Endogenous production of ghrelin and beneficial effects of its exogenous administration in monocrotaline-induced pulmonary hypertension

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Endogenous production of ghrelin and beneficial effects of its exogenous administration in monocrotaline-induced pulmonary hypertension. Am J Physiol Heart Circ Physiol 287: H2885–H2890, 2004; doi:10.1152/ajpheart.01122.2003.—We investigated the endogenous production of ghrelin as well as cardiac and pulmonary vascular effects of its administration in a rat model of monocrotaline (MCT)-induced pulmonary hypertension (PH). Adult Wistar rats randomly received a subcutaneous injection of MCT (60 mg/kg) or an equal volume of vehicle. One week later, animals were randomly assigned to receive a subcutaneous injection of ghrelin (100 μg/kg bid for 2 wk) or saline. Four groups were analyzed: normal rats treated with ghrelin (n = 7), normal rats injected with saline (n = 7), MCT rats treated with ghrelin (n = 9), and MCT rats injected with saline (n = 9). At 22–25 days, right (RV) and left ventricular (LV) pressures were measured, heart and lungs were weighted, and samples were collected for histological and molecular analysis. Endogenous production of ghrelin was almost abolished in normal rats treated with ghrelin. In MCT-treated animals, pulmonary expression of ghrelin was preserved, and RV myocardial expression was increased more than 20 times. In these animals, exogenous administration of ghrelin attenuated PH, RV hypertrophy, wall thickening of peripheral pulmonary arteries, and RV diastolic disturbances and ameliorated LV dysfunction, without affecting its endogenous production. In conclusion, decreased tissular expression of ghrelin in healthy animals but not in PH animals suggests a negative feedback in the former that is lost in the latter. A selective increase of ghrelin mRNA levels in the RV of animals with PH might indicate distinct regulation of its cardiac expression. Finally, ghrelin administration attenuated MCT-induced PH, pulmonary vascular remodeling, and RV hypertrophy, indicating that it may modulate PH and pulmonary hypertension; ventricular hemodynamics; pulmonary vascularization.

PULMONARY HYPERTENSION (PH) is a progressive disease associated with right ventricular (RV) hypertrophy that commonly progresses to heart failure. Endothelin (ET)-1 and several other neurohumoral agents play a central role in the complex pathophysiology of PH (11, 28) and are used as therapeutic targets for this entity. For instance, several authors observed that ET-1 antagonism decreases PH, restores endothelial metabolic function, inhibits RV hypertrophy, and improves survival both in experimental (5, 19, 26) and clinical (27) settings.

Ghrelin (Ghr) is an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHSR), originally isolated from the rat stomach (17). Acting on hypothalamic pituitary GH secretagogue type 1a, Ghr induces a potent release of GH. Various experimental studies have shown that the GH/insulin growth factor (IGF)-1 axis improves cardiac function both in normal and failing hearts (6, 8, 38), although human trials revealed contradictory results about the value of GH in heart failure therapy (9, 12, 31).

However, Ghr is much more than an endogenous GH secretagogue and has important direct cardiovascular effects. Ghr administration reduces cardiac afterload and increases cardiac output without increasing heart rate in both normal subjects and patients with dilated cardiomyopathy (20, 21). In addition, Ghr has potent vasodilator properties that are endothelium and GH independent (23, 37). The Ghr-GHSR signaling pathway is, therefore, presumably involved in the regulation of vascular tone (21, 37). There is increasing evidence supporting the hypothesis that cardiovascular effects of Ghr are mediated by multiple receptors, some of them still to be identified (2, 3).

The present study investigated the production of Ghr in the stomach, lung, and RV of normal rats and rats with monocrotaline (MCT)-induced pulmonary hypertension as well as the effects of its chronic administration in both groups. Our hypothesis was that Ghr production could be altered in PH and that its administration might have beneficial effects by improving cardiac and/or pulmonary vascular structure and function.

