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Effect of Recombinant Human Growth Hormone on Age-Related Hepatocyte Changes in Old Male and Female Wistar Rats

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Aging induces changes in several organs, such as the liver, and this process might be due to damage caused by free radicals and inflammatory mediators. The growth hormone (GH)/insulin-like growth factor-1 (IGF-1) axis shows a reduction with age, and this fact could be associated with some age-related changes. The aim of this study was to investigate the effect of GH administration on age-induced alterations in hepatocytes. Two and twenty two month-old male and female Wistar rats were used. Old rats were treated with human recombinant GH for 10 wk. At the end of the treatment, hepatocytes were isolated from the liver and cultured, and different parameters were measured in cells and medium. Plasma IGF-1 was also measured. Aging significantly decreased plasma IGF-1 in males. In females, plasma IGF-1 was also reduced, but not significantly. GH treatment restored plasma IGF-1 levels to values similar to young males. Aging was associated with a significant increase in lipid peroxidation (LPO), nitric oxide (NO), carbon monoxide (CO) and cyclic guanosyl-monophosphate (cGMP), as well as a reduction in adenosyl triphosphate (ATP) and phosphatidylcholine (PC) synthesis. GH administration partially prevented all these changes in males. In females, some of the parameters were significantly improved by GH (ATP, CO, cGMP), while others showed a tendency to improvement, although differences did not reach significance. In conclusion, GH administration could exert beneficial effects against age-related changes in hepatocytes, mainly in males.

Key Words: Growth hormone; aging; hepatocytes; estrogens.

Introduction

Liver plays a critical role in metabolism, and detoxifying processes. In the liver, aging is associated with several changes and alterations, such as a reduction in liver mass, blood and canalicular bile flow, impaired protein synthesis and degradation, changes in cholesterol and drugs metabolism, alterations in mitochondria, and changes in the expression and activity of different enzymes *(1–4)*. Moreover, the sensitivity of liver to different harmful agents, such as anoxia/reoxygenation *(5)* or toxic drugs *(6)*, is increased with aging. One theory is that the process of aging could be due to accumulative damage induced by reactive oxygen species (ROS) to cells and molecules *(7,8)*. On the other hand, several reports have addressed an increase in proinflammatory enzymes and molecules with aging, *(9)* suggesting that this process involves a prooxidant and a proinflammatory status.

An impairment in mitochondrial function with age has been reported *(10,11)*, which reduces ATP production and energy supply in old cells. The increase in oxidative stress that occurs with aging could be responsible for this fact *(12)*. On the other hand, nitric oxide (NO) and carbon monoxide (CO) are two molecules involved in oxidative damage and inflammatory response. NO can act as an inflammatory mediator and as a reactive nitrogen species (RNS), either directly or through peroxynitrites generated by its interaction with O_2^- *(13)*. NO and peroxynitrites in excess can alter mitochondrial function *(12)* and phosphatidylcholine (PC) synthesis *(14)*, which is an essential molecule for cell membrane function and integrity *(15)*. CO is one of the elements of the heme–oxygenase 1 (HO-1)–CO pathway, which could represent a defense system against oxidative and inflammatory damage *(16)*. Lipid peroxidation is another parameter related to oxidative stress. After its initiation by free radicals, it becomes a self-perpetuating chain reaction that could induce the peroxidation of all lipidic molecules of cell, leading to the alteration of membrane functions *(11)*. Moreover, lipid peroxidation is another source of CO production *(16)*.

Estrogens are known to exert protective effects on several organs and tissues *(17,18)*. These hormones have been shown to act as antioxidants *(19–21)* as well as anti-inflammatory agents *(22)* in several experimental models. Thus, it seems interesting to develop experiments that include male and female animals, in order to determine potential gender-related differences in the response to the agents that are being tested.

