Downregulation of the ACE2/Ang-(1–7)/Mas axis in transgenic mice overexpressing GH

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Abstract

The renin–angiotensin system (RAS) plays a crucial role in the regulation of physiological homeostasis and diseases such as hypertension, coronary artery disease, and chronic renal failure. In this cascade, the angiotensin-converting enzyme (ACE)/angiotensin II (Ang II)/AT1 receptor axis induces pathological effects, such as vasoconstriction, cell proliferation, and fibrosis, while the ACE2/Ang-(1–7)/Mas receptor axis is protective for end-organ damage. The altered function of the RAS could be a contributing factor to the cardiac and renal alterations induced by GH excess. To further explore this issue, we evaluated the consequences of chronic GH exposure on the *in vivo* levels of Ang II, Ang-(1–7), ACE, ACE2, and Mas receptor in the heart and the kidney of GH-transgenic mice (bovine GH (bGH) mice). At the age of 7–8 months, female bGH mice displayed increased systolic blood pressure (SBP), a high degree of both cardiac and renal fibrosis, as well as increased levels of markers of tubular and glomerular damage. Angiotensinogen abundance was increased in the liver and the heart of bGH mice, along with a concomitant increase in cardiac Ang II levels. Importantly, the levels of ACE2, Ang-(1–7), and Mas receptor were markedly decreased in both tissues. In addition, Ang-(1–7) administration reduced SBP to control values in GH-transgenic mice, indicating that the ACE2/Ang-(1–7)/Mas axis is involved in GH-mediated hypertension. The data indicate that the altered expression profile of the ACE2/Ang-(1–7) /Mas axis in the heart and the kidney of bGH mice could contribute to the increased incidence of hypertension, cardiovascular, and renal alterations observed in these animals.

Key Words
- renin–angiotensin system
- angiotensin
- growth hormone
- hypertension
- Mas receptor
- ACE2

Journal of Endocrinology (2014) **221**, 223–235

Journal of Endocrinology

Introduction

The growth hormone (GH)/insulin-like growth factor 1 (IGF1) axis has important physiological functions in the maintenance of 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; and control of heart, kidney, and brain functions (Waters *et al*. 2006, Vijayakumar *et al*. 2010). Derangements in the

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DOI: 10.1530/JOE-13-0497

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GH/IGF1 axis are associated with chronic renal failure (Roelfsema & Clark 2001). Accordingly, the expression of GH in transgenic mice is associated with the development of glomerular lesions characterized by a disproportionate increment in glomerular volume and severe glomerulosclerosis (Yang *et al*. 1993). Concerning the cardiovascular system, the GH/IGF1 system has also been implicated in the control of blood pressure and stimulation of components of the renin–angiotensin system (RAS; Giani *et al*. 2012). In addition, we have shown that transgenic mice overexpressing bovine GH (bGH) under the control of the phosphoenolpyruvate carboxykinase (PEPCK) promoter (bGH mice) display cardiomegaly and perivascular and interstitial fibrosis in the heart (Miquet *et al*. 2011).

In humans, chronic excess of GH occurs in acromegaly, a disease characterized by autonomous GH secretion from pituitary adenomas and subsequently elevated levels of IGF1 (Melmed 2009). Hypertension is frequent in acromegaly and patients suffer from an increased cardiovascular morbidity and mortality (Ho & Weissberger 1990, Lombardi *et al*. 2006). In contrast, although increased renal size and weight and increased glomerular diameter are known features of acromegaly in humans, renal failure is not a long-term hazard (Ritz *et al*. 1991). Elevation in the levels of circulating GH has been found to modulate the activity of the RAS. Early reports described unaltered function of the RAS in normotensive acromegalic patients and suppressed RAS and hyperaldosteronism in hypertensive acromegalic patients (Strauch *et al*. 1972). In a more recent study, Bielohuby *et al*. (2009) demonstrated that GH excess is associated with increased aldosterone in acromegalic patients. Similar results have been found in GH-overexpressing mice (Bielohuby *et al*. 2009). In GH-overexpressing mice, the increased serum GH levels have a different etiology from those seen in acromegalic patients. Besides, they are exposed to high GH levels throughout the animals lifetime, whereas in acromegaly excessive GH production arises much later in life. However, GH-transgenic mice appear to be a helpful model for studying the relationship between GH excess and the RAS, and understanding the underlying mechanisms.

As GH is used in therapy, the effects of GH administration have been a subject of study in this field. Some studies have concluded that GH administration does not affect the classic components of the RAS (Hoffman *et al*. 1996, Ekman *et al*. 2002). However, GH administration has been reported to stimulate the RAS and aldosterone secretion in healthy subjects (Ho & Weissberger 1990, Coulter *et al*. 1996, Hansen *et al*. 2001), in children with idiopathic short stature (Hanukoglu *et al*. 2001), and in hypopituitary adults (Weaver *et al*. 1994).

The RAS is classically conceived as a coordinated hormonal cascade in the control of cardiovascular, renal, and adrenal functions, mainly through the actions of angiotensin II (Ang II; Santos *et al*. 2013). This octapeptide hormone is not only generated in the circulation by renin and angiotensin-converting enzyme (ACE) but also is produced locally in numerous organs including kidney, blood vessels, heart, adrenal gland, eye, testis, and brain forming the so-called local RAS (Kumar *et al*. 2008). The description of the local RAS highlighted several non-hemodynamic effects of Ang II and led to the identification of new roles for other components of the RAS as members of paracrine or/and autocrine/intracrine systems implicated in physiological and pathophysiological processes, including inflammation, cell proliferation, and fibrosis (Hunyady & Catt 2006). In tissues such as kidney, heart, and vasculature, Ang II, through the AT1 receptor, promotes vasoconstriction, reactive oxygen species production, and extracellular matrix remodeling, and can activate multiple intracellular signaling pathways leading to inflammatory response and tissue injury (Mehta & Griendling 2007). In many cases, the AT2 receptor has been shown to counterbalance the actions exerted through the AT1 receptor (Hunyady & Catt 2006). Both systemic and local RAS also play a crucial role in other physiological processes including cell proliferation as well as pathophysiological conditions such as inflammation and tissue fibrosis (Mehta & Griendling 2007).

Advances in the field led to the recognition of other active components of the RAS metabolism, such as Ang-(1–7) (Santos *et al*. 2013), ACE2, a homolog of classic ACE that forms Ang-(1–7) directly from Ang II and indirectly from Ang I, and the Ang-(1–7)-specific G-protein-coupled receptor Mas (Santos *et al*. 2013). The ACE2/Ang-(1–7)/Mas receptor axis opposes the vascular and proliferative effects of Ang II and exerts complex renal actions in chronic renal diseases and hypertension (Ferrario & Varagic 2010). Alterations of the GH/IGF1 axis are commonly associated with cardiovascular diseases, but the basis for these alterations is not clear. In the current study, we used bGH mice as a model to analyze, by both immunohistochemistry and western blotting analysis, the effect of chronic exposure to high GH levels throughout a lifetime on the levels of ACE2/Ang-(1–7)/Mas receptor axis in the heart and the kidney. Also the role of the ACE2/Ang-(1–7)/Mas receptor axis in GH-induced hypertension was explored by treating bGH mice chronically with Ang-(1–7). In addition, both the cardiac and renal levels

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of angiotensinogen (AGT), Ang II and ACE were determined in these animals.