METHODS

Experimental Design

Animal experiments were performed according to the Portuguese law for animal welfare and conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). Adult male Wistar rats (Charles River Laboratories; Barcelona, Spain) weighing 180–200 g were housed in groups of 5 rats/cage in a controlled environment under a 12:12-h light-dark cycle at a room temperature of 22°C, with a free supply of food and water. Rats randomly received a subcutaneous injection of MCT (60 mg/kg body wt, Sigma; Barcelona, Spain) or an equal volume of vehicle (1 ml/kg body wt). One week later, animals were randomly assigned to receive a subcutaneous injection of Ghr (100 μg/kg bid for 2 wk) or saline. The first five residues of Ghr with the large hydrophobic group on the Ser3 side chain are sufficient to abolish the activity of endogenous Ghr (4). An active fragment of Ghr was obtained from Peptides (PGH-3628-PI, human, rat, 1–5), with a functional assay activation at 10 nM, relative to Ghr of 96 ± 7%. This protocol resulted in four groups: normal rats treated with Ghr (sham-Ghr, n = 7), normal rats injected with saline (sham, n = 7), MCT rats treated with Ghr (MCT-Ghr, n = 7), and MCT rats injected with saline (MCT, n = 7).
7), MCT-treated rats treated with Ghr (MCT-Ghr, n = 9), and MCT-treated rats injected with saline (MCT, n = 9).

Survival Studies

Analysis of survival rates was performed with another protocol, where two doses of MCT (50 or 60 mg/kg sc) and two doses of Ghr (100 or 200 μg/kg bid sc) were used. Ghr or saline was injected after day 7 after MCT or vehicle injection. This resulted in nine different groups: 1) sham (n = 10; no MCT, no Ghr); 2) sham-Ghr100 (n = 10; no MCT, 100 μg/kg Ghr); 3) sham-Ghr200 (n = 10; no MCT, 200 μg/kg Ghr); 4) MCT50 (n = 20; 50 mg/kg MCT, no Ghr); 5) MCT50-Ghr100 (n = 20; 50 mg/kg MCT, 100 μg/kg Ghr); 6) MCT50-Ghr200 (n = 20; 50 mg/kg MCT, 200 μg/kg Ghr); 7) MCT50 (n = 20; 60 mg/kg MCT, no Ghr); 8) MCT60-Ghr100 (n = 20; 60 mg/kg MCT, 100 μg/kg Ghr); and 9) MCT60-Ghr200 (n = 20; 60 mg/kg MCT, 200 μg/kg Ghr).

Hemodynamic Studies

The animals were anesthetized with pentobarbital (6 mg/100 g ip), placed over a heating pad, and tracheostomized for mechanical ventilation with room air (model 683 Harvard Small Animal Ventilator). Anesthesia was maintained with additional bolus of pentobarbital (6 mg/100 g ip), ventral surgical microcopy (Wild M651.MS-D, Leica; Herbrugg, Switzerland), the right jugular vein was cannulated for fluid administration (prewarmed 0.9% NaCl solution) to compensate for perioperative fluid losses. The heart was exposed through a median sternotomy, and the pericardium was widely opened. RV and left ventricular (LV) pressures were measured with a 2-Fr high-fidelity micromanometer (SPR-324, Millar Instruments) inserted into the RV and LV cavities, respectively. After complete instrumentation, the animal preparation was allowed to stabilize for 15 min. Hemodynamic recordings were made with respiration suspended at end expiration. Parameters were converted on-line to digital data with a sampling frequency of 1,000 Hz. RV and LV pressures were measured at end diastole and peak systole. Peak rates of RV and LV pressure rise (dP/dtmax) and pressure fall (dP/dtmin) were measured as well. The relaxation rate was estimated with the time constant τ by fitting the isovolumetric pressure fall to a monoexponential function.

Morphometric Analysis

Once collection of hemodynamic data was completed, cardiac arrest was induced by the injection of 7.5% KCl through the central venous catheter. The heart, lungs, and right gastrocnemius muscle were excised and weighed. The right tibia was also excised and measured using a millimetric ruler. Under binocular magnification (×3.5), the RV free wall was dissected from the LV and weighed separately. Heart, lung, RV, and LV + septal (LV + S) weights were normalized to body weight, whereas gastrocnemius weight was normalized to tibial length. RV weight was, in addition, normalized to that of LV + S.

