

Research article

## Effect of recombinant human growth hormone administration on body composition and vascular function and structure in old male Wistar rats

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### Abstract

The process of ageing affects negatively both cardiovascular system and body composition. On the other hand, the hormones of the somatotrophic axis, growth hormone (GH) and insulin-like growth factor-I (IGF-I), whose production is reduced by age, are involved in the regulation of the cardiovascular system. The aim of this study was to investigate the effect of GH on body composition, vascular function and structure in old male rats. Old (20 months) and adult (4 months) male Wistar rats were used. One group of old animals was treated with GH for 4 weeks. Periepididymary fat weight, Specific Gravity Index (SGI), dose responses to Acetylcholine (ACh), Isoproterenol (Iso), Phenylephrine (Phe) and ACh in the presence of *N*<sup>G</sup>-nitro-L-arginine methylester (L-NAME; ACh + L-NAME), as well as vascular morphology in aortic rings, were studied. Old rats showed increased fat weight and decreased SGI as compared to adult animals. GH increased SGI and tended to reduce fat weight. Old rats showed an impairment in the vasodilator response to ACh and Iso; GH significantly improved the vasodilatation induced by Iso, whereas the response to ACh was not significantly enhanced by GH treatment. There were no significant differences between adult and old rats in the contractile response to Phe, and GH did not show any effect. Contraction induced by ACh + L-NAME was higher in old rats as compared to adults, and treatment with GH significantly reduced this response. Aortic media area was increased in old rats, and GH administration reduced this parameter. In conclusion, GH shows beneficial effects on body composition, as well as on vascular function and morphology in old male rats.

**Abbreviations:** rhGH– recombinant human growth hormone; IGF-1– insulin-like growth factor 1; GHD– growth hormone deficiency; SGI– specific gravity index; KCl– potassium chloride; ACh– acetylcholine; Iso– isoproterenol; SNP– sodium nitroprusside; Phe– phenylephrine; L-NAME– *N*<sup>G</sup>-nitro-L-arginine methylester; NO– nitric oxide; PGI<sub>2</sub>– prostacyclin; EDHF– endothelial-derived hyperpolarizing factor; TXA<sub>2</sub>– thromboxane A<sub>2</sub>

### Introduction

It is known that the process of aging is associated with several changes and alterations in metabolism, body composition and organ function. More concretely, aging is associated with functional alterations in the vascular system,

such as a reduction in endothelium-dependent relaxation in response to different agonists and an increase in endothelium-dependent contraction (Mantelli et al. 1995; Nakajima et al. 1997; Tschudi and Lüscher 1995; Maeso et al. 1999; Matz et al. 2000). There is also an increase in media-intima thickness as well as changes in the

cellular component and in extracellular matrix of the vessel wall (Maeso et al. 1999).

The hormones of the somatotrophic axis, Growth Hormone (GH) and Insulin-like Growth Factor-1 (IGF-1), in addition to their effects on somatic growth and metabolism (García Barros and Devesa Múgica 2000), also exert some other actions on the cardiovascular system. These actions could be, at least in part, mediated by the ability of IGF-I to stimulate endothelial nitric oxide (NO) (Böger 1999). In this regard, adults with GH deficiency (GHD) show endothelial dysfunction (Evans et al. 1999; Capaldo et al. 2001; Smith et al. 2002) as well as changes in vascular structure, such as an increase in arterial stiffness (Irving et al. 2002; Smith et al. 2002) and in arterial wall thickness (Pfeifer et al. 1999; Irving et al. 2002). In fact, these patients develop early atherosclerosis and show higher rate of cardiovascular morbi-mortality (Rosen and Bengtsson 1990; McCallum et al. 2002). These data are supported by experimental results obtained in hypophysectomized rats, which show a reduction in endothelium-dependent vasodilatation as compared to control animals (Rossoni et al. 1999; Gustafsson et al. 2002). Treatment with GH improved all these parameters in both humans (Böger et al. 1996; Pfeifer et al. 1999; Evans et al. 2000; Smith et al. 2002) and experimental animals (Rossoni et al. 1999).

