Effects of Growth Hormone and Insulin-Like Growth Factor-1 on Hepatocyte Antioxidative Enzymes

HOLLY M. BROWN-BORG,[1] SHARLENE G. RAKOCZY, MARK A. ROMANICK, AND MELISSA A. KENNEDY

Department of Pharmacology, Physiology and Therapeutics, University of North Dakota School of Medicine and Health Sciences, Grand Forks, North Dakota 58203

The physiological decline that occurs in aging is thought to result, in part, from accumulation of oxidative damage generated by reactive oxygen species during normal metabolic processes. Elevated levels of antioxidative enzymes in liver tissues are present in the Ames dwarf, a growth hormone (GH)-deficient mouse that lives more than 1 year longer than wild-type mice from the same line. In contrast, transgenic mice that overexpress GH exhibit depressed hepatic levels of catalase and have significantly shortened life spans. In this study, we evaluated the *in vitro* effects of GH and insulin-like growth factor 1 (IGF-1) on antioxidative enzymes in mouse hepatocytes. Hepatocytes were isolated from wild-type mice following perfusion of livers with a collagenase-based buffer. Dispersed cells were plated on Matrigel and treated with rat GH (0.1, 1.0, or 10 μg/ml) or IGF-1 (0.5, 5.0, or 50 nM) for 24 hr. Hepatocytes were recovered and protein was extracted for immunoblotting and enzyme activity assays of catalase (CAT), glutathione peroxidase (GPX), and manganese superoxide dismutase (MnSOD). A 41% and 27% decrease in catalase activity was detected in cells treated with GH, whereas IGF-1 reduced CAT activity levels to a greater extent than GH ($P < 0.0001$). The activity and protein levels of GPX were also significantly depressed in cells treated with GH, whereas activity alone was decreased in cells treated with IGF-1 ($P < 0.04$). GH significantly suppressed MnSOD levels by 40% and 66% in 1.0 and 0.1 μg/ml concentrations, respectively. Similarly, IGF-1 decreased MnSOD protein levels (5 nM; $P < 0.05$). These results suggest that GH and IGF-1 may decrease the ability of hepatocytes to counter oxidative stress. In addition, these experiments provide an explanation for the differing antioxidative defense capacity of GH-deficient versus GH-overexpressing mice, and they suggest that GH is directly involved in antioxidant regulation and the aging process.

[Exp Biol Med Vol. 227(2):94–104, 2002]

Key words: hormones; aging; growth factors; catalase; glutathione peroxidase

Mechanisms that control the rate of aging and life span are not well understood. It is known, however, that factors that promote the generation of cellular reactive oxygen species (ROS) and/or impair antioxidative processes contribute to oxidative damage. Oxidative damage accumulates with aging and is likely responsible for the progressive decline in physiological systems that occurs in virtually all organisms. The identification of physiological regulators of antioxidative processes is critical to the understanding of aging and aging processes.

We have shown that mice with hereditary dwarfism (Ames dwarf) exhibit an upregulation of antioxidative defense capacity (1, 2) and a significant extension of life span compared with wild-type siblings (3). These mice are deficient in growth hormone (GH), prolactin (PRL), and thyroid stimulating hormone (TSH) due to a mutation in *Prop-1*, a factor required for appropriate differentiation of the pituitary gland during development (4, 5). Phenotypically identical dwarf mice (Snell; also deficient in GH, PRL, and TSH) also live longer than normal, wild-type siblings (6, 7). In contrast, high plasma GH levels in mice are associated with a depressed antioxidative defense capacity (2, 7) and a life span one-half that of wild-type mice (8). Our evidence in Ames dwarf mice along with reports in other species (yeast, nematodes, and flies) support the hypothesis that the GH/insulin-like growth factor (IGF)-1 pathway is involved in the regulation of aging. Therefore, the following studies were performed to evaluate the role of GH and IGF-1 on the expression and activity of antioxidative enzymes and proteins *in vitro*. The results of these studies suggest that, indeed, GH/IGF-1 can exert direct effects on cells and can alter expression of various antioxidative enzymes that may contribute to the rate of aging and life span in mammals.

This work was supported by the National Institute on Aging, by the American Federation of Aging Research, and by the University of North Dakota.

[1] To whom requests for reprints should be addressed at Department of Pharmacology, Physiology and Therapeutics, University of North Dakota School of Medicine and Health Sciences, 501 North Columbia Road, Grand Forks, North Dakota 58203. E-mail: brownbrg@medicine.nodak.edu

Received June 12, 2001.
Accepted October 4, 2001.

1535-3702/02/2272-0094$15.00

Materials and Methods

Two experiments were performed to determine the role of GH and IGF-1 on the expression of antioxidative enzymes. For Experiment 1, 6-month-old normal, wild-type mice (C57Bl/6J x C3H/J) mice were utilized for hepatocyte cultures. In Experiment 2, hepatocytes were isolated from 6-month-old Ames dwarf, 4-month-old GH transgenic, and corresponding groups of age-matched wild-type mice. All mice were maintained at the University of North Dakota vivarium facilities under controlled conditions of photoperiod (12:12-hr light:dark) and temperature (22° ± 1°C) with *ad libitum* access to food (PMI International, St. Louis, MO; Lab diet, ≥23% crude protein/≥ 4.5% fat) and water. The Ames dwarf mice used in these studies were derived from a closed colony (over 20 years) on a heterogeneous background. Ames dwarf (df/df) mice were produced by mating dwarf (df/df) or carrier ($df/^+$) males to carrier ($df/^+$) females. The GH transgenic and wild-type animals used in this study were kindly provided by Dr. Andrzej Bartke (Carbondale, Southern Illinois University) and were derived from a single male founder (strain B6SJL) produced by microinjection of the phospho*enol*pyruvate carboxykinase (PEPCK) promoter region (300 bp)/bGH hybrid gene into the male pronucleus of single-cell embryos. The production and initial characterization of the transgenic animals were described previously (9, 10). The Institutional Animal Care and Use Committee at the University of North Dakota approved all procedures utilizing animals. All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise noted.

Isolated hepatocyte cultures were prepared (utilizing modified procedures of Berry and coworkers [11]) with liver tissue isolated from 6-month-old male mice. Each animal was anesthetized with 2.5% tribromoethanol (i.p. 100 μl/10 g body wt). The portal vein was cannulated and the liver was preperfused with warm (37°C) calcium-free HEPES preperfusion buffer (pH 7.65; 160.8 mM NaCl, 3.15 mM KCl, 0.7 mM Na_2HPO_4-$7H_2O$, 33 mM HEPES, and 0.5 M EDTA/500 ml buffer) for 15 min followed by a ~15-min perfusion with collagenase solution (0.03% collagenase [Worthington, Lake Park, NJ], 1% glucose, and 1 mM $CaCl_2$ in preperfusion buffer [without EDTA]). Following perfusion, livers were removed and the cells were transferred, washed (Wash Media; Life Technologies, Rockville, MD), and cultured in serum-free HepatoStim Media (Becton Dickinson, Mountain View, CA). Trypan blue dye exclusion was used to assess cell viability and integrity. Hepatocyte suspensions prepared in this manner maintain high levels of integrity (12) and reflect the metabolic capabilities of cells *in vivo* (11). Culture dishes (60 x 15 mm) were coated with MatriGel (Becton Dickinson) for 30 min (37°C) prior to the addition of cells. Hepatocytes from three to five animals per line were pooled for each set of experiments. Cells were resuspended in Attachment medium (Life Technologies) containing 1% pen/strep and 5% FBS (HyClone, Logan, UT) and were seeded (2×10^6) into dishes coated with MatriGel. Cells were allowed to attach for 2 hr, at which time the medium was exchanged for serum-free HepatoStim media containing 1% pen/strep and 2 mM glutamine. The cells were then incubated overnight at 37°C and 5% CO_2. This time period allowed hepatocytes to recover from the isolation procedures. Following this incubation, fresh media was added, cells were allowed to equilibrate for 2 hr, and then the following hormone treatments were administered to cells for 24 hr. The concentrations of GH (rat GH-13; National Hormone and Pituitary Program [NHHP]) employed were 0.1, 1.0, and 10 μg/ml media. IGF-1 (recombinant human IGF-1, Genentech, San Francisco, CA) was used at concentrations of 0.5, 5.0, and 50 nM. An antagonist to IGF-1 (20 μg/ml, H1356, Bachem, King of Prussia, PA) was added to one-half of the 5.0 nM IGF-1 plates to inhibit the actions of IGF-1 (receptor blocker [RB]). Prolactin (PRL; ovine; oPRL-21; NHHP; 10, 100, and 1000 ng/ml) and luteinizing hormone (ovine; oLH-26; NHHP; 0.25, 2.5, and 25 ng/ml) were also examined to test hormone response specificity. Cells were washed with HEPES buffer, and MatriSperse (Becton Dickinson) was added to the plates to dissolve the MatriGel (4°C). Cell samples were washed in ice-cold HEPES, pelleted via centrifugation, and stored at –70°C until analyses were performed.