Materials and methods

Materials and reagents

The reagents and apparatus for SDS–PAGE and immunoblotting were obtained from Bio-Rad. The anti-ACE (sc-12187), anti-ACE2 (sc-17720), anti-podocin (sc-21009), anti-WT-1 (sc-192), anti-rabbit IgG conjugated with HRP, anti-goat IgG conjugated with HRP, and anti-mouse IgG-HRP antibodies were from Santa Cruz Biotechnology, Inc. The anti-Mas antibody was from Alomone Labs, Ltd, Jerusalem, Israel. The following primary antibodies: rabbit polyclonal antibody anti-Ang II (H002-12) and anti-Ang-(1–7) (H002-24) were from Phoenix Pharmaceutical, Inc. (Burlingame, CA, USA). The anti-AGT (ab108334) was from Abcam (Cambridge, UK). Protein loading in gels was evaluated with an anti-β tubulin antibody (ab6046, Abcam).

Animals and experimental design

Transgenic mice containing the bGH gene fused to the control sequences of rat PEPCK gene were used (McGrane *et al*. 1990). The hemizygous transgenic mice were derived from a founder male generously provided by Dr Thomas Wagner and were produced by mating transgenic males with normal C57BL/6×C3H F1 hybrid females purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mating produced approximately equal numbers of transgenic and normal progeny. Normal siblings of transgenic mice were used as controls. Transgenic animals had markedly accelerated post-weaning growth, leading to a significant increase in body weight (BW). The mice were housed three to five per cage in a room with controlled light (12 h light/day) and temperature (22±2 °C). The animals had free access to food (Lab Diet Formula 5001 containing a minimum of 23% protein, 4.5% fat, and a maximum of 6% crude fiber, from Purina Mills, Inc., St Louis, MO, USA), and tap water. Female mice at the age of 7–8 months old were used. The total number of animals used was 20 (ten transgenic and ten normal littermates). The experimental procedure was in compliance with federal and local laws and approved by the Southern Illinois University Animal Care and Use Committee.

To evaluate the effects of Ang-(1–7) on systolic blood pressure (SBP) of bGH mice, animals were divided into four groups: control treated with saline (control-saline) group ($n=8$), control treated with Ang-(1–7) (control-Ang-(1–7)) group ($n=8$), bGH treated with saline (bGH-saline) group ($n=8$), and bGH treated with Ang-(1–7) (bGH-Ang-(1–7)) group ($n=8$). For 2 weeks, all animals were treated according to the following schedule: C-saline (0.1 ml) and bGH-saline (0.2 ml)=receiving a daily i.p. volume of saline solution, C-Ang-(1–7) and bGH-Ang-(1–7)=receiving a daily i.p. dose of Ang-(1–7) (0.6 mg/kg; Bachem Americas, Inc., Torrance, CA, USA). Both the dose and administration interval of Ang-(1–7) were selected on the basis of our previous study carried out using stroke-prone spontaneously hypertensive rats (Giani *et al*. 2011).

Tissue collection and histological study

Mice were starved overnight and anesthetized with ketamine/xylazine mixture. After anesthesia was induced, the heart and kidneys were perfused with physiological saline solution through the abdominal aorta until they were free of blood. Thereafter, 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.

Four tissue sections were deparaffinized, stained with Masson's trichrome staining, and examined for myocardial and renal fibrosis, using an Olympus BX-51 microscope equipped with a digital camera, Olympus QColor 3 (Olympus, Tokyo, Japan). In ten randomly selected high-power fields, the degree of collagen deposition was estimated by the calculation of the percentage of blue-stained area corresponding to collagen deposition in relation to the total heart area using Image-Pro Plus 4.5 software (Media Cybernetics, Inc., Silver Spring, MD, USA). Sections (3 μm) were cut, stained with Sirius red, and illuminated with a polarized light.

Blood pressure determination

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

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Table 1 Characteristics of the experimental animals. Data are presented as mean ± S.E.M. ($n=10$)

	Control	bGH
GH (ng/ml)	6 ± 2	1630 ± 210*
SBP (mmHg)	102 ± 12	121 ± 12*
Glycemia (mg/dl)	126 ± 6	135 ± 5
Insulinemia (mIU/ml)	16 ± 4	95 ± 3*
Body weight (g)	33 ± 2	47 ± 2*
Heart weight (g)	0.138 ± 0.005	0.240 ± 0.009*
Heart weight/body weight (%)	0.34 ± 0.01	0.51 ± 0.01*
Kidney weight (g)	0.168 ± 0.006	0.337 ± 0.020*
Kidney weight/body weight (%)	0.49 ± 0.02	0.69 ± 0.02*

*$P<0.01$ vs control mice.

GH, glucose, and insulin measurements

All measurements were determined 12 h after food removal. GH levels were measured by RIA as described previously (Sotelo *et al.* 2008). Glucose levels were measured in blood using the blood One Touch Ultra 2 (Lifescan, Inc., Milpitas, CA, USA). Serum insulin levels were assessed using a rat insulin ELISA kit (Ultra Sensitive Rat Insulin ELISA Kit; Crystal Chem, Inc., Downers Grove, IL, USA).

Tissue solubilization

Tissue sections of heart and kidney were homogenized in solubilization buffer containing 1% w/v Triton X-100 together with phosphatase and protease inhibitors as described previously (Giani *et al.* 2012). Heart extracts were centrifuged at 100 000 ***g*** for 1 h at 4 °C to eliminate insoluble material, and protein concentration in the supernatants was measured using the bicinchoninic acid method (BCA Protein Assay Reagent, Thermo Scientific Pierce, Rockford, IL, USA). An aliquot of solubilized heart or kidney was diluted in Laemmli buffer, boiled for 5 min, and stored at −20 °C until they were used for immunoblotting.