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Table 1
IGF-1 Plasma Levels: Effect of Recombinant Human Growth Hormone (rhGH) Administration[a]

		Young	Old Control	Old GH
Plasma IGF-1	M:	1105.5 ± 61.5	559.2 ± 83.2*	1183 ± 96.6
(nmol/mL)	F:	649.6 ± 52[$]	490 ± 89	1115 ± 15.4*

[a]Values are expressed as mean ± SEM. *$p < 0.01$ vs rest of gender-matched groups; [$]$p < 0.01$ vs young male animals. M: males, F, females.

The process of aging is accompanied by several changes and alterations in metabolism, body composition, and organ function that resemble those found in adult patients with growth hormone deficiency (GHD) and in hypophysectomized experimental animals *(23–26)*. Furthermore, aging is associated with a physiological decline in the growth hormone/insulin-like growth factor 1 (GH/IGF-1) axis in humans and experimental animals *(27,28)*. Indeed, GH treatment has been shown to induce beneficial effects on different organs and tissues, such as bone, vascular system, lipid profile, etc., in aged animals and humans *(29–32)*. On the other hand, it has been demonstrated that GH administration and IGF-1 overexpression are able to prevent oxidative damage and inflammatory response, and induce antioxidant defenses in some experimental models *(33–35)*. However, the role of GH in the aging process is not without controversy *(36, 37)* and the effect of GH on some organs and tissues of aged individuals, such as the, liver has not been well established.

The aim of this study was to investigate the effect of human recombinant GH (rhGH) administration on several parameters related to oxidative damage in hepatocytes isolated from old male and female Wistar rats. It seems important to state that this study intends to be a substitutive treatment with GH, in a moment when the secretion of this hormone is impaired, and not a pharmacological treatment.

Results

IGF-1 levels were significantly reduced in old males when compared to young ones, and GH administration was able to restore these values (Table 1). In females, IGF-1 plasma levels of young animals were significantly lower than those of age-matched males, and aging also induced a decrease in its levels, but this reduction did not reach significance. GH administration significantly increased plasma IGF-1 in female rats, reaching values similar to those of young control and old GH-treated males.

As shown in Fig. 1, ATP content of cells showed a significant decrease in old males and females when compared to young rats, although this reduction was more evident in male rats. In fact, ATP concentration was significantly higher in old females than in males of the same age. When the animals were treated with GH, a significant increase in ATP could be observed in both gender groups, and these levels were also significantly higher in females, reaching values similar to those of young female animals.

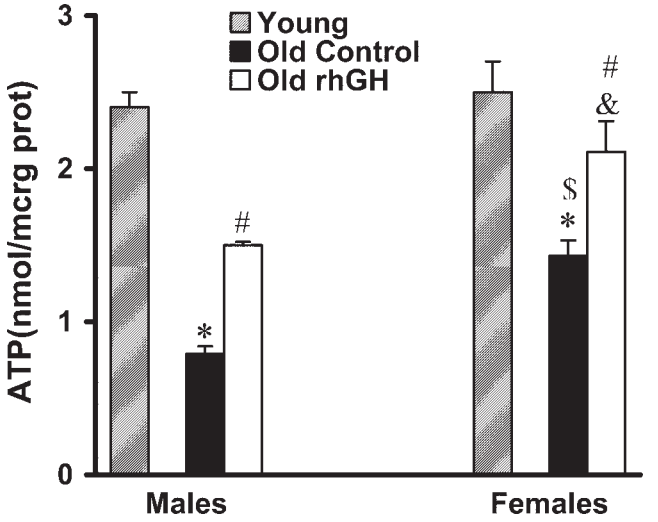

Fig. 1. Adenosyl triphosphate (ATP) content of hepatocytes isolated from old male and female Wistar rats (nmol/μg protein): Effect of recombinant human growth hormone (rhGH) administration. Values are expressed as mean ± SEM. *$p < 0.001$ vs young gender-matched rats; [#]$p < 0.001$ vs old control gender-matched rats; [$]$p < 0.01$ vs old control males; [&]$p < 0.05$ vs old males treated with rhGH.