Enzymes measured included catalase, which catalyzes the detoxification of H_2O_2 by disproportionating the H_2O_2 to H_2O and O_2, manganese superoxide dismutase (MnSOD), which catalyzes the dismutation of two superoxide anion molecules to form H_2O_2 and O_2 in mitochondria, and glutathione peroxidase (GPX), which performs functions similar to catalase in mitochondria. Activity and immunoblotting procedures are described below.

For enzyme assays, frozen hepatocyte samples were homogenized on ice with a Teflon pestle in buffer (20 mM MOPS, 300 mM sucrose, and 0.1 mM EDTA at pH 7.2). The homogenate was centrifuged for 30 min at 13,000*g* and the supernatant fraction was used for analysis of enzyme activities. Protein concentration was determined using the Bradford assay (13). The supernatant fractions were used to determine catalase enzyme activity (EC 1.11.1.6) (14) at 240 nm spectrophotometrically (1, 2) and GPX activity (340 nm) (1, 15). Activity values are calculated with respect to appropriate concurrently run blanks.

Human erythrocyte catalase antibody (Calbiochem, La Jolla, CA) was used to detect catalase levels (16, 17) using an ImmunStar chemiluminescence detection system (Bio-Rad, Hercules, CA) as previously described (2). Catalase was identified via comparison with molecular weight standards and purified human erythrocyte catalase (Sigma). Similar procedures were employed to detect GPX and MnSOD protein levels in these samples except that antibodies to GPX1 and MnSOD (kindly provided by Dr. Douglas Wallace, Emory University, Atlanta, GA) (18, 19) and SuperSignal West Pico substrate (chemiluminescence) were

employed along with molecular weight markers. Differences in catalase, GPX, and MnSOD protein were determined densitometrically (Bio-Rad Imaging Densitometer and Molecular Analyst software) following exposure of the membrane to x-ray film.

In each experiment, differences between means were assessed utilizing Prism (Graphpad, San Diego, CA). A simple one-way analysis of variance (ANOVA) was used to determine significant differences among means. When needed, a Newman-Keuls *post hoc* test was used to test for specific differences. For comparison of protein levels based on densitometric analysis, students *t* tests were employed. The accepted level of significance was $P < 0.05$.

Results

These experiments were conducted to evaluate the effect of both GH and IGF-1 on the activity and protein expression of enzymes involved in antioxidative processes. In Experiment 1, the data presented are derived from hepatocytes of normal, wild-type animals and are representative of a minimum of two experiments. The activity of the catalase enzyme in mouse hepatocytes was suppressed 41% and 27% in the presence of GH at 1.0 and 10.0 μg/ml, respectively ($P < 0.002$; Fig. 1). IGF-1, the major mediator of GH action, also directly decreased the activity of catalase by 49%, 34%, and 50% at 0.5, 5.0, and 50 nM, respectively ($P < 0.002$; Fig. 1). However, immunoblots of hepatocyte proteins revealed significant increases in catalase protein (Fig. 2) following GH treatment ($P < 0.02$ – 0.1 μg/ml; $P < 0.002$–1.0 and 10 μg/ml). In agreement, 24 hr following treatment, both 5 and 50 nM IGF-1 also increased the amount of catalase protein in cultured mouse hepatocytes ($P < 0.0007$; Fig. 2). The addition of an IGF-1 receptor blocker prevented the increase in catalase protein levels and maintained levels similar to that of the control.

The antioxidative enzyme GPX was also evaluated following *in vitro* treatment with GH or IGF-1. The activity of GPX was decreased 34%, 34%, and 18% by each concentration of GH (0.1, 1.0, and 10 μg/ml, respectively; $P < 0.002$), a response similar to that observed in the activity of catalase (Fig. 3). GH treatment resulted in an even greater suppression ($P < 0.05$) in GPX protein levels: 50%, 50%, and 46% in 0.1, 1.0, and 10 μg/ml GH, respectively (Fig. 4) compared with nontreated control cell protein levels. In response to IGF-1 treatment, the GPX activity of mouse hepatocytes also declined ($P < 0.04$) when compared with control hepatocytes. Along with IGF-1, lower levels of GPX activity were also maintained following the addition of a receptor blocker (Fig. 3). Hepatocyte GPX protein levels were only numerically lower following treatment with IGF-1 (Fig. 4). Likewise, the addition of an IGF receptor blocker did not significantly alter GPX protein levels, maintaining levels similar to that of control hepatocytes.

Hepatocyte MnSOD protein levels were decreased significantly by 0.1 and 1.0 μg/ml GH (66% and 40% respectively; $P < 0.01$; Fig. 5). In addition, IGF-1 at 5.0 and 50 nM concentrations depressed MnSOD protein ($P < 0.05$). A similar suppressive effect of GH on MnSOD protein expression (44% decrease) was observed in hepatocytes isolated directly from GH transgenic mice ($P < 0.05$; Fig. 6) compared with wild-type mouse hepatocytes.

To determine whether the antioxidative enzymes were

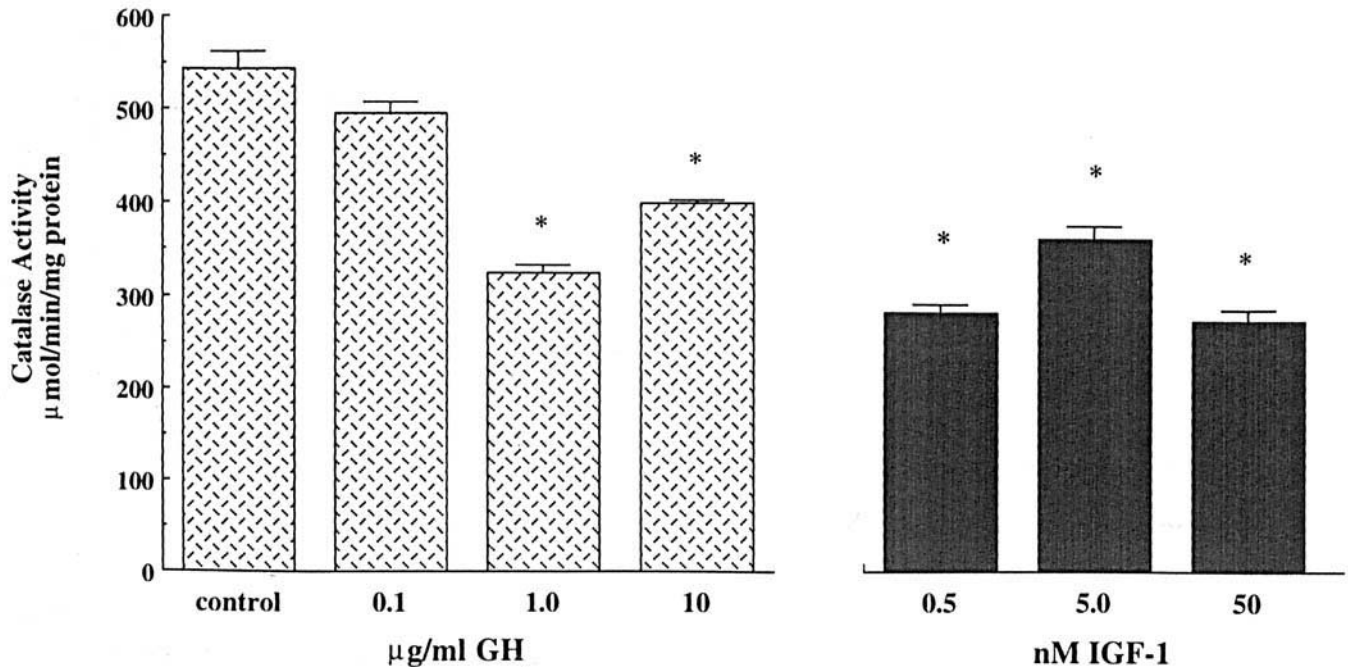

Figure 1. Hepatocyte catalase activity (micromoles per minute per milligram of protein) in normal, wild-type mice treated with GH or IGF-1. Values represent means ± SEM. An asterisk represents differences between treated and control, untreated hepatocytes. *$P < 0.002$.

