx1, and (d) lipid peroxidation (LP) in female GHR-KO (KO) and normal (N) mice subjected to caloric restriction (CR) or fed ad libitum (AL). Data reported as mean \pm SE. Groups that do not share a common superscript are significantly different ($p < 0.05$).

that Ames dwarf mice have extended life as compared to wild type animals (Brown-Borg et al 1996). Furthermore, CR extended the lifespan of long-lived Ames dwarves (Bartke et al, 2001). Studies on murine models suggested that CR significantly increases the activity of several antioxidant enzymes including catalase, superoxide dismutase, and glutathione peroxidase (Koizumi et al 1987, Rao et al 1990, Gomi et al 1998). Ames dwarf mice, having mutations for less pituitary secretions, exhibited enhanced activity of antioxidant enzymes in the liver and kidney than age matched normal controls which showed that increased activity of these enzymes have protective effect against oxidative damage and consequently increases the life span (Brown-Borg et al., 1999, 2000 and Hauck and Bartke, 2000). As mentioned earlier, CR did not increase the overall, median or average life span of GHRKO animals and it is suggested that somatotrophic signaling is important in mediating the effects of CR on life span of animals (Bonkowski et al., 2006). In the current study we sought to elucidate the effects of CR on the activity of antioxidant enzymes in the liver and kidney of these GHRKO animals.

Catalase is an antioxidant enzyme found in all aerobic cells that catalyzes the decomposition of hydrogen peroxide to oxy-

gen and water. A study by Perez et al. (2009) has shown that transgenic mice overexpressing SOD1, catalase or and manganese superoxide dismutase (MnSOD) did not extend the life span of animals. In our study, GHRKO animals had higher catalase activity in the liver and CR reduced it in both normal and GHRKO animals (Figure 2A). In contrast, in the kidney catalase activity was lower in GHRKO animals with no effect of diet (Figure 3A). these tissue specific differences might suggest different mechanisms of somatotrophic signaling and CR on antioxidant capacity. It also questions the notion that catalase mediates the longevity induced by CR.

CuZnSOD (SOD) is the major superoxide dismutase isozyme found in cells. Mice deficient in SOD1 showed 30% reduction in life span (Elchuri et al., 2005; Perez et al., 2009) However, overexpression of SOD did not affect the life span in mice (Huang et al, 2000). SOD1 in the kidney was shown to be protective against oxidative damage. Injury to diabetic rodent kidneys has been reduced by overexpression of the antioxidant SOD (Craven et al, 2001). Moreover, SOD activity was found to be reduced in diabetic nephropathy (Fujita et al, 2009). Nishikawa et al demonstrated the importance of ROS generation in mediating hyperglycemia-induced cellular damage (Nishikawa

et al, 2000). In the current study, GHRKO animals had higher SOD1 activity in the liver and CR reduced it in these animals (Figure 2B). In the kidney, GHRKO animals have significantly lower SOD activity with no effect of diet (Figure 3B). It was shown that increasing SOD concentrations in mammalian cells produce a paradoxical pro-oxidant action by which protection against oxidative stress is lost and injury was even exacerbated, with increased lipid peroxidation (reviewed in McCord and Edeas, 2005). Reduced SOD activity in GHRKO kidney might provide a balanced protection against ROS injury. These results also suggest different effects of GH/IGF-1 and CR on antioxidant capacity.

Studies in Gpx1-deficient mice have revealed many useful rules of Gpx1 in regulating acute oxidative stress. Gpx1 knockout mice appear to be phenotypically normal, However they are highly susceptible to injury induced by paraquat (a superoxide generator), cerebral ischemia-reperfusion (stroke), and cold-induced head trauma (De Haan et al, 2003). However, Gpx1 and SOD2 deficiency in mice does not induce lifespan reduction despite increased oxidative damage (Zhang et al, 2009). In our study, in contrast to catalase and SOD1, Gpx1 activity was lower in GHRKO liver but higher in the kidney (Figures 2C and 3C). It was previously shown that GHRKO and Ames dwarf mice have less severe glomerulonephritis as compared to normal animals (Ikeno et al, 2003). GHRKO kidneys appear to be protected against oxidative damage as indicated by increased Gpx1 activity. However, CR did increase Gpx1 activity in normal animals but decreased it in GHRKOs. This adds to the finding that CR did not further increase the lifespan of GHRKO animals (Bonkowski et al, 2006).

This study identified a high level of redundancy for antioxidant enzymes and oxidative stress as mediators of improved longevity in CR and GHRKO animals.

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