RV and right lung samples were immersion fixed in 10% buffered formalin and embedded in paraffin. Sections 7 μm thick were cut and stained with hematoxylin and eosin.

RV free wall specimens were obtained from each heart at midterm between the apex and base. Studied samples were photographed with a digital camera, and the muscle fiber area was measured, in each section, using a digital image analyzer (Olympus DP Software version 3.0). These measurements were made directly at ×400 magnification only in muscle fibers whose cross section included a nuclear profile (7). Five sections per sample were photographed, and the area of 10 muscle fibers was measured. The area of the 50 analyzed muscle fibers/sample was then averaged.

Pulmonary specimens of each animal were collected from the lower segments of the upper right lobe. External diameter and medial wall thickness in muscular arteries (12 arteries/lung) were analyzed at ×400 magnification. Orthogonal intercepts were used to generate 8 random measurements of external diameter of the vessels (distance between the external lamina) and 16 random measurements of medial thickness of the vessels (distance between the internal and external lamina). For each artery, medial hypertrophy was expressed as follows: %wall thickness = [(medial thickness × 2)/external diameters] × 100.

mRNA Quantification by Real-Time RT-PCR

Samples were collected from the RV free wall, lung parenchyma, and gastric fundus. Total mRNA was extracted through the guanidinium-thiocyanate selective silica-gel membrane-binding method (Qiagen 74124) according to the manufacturer’s instructions. Concentration and purity were assayed by spectrophotometry (Eppendorf 6131000.012).

Two-step real-time RT-PCR was used to perform relative quantification of mRNA. For each studied mRNA molecule, standard curves were generated from the correlation between the amount of starting total mRNA and the PCR threshold cycle (second derivative maximum method) of graded dilutions from a randomly selected tissue sample (IGF-1: r = 0.994; GAPDH: r = 0.993; Ghr: r = 0.990). For relative quantification of specific mRNA levels, 50 ng of total mRNA from each sample underwent two-step real-time RT-PCR. A melt curve analysis of each real-time PCR and 2% agarose gels (0.5 mg/ml ethidium bromide) was run to exclude primer-dimer formation and assay the purity of the amplification product. Experiments were run in triplicate with a PCR intra-assay variability of 18.4% and a RT-PCR interassay variability of 21.6%. The GAPDH mRNA level was similar in all groups and was used as an internal control gene. Results of mRNA quantification are expressed in an arbitrary unit (AU) set as the average value of the sham group (sham = 1 AU).

RT (10 min at 22°C, 50 min at 50°C, and 10 min at 95°C) was performed in a standard thermocycler (Whatman Biometra 050-901) with a total volume of 20 ml: 40 U reaction of reverse transcriptase (Invitrogen 18064-014), 20 U reaction of RNase inhibitor (Promega 1 AU).

Table 1. Effects of Ghr on somatic and cardiac growth

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham-Ghr</th>
<th>MCT</th>
<th>MCT-Ghr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>269±6</td>
<td>273±3</td>
<td>251±9</td>
<td>263±7</td>
</tr>
<tr>
<td>Heart weight/body weight, g/kg</td>
<td>3.03±0.06</td>
<td>2.70±0.07</td>
<td>4.10±0.12*</td>
<td>3.45±0.12*†‡</td>
</tr>
<tr>
<td>RV weight/body weight, g/kg</td>
<td>0.38±0.02</td>
<td>0.42±0.03</td>
<td>1.18±0.07*</td>
<td>0.74±0.08*†‡ $</td>
</tr>
<tr>
<td>LV + S weight/body weight, g/kg</td>
<td>1.76±0.05</td>
<td>1.83±0.05</td>
<td>2.01±0.05</td>
<td>1.90±0.06</td>
</tr>
<tr>
<td>RV/LV + S, g/g</td>
<td>0.21±0.01</td>
<td>0.23±0.01</td>
<td>0.58±0.03*</td>
<td>0.39±0.03*†‡ $</td>
</tr>
<tr>
<td>Lung weight/body weight, g/kg</td>
<td>4.60±0.15</td>
<td>3.85±0.14</td>
<td>9.69±0.76*</td>
<td>7.02±0.55*†‡</td>
</tr>
<tr>
<td>Gastrocnemius muscle weight/tibial length, mg/mm</td>
<td>36.1±1.8</td>
<td>43.2±0.8*</td>
<td>34.2±1.1</td>
<td>36.6±0.9‡ $</td>
</tr>
</tbody>
</table>