The analogies detected between some of the manifestations of GHD in adults and the changes induced by aging point to a possible relationship between age-related physical impairment and the GH/IGF-1 axis decline that physiologically occurs with age (Toogood et al. 1996; Toogood and Shallet 1998). In aged humans, GH administration is able to enhance lean body mass and muscular strength, reduces body fat (Cuttica et al. 1997; Blackman et al. 2002) and improves plasma lipid profile (Angelopoulos et al. 1998). Authors have previously shown that GH administration exerts beneficial effects on body composition, vascular function and structure in old intact and ovariectomized female rats (Castillo et al. 2003, 2005). Hence, the present study is an attempt to investigate whether the similar effects of GH treatment were reproducible in old male rats.

## Materials and methods

### *General procedure*

Eight adult (aged 4 months) and sixteen old (aged 20 months) male rats, maintained under controlled light and temperature conditions, were used in the study. The animals were fed a normal rat chow (A.04; Panlab, Barcelona, Spain) and had free access to tap water. Half of the old animals were treated with GH (2 mg/kg/d diluted in saline solution, divided into two subcutaneous injections, at 10:00 and 17:00 h.) for 4 weeks. The dose and pattern of administration of GH was the same to that used in previous experiments carried out in our laboratory (Castillo et al. 2003, 2005). Control animals were injected with the same amount of vehicle (saline solution) than GH-treated rats. At the end of the treatment period, the animals were sacrificed by decapitation, and troncular blood was collected and processed, in order to measure plasma IGF-I. Body composition, and vascular reactivity with morphometry of aortic rings have been studied, as described in the text ahead.

The study was conducted following recommendations from the institutional animal care and use committee, according to the Guidelines for Ethical Care of Experimental Animals of the European Union.

### *Body composition study*

All rats were weighted weekly, and the weight increase that the animals experienced from the beginning of the treatment period until the sacrifice date was calculated. After the rats were sacrificed, periepididymary fat was extracted and weighed. Total body fat was determined by the Specific Gravity Index (SGI), which shows the proportion between lean mass and body fat. It can be calculated comparing the animal's carcass weight (animal without head, hair and viscera) in the air ( $W_a$ ) and in the water ( $W_w$ ), using the following formula:  $SGI = W_a / [(W_a - W_w) \times 0.9979]$  (0.9979 = specific gravity of water at 21 °C) (López-Luna et al. 1986).

### *Hormone measurement*

Plasma IGF-I levels content were measured as previously described (Rol de Lama et al. 2000) by an specific radioimmunoassay (RIA), using reagents kindly provided by the National Hormone and Pituitary Program (NHPP) from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and a second antibody obtained in our laboratory.

### *Vascular reactivity*

The day of the experiment, the thoracic aorta was isolated, immediately transferred to ice-cold Krebs' bicarbonate solution (composition in mmol/l: NaCl 118.4, KCl 4.7, CaCl<sub>2</sub> 2.5, KH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25.0 MgSO<sub>4</sub> and glucose 11.1), gently cleaned from surrounding tissue and transversally cut in ring segments (3 mm long). Each ring was placed in a 5 ml tissue bath filled with Krebs' buffer (37 °C) bubbled with 95% O<sub>2</sub>–5% CO<sub>2</sub> and suspended between two L-shaped stainless steel hooks. The upper one was attached to a force transducer (FT03, Grass) coupled to a computerized system (Mc Lab 8E, AD Instruments) for measurement of isometric tension. Rings were allowed to equilibrate for 60 min approximately with changes of buffer every 15 min, and with several adjustments of length until baseline tension was stabilized at 2 g. In previous studies, we found that 2 g of resting tension are optimal for this type of experiments.

When tension was stable, the experiments were initiated by obtaining a reference contractile response to 30 min of 120 mmol/l KCl. Endothelium-dependent relaxations to acetylcholine (ACh; 10<sup>-9</sup>–10<sup>-5</sup> mol/l), and to the  $\beta$ -adrenoceptor agonist isoproterenol (Iso; 10<sup>-9</sup>–10<sup>-5</sup> mol/l) were carried out, as well as endothelium-independent relaxations to sodium nitroprusside (SNP; 10<sup>-10</sup>–10<sup>-6</sup> mol/l), after precontraction with phenylephrine (10<sup>-6</sup> mol/l). Dose-related contracting responses induced by phenylephrine (Phe; 10<sup>-9</sup>–10<sup>-5</sup> mol/l), and induced by ACh (10<sup>-8</sup>–10<sup>-4</sup> mol/l) after 20 min of incubation with N<sup>G</sup>-nitro-L-arginine methylester (L-NAME; incubation in the presence of L-NAME 10<sup>-4</sup> mol/l for 20 min), were assayed as well. L-NAME is a nitric oxide synthase (NOS) inhibitor.

