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Upregulation of the angiotensin-converting enzyme 2/angiotensin-(1–7)/Mas receptor axis in the heart and the kidney of growth hormone receptor knock-out mice

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ABSTRACT

Objective: Growth hormone (GH) resistance leads to enhanced insulin sensitivity, decreased systolic blood pressure and increased lifespan. The aim of this study was to determine if there is a shift in the balance of the renin-angiotensin system (RAS) towards the ACE2/Ang-(1–7)/Mas receptor axis in the heart and the kidney of a model of GH resistance and retarded aging, the GH receptor knockout (GHR−/−) mouse.
Design: RAS components were evaluated in the heart and the kidney of GHR−/− and control mice by immunohistochemistry and Western blotting (n = 12 for both groups).
Results: The immunostaining of Ang-(1–7) was increased in both the heart and the kidney of GHR−/− mice. These changes were concomitant with an increased immunostaining of the Mas receptor and ACE2 in both tissues. The immunostaining of AT1 receptor was reduced in heart and kidney of GHR−/− mice while that of AT2 receptor was increased in the heart and unaltered in the kidney. Ang II, ACE and angiotensinogen levels remained unaltered in the heart and the kidney of GH resistant mice. These results were confirmed by Western blotting and correlated with a significant increase in the abundance of the endothelial nitric oxide synthase in both tissues.
Conclusions: The shift within the RAS towards an exacerbation of the ACE2/Ang-(1–7)/Mas receptor axis observed in GHR−/− mice could be related to a protective role in cardiac and renal function; and thus, possibly contribute to the decreased incidence of cardiovascular diseases displayed by this animal model of longevity.

1. Introduction

The growth hormone (GH)/insulin-like growth factor-1 (IGF-1) axis has important physiological functions in maintaining normal growth, body composition, proliferation and differentiation of various cell types, regulation of lipid, carbohydrate and fat metabolism, development and maintenance of the immune system as well as control of heart, kidney and brain functions [1,2]. Deletion of growth hormone receptor (GHR) gene leads to GH resistance, very low levels of circulating IGF-1, reduced body size, signs of delayed aging, and a remarkable increase in longevity [3,4]. Growth hormone receptor deficient mice (GHR−/−) also display enhanced whole-animal insulin sensitivity [5–7], hypoinsulinemia and significantly lower fasting and nonfasting glucose levels in adult male and female (10 months old) GHR−/− mice compared with wild-type (WT) controls [5,7,8]. Moreover, GHR−/− mice show elevated percentage of adiposity [9], together with lower cholesterol, triglycerides and apolipoprotein B circulating levels than wild type mice [10,11].

The GH/IGF-1 system is also important for the maintenance of both renal and cardiovascular structure and function [12–14]. Derangements in the GH/IGF-1 axis are associated with chronic renal failure [13]. Accordingly, overexpression of GH in transgenic mice, is associated with development of severe glomerulosclerosis by about 6 months of age [15]. In contrast, GHR−/− mice are protected from diabetic nephropathy [16].

Previous studies described a close relationship between the GH/IGF-1 and the renin-angiotensin system (RAS). Growth hormone has been shown to stimulate the RAS as shown by an increase in circulating levels of angiotensinogen, aldosterone and plasma renin activity in human subjects [17,18] and dwarf rats [19]. In addition, it was demonstrated

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that GH can increase the AT1 receptor density in primary cultures of rat astrocytes [20] and in the remnant kidney of uninephrectomized male rats [21].

Although GHR −/− mice have reduced cardiac weight and volume, the preservation of health and the increased lifespan of these animals, suggest presence of compensatory mechanisms that tend to overcome the negative physiological effects on the cardiovascular system of a disrupted GH/IGF-1 axis [12,14]. This is demonstrated by echocardiography measurements that show that GHR −/− mice have a decreased systolic cardiac function that correlates with decreased systolic blood pressure observed in 18-week-old GHR −/− females [12] and in 9-month-old GHR −/− males [14], reduced levels of plasma renin and increased levels of circulating K^+ and aortic endothelial nitric oxide synthase (eNOS) expression, with no changes in total aldosterone levels [12].

The RAS is classically conceived as a coordinated hormonal cascade involved in the control of cardiovascular, renal, and adrenal functions, mainly through the actions of angiotensin (Ang) II [22], that is generated in the circulation and locally in numerous organs by renin and angiotensin-converting enzyme (ACE) [22,23]. The description of local RAS highlighted several non-hemodynamic effects of Ang II and led to the identification of new roles in physiological and pathophysiological processes, including inflammation, cell proliferation and fibrosis [22,24,25]. Angiotensin (Ang) II acts through two pharmacologically distinct G protein-coupled receptors, angiotensin type 1 (AT1) and the type 2 (AT2) receptors which have counter-regulatory actions in the cardiovascular and renal system [26,27]. Activation of the AT1 receptor, promotes vasoconstriction, reactive oxygen species (ROS) production; extracellular matrix remodeling and inflammation response, tissue injury and insulin resistance [26,28]. In line with these reports, it was also demonstrated that blockade of AT1 receptor could represent a crucial determinant of health and extended lifespan [29]. On the other hand, the AT2 receptor inhibits cell growth, inflammation and fibrosis; and exerts a cardio-protective role against ischemia–reperfusion injury and acute myocardial infarction [25,27]. Advances in the field led to the recognition of other active components of the RAS metabolism, such as Ang III, Ang IV, and Ang-(1–7) [30,31], the angiotensin-converting enzyme (ACE) 2, that forms Ang-(1–7) directly from Ang II and indirectly from Ang I [32], and the Ang-(1–7) specific G protein-coupled receptor Mas [33]. The ACE2/Ang-(1–7)/Mas receptor axis in general opposes the vascular and proliferative effects of Ang II [31]. Angiotensin-(1–7) arises as a potential regulator of endothelial function [31]; and several observations point towards an active role of Ang-(1–7) in metabolic actions [34–36].

Cardiovascular diseases are commonly associated with alterations of the GH/IGF-1 axis and involve an imbalance within the RAS. However, to date, there is scant information available regarding the effects of disturbances in the GH/IGF-1 axis on the in vivo expression of the main components of the RAS. Therefore, our hypothesis is that GHR −/− mice may display modifications on the expression of cardiac and renal main components of the RAS towards a protective status of this system that could explain at least in part their increased lifespan. This study was designed to evaluate local levels of Ang II and Ang-(1–7); AT1, AT2 and Mas receptor, as well as ACE, ACE2 angiotensinogen (AGT) and endothelial nitric oxide synthase (eNOS) in the heart and the kidney of GHR −/− mice by both immunohistochemistry and Western blotting analysis.

2. Materials and methods

2.1. Animals

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996), and the appropriateness of the experimental procedure, the required number of animals used, and the method of acquisition were approved by the Southern Illinois University Laboratory of Animal Care and Use Committee. Two to three-month old GHR −/− and normal male mice (n = 12) were produced in our breeding colony derived from GHR −/− animals provided by Dr. J. J. Kopchick (Ohio University, Athens, OH, USA). Wild-type littermates of GHR −/− mice served as controls for this study. Mice were housed three to five per cage in a room with controlled light (12 h light per day) and temperature (22 ± 2 °C). The animals had free access to food (Lab Diet Formula 5008, containing a minimum of 23% protein and 5% fat and a maximum of 5% fiber; Purina Mills Inc., St. Louis, MO, USA) and tap water.