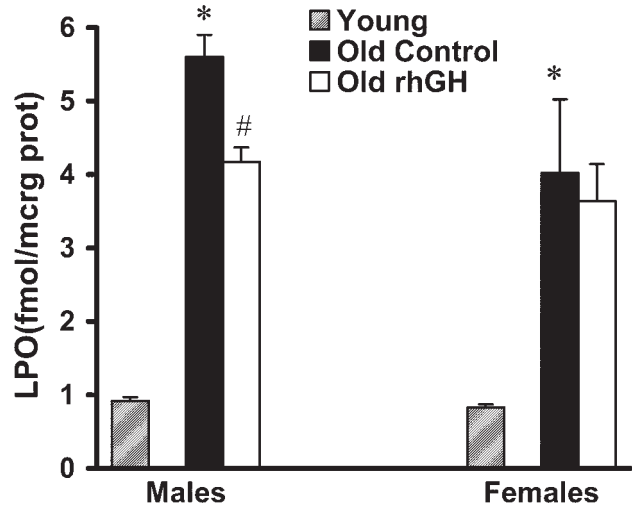

Fig. 2. Lipid peroxide (LPO) content of hepatocytes isolated from old male and female Wistar rats (fmol/μg protein): Effect of recombinant human growth hormone (rhGH) administration. Values are expressed as mean ± SEM. *$p < 0.001$ vs young gender-matched rats; [#]$p < 0.001$ vs old control gender-matched rats.

LPO content of cells was significantly increased by age in male and female rats (Fig. 2). Again, this increase was more evident in male rats than in females, although in this case the differences were not significant. GH treatment was able to significantly reduce LPO content of cells in males. In GH-treated females, values were no statistically different from those of old controls.

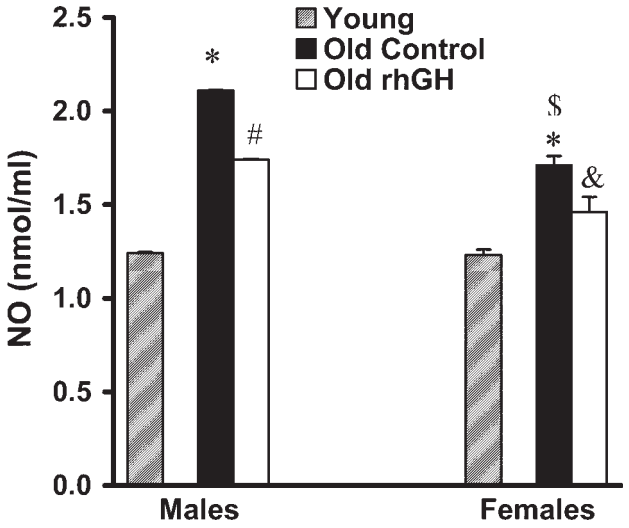

Fig. 3. Nitric oxide (NO) release from hepatocytes isolated from old male and female Wistar rats (nmol/mL): Effect of recombinant human growth hormone (rhGH) administration. Values are expressed as mean ± SEM. $^{*}p < 0.001$ vs young gender-matched rats; $^{\#}p < 0.001$ vs old control gender-matched rats; $^{\$}p < 0.01$ vs old control males; $^{\&}p < 0.01$ vs old males treated with rhGH.