### *Vascular morphometry*

Aortic segments were fixed in 10% sodium phosphate-buffered formaldehyde, processed, and cut in serial sections (5  $\mu$ m). Samples were submitted to hematoxylin–eosin staining. Images were taken with a video-camera module (SONY, SSC-C357P) coupled to a microscope (Leica, LEITZ DMRB) and analyzed with an image processing program (LEICA QWIN, Image processing and Analysis Software; Cambridge, UK).

### *Drugs*

Recombinant human GH (rhGH, Saizen<sup>®</sup>; Madrid, Spain) was kindly provided by Serono Laboratories. Other drugs and chemicals were purchased from Sigma Chemicals Co. (St. Louis, Missouri, USA). Stock solutions of drugs were initially prepared in distilled water and diluted to the desired concentration with Krebs' buffer immediately before the experiment. Products for morphological analysis were purchased from Merck (Darmstadt, Germany) and Panreac (Barcelona, Spain). Concentrations are expressed as the final molar concentration in the organ chamber.

### *Calculations and statistical analysis*

For agents that elicit contraction of aortic rings, results are expressed as the percentage change in isometric tension induced by 120 mM KCl. Relaxing responses are expressed as percent reduction of the phenylephrine (10<sup>-6</sup> mol/l)-precontracted state. All results are expressed as mean  $\pm$  SEM of 8 animals. Single variable comparisons were made using a one-way analysis of variance; all other data were analyzed by two-way analysis of variance for multiple comparisons. Newman–Keuls tests were performed if differences were noted. Data were analyzed with the SPSS statistical software package, version 10.0. The null hypothesis was rejected when *P* value was less than 0.05.

## **Results**

### *Body composition study*

Old controls lost weight along the treatment period as compared with adult animals, while GH

treatment produced a weight increase in old rats. Old control animals had significantly more relative periepididymary fat than adult animals, and GH treatment reduced this parameter. Referring to SGI, old rats showed a lower SGI than adult animals, and GH significantly increased this index (Table 1).

Old male rats showed significantly lower values of IGF-I plasma levels as compared to adult animals, and GH treatment increased these levels in old rats.

### *Vascular reactivity*

#### *Relaxing responses*

Dose-dependent relaxing responses to ACh and Iso were diminished in old rats as compared to those of adult animals. Treatment with GH significantly improved Iso relaxation in old rats (Figure 1a), whereas the response to ACh was only slightly and not-significantly improved (Figure 1b). Maximal endothelium-independent relaxing response to SNP was similar in old and adult animals, and no effect of GH treatment was detected (Figure 2).

#### *Contractile responses*

Concerning the response to Phe, no significant differences between adult and old rats were detected, and GH did not show any significant effect on Phe-induced contraction (Figure 3a).

As shown in Figure 3b, dose-dependent contracting response to ACh + L-NAME was higher in old than in adult rats, and treatment with GH significantly reduced this response.

### *Vascular morphometry*

As compared to adult animals, media cross-sectional area was significantly increased in old rats, and GH treatment reduced this area significantly (Table 2; Figure 4).

Vessel and lumen cross-sectional area were bigger in old rats than in adult animals, and treatment with GH didn't exert any significant effect on these parameters.

### **Discussion**

As we have previously described for old intact and ovariectomized female rats (Castillo et al. 2003, 2005), the present experiment shows the improvement in body composition, vascular function and vascular structure induced by GH treatment in old male rats.

First of all, it should be mentioned that the mean lifespan of a rat ranges from 2 years to 3.6 years. A male outbred lifespan is around 1000 days (2.7 years) and 1300 days for females (3.6 years), but inbreds have generally shorter lifespans (Pass and Freeth 1993). The rats of the present experiment were sacrificed when they were 20 months of age (almost 2 years), an age that can be assumed as "old", and which has been previously accepted as such (Castillo et al. 2003).

It has been previously reported that there is a decrease in GH and IGF-1 production with age (Florini and Roberts 1980; Corpas et al. 1993; Raynaud-Simon 2003), and this fact has been proposed to be related to some of the changes that accompany the process of aging. Similarly, in the present study a significant reduction of plasma IGF-I with age was found.