2.2. Tissue collection

The heart and kidneys from GHR −/− and control mice were perfused with physiological saline solution through the abdominal aorta until they were free of blood. Afterwards, tissues were removed and weighed. For immunohistochemical studies, whole heart as well as whole decapsulated kidneys were cut longitudinally, fixed in phosphate-buffered 10% formaldehyde (pH 7.2), and embedded in paraffin. A piece of each tissue was preserved at −80 °C for immunoblotting determinations.

2.3. Blood pressure determination

Systolic and diastolic blood pressures were measured using a computerized noninvasive tail-cuff system based on Volume Pressure Recording (Kent Scientific Corporation, Northwest Connecticut, USA). Conscious animals were allowed to enter a restraining holder freely and were kept in the cylinder for 10 min before the determination. The blood pressure session consisted of 50 cycles; the first 20 cycles were considered acclimatization cycles and were not recorded.

2.4. Immunohistochemistry

Paraffin-embedded tissues were cut at 3 μm and subjected to immunohistochemistry. Briefly, the sections were deparaffinized with xylene, rehydrated through graded series of ethanol to water, and then incubated in blocking solution (PBS plus 1% bovine serum) at room temperature for 1 h. Then, the sections were incubated overnight at 4 °C with one of the following primary antibodies: rabbit polyclonal antibody anti-Ang II (1:100 dilution; H002-12) and anti-Ang-(1–7) (1:50 dilution; H002-24; Phoenix Pharmaceutical, Inc., Burlingame, CA, USA); polyclonal anti-Ang-(1–7) Mas receptor (1:100 dilution; AAR-013; Alomone Labs, Ltd., Jerusalem, Israel); polyclonal anti-AT1 receptor (1:100 dilution; sc-579), anti-AT2 receptor (1:100 dilution; sc-9040), anti-ACE (1:100 dilution; sc-12187) and anti-ACE2 (1:100 dilution; sc-17720; Santa Cruz Biotechnology, Santa Cruz, CA). All antibodies were diluted with blocking solution. Immunostaining was carried out with an avidin-biotin-peroxidase complex kit and counterstained with hematoxilin [37]. Specificity of the Ang II and Ang-(1–7) staining was tested by preincubating the corresponding primary antibodies for 30 min at room temperature with a 1 μM solution of Ang II or Ang-(1–7) peptides (Bachem Americas, Torrance, CA, USA), [37]. Histological sections were studied in each animal using a light microscope Nikon E400 (Nikon Instrument Group, Melville, NY, USA). All tissue samples were evaluated independently by two investigators without prior knowledge of the group to which the mouse belonged. Histological evaluation of tissues was assessed on 20 consecutive microscopic fields at 400× magnification, where each field represents 1.13 mm2, resulting in a total explored area of 22.6 mm^2. Data were averaged and results were expressed as a percentage per mm^2. In the case of the kidney, independent analysis in glomeruli and tubules was performed in order to evaluate potential differences in the expression of Ang II, Ang 1–7, ACE, ACE2, AT1, AT2 and Mas receptor. All measurements were carried out using an image analyzer Image-Pro Plus ver. 4.5 for windows (Media Cybernetics, LP. Silver Spring, MD, USA).

2.5. Immunoblotting

Tissues were homogenized in 10 vol. of solubilization buffer [1% Triton, 100 mM HEPES, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 0.035 TIU/ml aprotinin (pH 7.4)] at 4 °C. Tissue homogenates were centrifuged at 100,000×*g* for 1 h at 4 °C to remove insoluble material and protein concentration of supernatants was determined by the bicinchoninic acid method [38]. To determine the protein abundance of AT1, AT2, Mas receptors, ACE and ACE2, equal amounts of solubilized proteins (40 μg) were denatured by boiling in reducing sample buffer, resolved by SDS-PAGE, and subjected to immunoblotting with the same antibodies used for immunohistochemistry (1:1,000 dilution) diluted in Tris-buffered saline 0.1% Tween-20 plus 1% BSA. To determine eNOS and angiotensinogen (AGT) protein expression, immunoblotting was performed with a rabbit monoclonal antibody against AGT (1:1,000 dilution; MABC123; Millipore, Billerica, MA, USA) or rabbit polyclonal antibody against eNOS (1:1,000 dilution; sc-654; Santa Cruz Biotechnology, USA) respectively. Finally, membrane blots were washed and incubated for 1 h at room temperature with goat anti-rabbit IgG-horseradish peroxidase (HRP) secondary antibody (1:20,000 dilution) or donkey anti-goat IgG-HRP secondary antibody (1:10,000 dilution; Santa Cruz Biotechnology, USA). Specific bands were visualized on Amersham Hyperfilm ECL using an enhanced chemiluminescence detection system (GE Healthcare Bio-Sciences, USA) and their intensities were quantitated by digital densitometry using Gel-Pro Analyzer 4.0 (Media Cybernetics, Bethesda, MD, USA). Protein loading in gels was evaluated by stripping and reblotting membranes with antibody anti-β-tubulin (1:3000 dilution; T3526; Sigma-Aldrich, St. Louis, MO). Membranes were then incubated with goat anti-rabbit IgG-HRP antibody, proteins detected by ECL and the intensities of specific bands were quantitated by optical densitometry.

2.6. Statistical analysis

Experiments were performed analyzing all groups of animals in parallel, with n representing the number of different individuals used in each group. Results are presented as mean ± S.D. Statistical analyses were performed by Mann Whitney test using the InStat statistical program by GraphPad Software, Inc. (San Diego, CA, USA). $P<0.05$ was required for statistical significance.

3. Results

Growth hormone receptor knockout mice were used to study the relationship between GH resistance and the abundance of different RAS components in the heart and the kidney. In agreement with previously published results [39], GHR −/− mice exhibited reduced body weight when compared with normal littermates (Table 1). Also, these animals displayed reduced absolute heart weight which did not differ from WT animals when normalized to body weight. However, compared to their normal siblings, GHR −/− mice had reduced kidney weight even after normalizing to body weight (Table 1). In contrast to previous observations [12], systolic and diastolic blood pressure were found to be not significantly different between GHR −/− and normal mice (Table 1).

In the heart and the kidney of normal and GHR −/− mice, the two main active peptides of the RAS, Ang II and Ang-(1–7), were evaluated by immunohistochemistry through the use of specific antibodies. As shown in Fig. 1, similar content of Ang II was observed between normal and GHR −/− mice in the heart (Fig. 1A, upper panel) and in the kidney (Fig. 1B, upper panel). However, the analysis of Ang-(1–7) revealed an important increased abundance of this heptapeptidic hormone both in the heart (Fig. 2A, upper panel) and the kidney (Fig. 2B, upper panel) of GHR −/− mice. Quantification of Ang II and Ang-(1–7) tissular abundance was expressed as percentage of positive staining per area of tissue (Table 2). Expression of these two hormones in the kidney was detected mainly in the tubular area while a small amount of positive staining was found in glomeruli (Table 2). Antibody specificity was confirmed by the use of anti-Ang II or anti-Ang-(1–7) antibodies previously blocked by preincubation with Ang II or Ang-(1–7) respectively (Figs. 1 and 2, lower panels).

Table 1
Body and organ weights and blood pressure of GHR −/− and normal mice.