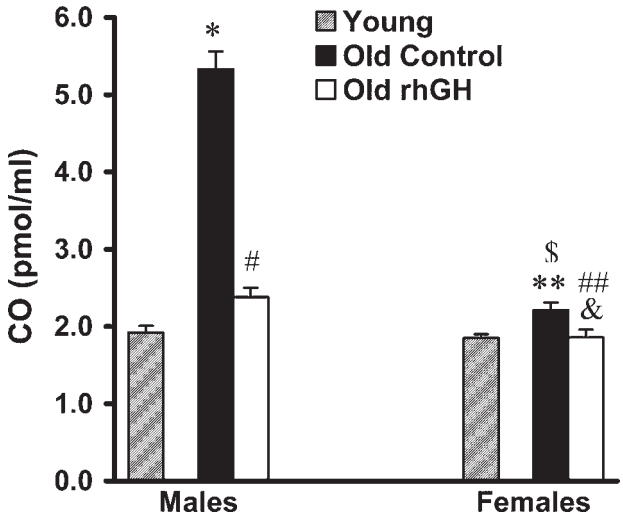

Fig. 4. Carbon monoxide (CO) release from hepatocytes isolated from old male and female Wistar rats (pmol/mL): Effect of recombinant human growth hormone (rhGH) administration. Values are expressed as mean ± SEM. $^{*}p < 0.001$ and $^{**}p < 0.05$ vs young gender-matched rats; $^{\#}p < 0.001$ and $^{\#\#}p < 0.05$ vs old control gender-matched rats; $^{\$}p < 0.001$ vs old control males; $^{\&}p < 0.05$ vs old males treated with rhGH.

When we measured NO release to the medium, we found a significant increase with age in males (Fig. 3). In females, this parameter was also significantly increased in old animals compared to 2-mo-old rats, but, as it was found for other parameters, these changes were not as evident as in males, and values were significantly lower than those of age-matched male rats. Administration of GH to old animals induced a significant decrease of NO release to the medium in males. There was also a reduction in NO release after GH treatment in females, although this decrease was not significant. However, NO release of GH-treated females was still lower than that of GH-treated males.

CO release showed a similar pattern in old males, although differences were more marked that those found for NO (Fig. 4). In females, this parameter was also significantly increased, and treatment with GH was able to significantly reduce CO release in males and females. Again, levels of CO in both groups of female rats were significantly lower than those of age-matched males.

Figure 5 shows that, as expected, cGMP content was significantly higher in hepatocytes isolated from old rats than those from young ones, and this increase was much more evident in aged males than in females, which showed values significantly lower than males. This increase was partially and significantly prevented by GH administration in both gender groups, inducing a significant reduction in cGMP content of cells isolated from old animals.

When PC *de novo* synthesis was tested, we found a marked decrease between young and old males. In females, a similar effect was observed. In this parameter, GH treatment induced a slight increase in PC *de novo* synthesis in males and females, although differences were significant only in the group of male rats (Fig. 6).

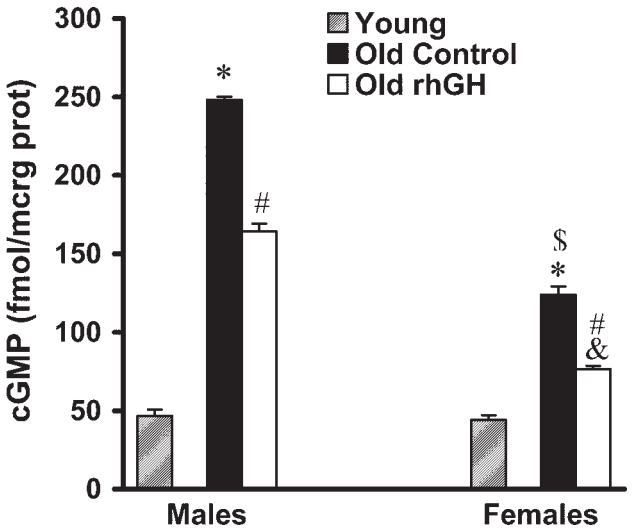

Fig. 5. Cyclic guanosyl monophosphate (cGMP) content of hepatocytes isolated from old male and female Wistar rats (fmol/µg protein): Effect of recombinant human growth hormone (rhGH) administration. Values are expressed as mean ± SEM. $^{*}p < 0.001$ vs young gender-matched rats; $^{\#}p < 0.001$ vs old control gender-matched rats; $^{\$}p < 0.001$ vs old control males; $^{\&}p < 0.001$ vs old males treated with rhGH.