Table 1. Body composition measurements and plasma IGF-1 levels in adult males and old male rats treated or not with GH.

	Adult control	Old control	Old GH
Weight increase (g)	20 ± 4	-62 ± 36**	9.3 ± 5 <sup>##</sup>
Periepididymary relative fat weight (g/100 g weight)	1.86 ± 0.2	2.8 ± 0.4*	2.4 ± 0.2
Periepididymary absolute fat weight (g)	8.5 ± 0.7 <sup>§</sup>	16.4 ± 3.2	15.1 ± 1.7
SGI	1.05 ± 0.9 × 10 <sup>-3</sup>	1.03 ± 0.006**	1.042 ± 0.003 <sup>##</sup>
IGF-1 plasma levels (ng/ml)	813 ± 68	598 ± 71*	1184 ± 93 <sup>##</sup>

Values are expressed as mean ± SEM.

\**P* < 0.05 and \*\**P* < 0.01 versus adult control.

#*P* < 0.05 and <sup>##</sup>*P* < 0.01 versus old control.

<sup>§</sup>*P* < 0.05 versus rest of groups.

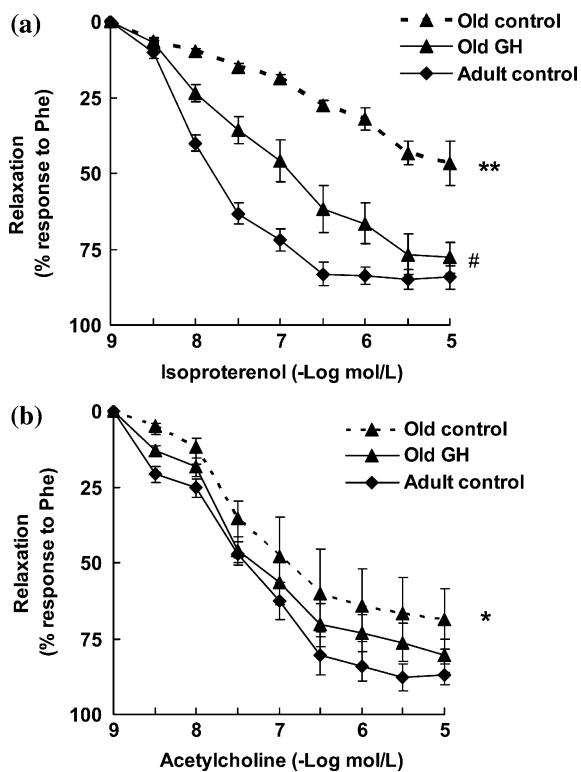


Figure 1. Relaxation induced by Acetylcholine (ACh  $10^{-9}$ – $10^{-5}$  mol/l; (a) and Isoproterenol (Iso  $10^{-9}$ – $10^{-5}$  mol/l; (b) in Phe-precontracted aortic rings (Phe;  $10^{-6}$  mol/l). Values are expressed as mean  $\pm$  SEM. \* $P$ <0.05 and \*\* $P$ <0.01 versus adult control rats. ( $P$ <0.05 versus old control animals).

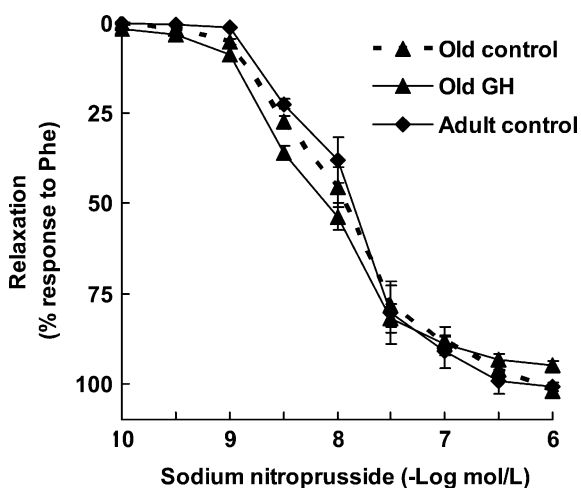


Figure 2. Relaxation induced by Sodium Nitroprusside (SNP  $10^{-10}$ – $10^{-6}$  mol/l) in Phe-precontracted aortic rings (Phe;  $10^{-6}$  mol/l). Values are expressed as mean  $\pm$  SEM.