	Normal	GHR −/−
Body weight (g)	27.7 ± 1.2	18.2 ± 1.0*
Heart weight (g)	0.110 ± 0.006	0.072 ± 0.003*
Heart wt/body wt (%)	0.40 ± 0.02	0.41 ± 0.03
Kidney weight (g)	0.176 ± 0.008	0.101 ± 0.003*
Kidney wt/body wt (%)	0.66 ± 0.03	0.55 ± 0.04*
Systolic blood pressure (mm Hg)	97.0 ± 2.4	88.6 ± 4.0
Diastolic blood pressure (mm Hg)	70.4 ± 4.0	65.7 ± 4.5

ACE: angiotensin converting enzyme; ACE2: angiotensin converting enzyme type 2; Ang: angiotensin; GHR −/−: growth hormone receptor knock-out mice; RAS: renin-angiotensin system. Data are presented as percentage of positive staining per area. Values are expressed as mean ± S.D. *$P<0.01$ vs. normal mice; n = 12; Mann Whitney test.

The analysis of tissue levels of these hormones was accompanied by measurements of the Ang II specific AT1 and AT2 receptors; and the Ang-(1–7) specific receptor Mas by immunohistochemistry and Western blot analysis. Both heart (Fig. 3A) and kidney (Fig. 3B) from GHR −/− displayed a significant reduction in the expression of AT1 receptor. However, the evaluation of AT2 receptor inmunostaining and abundance revealed that when compared with normal littermates, hearts from GHR −/− mice had an increased expression of this receptor (Fig. 4A), while no difference in AT2 receptor abundance in the kidney was observed between GHR −/− and normal mice (Fig. 4B). Interestingly, the abundance of the Mas receptor, specific for Ang-(1–7), was increased in both tissues analyzed (Fig. 5A and B). As shown in Table 2, immunohistochemical quantification of AT1, AT2 and Mas receptors local abundance was expressed as percentage of positive staining per area of tissue. Renal localization of these receptors was mainly tubular (Figs. 3, 4 and 5, and Table 2). All immunohistochemical results were confirmed by Western blot analysis of the heart (Figs. 3C, 4C and 5C) and the renal (Figs. 3D, 4D and 5D) homogenates.

Classical ACE and recently described ACE2 were determined as another approach to evaluate the main components of the RAS. Compared to WT animals, the levels of ACE, capable of synthesizing Ang II from Ang I were similar both in the heart (Fig. 6A) and the kidney (glomerular and tubular sections; Fig. 6B) of GHR −/− mice. Immunostaining of ACE2 in the heart was significantly increased, this result correlated well with the detection of higher ACE2 cardiac abundance as detected by Western blotting (Fig. 7A). Expression of ACE2 in the kidney (mainly tubular) was also increased in GHR −/− mice as determined by immunohistochemistry (Fig. 7B) and confirmed by Western blot (Fig. 7D). Since ACE2 is responsible for synthesizing Ang-(1–7), these observations could explain the increased expression of this heptapeptidic hormone in both tissues analyzed. Immunohistochemical quantification of ACE and ACE2 local abundance was expressed as percentage of positive staining per area of tissue (Table 2). As shown in Table 2, immunostaining for AT1, AT2 and Mas receptors, as well as immunostaining of ACE and ACE2 was detected mainly in the tubular area. Glomeruli displayed a very low percentage of positive staining for these proteins without statistically significant difference between GHR −/− and wild type mice (Table 2).

The analysis of cardiac and renal RAS components was accompanied by determination of AGT abundance by Western blot. As observed in Fig. 8, AGT abundance was similar between GHR −/− and normal mice both in the heart (Fig. 8A) and kidney (Fig. 8B), showing that initial steps of the proteolytic cascade within the RAS seems to be conserved between GHR −/− and wild type mice.

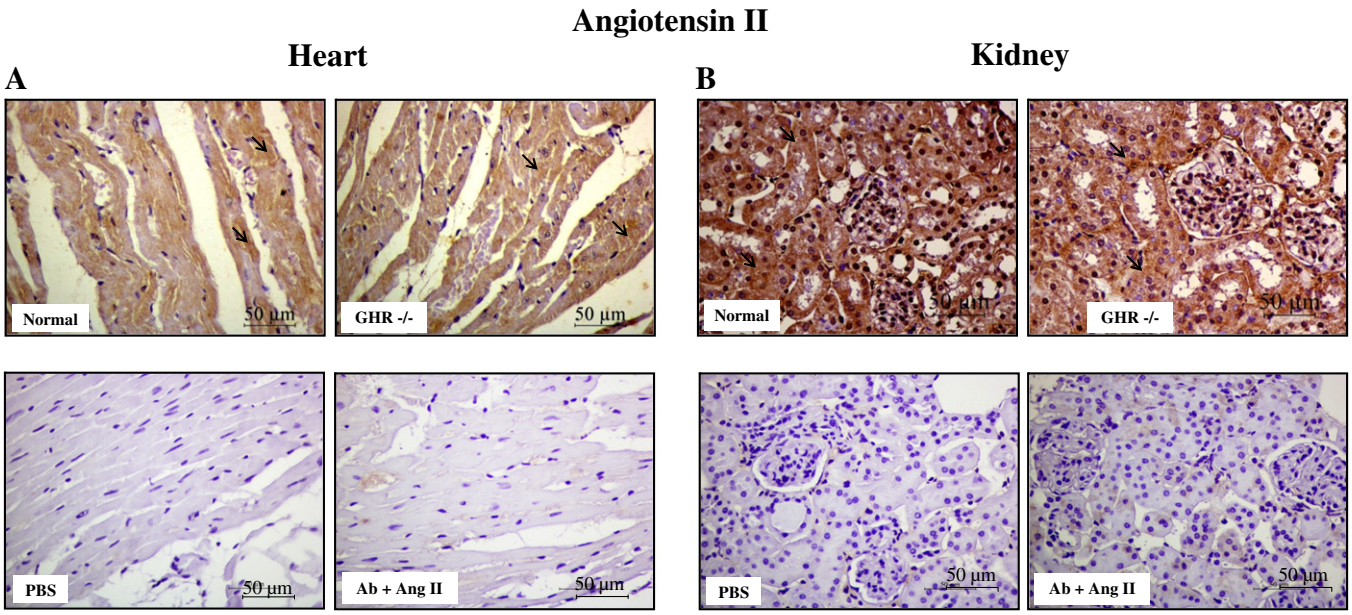

Fig. 1. Representative images showing the immunohistochemical staining of angiotensin II in the heart (A) and the kidney (B) from GHR−/− (n = 12) and normal mice (n = 12). Images are shown at 400× magnification; black arrows indicate specific staining in cardiomyocytes (A) as well as in glomerular and tubular sections in the kidney (B). Lower panels show cardiac (A) and renal (B) sections incubated with either PBS (control of non-specific staining) or with anti-Ang II antibody (Ab) previously blocked by preincubation with Ang II (control of antibody specificity).

Since eNOS could represent a potential effector of beneficial cardiovascular actions of the ACE2/Ang-(1–7)/Mas receptor axis [31], we analyzed the protein abundance of this enzyme as a measurement of its expression in heart and kidney of GHR−/− mice. As observed by Western blotting, a significantly increased abundance of eNOS was found both in the heart (Fig. 9A) and the kidney (Fig. 9B) of GHR−/− mice compared to control animals.