Data are means ± SE. RV, right ventricle; LV + S, left ventricle plus septum; Ghr, ghrelin; MCT, monocrotaline. *P < 0.05 vs. sham; †P < 0.05 vs. sham-Ghr; ‡P < 0.05 vs. MCT, §significant interaction.
Specific PCR primer pairs for the studied genes were as follows: green (Qiagen 204143) according to the manufacturer’s instructions.

N2515), 30 ng/ml random primers (Invitrogen 48190–011), 0.5 mM nucleotide mix (MBI Fermentas R0192), 1.9 mM MgCl₂, and 10 mM DTT. Ten percent of the cDNA yield was used as a template for PCR. N2515), 30 ng/ml random primers (Invitrogen 48190–011), 0.5 mM nucleotide mix (MBI Fermentas R0192), 1.9 mM MgCl₂, and 10 mM DTT. Ten percent of the cDNA yield was used as a template for PCR.

Statistical Analysis

Statistical analysis was performed using SPSS 12.0 software.

RESULTS

Somatic and Cardiac Growth

Data related to somatic and cardiac growth are summarized in Table 1. Compared with the sham groups, heart and lung weights were significantly increased in the MCT group. Heart weight augmentation was mainly due to RV growth. Treatment with Ghr attenuated the effects of MCT on cardiac and lung weights, whereas in sham animals it only increased the gastrocnemius weight-to-tibial length ratio. Interaction analysis revealed that effects of Ghr on RV and gastrocnemius weights were significantly different in sham and MCT rats.

RV and LV Hemodynamics

Hemodynamic data are summarized in Table 2. Peak systolic RV pressure (RVPmax), which was used to estimate PH, was significantly increased in the MCT group. Treatment with Ghr markedly attenuated this effect. MCT treatment also significantly increased RV dP/dt max, whereas in sham animals it only increased the gastrocnemius weight-to-tibial length ratio. Interaction analysis revealed that effects of Ghr on RV and gastrocnemius weights were significantly different in sham and MCT rats.

Table 2. Effects of Ghr on hemodynamic parameters

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham-Ghr</th>
<th>MCT</th>
<th>MCT-Ghr</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RVPmax, mmHg</td>
<td>24.6±1.8</td>
<td>20.4±1.0</td>
<td>49.1±1.6*</td>
<td>36.4±1.8*†‡§</td>
</tr>
<tr>
<td>RVEDP, mmHg</td>
<td>1.3±0.3</td>
<td>1.6±0.2</td>
<td>3.0±0.3*</td>
<td>1.7±0.2*‡§</td>
</tr>
<tr>
<td>dP/dmax, mmHg/s</td>
<td>1.227±80</td>
<td>1.139±185</td>
<td>1.602±91*</td>
<td>1.692±113*†</td>
</tr>
<tr>
<td>dP/dmin, mmHg/s</td>
<td>−865±126</td>
<td>−721±49</td>
<td>−1.087±52</td>
<td>−1.627±141*†‡§</td>
</tr>
<tr>
<td>τ, ms</td>
<td>7±1</td>
<td>11±1</td>
<td>12±2†‡§</td>
<td>17±2*‡§</td>
</tr>
<tr>
<td>LV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVPmax, mmHg</td>
<td>99.4±4.9</td>
<td>109.7±2.1</td>
<td>67.4±3.5*</td>
<td>85.0±4.2†‡§</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>2.1±0.6</td>
<td>2.6±0.6</td>
<td>2.3±0.3</td>
<td>1.8±0.3</td>
</tr>
<tr>
<td>dP/dmax, mmHg/s</td>
<td>5.624±631</td>
<td>7.297±351*</td>
<td>3.442±325*</td>
<td>5.510±604‡§</td>
</tr>
<tr>
<td>dP/dmin, mmHg/s</td>
<td>−4.277±495</td>
<td>−4.203±280</td>
<td>−2.340±214*</td>
<td>−4.019±442‡§</td>
</tr>
<tr>
<td>τ, ms</td>
<td>18±1</td>
<td>14±1</td>
<td>22±1</td>
<td>16±2†‡§</td>
</tr>
</tbody>
</table>