Western blotting analysis

Samples were subjected to electrophoresis in SDS–polyacrylamide gels using BioRad Mini Protean apparatus (Bio-Rad Laboratories). Equal amount of total protein was loaded in each lane. Electrotransference of proteins from gel to polyvinylidene difluoride membranes was carried out for 1 h at 100 mA per transferred membrane (constant current) using the V20-SDB semi-dry blotting apparatus (Scie-Plas, Cambridge, UK) in 0.025 mol/l Tris, 0.192 mol/l glycine, 20% (v/v) methanol, and 0.03% (w/v) SDS (pH 8.3). To reduce non-specific antibody binding, membranes were incubated for 1 h at room temperature in T-TBS buffer (0.01 mol/l Tris–HCl, 0.15 mol/l NaCl, and 0.02% w/v Tween 20, pH 7.6) containing 3% w/v BSA. The membranes were then incubated overnight at 4 °C with the primary antibody. After washing with T-TBS, the membranes were incubated with a secondary antibody conjugated with HRP for 1 h at room temperature and washed in T-TBS. Immunoreactive proteins were revealed by enhanced chemiluminescence (ECL-Plus, Amersham Biosciences). Band intensities were quantified using Gel-Pro Analyzer 4.1 software (Media Cybernetics). To determine the protein abundance of Mas receptor, ACE2, ACE, AGT, podocin, neutrophil gelatinase-associated lipocalin (NGAL), and Wilms' tumor 1 (WT-1), equal amounts of solubilized proteins (40 μg) were denatured by being boiled in reducing sample buffer, resolved by SDS–PAGE, and subjected to immunoblotting with the following antibodies diluted in Tris-buffered saline 0.1% Tween-20 containing 1% BSA, Mas receptor (1:4000), ACE (1:1000), ACE2 (1:1000), AGT (1:1000), podocin (1:2000), NGAL (1:2000), WT-1 (1:1000), and tubulin (1:5000). Finally, membrane blots were washed and incubated for 1 h at room temperature with goat anti-rabbit IgG-HRP secondary antibody (1:20 000 dilution) or donkey anti-goat IgG-HRP secondary antibody (1:10 000 dilution; Santa Cruz Biotechnology).

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% BSA) 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.), polyclonal anti-Mas receptor (1:100 dilution), anti-ACE2 (1:100), anti-ACE (1:100), and anti-WT-1 (1:100).

Table 2 Effect of Ang-(1–7) on SBP. Data are presented as mean ± S.E.M. ($n=8$). Comparisons among groups were carried out with one-way ANOVA followed by Bonferroni's test

	Control-saline	Control-Ang-(1–7)	bGH-saline	bGH-Ang-(1–7)
SBP (mmHg)	113 ± 5	110 ± 3	142 ± 5*	119 ± 4

*$P<0.01$ vs all groups. A value of $P<0.05$ was considered significant. bGH, transgenic mice expressing bovine GH; Ang, angiotensin; SBP, systolic blood pressure.

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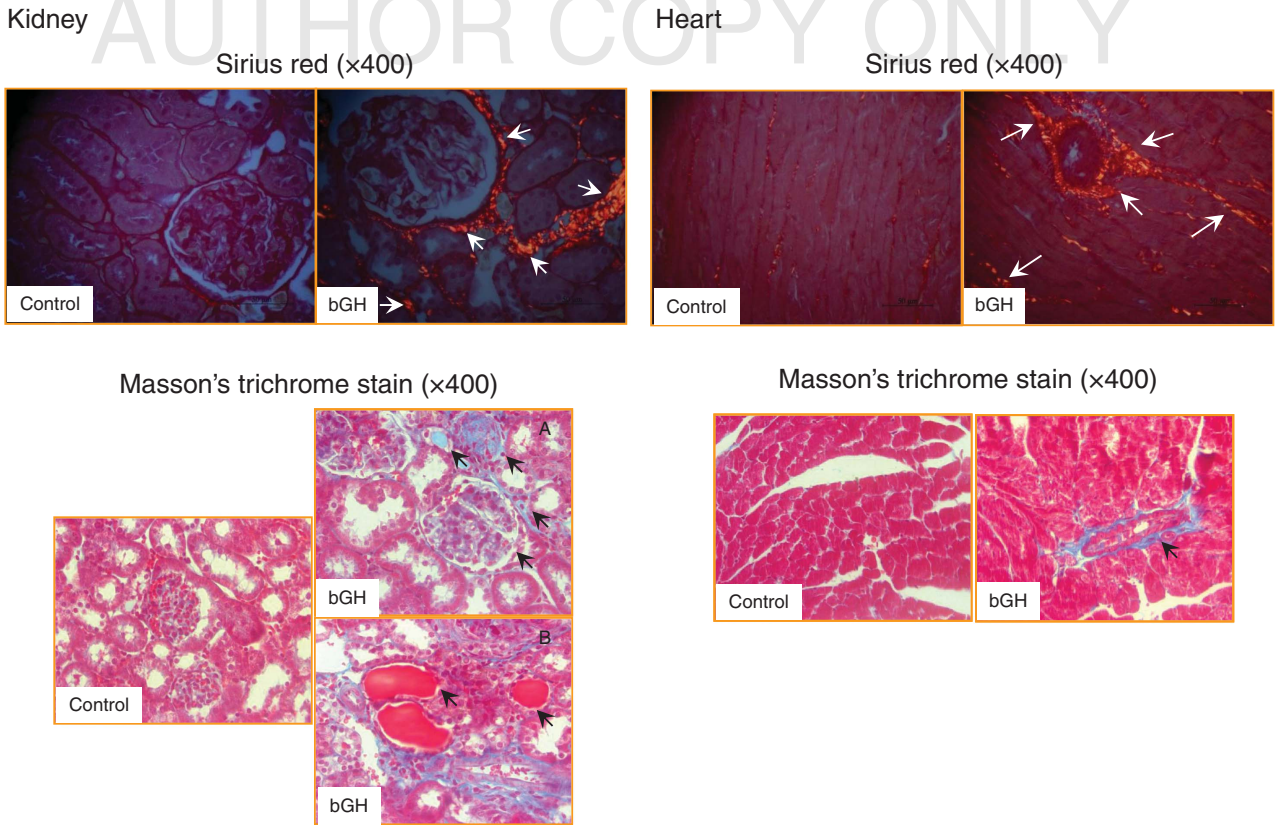

Figure 1
Renal and myocardial fibroses evaluation. Sirius red staining illuminated with polarized light and Masson's trichrome were used to evidence collagen accumulation and extracellular expansion (fibrosis), respectively, in the kidney and the heart of control and transgenic (bGH) mice (400×), as indicated by arrows. Kidney sections show an increase in extracellular matrix protein deposition, glomerular size (A), and tubular protein casts (B) in bGH mice (indicated by arrows) (400×); (n=8–10 for all determinations). Scale bar: 50 μm. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-13-0497.

All antibodies were diluted with blocking solution. Immunostaining was carried out with an avidin–biotin–peroxidase complex kit and counterstaining with hematoxylin (Giani *et al*. 2012). The 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) (Giani *et al*. 2012). 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 ten consecutive microscopic fields at 400× magnification. Data were averaged and results were expressed as a percentage per area. All measurements were carried out using an image analyzer Image-Pro Plus version 4.5 for windows (Media Cybernetics).

Statistical analysis

Experiments were carried out by analyzing all groups of animals in parallel, with n representing the number of

Table 3 Quantification of immunohistochemical staining of RAS components in the heart and the kidney of bGH and control mice

	Kidney		Heart	
	Control	bGH	Control	bGH
Ang II	37.8±0.9	36.7±0.7	5.7±0.4	8.5±1.4 *
Ang-(1–7)	19.9±0.9	16.3±0.8*	5.6±0.5	2.8±0.7*
Mas receptor	18.9±0.7	8.1±0.5*	26.2±1.5	11.1±0.5 *
ACE2	21±1	12.6±0.9*	13.9±0.5	10.2±0.5*
Collagen deposition	2.9±0.3	12.4±0.6*	1.7±0.2	7.2±0.5*
Glomerular size (μm)	54±4	91±3*	–	–

ACE2, angiotensin-converting enzyme type 2; Ang, angiotensin; bGH, transgenic mice expressing bovine GH; RAS, renin–angiotensin system. Data are presented as percentage of positive staining per area. Values are expressed as mean±S.E.M. *P<0.01 vs control mice; n=8–10.