4. Discussion

The key finding of the present study is that long-lived GHR−/− mice exhibit an imbalance within the RAS in both the heart and the kidney, characterized by a reduced abundance of AT1 receptors accompanied by a substantial over-expression of RAS components that belong to the ACE2/Ang-(1–7)/Mas receptor axis. In agreement with previous studies [14,39], our current results showed a reduction in both absolute and relative kidney weight in GHR−/− mice. In contrast, the relative heart weight was not significantly altered in GHR−/− mice. The RAS is activated during neonatal growth and differentiation of the kidney, highlighting the importance of this system in normal renal maturation [40]. Lack of neonatal Ang II-induced AT1 receptor stimulation produces renal abnormalities characterized by papillary atrophy and impaired urinary concentrating ability, together with a downregulation of genes encoding components of cytoskeleton and

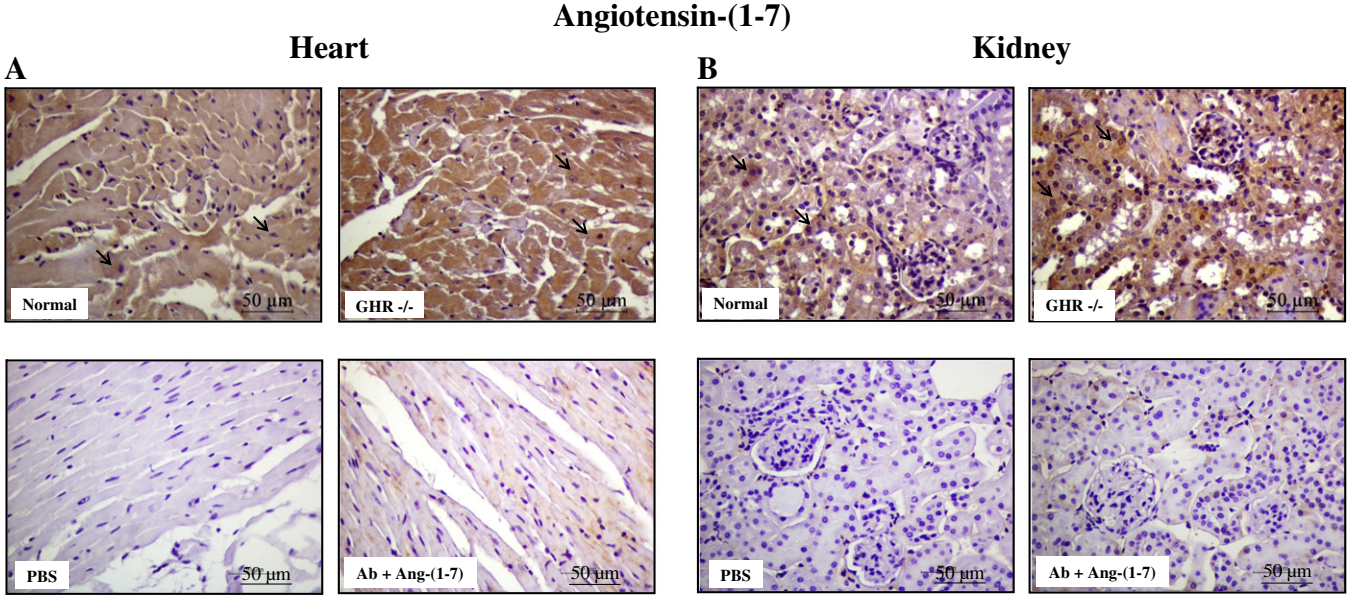

Fig. 2. Representative images showing the immunohistochemical staining of angiotensin-(1–7) in the heart (A) and the kidney (B) from GHR−/− (n = 12) and normal mice (n = 12). Images are shown at 400× magnification; black arrows indicate specific staining in cardiomyocytes (A) as well as in glomerular and tubular sections (B). Lower panels show cardiac (A) and renal (B) sections incubated with either PBS (control of non-specific staining) or with anti-Ang-(1–7) antibody (Ab) previously blocked by preincubation with Ang-(1–7) (control of antibody specificity).

Table 2
Quantification of immunohistochemical staining of RAS components in the heart and the kidney of GHR−/− and normal mice.

	Heart		Kidney			
			Glomerular		Tubular	
	Normal	GHR−/−	Normal	GHR−/−	Normal	GHR−/−
Ang II	16.6 ± 2.6	17.9 ± 1.9	3.9 ± 0.7	3.7 ± 0.5	29.7 ± 2.1	28.6 ± 2.7
Ang-(1–7)	4.1 ± 1.9	26.2 ± 3.7*	1.3 ± 0.1	2.1 ± 0.4*	8.9 ± 1.4	17.4 ± 1.3*
AT1 receptor	17.3 ± 2.2	4.2 ± 2.7*	0.2 ± 0.3	0.1 ± 0.2	15.9 ± 2.2	2.7 ± 0.5*
AT2 receptor	19.7 ± 1.7	24.2 ± 1.5*	0.1 ± 0.1	0.1 ± 0.3	18.1 ± 1.0	18.9 ± 1.9
MAS receptor	12.5 ± 2.0	22.4 ± 2.5*	0.1 ± 0.1	0.1 ± 0.1	5.0 ± 0.9	8.4 ± 1.0*
ACE	23.6 ± 3.1	21.7 ± 2.9	0.2 ± 0.2	0.2 ± 0.1	30.1 ± 1.6	29.5 ± 2.3
ACE2	13.2 ± 1.4	18.4 ± 1.7*	0.2 ± 0.1	0.3 ± 0.5	9.3 ± 1.0	26.9 ± 1.5*

ACE: angiotensin converting enzyme; ACE2: angiotensin converting enzyme type 2; Ang: angiotensin; GHR−/−: growth hormone receptor knock-out mice; RAS: renin-angiotensin system. Data are presented as percentage of positive staining per area. Values are expressed as mean ± S.D. *$P<0.01$ vs. normal mice; n = 12; Mann Whitney test.

cytoskeleton-associated proteins, extracellular matrix, and enzymes involved in extracellular matrix maturation or turnover [41]. Thus, the reduced expression of AT1 receptor in the kidney of GHR−/− mice could be a contributing factor to reduced kidney weight in these animals. Additionally, Egecioglu and collaborators showed that, besides a reduction in blood pressure, GHR−/− mice displayed an unexpected significant reduction in plasma renin activity together with an increase in plasma potassium levels [11]. Taken together, these findings indicate an abnormal kidney response towards extracellular volume changes in GHR−/− mice. However, delayed aging and increased life expectancy of GHR−/− mice, suggest the existence of compensatory mechanisms that tend to overcome the negative physiological effects of a disrupted GH/IGF-1 axis on the cardiovascular and renal system. In the current study growth hormone receptor knockout mice show a tendency to reduced blood pressure; however, this change did not reach statistical significance. In a previous study, reduced systolic blood pressure was reported in 9 month female GHR −/− mice [12]. Possible contributing factors to this inconsistency could be differences in gender (male in the current study) and/or age of the animals used (2–3 months in the current study).