Discussion

It is well known that aging is accompanied by an alteration of the GH/IGF-1 axis, with a decrease in GH secretion

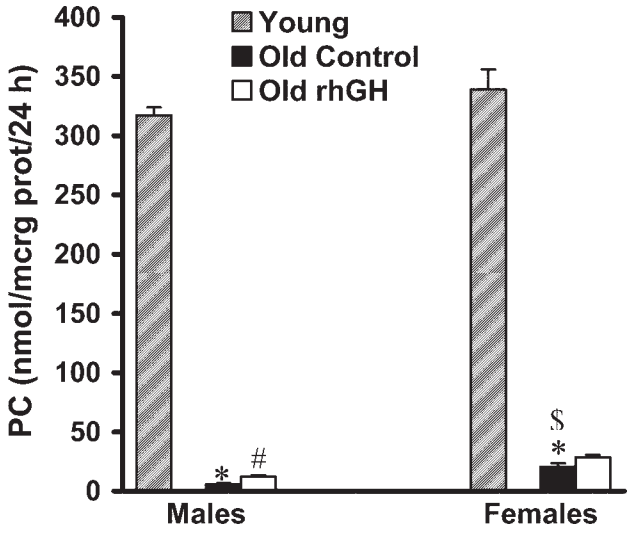

Fig. 6. Phosphatidylcholine (PC) synthesis of hepatocytes isolated from old male and female Wistar rats (pmol/μg protein/24 h): Effect of recombinant human growth hormone (rhGH) administration. Values are expressed as mean ± SEM. $^{*}p < 0.001$ vs young gender-matched rats; $^{\#}p < 0.05$ vs old control gender-matched rats; $^{\$}p < 0.05$ vs old control males.

and IGF-1 plasma levels *(23,27,28)*, and it has been proposed that some alterations related to physiological aging could be, at least in part, due to this decline in the somatotrophic axis *(29,38)*. The results obtained in the present study are in accordance with these findings, since old animals showed lower IGF-1 plasma levels than their young counterparts, although gender differences in absolute values of IGF-1 can be seen.

On the other hand, the role of ROS in the aging process has been widely discussed, and many studies support this hypothesis. ROS production has been found to increase with age, thus enhancing the oxidative damage induced to lipids, proteins and DNA *(7,8,39)*.

An impairment in mitochondrial function with age has been reported. In this regard, an age-related decrease in the activity of several mitochondrial enzymes and in some elements of the mitochondrial electron transport chain in different tissues has been described *(10,11)*, as well as a decrease in mitochondrial membrane potential *(12)*. In fact, the expression of several genes encoding proteins involved in ATP synthesis was reduced with aging *(3)*. These observations support the results obtained in the present study, in which a decrease with age in ATP content of cells has been found. Oxidative damage could be involved in this phenomenon, since it is able to inhibit mitochondrial respiration *(11)*. Lipid peroxidation is an autocatalitic process and it induces decreased membrane fluidity and can alter closely located proteins, which negatively affects membrane functions *(14)*. In the present study, the age-related ATP depletion was accompanied by an increase in LPO content of hepatocytes, which is a cellular marker of oxidative stress. Our findings are in accordance with previous studies, in which an increase in lipid peroxidation with age has been reported in several tissues *(40,41)*.