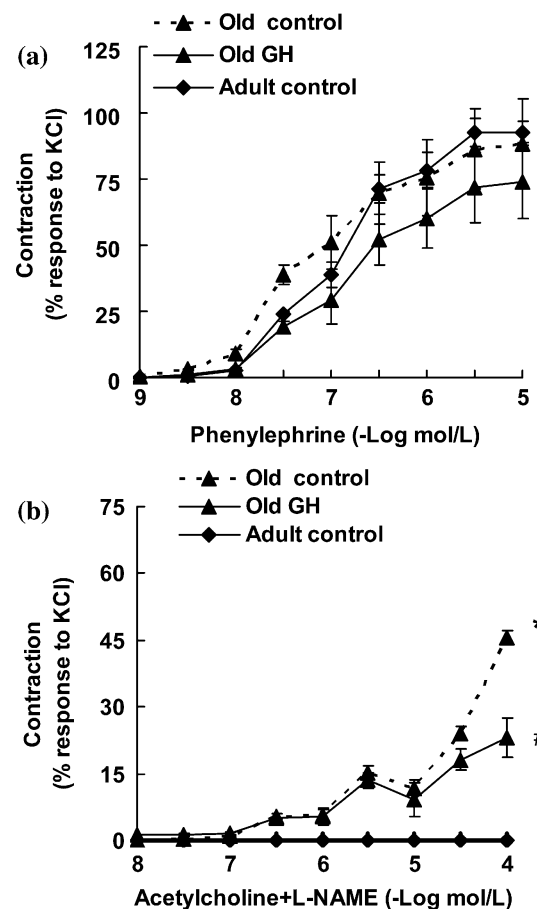


Figure 3. Contraction induced by Phenylephrine (Phe  $10^{-9}$ – $10^{-5}$  mol/l; (a) and Acetylcholine after 20 min of incubation with L-NAME (L-NAME  $10^{-4}$  + ACh  $10^{-8}$ – $10^{-4}$  mol/l; (b). Values are expressed as mean  $\pm$  SEM. \* $P$ <0.05 versus adult control rats. ( $P$ <0.05 versus old control animals).

Referring to body composition, our data are consistent with previous studies obtained in our laboratory using female rats (Castillo et al. 2003, 2005). It is known that GHD and elderly people show an increase in total body fat and visceral fat with reduced lean body mass (Toogood and Shalet 1998; McCallum et al. 2002; Nassis and Geladas 2003; Toogood 2003). In our study, old male rats showed a reduction in the SGI of animals' carcass together with an increase in peri-epididymary fat, which means that adiposity is augmented and lean body mass reduced. SGI, as was explained before, is an index that relates lean body mass and fat mass; the higher it is, the less fat the animal has. Our data also show that GH

Table 2. Media, vessel and lumen areas of aortic segments from adult male rats and old male rats treated or not with GH (2 mg/kg/day).

	Adult control	Old control	Old GH
Media area (mm <sup>2</sup> )	0.48 ± 0.01	0.84 ± 0.07*	0.68 ± 0.03**
Vessel area (mm <sup>2</sup> )	2.25 ± 0.13***	3.63 ± 0.24	3.44 ± 0.04
Lumen area (mm <sup>2</sup> )	1.77 ± 0.13***	2.79 ± 0.16	2.74 ± 0.04

Values are expressed as mean ± SEM.

\* $P < 0.01$  versus adult control.

\*\* $P < 0.05$  versus old control.

\*\*\* $P < 0.001$  versus rest of groups.