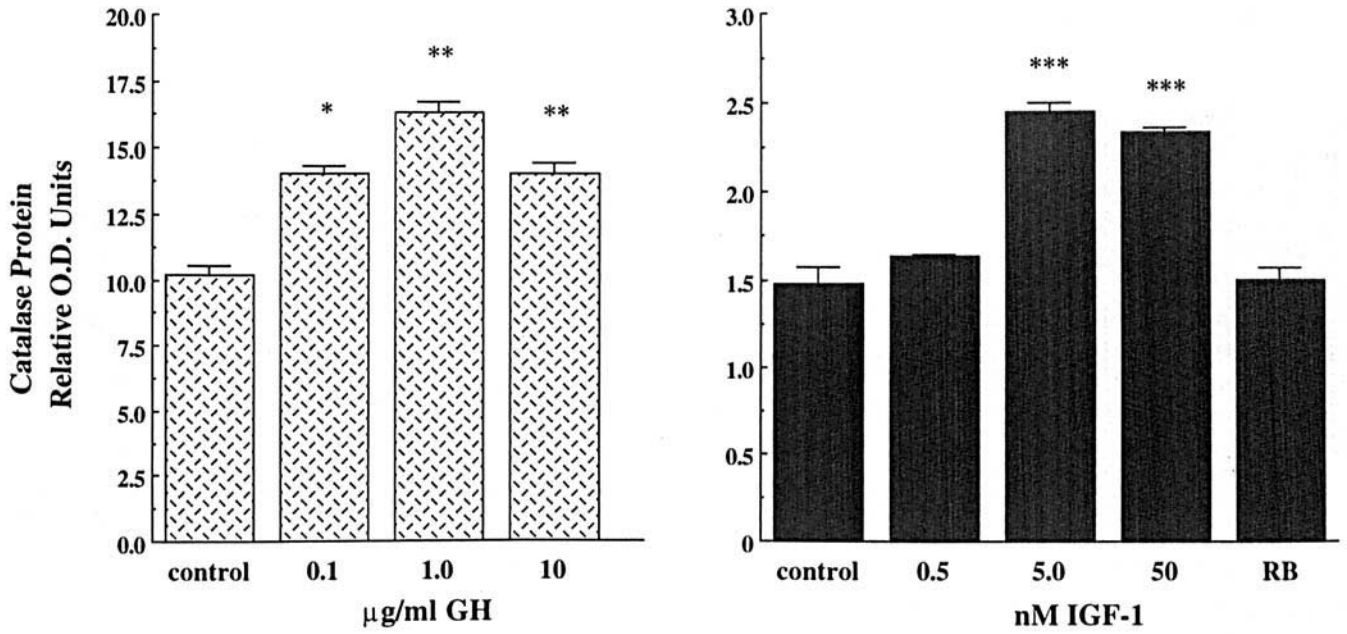

Figure 2. Catalase protein levels determined via immunoblotting (relative O.D. units) in normal, wild-type mouse hepatocytes treated with GH or IGF-1. Values represent means ± SEM. An asterisk represents differences between treated and control, untreated cells. $^{*}P < 0.02$; $^{**}P < 0.002$; $^{***}P < 0.0007$.

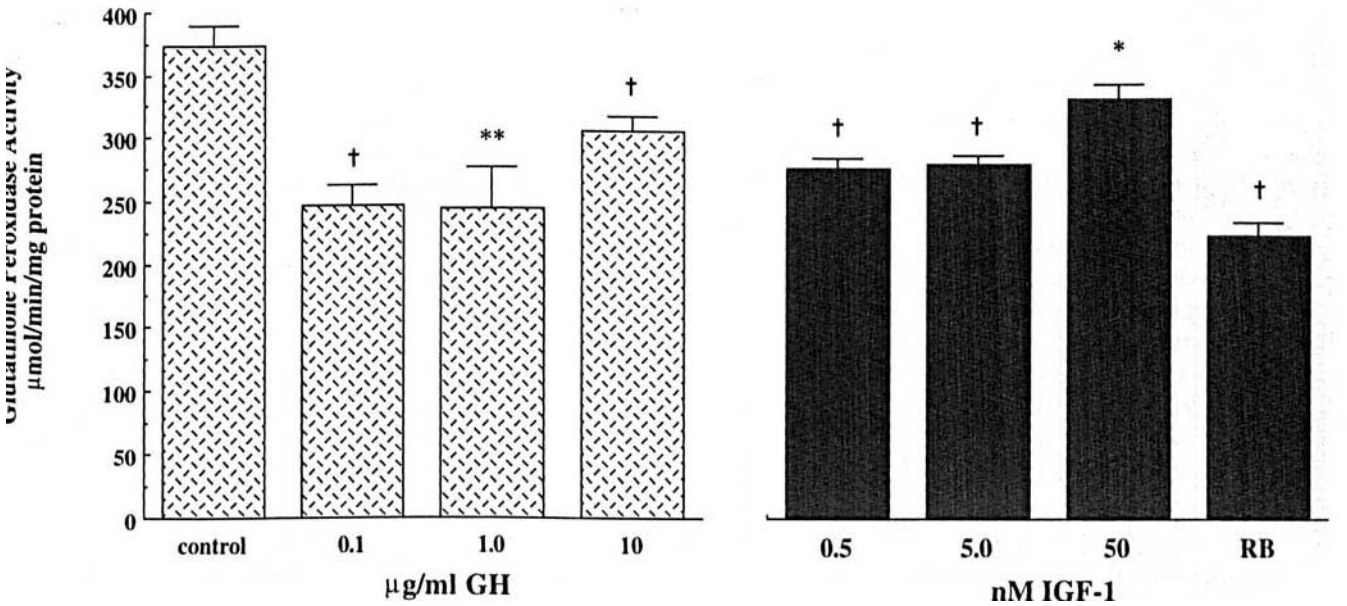

Figure 3. Hepatocyte glutathione peroxidase activity (micromoles per minute per milligram of protein) in normal, wild-type mice treated with GH or IGF-1. Values represent means ± SEM. An asterisk represents differences between treated and control, untreated hepatocytes. $^{*}P < 0.04$; $^{**}P < 0.002$, $^{***}P < 0.0005$.

specifically downregulated by GH and IGF-1, other protein hormones were administered to hepatocytes in various concentrations. In contrast to GH and IGF-1, PRL, a structurally similar hormone, and luteinizing hormone (LH), had no significant effect on catalase activity or protein (data not shown). Furthermore, PRL treatment did not significantly alter hepatocyte GPX activity, although the 25 ng/ml concentration of LH decreased the activity of this enzyme in cultured hepatocytes (data not shown) when compared with control, nontreated cells. No major differences in the levels of GPX protein were revealed by immunoblotting following treatment of hepatocytes with PRL or LH (data not shown).

In Experiment 2, the effects of hormone treatment on enzyme expression in GH-deficient Ames dwarf hepatocytes were evaluated. The enzyme activity levels in untreated (control) cells of catalase (440.2 ± 53.7 vs. 312.9 ± 23.6 μmol/min/mg protein; $P < 0.02$) and GPX (372.1 ± 31.0 vs. 269.8 ± 15.3 μmol/min/mg protein; $P < 0.002$) were higher in normal, wild-type versus dwarf hepatocytes, respectively. When dwarf hepatocytes were treated with