Some age-related pathologies, such as hypertension *(42)*, atherosclerosis *(43)*, and neurodegenarative diseases *(44)*, are now considered to have an inflammatory component in its pathogenesis. In fact, proinflammatory enzymes and molecules, such as COX-2, cytokines, and prostaglandins, are increased with age *(11)*. Nitric oxide (NO) is one molecular mediator involved in the inflammatory response *(45)* that also induces oxidative damage, either directly or through the production of peroxynitrites *(13)*. In the present study, NO release was increased in hepatocytes isolated from old rats, in accordance with other reports, in which age induced a rise in iNOS activity *(9)*. The present study also shows that age induces an increase in local CO production in hepatocytes, and this could mean that this pathway has been activated by the increase in age-associated ROS and proinflammatory molecules, such NO. CO is a physiologically synthesized molecule that shares some of the mechanisms of action and physiological effects of NO *(16,46)*. The main endogenous source of CO is heme metabolism by HO *(16, 46)*. The HO–CO pathway has been proposed to be involved in the defense against oxidative stress and the deleterious effects of NO, and could be activated to counteract an excess of oxidant and inflammatory agents *(16,47)*. Furthermore, the increase in LPO induced by age found in the present study could account for some part of this increase in CO release. Concerning the cGMP content of cells, it is higher in those isolated from old rats compared to young ones, as could be expected, since both NO and CO, which are increased by age, act through soluble guanylate cyclase activation and cGMP rise *(46)*.

Phospholipids and, more specifically, PC, are essential components of cell membranes. PC plays a critical role in membrane fluidity, cell surface charge and signal transmission *(15,48)*. In the present study an age-induced decrease in PC *de novo* synthesis was found. This finding is in accordance with previous studies developed in other experimental models, in which oxidative stress and inflammation inhibited PC synthesis *(14)*. We can speculate that the age-induced increase in oxidative damage found in the present study (as shown by the increase in LPO) and NO release could be responsible for the reduction in PC synthesis.

GH administration (either directly or through IGF-1 production) has been shown to exert beneficial effects on some organs and tissues deteriorated by aging, such as bone, body composition, cardiovascular system, and central nervous system *(29–32,49,50)*. Moreover, it has been shown that the reduction in the expression of some components of the mitochondrial respiratory chain induced by age was prevented by GH administration *(3)*. These findings support the results obtained in the present study, in which GH was able to partially revert the age-related reduction in ATP content of cells.

On the other hand, GH and IGF-1 administration have been shown to reduce oxidative stress and to improve antioxidant defenses in several experimental models in which oxidative damage is involved, such as CCl_4-induced liver cirrhosis *(33)* or thermal injury *(51)*. The transgenic Mini rats, in which GH production is suppressed, show increased susceptibility to hepatotoxic agents that induce oxidative damage, such as CCl_4 and thioacetamide *(52,53)*. In humans, patients with GH deficiency exhibit higher free radicals and oxidative stress, and this situation was improved by GH administration *(54)*. GH and IGF-1 administration have also been demonstrated to be able to modulate hepatic acute phase response and cytokine production in experimental animals *(34,55)* and in humans *(56)*. All these observations support some of the findings in our experiment, in which GH administration was able to reduce LPO, an indicator of oxidative stress, as well as NO and CO release, that seem to be related to oxidative damage and inflammation. Furthermore, GH treatment was able to slightly improve the dramatic age-related decrease in PC synthesis. Thus, GH and its mediator IGF-1 seem to play a role in the regulation of oxidative stress and inflammation in the aged liver, although the intervention of other hormones or growth factors can not be excluded.

However, there is a big controversy concerning the relationship among aging, GH and oxidative stress. Several reports have addressed an increase in oxidative stress and a reduction in antioxidant defenses and life span in transgenic mice overexpressing GH, and the opposite situation was found in those lacking GH, IGF-1 or their effects *(36, 37,57)*. Nevertheless, these findings are not conclusive and cannot be extrapolated to the physiological process of aging, because these transgenic animals are exposed to abnormally high levels of GH/IGF-1 during all their lives, and they usually exhibit alterations in other hormones apart from GH, such as prolactin and thyroid stimulating hormone. Furthermore, some of the discrepancies concerning the beneficial or harmful actions of GH on aging may be related to a differential effect of this hormone depending on the doses administered or plasma levels obtained: physiological amounts of GH can exert beneficial effects, whereas pathological excess can lead to disease and accelerated aging *(36,37)*.