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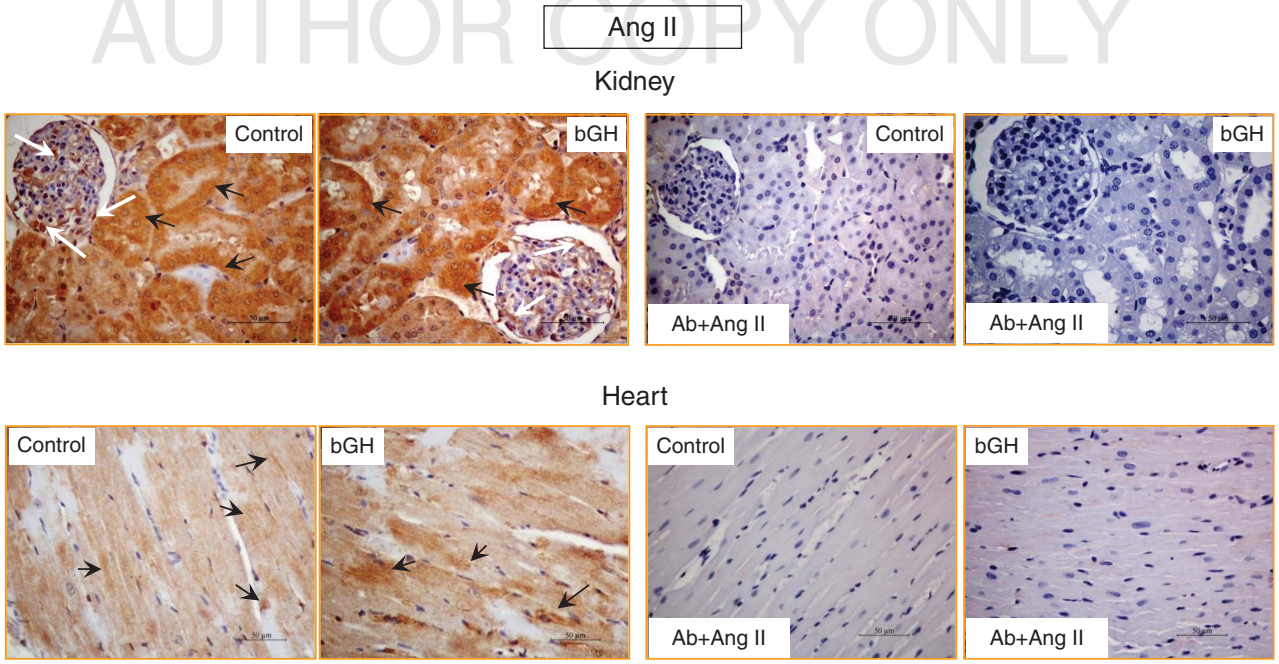

Figure 2
Representative images showing the immunohistochemical staining of Ang II in the kidney (upper panels) and the heart (lower panels) of control and bGH mice (n=8–10 for both groups). Images are shown at 400× magnification; black arrows indicate specific staining in glomerular sections and tubular sections as well as in cardiomyocytes. White arrows indicate the localization of Ang II in glomerular epithelial cells. Right panels show renal and cardiac sections from control and bGH mice incubated with anti-Ang II antibody (Ab) previously blocked by preincubation with Ang II (control for antibody specificity). A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-13-0497.

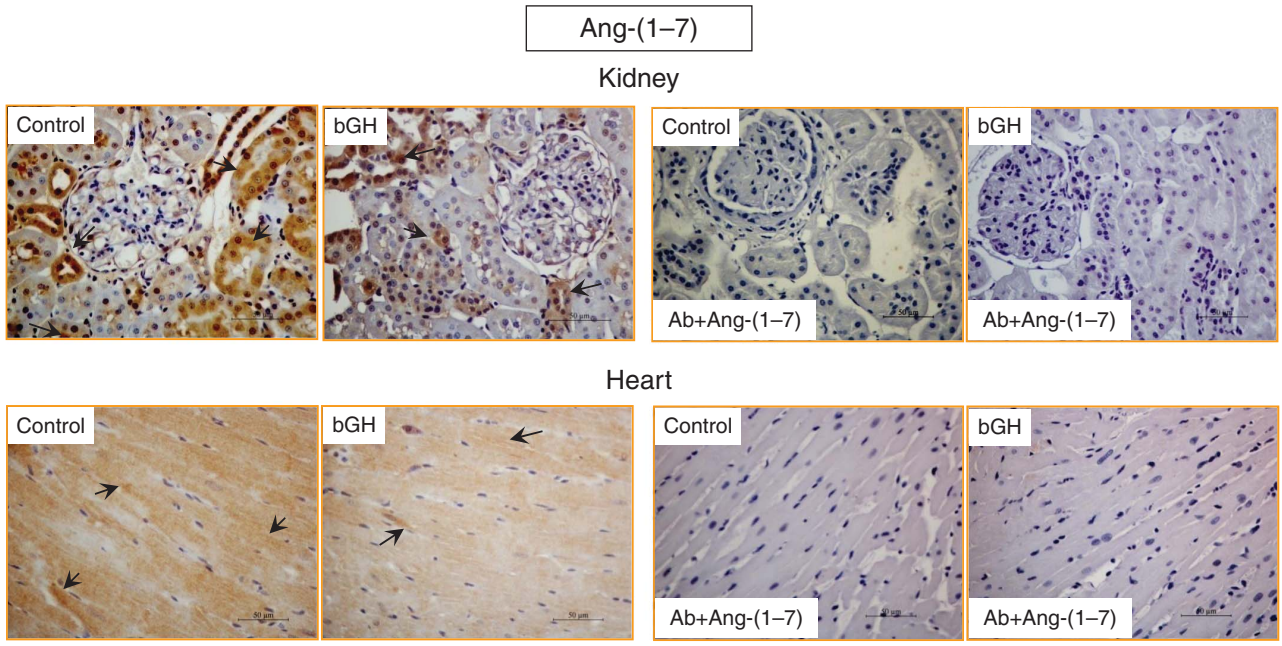

Figure 3
Representative images showing the immunohistochemical staining of Ang-(1–7) in the kidney (upper panels) and the heart (lower panels) of control and bGH mice (n=8–10 for both groups). Images are shown at 400× magnification; black arrows indicate specific staining in glomerular and tubular sections as well as in cardiomyocytes. Right panels show renal and cardiac sections incubated with anti-Ang-(1–7) antibody (Ab) previously blocked by preincubation with Ang-(1–7) (control for antibody specificity). A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-13-0497.