Data are means ± SE. RVPmax and LVPmax, RV and LV peak systolic pressures, respectively; RVEDP and LVEDP, RV and LV end-diastolic pressures, respectively; dP/dmax and dP/dmin, peak rates of ventricular pressure rise and fall, respectively; τ, time constant. *P < 0.05 vs. sham; †P < 0.05 vs. sham-Ghr; ‡P < 0.05 vs. MCT; §significant interaction.
Effects of Ghr on RV hemodynamics were, therefore, significantly different in sham and MCT animals, as confirmed by interaction analysis.

With regard to the LV, the MCT group presented, compared with the sham group, lower systolic pressures and smaller dP/dt max and dP/dt min. Treatment with Ghr blunted all these effects. LV filling pressures and τ were not significantly altered by MCT, but the latter significantly decreased in the animals treated with Ghr. In sham animals, the only significant effect of Ghr was an increase of dP/dt max.

Morphometric Analysis

RV chronic pressure overload secondary to PH in the MCT group resulted in RV hypertrophy, which was attenuated by Ghr, as expressed by RV/LV + S and RV myocyte cross-sectional area (Table 1 and Fig. 1).

Pulmonary vessels showed a significant hypertrophy of the media in the MCT group, which was significantly reduced by Ghr, as illustrated in Fig. 1. This was true both for larger and smaller peripheral arteries.

In sham animals, no significant effects of Ghr on those parameters were detected. Interaction analysis confirmed that effects of Ghr on such parameters were significantly different in sham and MCT animals.

Real-time RT-PCR Analysis

Expression of Ghr. The expression of Ghr is shown in Fig. 2. We detected expression of Ghr mRNA in the RV, lung, and gastric fundus of sham animals, but this expression was almost abolished by Ghr treatment. MCT treatment increased the expression of Ghr more than 20-fold in the RV but did not significantly alter it in the lung and gastric fundus. In contrast to sham animals, Ghr administration did not significantly alter expression of its mRNA in MCT-treated rats, although interaction analysis revealed significant differences in the lung.

Expression of IGF-1. The expression of IGF-1 is shown in Fig. 3. Ghr treatment significantly increased IGF-1 mRNA levels in the lung but not in the RV of sham animals. MCT treatment increased IGF-1 mRNA levels both in the RV and lung. In PH, Ghr administration normalized RV, but not pulmonary, IGF-1 mRNA expression. Interaction analysis confirmed that Ghr distinctly affects IGF-1 mRNA expression in sham and MCT animals in both the RV and lung.

Survival Analysis

At the end of the third week, there was a trend for Ghr to dose dependently increase the survival of MCT-injected rats: MCT50 70%, MCT50-Ghr100 80%, MCT50-Ghr200 90%, MCT60 45%, MCT60-Ghr100 55%, and MCT60-Ghr200 60%.
This tendency disappeared, however, at the end of study (day 35), with no significant differences in survival, which averaged 9 ± 2% in all MCT-treated animals. All sham animals were alive at the end of day 35.

**DISCUSSION**

In the present study, we documented endogenous production of Ghr in the RV, lung, and gastric fundus, which is consistent with previous reports showing production of Ghr in these organs (14, 17, 25). Such production was inhibited by exogenous administration of Ghr in healthy animals, suggesting that systemic levels of Ghr might regulate its local production. No deleterious effects were detected as a result of this suppression: these animals had no mortality and cardiac function was normal.