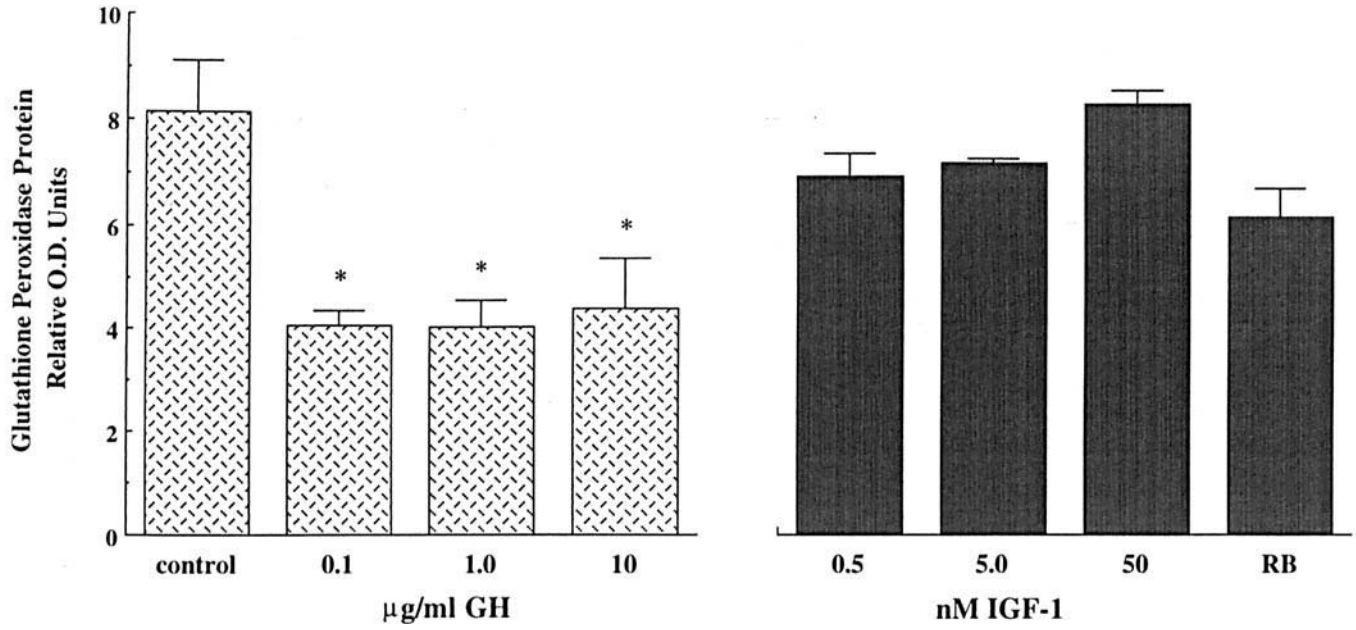

Figure 4. Glutathione peroxidase protein levels determined via immunoblotting (relative O.D. units) in normal, wild-type mouse hepatocytes treated with GH or IGF-1. Values represent means ± SEM. An asterisk represents differences between treated and control, untreated cells. $^{*}P < 0.05$.

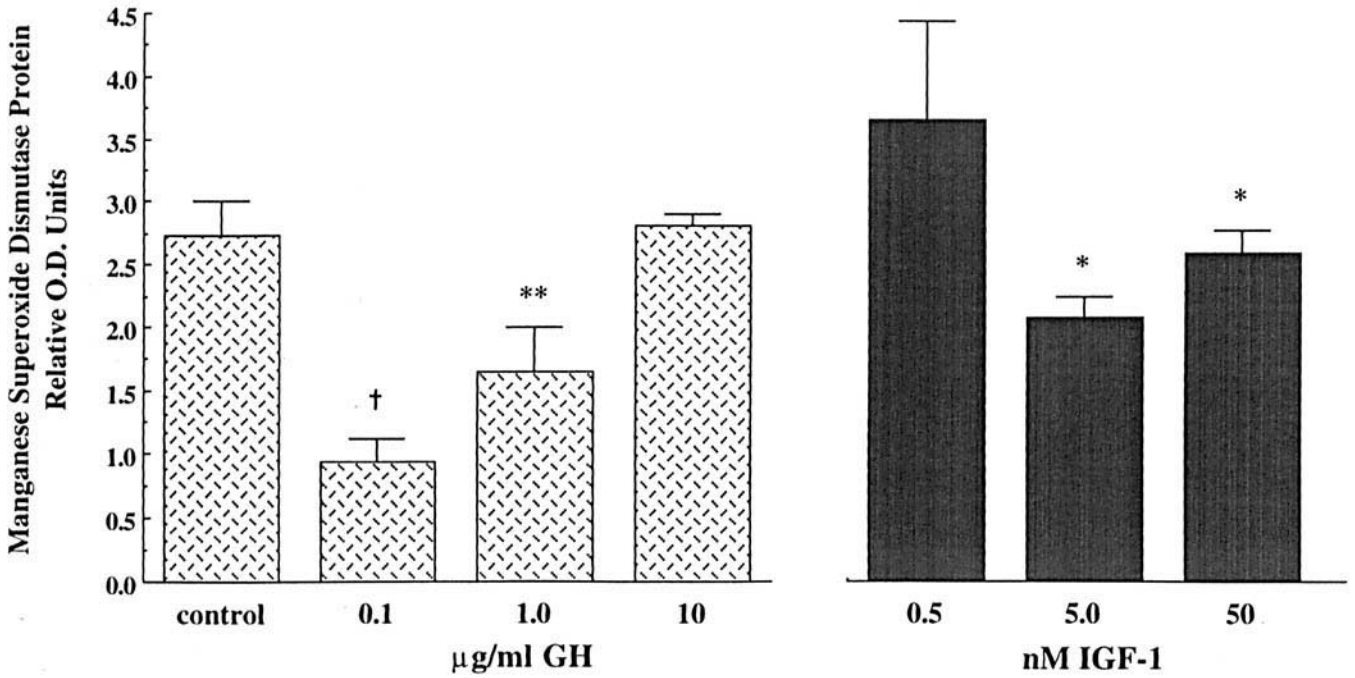

Figure 5. Manganese superoxide dismutase protein levels determined via immunoblotting (relative O.D. units) in normal, wild-type mouse hepatocytes treated with GH or IGF-1. Values represent means ± SEM. N asterisk represents differences between treated and control, untreated cells. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

GH, no significant changes in catalase activity were observed (Fig. 7). Although the levels of catalase protein in dwarf control hepatocytes tended to be higher following GH treatment, only the 1.0 μg/ml concentration was significant (Fig. 8). Administration of IGF-1 (5.0 nM) slightly decreased catalase activity in dwarf cells (14%; $P < 0.05$; Fig. 7) and tended to decrease catalase protein levels (Fig. 8), although not significantly. The IGF receptor blocker returned catalase protein levels to that of the control. In contrast, GH decreased catalase activity in normal, wild-type hepatocytes from the dwarf line (data not shown) similar to that observed in experiments presented in Figure 1. Overall, catalase activity tended to be lower in hepatocytes from dwarf mice when compared with normal, wild-type cells cultured and treated at the same time. With the exception of 10 μg/ml GH and 0.5 nM IGF-1 (concentrations that were

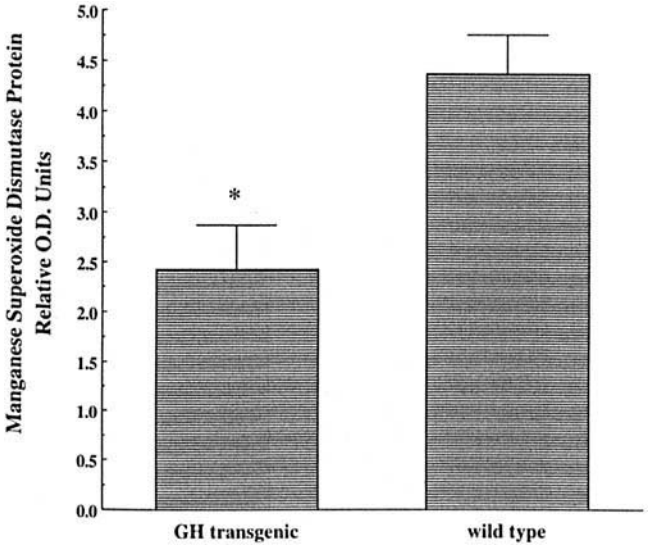

Figure 6. Liver manganese superoxide protein levels determined via immunoblotting (relative O.D. units) in GH transgenic versus wild-type mouse hepatocytes. Values represent means ± SEM. An asterisk represents differences between transgenic and wild-type mice. $^*P < 0.05$.

not significantly different from respective controls), the percent decrease in catalase activity (compared with control, nontreated cells) was less in dwarf growth factor-treated hepatocytes compared with wild-type growth factor-treated hepatocytes.

GPX activity, on the other hand, was increased 67%, 82%, and 47% in dwarf hepatocytes treated with GH at each concentration tested (0.1, 1.0, and 10 μg/ml respectively; $P < 0.0001$; Fig. 9) compared with control, untreated dwarf cells. Corresponding levels of GPX protein, however, did not differ significantly following the addition of GH (data not shown). Conversely, neither IGF-1 nor the receptor blocker significantly altered GPX activity (Fig. 9) or GPX protein levels of dwarf hepatocytes (data not shown).