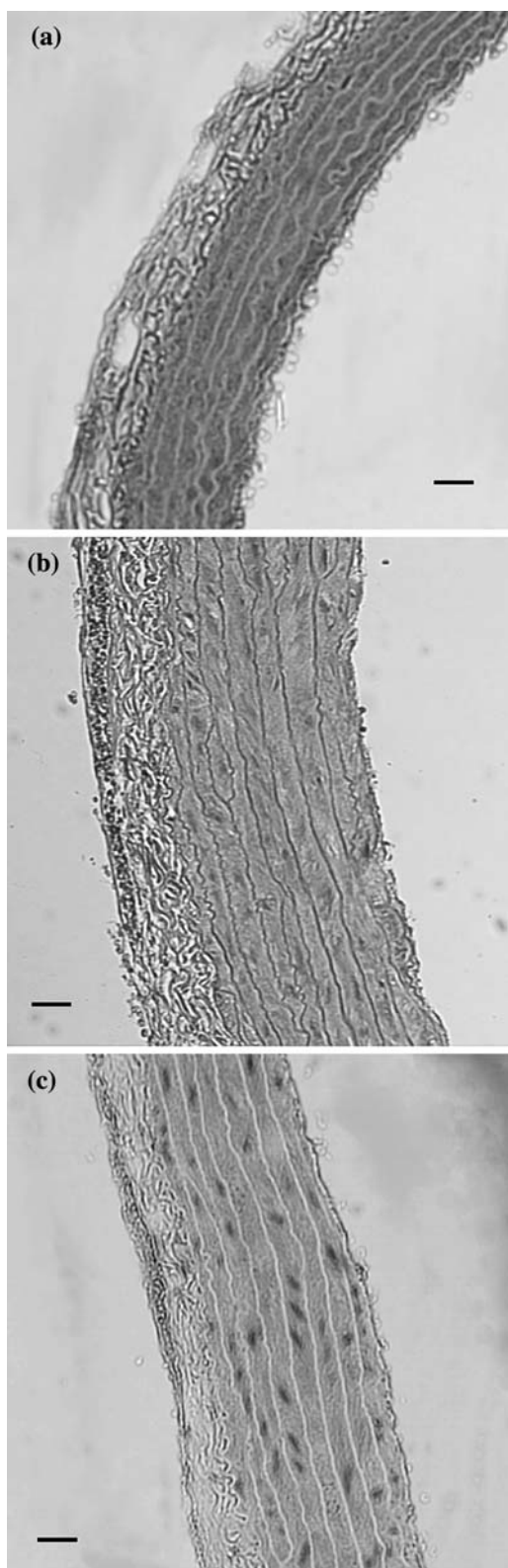
administration significantly increases SGI and tends to reduce periepididymary relative fat weight in old male rats, which means that GH, through its anabolic, antilipogenic and lipolytic properties, is able to increase muscle mass and reduce body fat (Richelsen 1997; Malmlof et al. 2002). It has been demonstrated that GH treatment to GHD adults, elderly people and old experimental animals is able to improve several parameters related to body composition (Rudman et al. 1990; Bengtsson et al. 1993; Cuttica et al. 1997; Jorgensen et al. 1997; Blackman et al. 2002; Malmlof et al. 2002; McCallum et al. 2002), for example reducing abdominal obesity, which is a strong predictor of cardiovascular risk (Despres et al. 2001). These findings are in consonance with the results obtained by our group in the present and in previous studies, in which GH administration was able to improve body composition in old intact and ovariectomized female rats (Castillo et al. 2003, 2005).

It has been demonstrated that, in rats (Tominaga et al. 1994; Castillo et al. 2003, 2005) and humans (Andrawis et al. 2000), aging is associated with an impaired endothelium-dependent vasodilatation without changes in endothelium-independent relaxation induced by nitric oxide (NO) donors (Tominaga et al. 1994; Maeso et al. 1999). Our results in males confirm these findings, as suggested by the reduced response to both ACh and Iso and by the absence of differences in the response to SNP in old rats as compared to adult ones. Alterations in the bioavailability of endothelial relaxing factors, such as prostacyclin (PGI<sub>2</sub>), endothelial-derived hyperpolarizing factor (EDHF) and NO, have been suggested to be involved in this phenomenon

(Mantelli et al. 1995; Tschudi et al. 1996; Nakajima et al. 1997; Maeso 1999; Matz et al. 2000; Andrawis et al. 2000). More concretely, a decrease in endothelial NO availability due to reduced synthesis and/or major degradation induced by increased oxidative stress could be an important mechanism underlying the altered response to endothelium-dependent agents during aging (Gryglewski et al. 1986; Tschudi et al. 1996; Cernadas et al. 1998; Maeso et al. 1999; Matz et al. 2000; Van der Loo et al. 2000). In addition to these mechanisms, an increase in contracting factors, which could counteract the effect of relaxing ones, might also be involved in this altered endothelial function. This hypothesis is supported by the findings of the present study, in which old animals showed a higher contractile response induced by ACh+L-NAME than adults. L-NAME is an agent that inhibits NOS, thus avoiding NO release and unmasking the effect of endothelium-derived contracting factors. Endothelial dysfunction of different aetiologies, such as aging (Koga et al. 1989; Kung and Luscher 1995; Matz et al. 2000), or hypertension (Rapoport and Williams 1996) show a similar pattern, with an increase in endothelium-dependent contraction. Arachidonic acid metabolites of the cyclooxygenase pathway, such as thromboxane A<sub>2</sub> (TXA<sub>2</sub>) or prostaglandin H<sub>2</sub> (PGH<sub>2</sub>), have been proposed to be the responsible agents for this increase in contraction under these situations (Matz et al. 2000).