Interestingly, such an ability of systemic Ghr to regulate its local production was apparently lost in PH. Furthermore, in PH, expression of Ghr was markedly increased in the RV but not in the lung and gastric fundus, which might indicate a distinct regulation of its cardiac expression. Given the well-known vasodilating properties of Ghr (21, 37), one might wonder if its increased production in PH is not a compensatory response of the RV to increased afterload, to produce a vasodilator substance that could reach the hypertensive lung and locally modify the excess production of vasoconstrictors.

Most actions of Ghr have been explained by stimulation of specific receptors (GHSR-1a), which are responsible not only for its direct effects (e.g., vasodilatation) but also for the indirect effects secondary to GH release (16, 17, 24). In the present study, we demonstrated that Ghr administration decreased pulmonary hypertension and attenuated RV hypertrophy. Rather disappointing, however, were the modest effects of Ghr on animal survival. In fact, given the impressive effects of Ghr on myocardial and pulmonary vascular remodeling, improved survival was marginal compared with other vasodilating drugs (5, 22, 26, 29), which often have less important pulmonary and cardiac effects.

Potential explanations for the beneficial effects of Ghr include actions both at pulmonary and cardiac levels. At the pulmonary level, Ghr seems to have a determinant role in fetal lung development (35), whereas the adult human lung is a major source of Ghr mRNA gene expression (13). Additionally, GH secretagogue-binding sites have been demonstrated in the adult lung parenchyma and pulmonary artery wall (16, 24). Taking together these evidences with our results, it seems reasonable to propose that Ghr may modulate PH. The mechanisms for such role include pulmonary vasodilatation and vascular remodeling. Shimizu et al. (30) demonstrated that Ghr improves endothelial dysfunction and increases endothelial nitric oxide synthase expression through a GH-independent mechanism. Because MCT induces endothelial dysfunction, the beneficial effects of Ghr in the lung, as observed in our study, could be due to improvement of pulmonary endothelial function. On the other hand, the Ghr-GHSR signaling pathway can revert ET-1-induced vasoconstriction by mechanisms that act by GH/IGF-1 and endothelium-independent pathways (21, 37). It should be emphasized that ET-1 is increased in PH and seems to be largely involved in MCT-induced PH (10). With regard to pulmonary vascular remodeling, the present study showed that the medial area in peripheral pulmonary arteries was significantly reduced by Ghr, indicating that attenuation of PH is, at least partially, mediated by this mechanism. This mechanism could also contribute to the effects of Ghr at the pulmonary level.

At the cardiac level, Ghr improved RV and LV hemodynamic, morphological, and molecular parameters. Such improvement might be due either to decreased RV overload or to myocardial actions of Ghr. Decreased RV overload will primarily favor recovery of RV function and structure. With regard to the LV, previous studies also showed that chronic administration of Ghr was able to improve LV dysfunction and to attenuate the development of LV remodeling and cardiac cachexia in rats with heart failure (23).

With regard to the potential role of the GH/IGF1 axis, cardiac production of IGF-1 is increased in RV volume overload and seems to be related with systolic wall stress (15). Additionally, activation of the GH/IGF-1 axis increased myocardial contractility, decreased peripheral vascular resistance (1, 6, 8, 32, 38), promoted physiological myocardial hypertrophy (34), and upregulated sarco(endo)plasmic reticulum Ca2+. ATPase 2a (33) in heart failure. In the present study, RV IGF-1 mRNA levels were increased in the MCT group, which might suggest a local increase, dependent of RV overload. Interestingly, treatment with Ghr decreased PH and RV local IGF-1 mRNA levels, which further reinforces a potential load-dependent regulation of cardiac IGF-1 expression. In the lung, IGF-1 levels were increased by Ghr in sham animals and were similarly elevated in PH. However, in PH, IGF-1 levels were not significantly altered by Ghr, indicating that one cannot explain the observed effects of exogenous Ghr at the pulmonary level by changes in IGF-1 expression.

**Limitations of This Study**

Although PH induced by MCT has no equivalent in humans, there are some similarities between this model and some forms of human PH. MCT treatment results in endothelial dysfunction, medial thickness increasing, peripheral artery loss, pulmonary vasculature resistance elevation, and RV hypertrophy that mirror human PH (18, 36).

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