Discussion

The results of these experiments strongly suggest that GH and/or IGF-1 exert direct effects on components of the antioxidative defense system in mice. Our previous studies have shown that mice deficient in both GH and IGF-1 (Ames dwarf) (20) exhibit heightened antioxidative defenses (1, 2) and less oxidative damage to proteins and DNA when compared with wild-type mice (21). These mice are extremely long-lived, living 49%–64% longer than male and female wild-type siblings, respectively (3). There is also behavioral evidence demonstrating that aging is delayed significantly; learning, memory, and locomotor activities remain intact compared with age-matched wild-type controls that exhibit the normal age-related decline in these functions (22).

For these studies, there were several reasons for choosing isolated hepatocytes to test the effects of GH and IGF-1 on antioxidative capacity. The liver is the major target organ of GH and is, metabolically, a very active tissue. In addition, previous characterization of these enzymes was performed in intact livers of each of the mice described in the current experiments. Primary hepatocyte culture is a useful model for studying cellular physiology and biochemistry, as these cells retain a variety of normal functions, reflecting the metabolic capabilities of cells *in vivo* (11) and they maintain a high level of cellular integrity (12). The culture procedures and 24-hr time period utilized in these experiments allowed hepatocytes to recover from the isolation procedures, including possible restoration of hormone receptors, restoration of normal intracellular ions and glutathione levels, repair of DNA damaged during isolation, removal of proteolytic enzymes, and removal of damaged hepatocytes.

The suppressive effects of *in vitro* GH and IGF-1 on the activity of the catalase and GPX enzymes are striking. This effect was not limited to one strain of mice, but also was observed in wild-type mice from the Ames dwarf line. In agreement, *in vivo* evidence shows that mice with lifelong elevated plasma GH (due to transgene expression), have suppressed levels of catalase activity, protein, and mRNA in liver, kidney, and heart tissues (2, 23). Furthermore, high plasma GH is associated with increased levels of ROS and increased oxidative damage to tissues (21, 24). Reduced activity of antioxidative enzymes may be responsible for some of the pathologies associated with disorders of GH excess such as acromegaly.

In stark contrast to animals overexpressing GH, in the absence of plasma GH and IGF-1, catalase activity is significantly higher in several tissues of dwarf mice, including the liver (1, 2). In addition, GH receptor knockout mice also exhibit altered antioxidative defenses and long life (7, 25). However, one report has shown that short-term *in vivo* GH treatment elevates catalase activity in rats (26). In our study, protein levels of catalase were increased in the presence of GH and high concentrations of IGF-1 in two different strains of mice. The observed increase in catalase protein may reflect an adaptive response of the cells to the apparent depressed activity of the enzyme in the presence of GH. The loss of activity may reflect protein instability due to potential actions of GH on degradation enzymes or perhaps activity is not strongly dependent on amounts of enzyme. However, GH and IGF-1 are known to stimulate general protein synthesis, which may result in an increase in catalase protein levels. Analysis of catalase gene expression will be evaluated in future experiments. Additionally, an overall increase in metabolism associated with GH treatment could increase the production of free radicals, potentially overwhelm the system, and/or alter activity of other enzymes such that the system is not able to appropriately counter the oxidative attack.

GPX plays a major role in the elimination of hydrogen peroxide (H_2O_2) in mitochondria, serving a function similar to catalase. The activity and protein level of GPX declined following treatment with GH and IGF-1, suggesting that

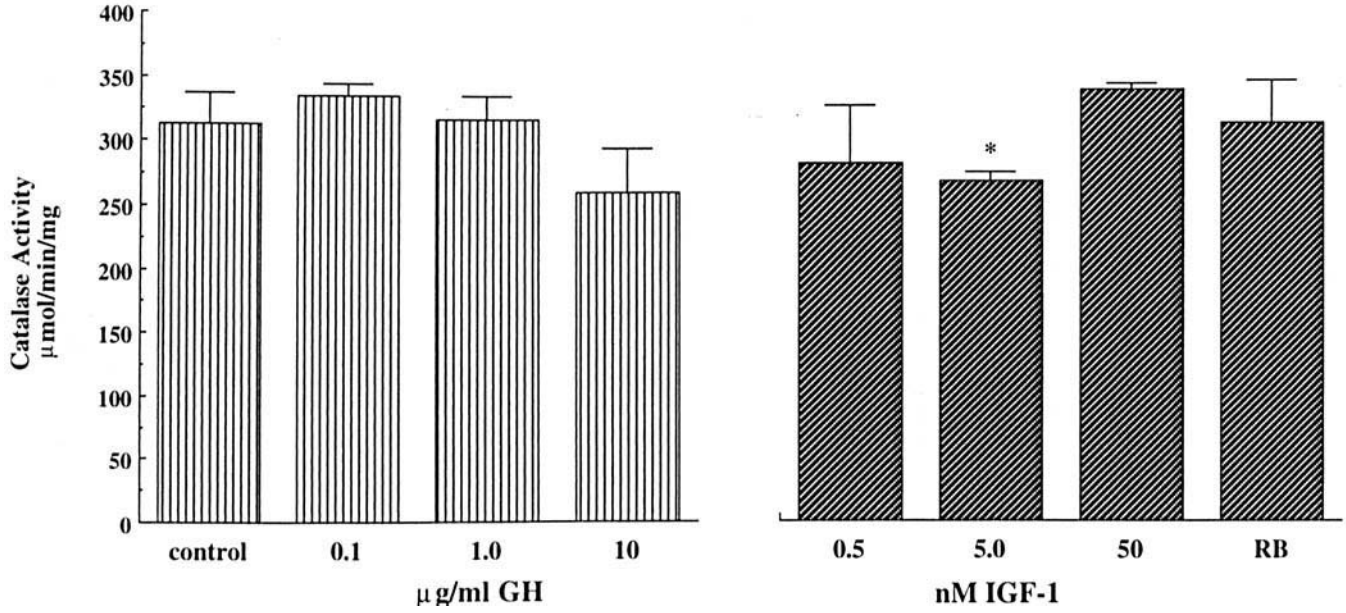

Figure 7. Hepatocyte catalase activity (micromoles per minute per milligram of protein) levels in Ames dwarf mice treated with GH or IGF-1. Values represent means ± SEM. An asterisk represents differences between treated and control, untreated cells. $^*P < 0.05$.

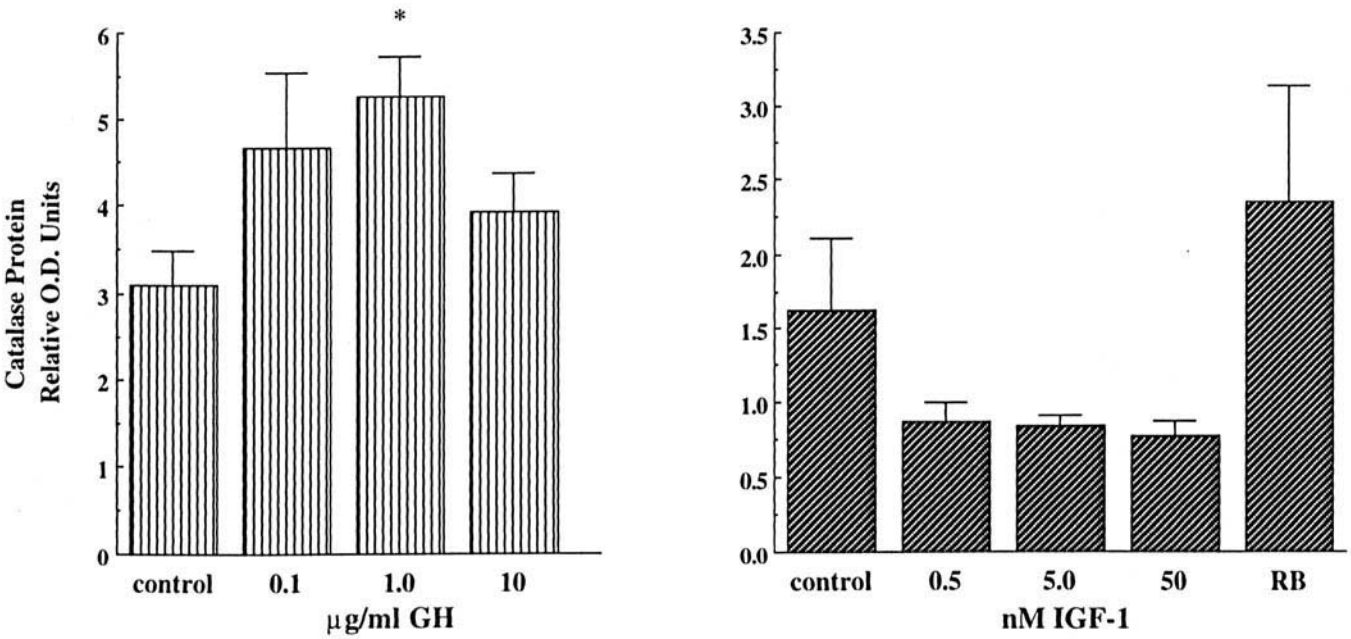

Figure 8. Catalase protein levels determined via immunoblotting (relative O.D. units) in Ames dwarf hepatocytes treated with GH or IGF-1. Values represent means ± SEM. An asterisk represents differences between treated and control, untreated cells. $^*P < 0.05$.