Comparing the results obtained in females and males, we can speculate about a protective effect of estrogens on oxidative and inflammatory age-induced liver changes, since the deterioration that accompanies aging is of a lesser extent in intact females than in age-matched males. These findings may be explained by the fact that estrogens have been shown to exert both antioxidant and anti-inflammatory properties in several experimental models *(19–22)*, and female rats maintain a certain degree of estrogen secretion until late in their lives *(58)*. Other authors have also suggested that females, owing to their maintained estrogenic secretion, are partially protected against some age-related changes *(59)*. These facts could also explain the differences in the effects of GH between males and intact females. GH administration seems to be more beneficial in males, whereas in females the improvements only reached significance in three parameters, but not in the other three. Since the alterations induced by age were more pronounced in males than in intact females, the beneficial effects were also more evident in the former. In fact, we have found in other experiments from our group that, when female rats are ovariectomized, they show more evident changes related to aging, approaching the situation found in males, and in these rats, GH administration is able to significantly improve all the parameters studied. Being the alterations more marked, the beneficial effect of GH is also more evident than in intact females *(60)*. The differences found between males and females in the response to GH could also be due to the fact that IGF-1 levels were much more altered in old males than in intact females when compared with their gender-matched young counterparts. A more evident deterioration in IGF-1 levels with age would make old male rats more responsive to GH treatment.

In conclusion, these results suggest that GH could improve some parameters related to the oxidative and inflammatory damage associated with aging in hepatocytes isolated from old rats, and that this effect is apparently more marked in males, owing to the fact that alterations are also more evident in this gender. The controversy concerning the effects of GH on aging suggests the necessity of investigating the mechanisms of the effects of GH found in the present study, to determine the possible risks of such treatment and elucidate whether GH administration is capable to preserve hepatic function in aging individuals.

Materials and Methods

Animals

Sixteen males and sixteen female Wistar rats of 22 mo of age were used in the present study. The animals were given a standard laboratory rat diet (A.04; Panlab, Barcelona, Spain) and water *ad libitum,* in a light- and temperature-controlled room, and were divided into four experimental groups (n = 8 each group): saline-treated male rats, males treated with human recombinant GH (rhGH, 2 mg/kg/d, sc, in two daily injections at 10:00 h and 17:00 h), saline-treated female rats, and female animals treated with rhGH in the same way that males. Previous data obtained by our group, both on GH effects on growth *(61)* and on vascular aging *(32)*, indicated that this is an appropriate administration schedule to obtain a GH substitution treatment. Treatments were performed during 10 wk. Eight males and eight intact female rats of 2 mo of age were administered vehicle and used as reference groups. All of the animals received humane care according to the Guidelines for Ethical Care of Experimental Animals of the European Union, and the study was approved by the Ethical Committee for Animal Studies of the School of Medicine of the Complutense University (Madrid, Spain). At the end of the treatment period,

rats were sacrificed by decapitation, and blood and liver were collected and processed as described later.

IGF-1 Plasma Levels

Trunk blood was collected and centrifuged to obtain plasma. Plasma IGF-1 levels were measured after acid alcohol extraction as previously described *(61)* by a specific radioimmunoassay (RIA), using reagents kindly provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDKD) and a second antibody obtained in our laboratory.

Hepatocyte Isolation and Culture

Hepatocytes were isolated by collagenase digestion and prepared as previously described *(62)*. After isolation, hepatocytes were cultured by inoculating 5×10^5 cells in Falcon dishes each containing 5 mL of RPMI 1640 medium (10% fetal calf serum, 100 IU/mL penicillin G, 50 µg gentamicin). The viability of cells was 96 ± 2% (trypan blue exclusion test). Eighteen to twenty hours after inoculation, the cells were attached to the bottom with a plating efficiency of 70–80%. The unattached and nonviable cells were removed, media were changed, and cells were cultured for 24 h more. At the end of the culture period, media and cells were separately collected to perform different measurements. In a parallel experiment, cells were incubated in the presence of 10 m*M* D-[U-^{14}C]glucose (12.5 Ci/mol; Radiochemichal Center, Amersham, Buckinghamshire, UK), for 24 h and the incorporation of D-[U-^{14}C]glucose into *de novo* synthesized PC was measured as described elsewhere *(63)*.