Insulin sensitivity has also been proposed as a major contributor to aging. Insulin regulates metabolism in cardiovascular tissue by modulating glucose uptake and utilization, glycogen synthesis, lipid metabolism, proliferation, contractility, remodeling, and apoptosis in cardiomyocytes. In the kidney, insulin has been shown to increase sodium reabsorption in the proximal tubule, the thick ascending limb, and the distal tubule including the collecting duct in animals and humans [42]. Hyperinsulinemia resulting from insulin resistance is associated with pathological cardiomyocyte hypertrophy and increased blood pressure in both animals and humans [43,44]. An increasing body of evidence supports a role of Ang II in the multifactorial etiology of insulin resistance [22,43]. The critical role of Ang II in the etiology of insulin resistance has been addressed by using selective antagonist of AT1 receptors which elicited significant improvements of whole body insulin action in several distinct insulin-resistant rodent models, including the obese Zucker rat, the spontaneously hypertensive rat,

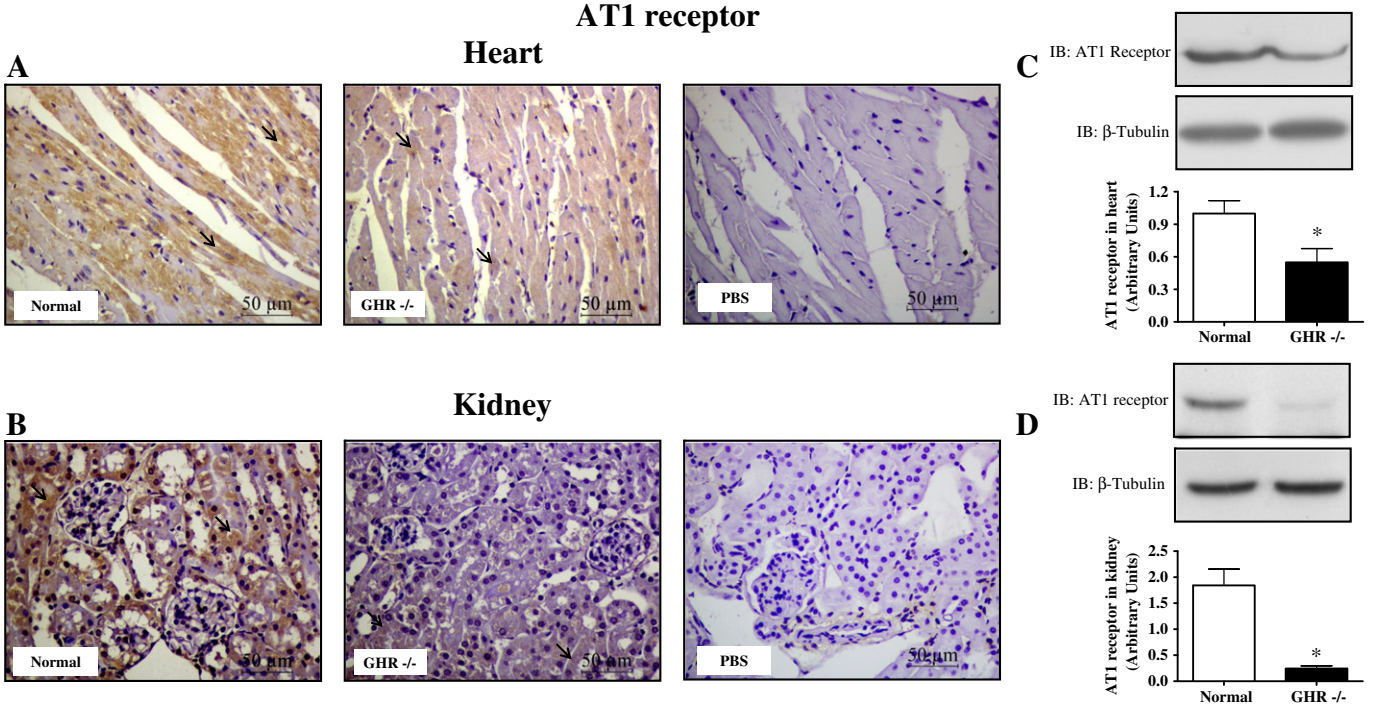

Fig. 3. Representative images showing the immunohistochemical staining of AT1 receptor in the heart (A) and the kidney (B) from GHR−/− and normal mice. Images are shown at 400× magnification; black arrows indicate positive staining in cardiomyocytes (A) and in tubular epithelial cells (B). Tissue sections were incubated with PBS instead of primary antibody as a control of non-specific staining. Results were confirmed by submitting tissue homogenates to Western blot analysis. Representative images and bar charts showing the quantification of AT1 receptor in the heart (C) and the kidney (D) are shown for each group. Data are shown as mean ± S.D. *$P<0.05$ vs. normal group (n = 12); Mann Whitney test. The calculated molecular weight for the specific bands corresponding to the AT1 receptor and β-tubulin was approximately 44 and 50 kDa respectively in both tissues. IB: immunoblotting.

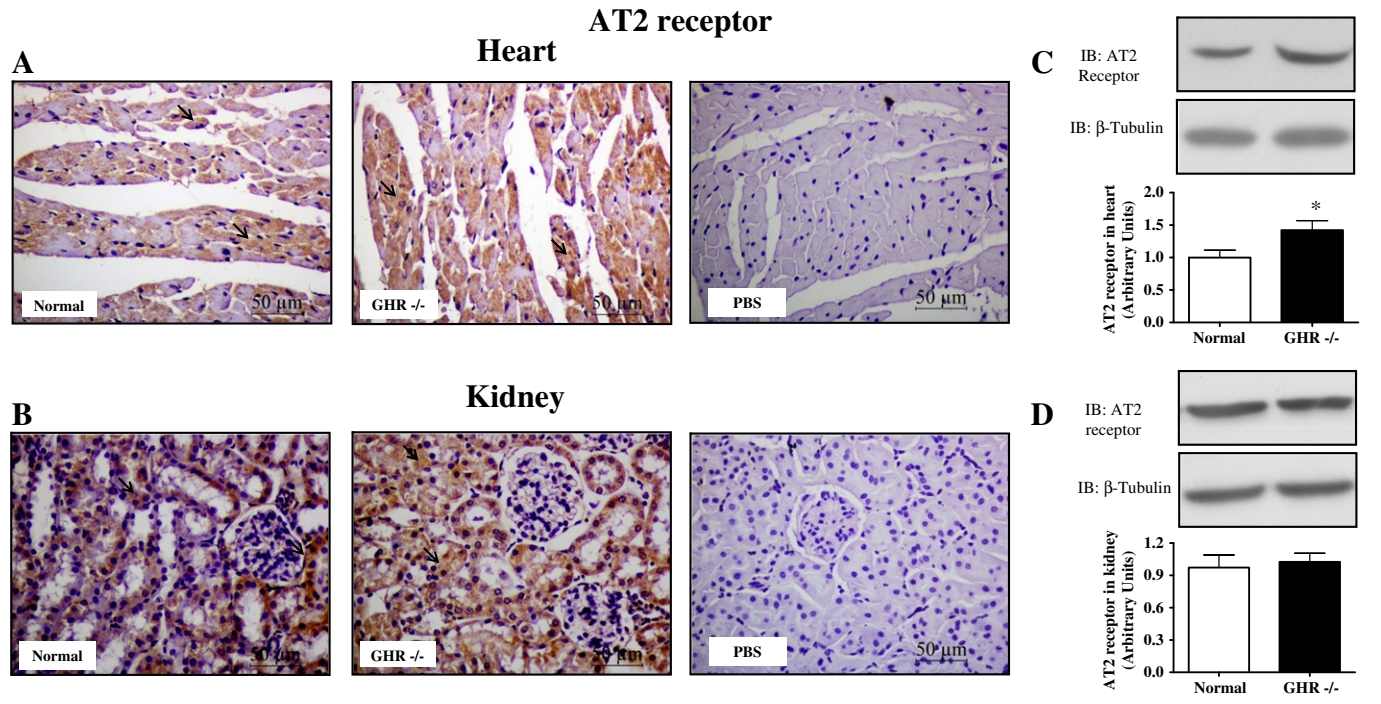

Fig. 4. Representative images showing the immunohistochemical staining of AT2 receptor in heart (A) and kidney (B) from GHR −/− and normal mice. Images are shown at 400× magnification; black arrows indicate positive staining in cardiomyocytes (A) and in tubular epithelial cells (B). Tissue sections were incubated with PBS instead of primary antibody as a control of non-specific staining. Results were confirmed by submitting tissue homogenates to Western blot analysis. Representative images and bar charts showing the quantification of AT2 receptor in heart (C) and kidney (D) are shown for each group. Data are shown as mean ± S.D. *$P<0.05$ vs. normal group (n = 12); Mann Whitney test. The calculated molecular weight for the specific band corresponding to the AT2 receptor was approximately 50 kDa in both tissues. IB: immunoblotting.