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different individuals used in each group. Results are presented as mean ± S.E.M. First we carried out a normality test using the WinSTAT version 2012.1. If the *P* value was high, statistical analyses were performed by Student's *t*-test. If the *P* value was low, demonstrating that the data did not follow a Gaussian distribution, statistical analyses were performed by Mann–Whitney *U* test using the InStat statistical program by GraphPad Software, Inc. (San Diego, CA, USA). Data were considered significantly different when $P<0.05$.

Results

The salient features of bGH mice are their increased BW and very high circulating levels of GH. At the age of 7–8 months, these animals displayed approximately a 40% increase in BW compared with their normal counterparts and an ~270-fold increase in circulating GH levels (Table 1). This was concomitant with a state of insulin resistance, as shown by increased insulin levels (sixfold above control values) together with normal glucose levels (Table 1). Also, these animals displayed a marked increase in absolute heart and kidney weight when compared with control animals. This change was also present after normalizing to BW (Table 1). Notably, SBP was found to be significantly increased in bGH mice when compared with values for control mice (Table 1). Interestingly, chronic administration of Ang-(1–7) induced a significant reduction in SBP in bGH mice (Table 2), indicating an important role of this heptapeptide hormone in the control of GH-induced hypertension. The same treatment did not affect SBP in control animals (Table 2).

As demonstrated by both Sirius red and Masson's trichrome staining, large areas of interstitial fibrosis were detected in the kidney and heart of bGH mice, which displayed a significantly greater renal and cardiac collagen

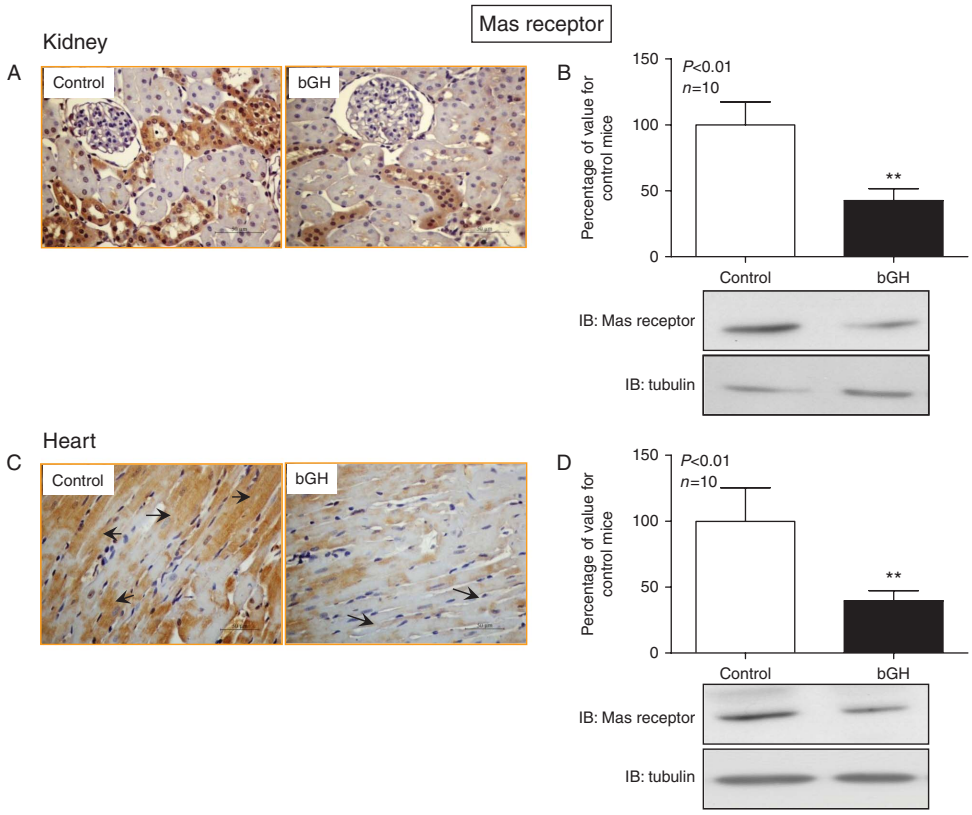

Figure 4
Representative images showing the immunohistochemical staining of Mas receptor (Mas) in kidney and heart from control and bGH mice. Images are shown at 400× magnification; tubular epithelial cells (A) and in cardiomyocytes (C). Black arrows indicate positive staining. Results were confirmed by submitting tissue homogenates to immunoblotting (IB). Representative images and bar charts showing the quantification of Mas receptor in kidney (B) and heart (D) are shown for each group. The calculated molecular weights for the immunoreactive bands are ~37 kDa for the Mas receptor and 50 kDa for tubulin in both tissues. Data are shown as mean ± S.E.M. $^{**}P<0.01$ vs control group ($n=8$–10). A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-13-0497.

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deposition than that observed in the control group (Fig. 1 and Table 3). Increased renal fibrosis in bGH mice was associated with a marked (1.7-fold; $P<0.01$) increase in glomerular size in these animals (Fig. 1 and Table 3). In addition, transgenic mice showed dilated tubules with noticeable protein accumulation within tubular lumen, thus indicating a variable degree of proteinuria.

The levels of the two main active peptides of the RAS, Ang II and Ang-(1–7), were measured in the kidney and the heart of control and bGH mice by immunohistochemistry through the use of specific antibodies. As shown in Fig. 2, bGH mice displayed increased Ang II levels in the heart (Fig. 2, lower panels), although levels of this hormone were not altered in the kidney (Fig. 2, upper panels). The analysis of the local levels of Ang-(1–7) revealed a significant decreased abundance of this heptapeptidic hormone in both tissues analyzed of bGH mice (Fig. 3). Quantification of Ang II and Ang-(1–7) tissular abundance was expressed as percentage of positive staining per area of tissue (Table 3). 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 2 and 3, right panels).

As determined by both immunohistochemistry and western blot analysis, bGH mice displayed a marked and significant reduction in the content of both renal (Fig. 4A and B) and cardiac (Fig. 4C and D) Mas receptors. As shown in Table 3, immunohistochemical quantification of Mas receptor local abundance was expressed as percentage of positive staining per area of tissue. Renal localization of the Mas receptor was mainly tubular (Fig. 4 and Table 2). All immunohistochemical results were confirmed by western blot analysis using kidney (Fig. 4B) and heart (Fig. 4D) homogenates. Protein loading in gels was evaluated with an anti-β tubulin antibody.