GPX may also be hormonally regulated. In contrast to catalase, however, GPX protein was not elevated, rather, it was depressed, indicating potential differential regulatory pathways between GPX and catalase or possibly differences in protein stability. The antibody employed to detect GPX is specific for GPX1, the primary selenium-dependent, glutathione-utilizing peroxidase in the liver (18, 19). The liver is highly dependent upon GPX1 for mitochondrial antioxidant defense (18, 19, 27). Therefore, the reduction in both activity and protein levels of this critical detoxification enzyme may be key in the overall antioxidant status in GH-deficient and GH-overexpressed states. Also, within the mitochondria, MnSOD is responsible for the dismutation of superoxide anion into H_2O_2 and O_2. Both GH and IGF-1 altered MnSOD in a manner similar to catalase and GPX. We have previously shown that both MnSOD and catalase are also downregulated in transgenic mice that overexpress GH (Ref. 2 and H. Brown-Borg, unpublished data).

Few reports outside of our current study have evaluated the effects of GH/IGF-1 on antioxidative enzymes in hepatocytes. The administration of IGF-1 to a rat model of liver cirrhosis improved liver function and reduced oxidative

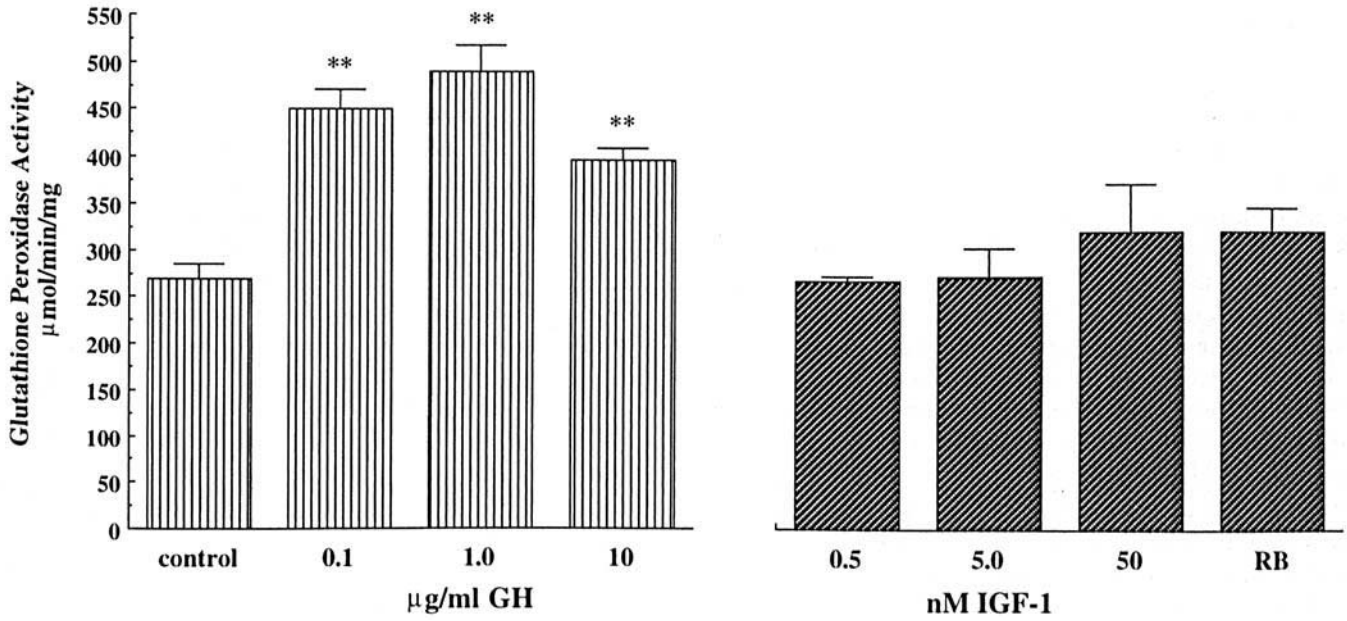

Figure 9. Hepatocyte glutathione peroxidase activity (micromoles per minute per milligram of protein) in Ames dwarf mice treated with GH or IGF-1. Values represent means ± SEM. An asterisk represents differences between treated and control, untreated hepatocytes. $^{**}P < 0.0001$.

liver damage and fibrosis by preventing the reduction in antioxidative enzymes (catalase, SOD, and GPX) that normally accompanies this condition (28). Although the positive changes in liver function may be due to direct effects of IGF-1 on antioxidative enzymes, the authors suggested that in this experimental paradigm, the observed changes in enzyme activities may reflect a general effect of the hormone in promoting protein synthesis in damaged liver cells. The hepatocytes in our system originated from healthy mice, and changes in enzyme activities were specific for GH and/or IGF-1.

Multiple reports demonstrate potential modulation of antioxidative enzymes (levels, activity, or both) by hormones (reviewed in Ref. 29), including but not limited to GH, PRL, TSH, insulin, estrogen, and glucocorticoids. Alterations in antioxidant enzymes in response to hormone may be tissue specific and dose dependent. For example, several genes involved in oxidative metabolism are upregulated by thyroid hormone (30), and hyperthyroidism results in marked increases in SOD, catalase, and GPX compared with controls (31). In contrast, no adverse reactions mediated by ROS are found in hypothyroid rats, and hypothyroidism in rats does not alter lipid peroxidation (32). PRL, a hormone with some structural and functional similarities to GH, has been shown to stimulate expression of both CuZn-SOD and MnSOD mRNA in rats (33). In addition, levels of both GH and PRL are positively correlated with increased SOD activity, whereas follicle stimulating hormone is associated with increased catalase activity in mammary tissue (34, 35).

High concentrations of insulin, which can bind to IGF type I receptors, suppress MnSOD and catalase activity in rat brainstem (36). Conversely, low-dose insulin stimulates MnSOD and has no effect on catalase, regardless of dose (37), in brown adipose tissue. Other reports present evidence of a strong negative correlation between serum insulin levels and catalase activity showing that insulin decreases the rate of liver catalase synthesis without affecting degradation (38). Indeed, hyperinsulinemia is strongly associated with the development of significant age-related diseases (39–41) and enhanced mortality (42). Our study, along with others, indicates that hormones modulate antioxidative defense and therefore, hypo- or hyperhormone states can influence overall antioxidative status. Furthermore, peroxisomal enzymes may be regulated differentially in humans and rodents (43), therefore, the direct implications of reduced enzyme activity remain to be determined.