and fructose-fed rats [44]. Several studies demonstrated that RAS blockade could represent a crucial determinant of health and lifespan; mainly, by a mechanism that involve a reduction of mitochondrial-derived reactive oxygen species. Interestingly, these observations are also accompanied by a substantial reduction of insulin resistance [29,45]. Thus, the reduced expression of AT1 receptor in heart and

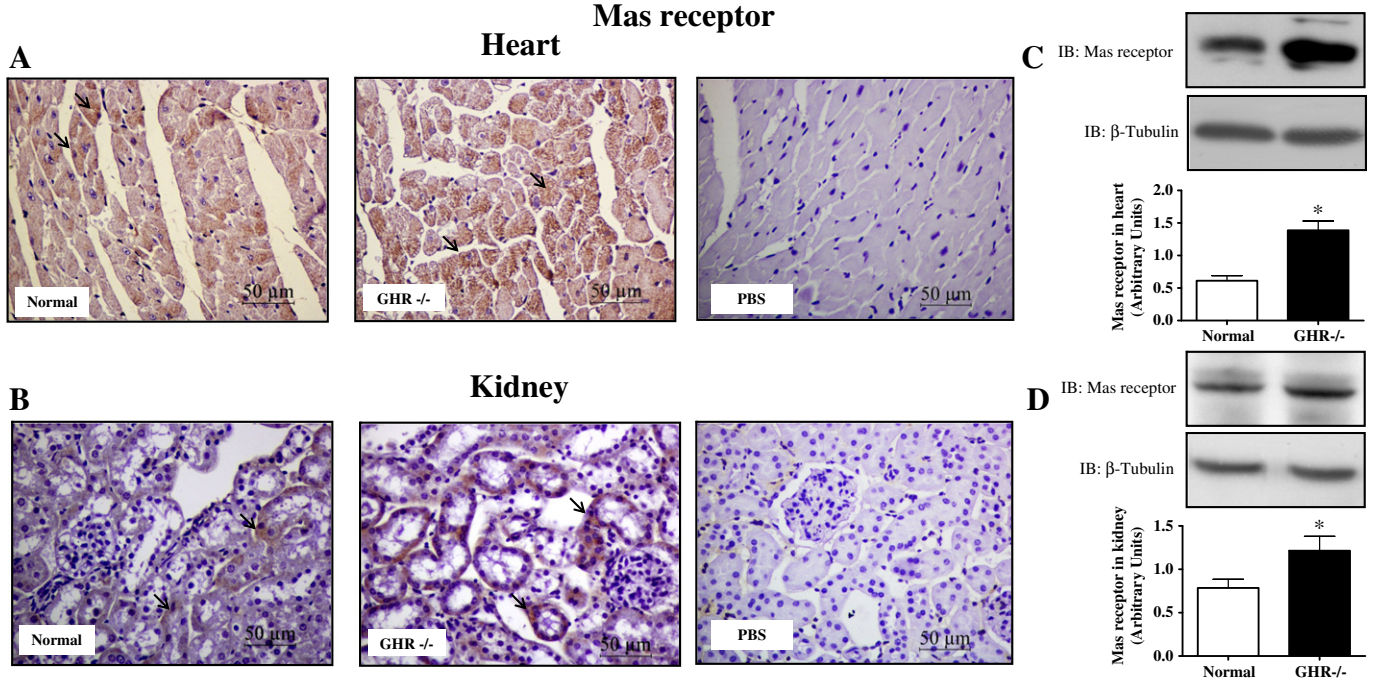

Fig. 5. Representative images showing the immunohistochemical staining of Mas receptor in heart (A) and kidney (B) from GHR −/− and normal mice. Images are shown at 400× magnification; black arrows indicate positive staining in cardiomyocytes (A) as well as in both glomerular and tubular sections in the kidney (B). Tissue sections were incubated with PBS instead of primary antibody as a control of non-specific staining. Results were confirmed by submitting tissue homogenates to Western blot analysis. Representative images and bar charts showing the quantification of Mas receptor in heart (C) and kidney (D) are shown for each group. Data are shown as mean ± S.D. *$P<0.05$ vs. normal group (n = 12); Mann Whitney test. The calculated molecular weight for the specific band corresponding to the Mas receptor was approximately 45 kDa in both tissues. IB: immunoblotting.