Classical ACE and the recently described ACE2 were determined as another approach to evaluate the main components of the RAS. Expression of ACE2 in the kidney

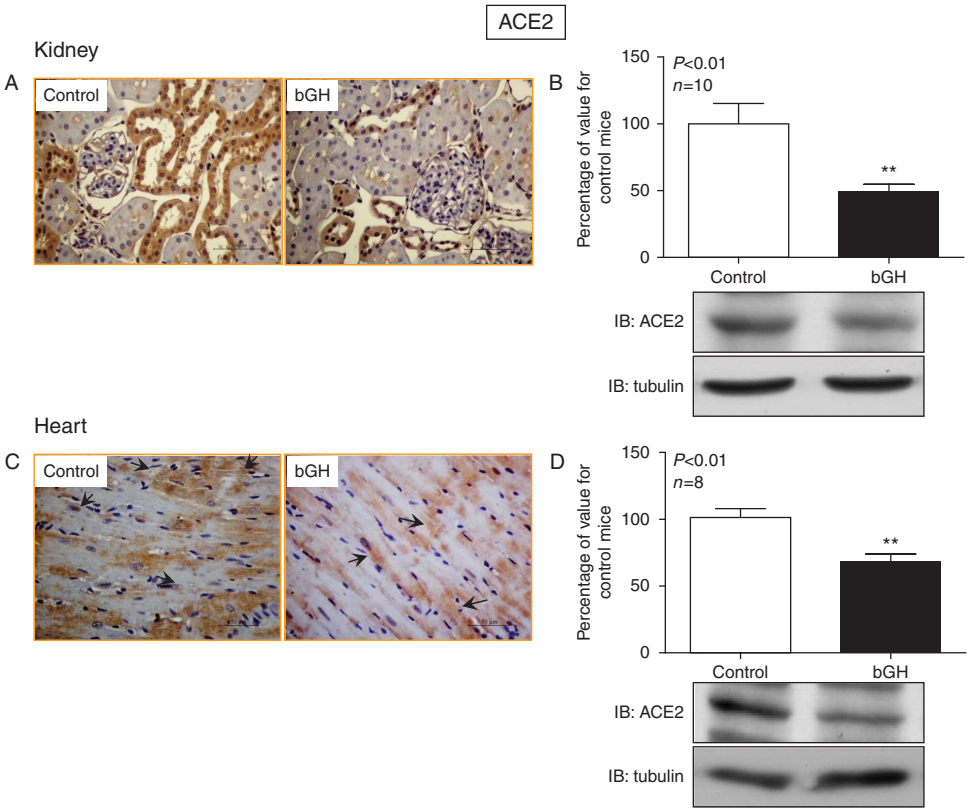

Figure 5
Representative images showing the immunohistochemical staining of angiotensin-converting enzyme type 2 (ACE2) in kidney and heart of control and bGH mice ($n=10$ for both groups). Images are shown at 400× magnification; tubular epithelial cells (A) and cardiomyocytes (C). Black arrows indicate positive staining. Results were confirmed by submitting tissue homogenates to immunoblotting (IB). Representative images and bar charts showing the quantification of ACE2 in kidney (B) and heart (D) are shown for each group. The calculated molecular weights for the immunoreactive bands are ~89 kDa for ACE2 and 50 kDa for tubulin in both tissues. Data are shown as mean±S.E.M. $^{**}P<0.01$ vs control group ($n=8$–10). A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-13-0497.

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(mainly tubular) as determined by immunohistochemistry was greatly decreased in bGH mice (Fig. 5A). This was confirmed by western blot analysis (Fig. 5B). Cardiac immunostaining of ACE2 (Fig. 5C) was also significantly reduced in bGH mice. This correlated well with results obtained by western blotting (Fig. 5D). As ACE2 is mainly responsible for synthesizing Ang-(1–7), these observations could explain the decreased expression of this heptapeptidic hormone in both tissues analyzed. Immunohistochemical quantification of local abundance of ACE2 was expressed as percentage of positive staining per area of tissue (Table 3). Compared with WT animals, the levels of ACE, as detected by both immunohistochemistry and western blotting, capable of synthesizing Ang II from Ang I were similar both in the kidney (glomerular and tubular sections) and in the heart for bGH mice (Fig. 6).

The analysis of renal and cardiac RAS components was accompanied by determination of the AGT abundance by western blotting analysis (Fig. 7). As shown in Fig. 7C, AGT abundance was significantly increased in the heart of bGH mice, showing that the initial steps of the proteolytic cascade within the RAS are overactivated under the conditions of GH excess. This increase in AGT abundance was also displayed in liver (the main source of AGT) of bGH mice (Fig. 7A). There was a slight increase in AGT protein content in the kidneys from these animals, although this change was not statistically significant (Fig. 7B).

To further characterize bGH mice in terms of renal damage, the local expression of NGAL, a marker of renal tubular damage (Mishra *et al.* 2003), was examined by immunoblotting analysis. As displayed in Fig. 8A, bGH mice presented an increased renal expression of NGAL compared with the control group. Besides increased renal fibrosis, bGH mice presented a profound reduction in the levels of the glomerular podocyte structural protein podocin (Fig. 8B), as well as marked decrease levels of WT-1 (Guo *et al.* 2002), a specific marker of podocytes (Fig. 8C and D). These results are indicative of podocyte lesions.

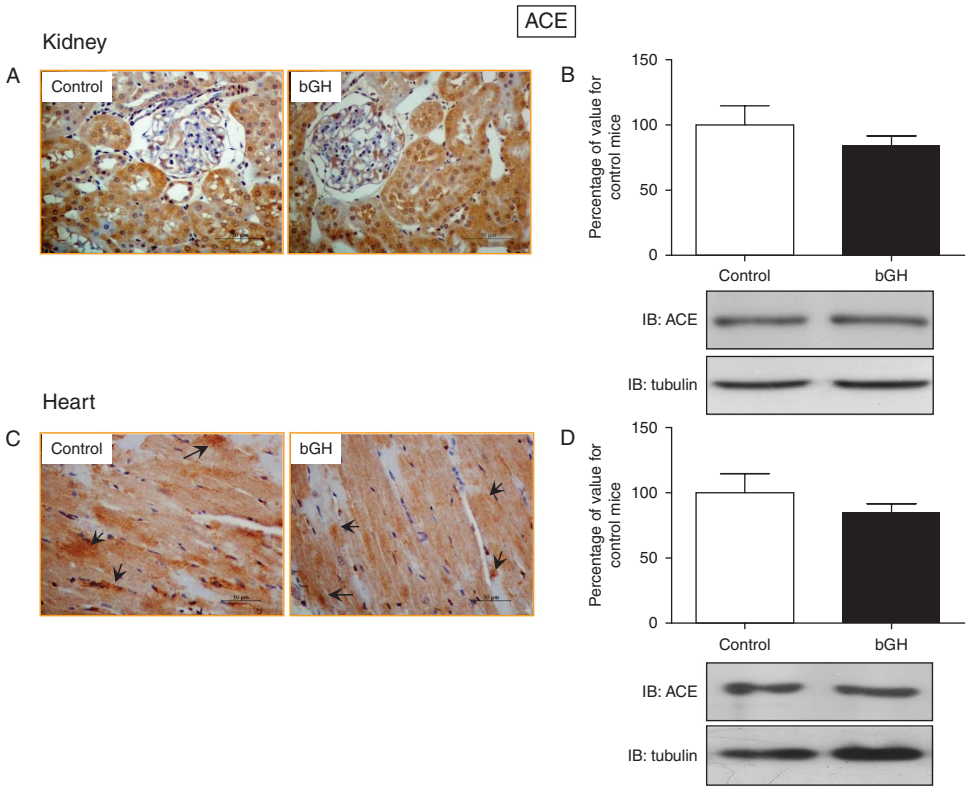

Figure 6
Representative images showing the immunohistochemical staining of angiotensin-converting enzyme type 1 (ACE) in the kidney and heart of control and bGH mice. Images are shown at 400× magnification; tubular epithelial cells (A) and cardiomyocytes (C). Black arrows indicate positive staining. Results were confirmed by submitting tissue homogenates to immunoblotting (IB). Representative images and bar charts showing the quantification of ACE in kidney (B) and heart (D) are shown for each group. The calculated molecular weights for the immunoreactive bands are ~188 kDa for ACE and 50 kDa for tubulin in both tissues. Data are shown as mean±S.E.M. A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-13-0497.