Biochemical Determinations

ATP content of cells was measured by a commercially available kit (Sigma, St. Louis, MO, USA). ATP measurement was based on two consecutive reactions: in the first one, ATP is transformed to adenosyl diphosphate (ADP) and 1,3-diphosphoglycerate in the presence of 3-phosphoglycerate by the enzyme phosphoglycerate phosphokinase (PGK); in the following reaction, catalyzed by glyceraldehyde–phosphate-dehydrogenase (GAPD), 1,3-diphosphoglycerate in the presence of NADH+H is transformed in glyceraldehydes-3-P and NAD+P. The reduction of absorbance at 340 nm due to the oxidation of NADH to NAD is proportional to the amount of ATP in the sample.

The basis of the lipid peroxide (LPO) determination is the reaction of hydroperoxides with 10-*N*-metylcarbamoil-3,7-dimetylamino-10-10-fenotiacine (MCDP), catalyzed by hemoglobin, which leads to methylene blue formation. The methylene blue formed was then measured colorimetrically.

NO release to the medium was measured by the Griess reaction as NO_2^- concentration after NO_3^- reduction to NO_2^-. Briefly, samples were deproteinized by the addition of sulfosalicylic acid, were then incubated for 30 min at 4°C, and subsequently centrifuged for 20 min at 12,000*g*. After incubation of the supernatants with *Escherichia coli* NO_3^- reductase (37°C, 30 min), 1 mL of Griess reagent (0.5% naphthylenediamine dihydrochloride, 5% sulfonylamide, 25% H_3PO_4) was added. The reaction was performed at 22°C for 20 min, and the absorbance at 546 nm was measured, using $NaNO_2$ solution as standard. The measured signal is linear from 1 to 150 µ*M* ($r = 0.994$, $p < 0.001$, $n = 5$), and the detection threshold is approx 2 µ*M*.

To quantify the amount of CO released, the ratio of carboxyhemoglobin after hemoglobin addition was measured. Hemoglobin (4 µ*M*) was added to samples and the mixture was allowed to react for 1 min, to be sure of maximum binding of CO to hemoglobin. Then, samples were diluted with a solution containing phosphate buffer (0.01mol/L monobasic potassium phosphate/dibasic potassium phosphate, pH 6.85) containing sodium dithionite, and after 10 min at room temperature, absorbance was measured at 420 and 432 nm against a matched curve containing only buffer.

Cyclic guanosyl monophosphate (cGMP) content of cells was determined with a RIA kit commercially available (^{125}I-RIA Kit, Radiochemical Center, Amersham, Bucks, England). Protein determination was performed by the Bradford method. The basis of this method is the addition of Coomassie brilliant blue dye/colorant to proteins. This union induces a shift in maximum dye absorbance from 465 to 595 nm. Absorbance is measured at 595 nm, comparing to a known standard curve.

Reproducibility within the assays was evaluated in three independent experiments. Each assay was carried out with three replicates. The overall intraassay coefficient of variation has been calculated to be <5%. Assay to assay reproducibility was evaluated in three independent experiments. The overall interassay coefficient of variation has been calculated to be <6%.

Statistical Analysis

Results are expressed as the mean ± SEM, from $n = 8$. Mean comparison was done by the Friedman's analysis of variance followed by a two-tailed Wilcoxon's test for paired data; a confidence level of 95% ($p < 0.05$) was considered significant.

Acknowledgments

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