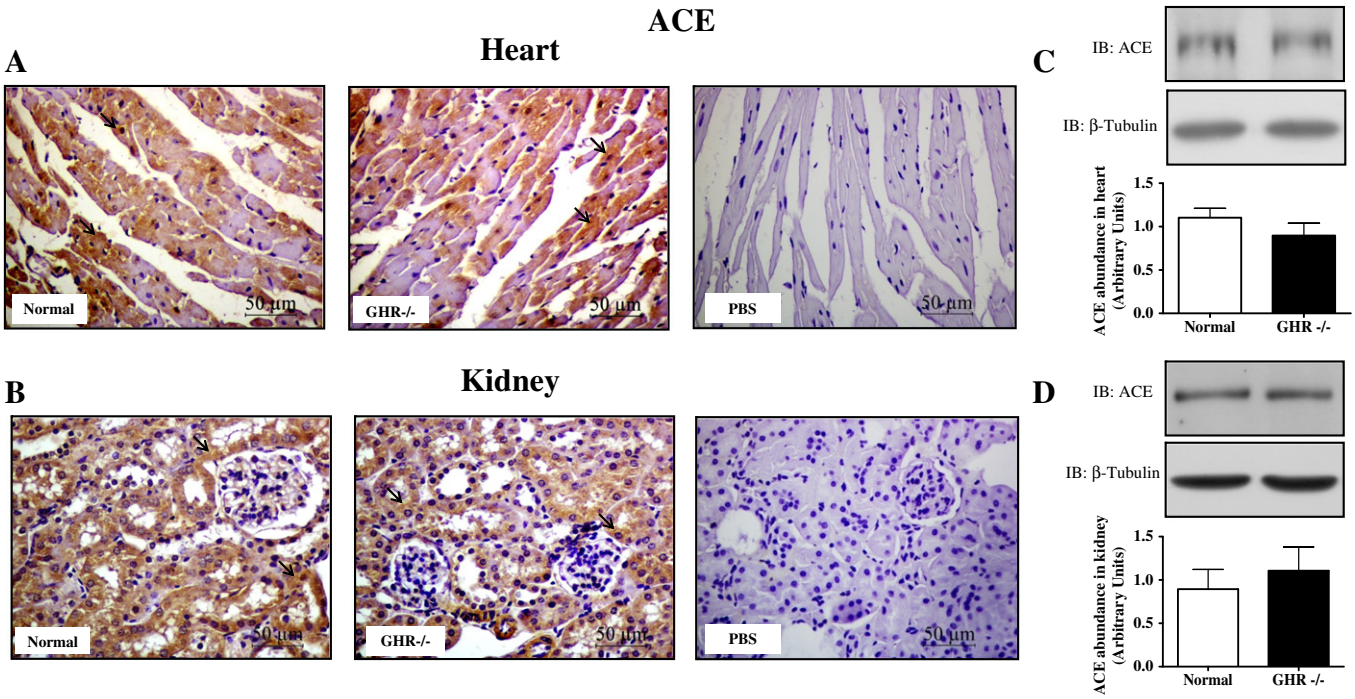

Fig. 6. Representative images showing the immunohistochemical staining of angiotensin-converting enzyme (ACE) in heart (A) and kidney (B) from GHR −/− and normal mice. Images are shown at 400× magnification; black arrows indicate positive staining in cardiomyocytes (A) and in both glomerular and tubular sections of the kidney (B). Tissue sections were incubated with PBS instead of primary antibody as a control of non-specific staining. Results were confirmed by submitting tissue homogenates to Western blot analysis. Representative images and bar charts showing the quantification of ACE in heart (C) and kidney (D) are shown for each group. Data are shown as mean ± S.D. The calculated molecular weight for ACE was approximately 188 kDa in both tissues. IB: immunoblotting.

kidney of GHR −/− mice could be a contributory factor to the improvement of insulin sensitivity and increased longevity observed in these long-lived mutant mice.

Although both AT1 and AT2 receptors are present on cardiac and renal tissue, the adverse effects of Ang II are mediated mostly through AT1 receptors [27]. Studies have shown that AT2 receptors inhibit

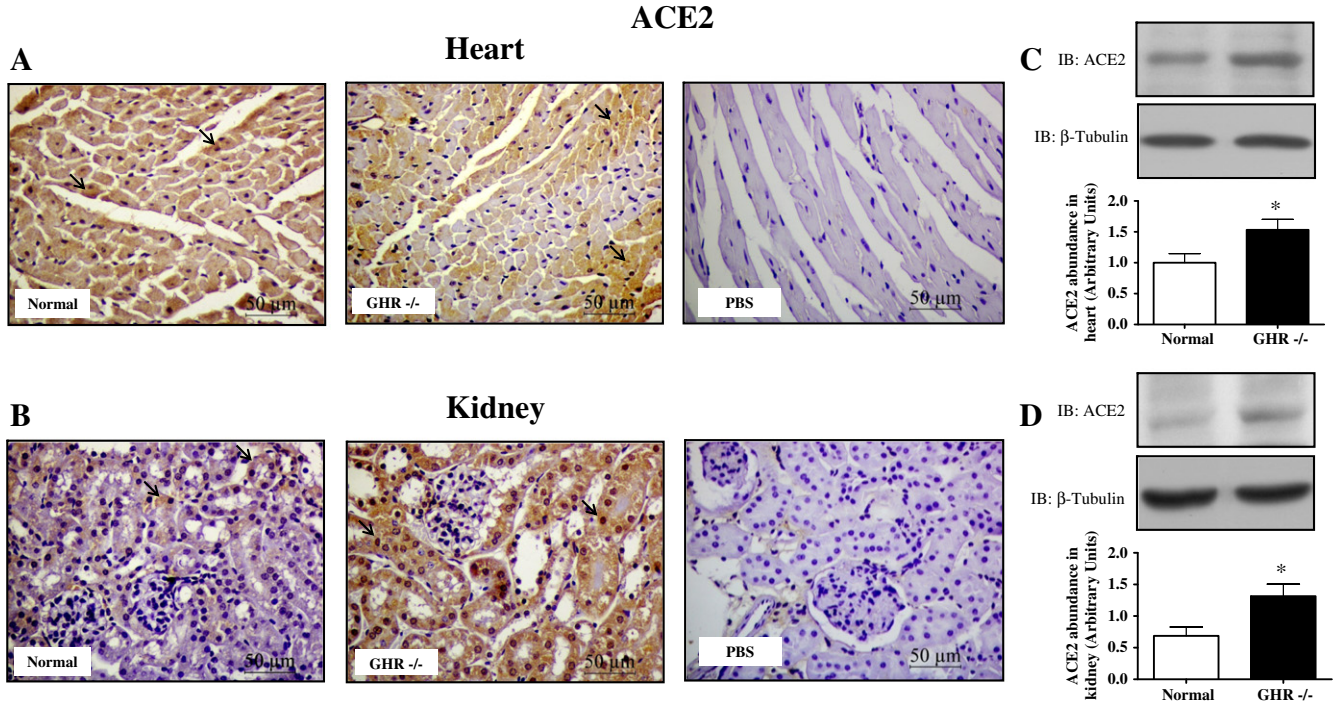

Fig. 7. Representative images showing the immunohistochemical staining of angiotensin-converting enzyme type 2 (ACE2) in heart (A) and kidney (B) from GHR −/− and normal mice. Images are shown at 400× magnification; black arrows indicate positive staining in cardiomyocytes (A) and in tubular epithelial cells (B). Tissue sections were incubated with PBS instead of primary antibody as a control of non-specific staining. Results were confirmed by submitting tissue homogenates to western blot analysis. Representative images and bar charts showing the quantification of ACE2 in heart (C) and kidney (D) are shown for each group. Data are shown as mean ± S.D. *$P<0.05$ vs. normal group (n = 12); Mann Whitney test. The calculated molecular weight for ACE2 was approximately 89 kDa in both tissues. IB: immunoblotting.

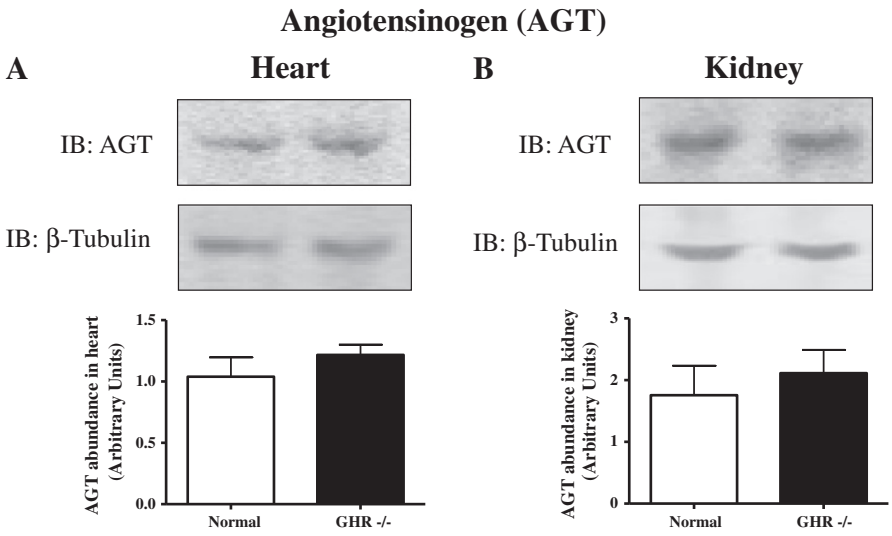

Fig. 8. Representative images and bar charts showing the quantification of angiotensinogen (AGT) in heart (A) and kidney (B) are shown for each group. Data are shown as mean ± S.D. (n = 12); Mann Whitney test. The calculated molecular weight for the specific band corresponding to AGT was approximately 50 kDa in the heart and 52 kDa in the kidney. IB: immunoblotting.