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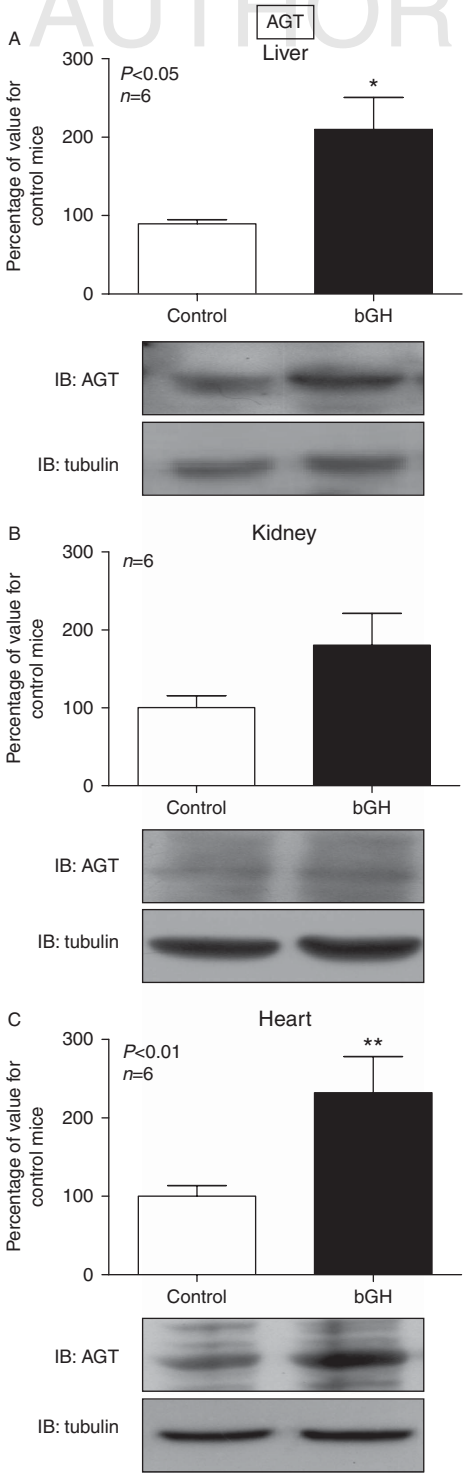

Figure 7
Representative images and bar charts showing the quantification of angiotensinogen (AGT) in liver (A), kidney (B), and heart (C) of control and bGH mice. Data are mean ± S.D. ($n=6$; *$P<0.05$; **$P<0.01$). The calculated molecular weights for the immunoreactive bands are ~55 kDa for AGT and 50 kDa for tubulin in all tissues. Data are mean ± S.E.M. ($n=6$; *$P<0.05$; **$P<0.01$). IB, immunoblotting.

Discussion

The present study was designed to examine the effects of chronic exposure to increased GH levels on the newly described axis of the RAS composed of ACE2, Ang-(1–7), and its specific receptor Mas. To that end, we used transgenic mice that overexpress bGH that are exposed since birth to high and continuous circulating levels of GH. One of the major findings of this study is that bGH mice exhibit downregulation of the ACE2/Ang-(1–7)/Mas receptor axis, both in the heart and the kidney.

In agreement with previously published studies (Yang *et al.* 1993, Kopchick *et al.* 1999, Miquet *et al.* 2011), our current results showed that at the age of 7–8 months, bGH mice displayed an increase in both absolute and relative kidney and heart weight, increased SBP, together with cardiac and renal fibrosis, indicative of altered function of these organs.

Ang-(1–7), generated from either Ang I or Ang II, opposes the vasoconstrictor, proliferative, and profibrotic actions of Ang II (Santos *et al.* 2013). This heptapeptidic hormone is generated primarily from Ang II through the hydrolytic activity of ACE2, acting as a monocarboxypeptidase, although tissue endopeptidases (neutral endopeptidase 24.11, prolyl-endopeptidase 24.26, and thimet oligopeptidase 24.15) also participate in its generation (Santos *et al.* 2013). ACE2 is present in cardiac myocytes and in the kidney it is found primarily in the luminal surface of the tubular epithelium (Brosnihan *et al.* 2003), this contrasts with the more generalized distribution of ACE in this tissue (Warner *et al.* 2005). An increased ACE:ACE2 activity ratio would lead to decreased generation of Ang-(1–7) leading to attenuation of the beneficial effects induced by this hormone (Ferrario & Varagic 2010). Accordingly, bGH mice displayed diminished local levels of Ang-(1–7), both in the kidney and in the heart, possibly as a result of the decreased expression of ACE2 found in these tissues. The pattern of expression of Ang II in the heart and the kidney of bGH mice coincided with those of AGT in these tissues (both proteins were increased in the heart but normal in the kidney). This could explain why renal Ang II immunostaining levels did not differ between normal and bGH mice. Because the circulating levels of AGT are close to the Michaelis–Menten constant for renin, changes in AGT levels have been shown to control directly the activity of the RAS (Ferrario & Varagic 2010). In agreement with this, plasma renin activity was found to remain unaltered in bGH mice in a previous study (Bielohuby *et al.* 2009).