The observation that catalase activity was not altered by GH treatment of hepatocytes from dwarf mice was unexpected. We hypothesized that cells from GH-deficient mice would respond to *in vitro* GH treatment and reduce catalase activity. However, dwarf mice lack GH throughout life, which may preclude the hepatocyte from expressing GH receptors. Therefore, the overall numbers of GH receptors may differ tremendously between dwarf and normal cells. The short-term nature of the hepatocyte culture and environment may not have allowed appropriate time for upregulation of receptors in dwarf cells, thus prolonging the time necessary for a cellular response equivalent to a normal hepatocyte. Conversely, the activity of GPX was consistently elevated in GH-treated dwarf hepatocytes. As shown in wild-type hepatocytes, catalase and GPX may be differentially regulated by GH or IGF-1. Exogenous GH administration to Ames dwarf mice has been shown to increase body weight, increase plasma IGF-1, and modulate basal LH secretions, thereby increasing circulating testosterone levels and enhancing steroidogenic capacity of the testes (20). In addition, the altered responses in the dwarf may

result from culture conditions that may have "normalized" the environment for the dwarf cells in that the levels of growth factors, insulin, glucose, etc. were different than those they are exposed to *in vivo*. Unfortunately, the formulation of the serum-free media used for these studies is proprietary knowledge (HepatoStim Media; Becton Dickinson). However, we were assured that GH and IGF-1 are not key components of this media (Becton-Dickinson representative, personal communication), but it is possible that key factors of serum are required to maintain appropriate response to some factors *in vitro*. Moreover, the lack of GH and IGF-1 throughout life in Ames dwarf mice has obvious effects on development—clearly observed are those effects on growth, body size, and sexual maturation. In calorie-restricted rodents, plasma IGF-1 concentrations are also significantly reduced, resulting in a similar phenotype. The reduced GH signaling in these animals may have significant metabolic effects that we are just now beginning to examine and understand.

Both GH and IGF-1 specifically suppressed antioxidant enzyme activities, whereas other protein hormones tested (PRL and LH) had little if any effect. It is possible that the downregulation of antioxidative enzymes in hepatocytes from IGF-1 directly represents an *in vitro* artifact. It is known that very few IGF-1 receptors are expressed on liver cells *in vivo* (44) as the liver is thought to respond directly to insulin. However, *in vitro* hepatocytes do maintain the ability to respond to GH (45).

Possible mechanisms of GH/IGF-1 actions on antioxidative enzymes include modulating regulatory pathways (synthesis, degradation, and enzyme kinetics); reducing the generation of ROS, thereby reducing the need for antioxidative enzymes; impairing repair pathways, resulting in more oxidative stress/damage; or generally decreasing metabolic activity of cultured hepatocytes. In some tissues, it has been shown that the activity of catalase is directly proportional to substrate levels (46). In brown fat tissue, insulin treatment did not change catalase activity, suggesting that H_2O_2 levels were maintained by other mechanisms. In adipocytes, insulin stimulates intracellular H_2O_2 production (47) and may serve as an intracellular signaling molecule. The incidence of hyperinsulinemia (and increased insulin resistance) increases with aging, whereas catalase synthesis decreases with aging (48). Such changes are associated with an increase in age-related disease. If this is true in hepatocytes, GH may be reducing overall H_2O_2 production. This seems unlikely as GH generally upregulates metabolic activity and thus, H_2O_2 generation. Furthermore, there are reports showing that GH transgenic mice have elevated superoxide radicals, increased lipid peroxidation (24), and oxidative protein damage (21) when compared with normal, wild-type mice.

In the context of aging, this data strongly supports observations correlating life span with levels of GH and GH/IGF-1 signaling. GH-deficient mice exhibit heightened antioxidant defenses, less oxidative damage, and they live longer than wild-type siblings (1–3, 6). In sharp contrast, mice with a GH transgene express high levels of GH, they have reduced antioxidant capacity, and they experience more oxidative damage than wild-type littermates (2, 8, 21, 23). In fact, it is well recognized that individuals (within a species) with disrupted GH/IGF/insulin signaling live longer than wild-type individuals. This concept is sustained in species ranging from mice (Ames [*Prop-1* mutant; Ref. 3] and Snell [*Pit-1* mutant; Ref. 6] dwarfs, and GH receptor knockout dwarf [25, 49]) to flies (insulin receptor mutant [50] and insulin receptor substrate [*chico*] mutant [51]) to nematodes (*daf-2* [52] and *age-1* mutants [53]; both components of insulin/IGF pathway) to yeast (*PKA* and *Sch9* [54]; both involved in nutrient utilization/Akt/PKB). Interestingly, a human correlate of the Ames dwarf residing on the Island of Krk exhibit mutations in the *Prop-1* gene and live significantly longer than normal individuals within this same population (55). Therefore, the control of metabolic activity by the GH/IGF/insulin signaling pathway may be a universal regulator of aging across species. Finally, although aging is associated with an overall decline in antioxidative enzyme activities and a striking increase in H_2O_2 production, based on the results of the current study, the decline in GH that occurs with aging may provide a mechanism that prevents further suppression of antioxidant protection.

The authors would like to extend appreciation to Dr. Andrzej Bartke for providing the GH transgenic mice, to Dr. Douglas Wallace for the GPX and MnSOD antibodies, and to the National Hormone and Pituitary Program for providing the rat growth hormone.

1. Brown-Borg HM, Bode AM, Bartke A. Antioxidative mechanisms and plasma growth hormone levels: potential relationship in the aging process. Endocrine **11**:41–48, 1999.
2. Brown-Borg HM, Rakoczy SG. Catalase expression in delayed and premature aging mouse models. Exp Gerontol **35**:199–212, 2000.
3. Brown-Borg HM, Borg KE, Meliska CJ, Bartke A. Dwarf mice and the aging process. Nature **384**:33, 1996.
4. Bartke A. Genetic models in the study of anterior pituitary hormones In: Shire JGM, Ed. Genetic Variation in Hormone Systems. Boca Raton, FL: CRC Press, pp113–126, 1979.
5. Sornson MW, Wu W, Dasen JS, Flynn SE, Norman DJ, O'Connell SM, Gukovsky I, Carriere C, Ryan AK, Miller AP. Pituitary lineage determination by the prophet of pit-1 homeodomain factor defective in Ames dwarfism. Nature **384**:327–333, 1996.
6. Flurkey K, Papconstantino J, Miller RA, Harrison DA. Lifespan extension and delayed immune and collagen aging in mutant mice with defects in growth hormone production. Proc Natl Acad Sci U S A **98**:6736–6741, 2001.
7. Hauck S, Bartke A. Effects of growth hormone on hypothalamic catalase and CuZn superoxide dismutase. Free Rad Biol Med **28**:970–978, 2000.
8. Steger RW, Bartke A, Cecim M. Premature aging in transgenic mice expressing growth hormone genes. J Reprod Fertil Suppl **46**:61–75, 1993.
9. McGrane MM, DeVente J, Yun J, Bloom J, Park E, Wynshaw-Boris A, Wagner T, Rottman FM, Hanson FW. Tissue-specific expression and dietary regulation of a chimeric phospho*enol*pyruvate carboxyki-