actions perpetuated by the AT1 receptors, possibly via activation of tyrosine or serine/threonine phosphatases [27]. The binding of Ang II to AT2 receptors generates vasorelaxation of conduit and resistant arteries and improves resistance arteries remodeling, promotes cardiovascular protection against ischemia–reperfusion injury and acute myocardial infarction, inhibits cardiac fibrosis, and protects the kidney from ischemic injury [27]. Growth hormone receptor knockout mice are apparently protected from several diseases, they appear not to have cardiac abnormalities, do not develop nephropathy when type 1 diabetes is induced, and are resistant to the development of certain types of cancers, which may contribute to their increased longevity [39]. On the other hand, transgenic mice overexpressing bovine GH were shown to have cardiomegaly, impaired cardiac function and an increase in mean arterial blood pressure associated with greater peripheral vascular resistance [46]. Previous studies demonstrated the ability of GH to increase the expression of AT1 receptors [19–21]. Thus, an imbalance within the RAS could represent, at least in part, a possible mechanism behind cardiovascular diseases commonly associated with alterations of the GH/IGF-1 axis. In line with these reports, results obtained in this study showing reduced AT1 receptor expression both in kidney and heart and increased expression of AT2 receptor in heart of GHR−/− mice provide novel insights into possible mechanisms of reduced aging and extended longevity in these animals. Since similar levels of local Ang II were observed both in heart and kidney of GHR−/− mice and normal littermates, Ang II could be exerting additional beneficial effects in GHR−/− mice through its ability to bind to AT2 receptors.

Currently, the RAS can be envisioned as a dual function system in which the vasoconstrictor/proliferative or vasodilator/antiproliferative actions are primarily driven by the ACE:ACE2 balance [47]. Thus, an increased ACE:ACE2 activity ratio would lead to increased Ang II generation and increased catabolism of Ang-(1–7), while an opposite ratio would decrease Ang II and increase Ang-(1–7) levels leading to its

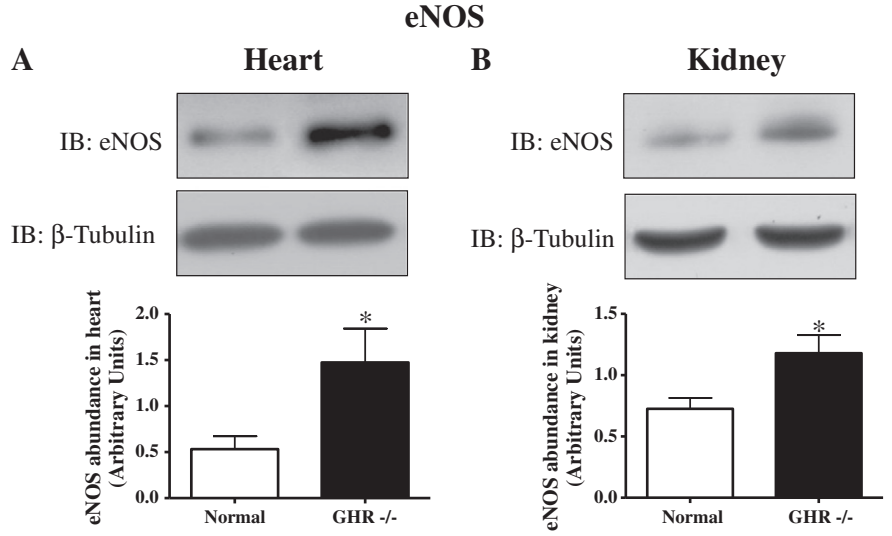

Fig. 9. Representative images and bar charts showing the quantification of endothelial nitric oxide synthase (eNOS) in heart (A) and kidney (B) are shown for each group. Data are shown as mean ± S.D. *$P<0.05$ vs. normal group (n = 12); Mann Whitney test. The calculated molecular weight for the specific band corresponding to eNOS was approximately 138 kDa in both tissues. IB: immunoblotting.

beneficial effects [31]. Growth hormone receptor deficient mice exhibited augmented local levels of Ang-(1–7), in both the kidney and the heart possibly, as a result of an increased expression of ACE2 in both kidney and heart. However, no differences were observed in ACE expression, which could explain why local immunostaining for Ang II did not differ between normal and GHR −/− animals.

While a chronic increase of Ang II can induce many deleterious effects on the heart and the kidney, Ang-(1–7), through its specific Mas receptor, appears to exert a protective role in these tissues. Angiotensin-(1–7) ameliorates insulin resistance [35] and can reduce or prevent cardiac remodeling by decreasing hypertrophy and fibrosis [31,48]. Chronic treatment with Ang-(1–7) lessens NAD(P)H-mediated oxidative stress and reduce renal dysfunction in diabetic hypertensive rats [49] and spontaneously hypertensive rats [37]. Recent reports showing that genetic deletion of the Mas receptor impairs heart function and induces a profibrotic state [50] further strengthen the putative cardioprotective role of ACE2/Ang-(1–7)/Mas receptor axis. Angiotensin-converting enzyme 2 is present in several tissues including heart and kidney, where it is thought to be the main Ang-(1–7)-forming enzyme. A close relationship has been established between Ang II and ACE2 Accordingly, loss of ACE2 leads to an increase in blood pressure, age-dependent progressive ventricular dilatation and reduced systolic performance accompanied with increased Ang II abundance in the heart [51]. It was suggested that AT1 receptor stimulation by Ang II reduces ACE2 expression in the kidney and astrocytes, in this way, the reduced expression of renal AT1 receptor observed in GHR −/− could contribute, at least in part, to the increased abundance of ACE2 local levels [52,53]. Also, cardiac ACE2 overexpression is able to protect the heart against myocardial injuries induced by Ang II infusion [54]. Oudit and co-workers reported that deletion of the ACE2 gene leads to development of Ang II-dependent glomerular injury in male mice [55]. In keeping with these studies, loss of ACE2 enhances renal Ang II levels and Ang II-induced renal oxidative stress, resulting in greater renal injury, whereas recombinant human ACE2 prevents Ang II-induced hypertension, renal oxidative stress, and tubulointerstitial fibrosis [56].

In an attempt to complete the evaluation of RAS components between GHR −/− and normal mice, AGT abundance was measured in the heart and the kidney by the Western blot approach. Results regarding this protein revealed no differences in AGT abundance in any analyzed tissue showing that initial steps of the proteolytic cascade within the RAS seems to be conserved between GHR −/− and wild type mice.

Furthermore, we determined that GHR −/− exhibit increased expression of eNOS in the heart and the kidney. In line with current results, it was demonstrated that the stimulation of the cardiac and renal ACE2/Ang-(1–7)/Mas receptor axis acts as a potential regulator of endothelial function since its ability to cause endothelial-dependent vasodilation mediated, in part, by activating eNOS and consequently stimulating NO production [57].

In conclusion, the increase in both AT2 receptor and eNOS expression together with the exacerbation of the ACE2/Ang-(1–7)/Mas receptor axis and the reduction of AT1 receptor abundance observed both in the heart and the kidney of GHR −/− mice could play a protective role in metabolic, cardiac and renal function; possibly contributing to delayed aging and extended longevity of these GH resistant animals.

Disclosure statement

The authors have nothing to declare.

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