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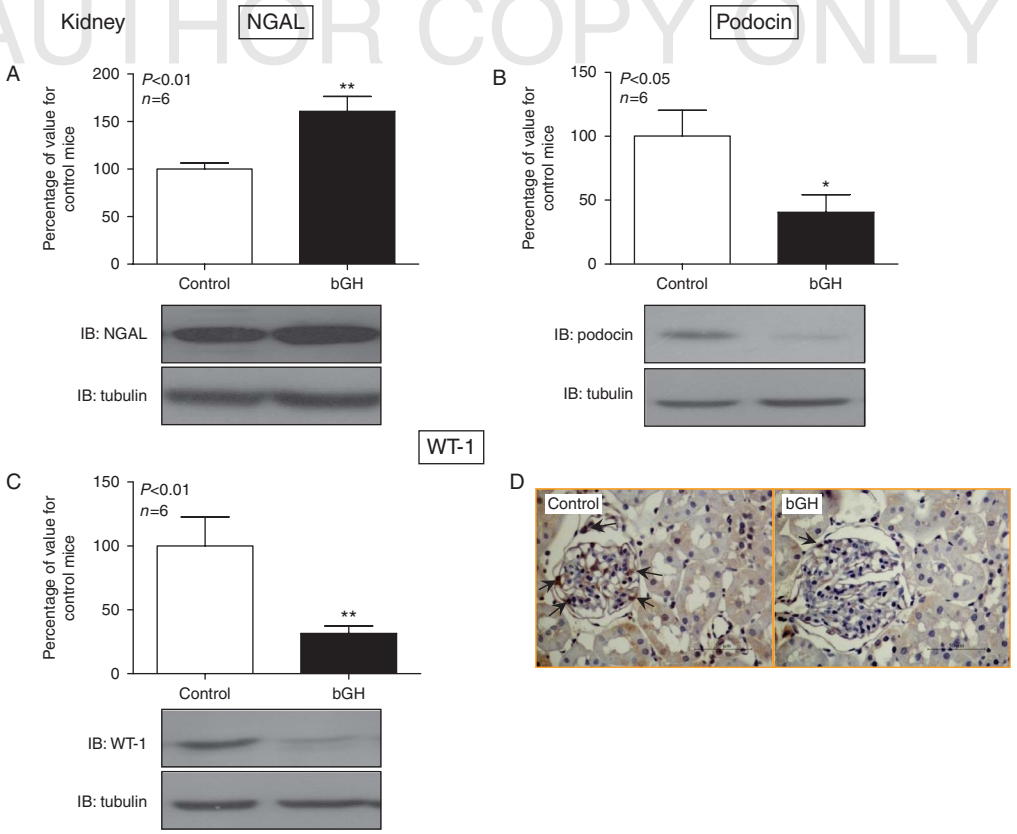

Figure 8

Representative images and bar charts showing the quantification of neutrophil gelatinase-associated lipocalin (NGAL) (A), podocin (B), and Wilms' tumor-1 (WT-1) (C) in the kidney as determined by immunoblotting (IB) are shown for each group. Data are shown as mean±S.E.M. ($n=6$; *$P<0.05$; **$P<0.01$). Localization and immunostaining of WT-1. Control mice showed an intense nuclear staining of glomerular visceral epithelial cells but weak staining of the tubules, while renal tissue from bGH showed a scanty immunostaining (black arrows indicate positive nuclear staining) (D). The calculated molecular weights for the immunoreactive bands are ~68 kDa for NGAL, 47 kDa for podocin, 50 kDa for WT-1, and 50 kDa for tubulin. Data are shown as mean±S.E.M. **$P<0.01$ vs control group ($n=8$–10). A full colour version of this figure is available at http://dx.doi.org/10.1530/JOE-13-0497.

Although 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. Specifically, Ang-(1–7) reduces or prevents cardiac remodeling by decreasing hypertrophy and fibrosis (Grobe *et al.* 2007, Giani *et al.* 2010), attenuating oxidative stress, and also reducing renal dysfunction in several models including diabetic-hypertensive rats and stroke-prone hypertensive rats (Benter *et al.* 2006, Giani *et al.* 2011). In concordance, genetic deletion of the Mas receptor impairs heart function and induces a profibrotic state (Santos *et al.* 2013). Consistent with this evidence, the cardiac and renal hypertrophy and fibrosis found in bGH mice were associated with decreased protein levels of the Mas receptor in the heart and the kidney.

In acromegalic humans, increased renal size and weight and increased glomerular diameter are well known, whereas renal failure is infrequent. However, ourselves and others have shown that, in addition to cardiac fibrosis, bGH mice develop renal damage (Doi *et al.* 1991, Yang *et al.* 1993, Miquet *et al.* 2011). The podocyte has emerged as a critical cell type in regulating the progression of kidney disease (Shankland 2006). Podocin is one of the major proteins of the podocyte and has a central role in the maintenance of slit diaphragm homeostasis. WT-1 is a well-known zinc-finger-containing transcription factor required for normal kidney development, and loss of renal WT-1 is associated with many glomerular diseases (Niaudet & Gubler 2006). Decreases in WT-1 staining have been used to confirm podocyte injury in biopsies (Su *et al.* 2010). In line with these reports, in the current study, we showed that bGH mice displayed a reduction in both podocin and WT-1 abundance, implying the existence of podocyte injury in these animals. NGAL is a protein of the lipocalin family and is normally

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secreted in low amounts in various tissues (Schmidt-Ott *et al*. 2007). In the kidney, NGAL is produced and secreted by renal tubular cells in response to various injuries and its levels predict the appearance of acute kidney injury and even the acute worsening of unstable nephropathies (Bolignano *et al*. 2008). Recent evidence also indicates that NGAL may somehow be involved in the pathophysiological process of chronic renal diseases such as polycystic kidney disease and glomerulonephritis (Bolignano *et al*. 2008). As bGH mice develop progressive glomerulosclerosis, our present results showing upregulation of NGAL in the kidney are consistent with renal damage.

In the current study, chronic treatment with Ang-(1–7) induced normalization of SBP in bGH mice. This experimental evidence strongly indicates that the ACE2/Ang-(1–7)/Mas axis is involved in GH-mediated hypertension. Given the well-demonstrated beneficial effect of Ang-(1–7) in terms of reversal of cardiac hypertrophy (Grobe *et al*. 2007, Giani *et al*. 2010) and renal damage (Benter *et al*. 2006, Giani *et al*. 2011, Zimmerman & Burns 2012), it is possible that the hypotensive effect of Ang-(1–7) could be associated with reversal of tissue alterations in bGH mice. However, this remains to be determined.

In conclusion, this study shows that in bGH mice, chronic exposure to continuously elevated GH levels is associated with increased SBP, a high degree of both cardiac and renal fibrosis as well as increased levels of markers of tubular and glomerular damage. Interestingly, this is concomitant with a shift within the RAS toward an attenuation of the ACE2/Ang-(1–7)/Mas receptor axis both in the heart and in the kidney of these animals. We hypothesize that these changes may contribute to the increased incidence of hypertension, cardiovascular and renal disfunction displayed by this animal model of acromegaly. This hypothesis is substantiated in part by our current results showing that chronic Ang-(1–7) induced a normalization of SBP in this model of GH excess.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

The present work was supported by grants from Universidad de Buenos Aires (20020100100207) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (PIP 114-200801-00374) to F P D; Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT) (PICT 2011-0387) to F P D; National Institute on Aging (NIA) (AGO32290) and Polish Ministry of Science and Higher Education (N N401 042638) to M C M, J G M, J E T, and F P D are researchers at the National Research Council of Argentina (CONICET). J F G is a postdoctoral fellow and V B is doctoral fellow of the Agencia Nacional de Promoción Científica y Tecnológica. The Fulbright Commission and the CONICET awarded Dr J G M a grant for a postdoctoral research stay at the Southern Illinois University.

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Received in final form 29 January 2014
Accepted 18 February 2014

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-13-0497

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