nase/bovine growth hormone gene in transgenic mice. J Biol Chem **263:**11443–11451, 1988.
10. Steger RW, Bartke A, Parkening TA, Collins T, Cerven R, Jun JS, Wagner TE. Effects of chronic exposure to bovine growth hormone (bGH) on the hypothalamic-pituitary axis in transgenic mice: relationship to the degree of expression of the PEPCK.bGH hybrid gene. Transgenics **1:**245–253, 1994.
11. Berry MN, Edwards AM, Barritt GJ. Isolated hepatocytes preparation, properties and applications. In: Burdon RH, van Knippenberg PH, Eds. Laboratory Techniques in Biochemistry and Molecular Biology. New York: Elsevier, Vol. **21**, 1991.
12. Kreamer BL, Staecker JL, Sawada N, Sattler GL, Hsia MTS, Pitot HC. Use of a low-speed, iso-density Percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. In Vitro Cell Dev Biol **22:**201, 1986.
13. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:**248–254, 1976.
14. Aebi H. Catalase in vitro. In: Packer L, Ed. Methods in Enzymology. Orlando, FL: Academic Press, pp121–126, 1984.
15. Flohe L, Gunzler W. Assays of glutathione peroxidase. In: Packer L, Ed. Methods in Enzymology. Orlando, FL: Academic Press, pp114–121, 1984.
16. Yan T, Jiang X, Zhang HJ, Li S, Oberley LW. Use of commercial antibodies for detection of the primary antioxidant enzymes. Free Rad Biol Med **25:**688–693, 1998.
17. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A **76:**4350–4354, 1979.
18. Esposito LA, Melov S, Panov A, Cottrell BA, Wallace DC. Mitochondrial disease in mouse results in increased oxidative stress. Proc Natl Acad Sci U S A **96:**4820–4825, 1999.
19. Esposito LA, Kokoszka JE, Waymire KG, Cottrell B, MacGregor GR, Wallace DC. Mitochondrial oxidative stress in mice lacking the glutathione peroxidase-1 gene.Free Rad Biol Med **28:**754–766, 2000.
20. Chandrashekar V, Bartke A. Induction of endogenous insulin-like growth factor-I secretion alters the hypothalamic-pituitary-testicular function in growth hormone-deficient adult dwarf mice. Biol Reprod **48:**544–551, 1993.
21. Brown-Borg HM, Johnson WT, Rakoczy SG, Romanick MA. Mitochondrial oxidant production and oxidative damage in Ames dwarf mice. J Am Aging Assoc (in press).
22. Kinney BA, Meliska CJ, Steger RW, Bartke A. Evidence that Ames dwarf mice age differently from their normal siblings in behavioral and learning and memory parameters. Horm Behav **39:**277–285, 2001.
23. Hauck S, Bartke A. Free radical defenses in the liver and kidney of human growth hormone transgenic mice: possible mechanisms of early mortality. J Gerontol Bio Sci **56A:**B153–B162, 2001.
24. Rollo CD, Carlson J, Sawada M. Accelerated aging of giant transgenic mice is associated with elevated free radical processes. Can J Zool **74:**606–620, 1996.
25. Coschigano KT, Clemmons D, Bellush LL, Kopchick JJ. Assessment of growth parameters and life span of GHR/BP gene-disrupted mice. Endocrinology. **141:**2608–2613, 2000.
26. Youn YK, Suh GJ, Jung SE, Oh SK, Demling R. Recombinant human growth hormone decreases lung and liver tissue lipid peroxidation and increases antioxidantactivity after thermal injury in rats. J Burn Care Rehabil **19:**542–548, 1998.
27. Esworthy RS, Swiderek KM, Ho YS, Chu FF. Selenium-dependent glutathione peroxidase-G1 is a major glutathione peroxidase activity in the mucosal epithelium of rodent intestine. Biochim Biophys Acta **1381:**213–226, 1998.
28. Castilla-Cortazar I, Garcia M, Muguerza B, Quiroga J, Perez R, Santidrian S, Prieto J. Hepatoprotective effects of insulin-like growth factor 1 in rats with carbon tetrachloride-induced cirrhosis. Gastroenterology **113:**1682–1691, 1997.
29. Brown-Borg HM, Harman SM. Hormones and oxidative stress In: Cutler RG, Rodriguez H, Eds. Oxidative Stress and Aging: Advances in Basic Science, Diagnostics and Intervention. River Edge, New Jersey: World Scientific Publishers, 2002.
30. Jameson JL, DeGroot LJ. Mechanisms of thyroid hormone action. In: Groot LG, Ed. Endocrinology. Philadelphia: Harcourt Brace, pp587, 1995.
31. Komosinska-Vassev K, Olczyk K, Kucharz EJ, Marcisz C, Winsz-Szczotka K, Kotulska A. Free radical activity and antioxidant defense mechanisms in patients with hyperthyroidism due to Graves' disease during therapy. Clin Chim Acta **300:**107–117, 2000.
32. Asayama K, Dobashi K, Hayashibe H, Megata Y, Kato K. Lipid peroxidation and free radical scavengers in thyroid dysfunction in the rat: a possible mechanism of injury to heart and skeletal muscle in hyperthyroidism. Endocrinology **121:**2112–2118, 1987.
33. Sugino N, Hirosawa-Takamori M, Zhong L, Telleria CM, Shiota K, Gibori G. Hormonal regulation of copper-zinc superoxide dismutase and manganese superoxide dismutase messenger ribonucleic acid in the rat corpus luteum: induction by prolactin and placental lactogens. Biol Reprod **59:**599–605, 1998.
34. Bolzan AD, Brown OA, Goya RG, Bianchi MS. Hormonal modulation of antioxidant enzyme activities in young and old rats. Exp Gerontol **30:**169–175, 1995.
35. Bolzan AD, Bianchi MS, Console GM, Goya RG. Relationship between pituitary hormones, antioxidant enzymes, and histopathological changes in the mammary gland of senescent rats. Exp Gerontol **32:**297–304, 1997.
36. Radojicic R, Cvijic G, Djordjevic J, Djurasevic S, Davidovic V. Chronic effect of insulin on monoamine oxidase and antioxidant enzyme activities in the rat brainstem. Comp Biochem Physiol **117C:**187–192, 1997.
37. Davidovic V, Radojicic R, Cvijic G, Durasevic S, Petrovic N. Activities of antioxidant enzymes and monoamine oxidase-A in the rat interscapular brown adipose tissue: effect of insulin and 6-hydroxydopamine. Comp Biochem Physiol **117C:**181–186, 1997.
38. Xu L, Badr MZ. Enhanced potential for oxidative stress in hyperinsulinemic rats: imbalance between hepatic peroxisomal hydrogen peroxide production and decomposition due to hyperinsulinemia. Horm Metab Res **31:**278–282, 1999.
39. Ferannini ES, Hoffner M, Mitchell BD, Stern MP. Hyperinsulinemia: the key feature of a cardiovascular and metabolic syndrome. Diabetologia **34:**416–422, 1991.
40. Lillioja S, Mott DM, Spraul M, Ferraro R, Foley JE, Ravussin E, Knowler WC, Bennett PH, Bogardus C. Insulin resistance and insulin secretory dysfunction as precursors of non-insulin dependent diabetes mellitus. N Engl J Med **329:**1988–1992, 1993.
41. Facchini FS, Hua NW, Reaven GM, Stoohs RA. Hyperinsulinemia: the missing link among oxidative stress and age-related disease. Free Rad Biol Med **29:**1302–1306, 2001.
42. Xu L, Ash M, Abdel-aleem S, Lowe JE, Badr MZ. Hyperinsulinemia inhibits hepatic peroxisomal β-oxidation in rats. Horm Metab Res **27:**76–78, 1995.
43. Youssef J, Badr M. Extraperoxisomal targets of peroxisome proliferators: mitochondrial, microsomal and cytosolic effects: implications for health and disease. Crit Rev Toxicol **28:**1–33, 1998.
44. Caro JF, Poulos J, Ittoop O, Pories WJ, Flickinger EG, Sinha MK. Insulin-like growth factor 1 binding in hepatocytes from human liver, human hepatoma, and normal regenerating and fetal rat liver. J Clin Invest **81:**976–981, 1988.
45. Paul C, Seiliez I, Thissen JP, Le Cam A. Regulation of the expression of the rat SOCS-3 gene in hepatocytes by growth hormone, interleukin-6 and glucocorticoids mRNA analysis and promoter characterization. Eur J Biochem **267:**5849–5857, 2000.
46. Nedergaard J, Alexon S, Cannon B. Cold adaptation in the rat: increased brown fat peroxisomal β-oxidation relative to maximal mitochondrial oxidative capacity. Am J Physiol **239:**C208–C216, 1980.
47. May JM, de Haen C. Insulin-stimulated intracellular hydrogen perox-

ide production in rat epididymal fat cells. J Biol Chem **254:**2214–2220, 1979.

48. Haining JL, Legan JS. Catalase turnover in rat liver and kidney as a function of age. Exp Gerontol **8:**85–91, 1973.
49. Zhou Y, Xu BC, Maheshwari HG, He L, Reed M, Lozykowski M, Okada S, Wagner TE, Cataldo LA, Coschigano K, Baumann G, Kopchick JJ. A mammalian model of Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (The Laron mouse). Proc Natl Acad Sci U S A **94:**13215–13220, 1997.
50. Tatar M, Kopelman A, Epstein D, Tu MP, Yin CM, Garofalo RS. A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function. Science **292:**107–110, 2001.
51. Clancy DJ, Gems D, Harshman LG, Oldham S, Stocker H, Hafen E, Leevers SJ, Partridge L. Extension of life-span by loss of CHICO, a *Drosophila* insulin receptor substrate protein. Science **292:**104–106, 2001.
52. Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang RA. *Caenorhabiditis elegans* mutant that lives twice as long as wild type. Nature **266:**461, 1993.
53. Johnson TE, Hutchinson EW. Aging in *Caenorhabditis elegans*: update 1988. In: Rothstein M, Ed. Review of Biological Research in Aging. New York: Alan R. Liss, Vol **4**, 1990.
54. Fabrizio P, Pozza F, Pletcher SD, Gendron CM, Longo VD. Regulation of longevity and stress resistance by Sch9 in yeast. Science **292:**288–290, 2001.
55. Krzisnik C, Kolacio Z, Battelino T, Brown M, Parks JS, Laron Z. The 'little people' of the Island of Krk revisited: etiology of hypopituitarism revealed. J Endo Genet **1:**9–19, 1999.