Concerning Phe-induced contractile response, no significant changes with age were found. There are contradictory results in the literature about the effect of aging on vascular response to  $\alpha$ -adrenergic agents. Different studies have shown disparate effects of aging on vasoconstrictor factors since no changes, increases or decreases have been reported. Differences in the age, type of vessel, agonist, species and even gender could rely on this discrepancy (Maeso et al. 1999).

Changes in vascular structure have been reported in both aged and GHD humans. GHD patients show an increase in intima-media thickness, even previous to the instauration of atherosclerotic disease (Pfeifer et al. 1999). Similarly, aging is associated with changes in vascular structure and composition, such as cellular proliferation in the intima and media of the



arteries, collagen deposition, increase in wall artery thickness, reduction of elasticity and changes in extracellular matrix composition (Maeso et al. 1999; Kunz 2000). In female rats, we have previously described an increase in media cross sectional area with age (Castillo et al. 2003). Our present results confirm in old male rats some of these data, as we have also found an evident increase in media cross-sectional in these animals as compared to adults.

In our study, the administration of GH to old male rats induced, as expected, an increase in IGF-1 production, and this fact was accompanied by an improvement of endothelial function and vessel structure, as shown by the enhancement in endothelium-dependent vasodilatation, the reduction of endothelium-dependent vasoconstriction and the decrease in media cross-sectional area. These findings were similar to those previously reported by our group in old intact and ovariectomized female rats (Castillo et al. 2003, 2005).

The mechanisms underlying the beneficial effects exerted by GH administration could involve an increase in endothelial NO availability. Several studies have shown that IGF-1 is able to induce vasodilatation in humans and experimental animals (Copeland and Nair 1994; Hasdai et al. 1998). In fact, GH administration is able to improve endothelial function in patients with GHD, a parameter that is impaired in these patients (Evans et al. 2000). The possible mechanisms responsible for these effects seem to be related to an increase in NO bioavailability, since GH and IGF-1 induce NO synthesis by endothelial cells (Böger et al. 1996; Böger 1999; Thum et al. 2003) and also decreases NO degradation by reducing oxidative stress (Evans et al. 2000; Thum et al. 2003). This enhancement in NO availability could be also positively influencing vascular function and structure in old GH-treated male rats. Another possible mechanism could involve a reduction in the release of vasoconstrictor agents, such as  $\text{TXA}_2$  or  $\text{PGH}_2$ , which could

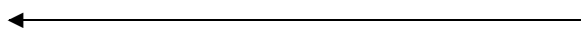


Figure 4. Representative microphotographs of hematoxylin-eosin-stained sections of aorta from adult (a), old control (b), and old GH-treated (c) male rats. Scale bar represents 40  $\mu\text{m}$ .

counteract the beneficial effects of NO. This hypothesis could be supported by the finding of a reduction of endothelium-dependent contraction in old GH-treated male rats.

GH treatment also seems to exert beneficial effects on vascular structure, since it reduces media cross-sectional area in old male rats. This structural improvement could be related to the enhancement in endothelial function, since it is known that endothelial cells, through paracrine mechanisms, are able to regulate vascular structure, as they modulate cellular growth, apoptosis, cell migration and extracellular matrix composition (Griendling and Alexander 1996; Jaffe 1996). Endothelial dysfunction may contribute to alterations in vascular structure; therefore, any intervention capable of improving endothelial function could be able to influence vessel structure. In fact, GHD patients submitted to GH treatment show a reversion early structural changes, this is, they show a decrease in intima-media thickness, which is an incipient change in vascular structure, and this beneficial effect could be probably due to the improvement in NO production that this treatment induces (Pfeifer et al. 1999).

In summary, our study shows that GH has beneficial effects in body composition, endothelial function and vascular structure in old male rats. More studies are needed in order to evaluate the possible application of this treatment in elderly people and to estimate the benefit/risk of such intervention.

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