Cardiovascular and hormonal effects of subcutaneous administration of ghrelin, a novel growth hormone-releasing peptide, in healthy humans

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ABSTRACT

Ghrelin is a novel GH (growth hormone)-releasing peptide isolated from the stomach. The cardiovascular and hormonal effects of the subcutaneous administration of ghrelin in humans remain unknown. Six healthy volunteers each received subcutaneous administration of three doses of ghrelin (1, 5 or 10 µg/kg) and placebo; the order of administration was randomized, and separate doses were given at least 24 h apart. The serum GH level dose-dependently increased from 0.5 ± 0.4 to 3.6 ± 2.1 ng/ml (1 µg/kg ghrelin; P = 0.99 compared with baseline), 27.1 ± 12.0 ng/ml (5 µg/kg; P < 0.01 compared with baseline) and 45.4 ± 12.8 ng/ml (10 µg/kg; P < 0.01 compared with baseline) 30 min after ghrelin administration. Subcutaneous administration of ghrelin did not significantly alter circulating levels of corticotropin, cortisol, insulin-like growth factor-1, noradrenaline or adrenaline, although 10 µg/kg ghrelin slightly increased the prolactin level. No significant changes in heart rate or mean arterial pressure were observed. In contrast, the left ventricular ejection fraction, as assessed by echocardiography, increased dose-dependently from 63.5 ± 0.6 % to 65.1 ± 0.9 % (1 µg/kg ghrelin; P = 0.97 compared with baseline), 69.6 ± 1.3 % (5 µg/kg; P < 0.01 compared with baseline) and 71.5 ± 0.9 % (10 µg/kg; P < 0.01 compared with baseline) 30 min after ghrelin administration. These haemodynamic and hormonal changes were still apparent 60 min after ghrelin injection. In conclusion, subcutaneous administration of ghrelin dose-dependently induced relatively specific GH release and enhanced cardiac performance in humans.

INTRODUCTION

Ghrelin is a novel GH (growth hormone)-releasing peptide, isolated from the stomach, which has been identified as an endogenous ligand for the GHS-R (GH secretagogue receptor) [1]. Human ghrelin is a 28-amino-acid peptide containing an n-octanoyl modification at serine-3, and is identical to rat ghrelin apart from two amino acids. The intravenous administration of ghrelin has been shown to dose-dependently induce GH release in rats and humans [1–3]. In addition, the GH-releasing effect of ghrelin has been shown to be more potent

Key words: cardiac function, ghrelin, growth hormone, haemodynamics.

Abbreviations: ACTH, adrenocorticotropic hormone; GH, growth hormone; GHS-R, GH secretagogue receptor; IGF-1, insulin-like growth factor-1; LV, left ventricular.

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than that of hypothalamic GH-releasing hormone. On the other hand, ghrelin peptide and mRNA have also been detected in blood vessels and the heart, where GHS-R mRNA is also expressed [4–6]. In fact, we have shown recently that intravenous injection of ghrelin significantly decreased mean arterial pressure and increased cardiac output in healthy volunteers [6]. These findings suggest that ghrelin may be involved in the regulation of the cardiovascular system and hormonal balance as a circulating factor as well as a paracrine and/or autocrine factor. While such beneficial effects of intravenous ghrelin have been examined, the effects of subcutaneous administration of ghrelin remain unclear. The subcutaneous delivery route is important for the long-term administration of drug in clinical settings. Thus the purpose of the present study was to investigate the cardiovascular and hormonal effects of the subcutaneous administration of ghrelin in healthy humans.

METHODS

Subjects
We studied six healthy male hospital staff, aged 32 ± 3 years, with a body weight of 65.6 ± 7 kg. None of them had any history of cardiovascular, renal, respiratory, hepatic or metabolic disease, and none were taking any drugs. Physical examination and electrocardiographic and echocardiographic findings were also normal. Subjects were requested to refrain from sodium- and glucose-rich foods, caffeine, alcohol, drugs such as non-steroidal anti-inflammatory agents and nicotine for several hours before the experiments. The study was approved by the ethical committee of the National Cardiovascular Center, and all subjects gave written informed consent.

Study protocol
The six healthy volunteers each received subcutaneous administration of three doses of ghrelin (1, 5 or 10 µg/kg) and placebo; the order of administration was randomized, and separate doses were given at least 24 h apart. The protocol was started at 08.00 hours after an overnight fast. A 22-gauge catheter was inserted into an antecubital vein for blood sampling. After an equilibration period of 30 min, ghrelin (1, 5 or 10 µg/kg) or placebo (0.9 % saline) was administered subcutaneously. To assess LV (left ventricular) function, echocardiography was performed at baseline and 30, 60, 120 and 180 min after ghrelin injection. Blood sampling was repeated for measurements of circulating levels of ghrelin, GH, IGF-1 (insulin-like growth factor-1), prolactin, ACTH (adrenocorticotropin; corticotropin), cortisol, noradrenaline and adrenaline. Systemic blood pressure and heart rate were monitored simultaneously using an automatic sphygmomanometer.

Preparation of synthetic human ghrelin
Human ghrelin was obtained from the Peptide Institute Inc., Osaka, Japan. The homogeneity of human ghrelin was confirmed by reverse-phase HPLC and amino acid analysis. Ghrelin was dissolved in saline with 4 % (w/v) D-mannitol and was sterilized by passage through a 0.22-µm filter (Millipore Co.). Randomly selected vials were then submitted for sterility and pyrogen testing. Ghrelin was stored in aliquots of 1 ml (each containing 600 µg of ghrelin) at −80 °C until the time of preparation for administration.

Echocardiographic evaluation
Echocardiographic studies were conducted by an expert cardiologist who was blinded to the dose of ghrelin or placebo. Two-dimensional targeted M-mode tracings were obtained at the level of the papillary muscles using an echocardiographic system equipped with a 2.5 MHz transducer (model SSD-9000; Aloka, Tokyo, Japan). LV end-diastolic and end-systolic dimensions were measured using the American Society for Echocardiology leading-edge method [7] from at least three consecutive cardiac cycles. LV end-systolic and end-diastolic volume and LV ejection fraction were calculated from echocardiographic M-mode dimensions using the formula of Teichholz (see [8]).

Assay for plasma ghrelin
Blood was transferred immediately into a chilled glass tube containing disodium EDTA (1 mg/ml) and aprotinin (500 units/ml) and centrifuged immediately at 4 °C. The plasma samples were frozen and stored at −80 °C, and then were extracted before RIA. Briefly, Sep-Pak C18 cartridges (Waters, Milford, MA, U.S.A.) were preconditioned with 5 ml each of chloroform, methanol, 60 % (v/v) acetoniitrile containing 0.1 % (v/v) trifluoroacetic acid and saline. Plasma (1 ml) was diluted with 1 ml of saline, and then loaded into a Sep-Pak C18 cartridge. After the column had been washed with 5 ml each of saline and 5 % (v/v) acetoniitrile containing 0.1 % trifluoroacetic acid, the absorbed materials were eluted with 3 ml of 60 % (v/v) acetoniitrile containing 0.1 % trifluoroacetic acid. The eluate was then lyophilized.

RIA for plasma ghrelin was performed as described previously [9]. In brief, a polyclonal antibody was raised against the C-terminal fragment (residues 13–28) of rat ghrelin in a rabbit. A maleimide-activated mariculture keyhole-limpet haemocyanin–[Cys3]ghrelin-(13–28) conjugate was used for immunization. Rat [Tyr3]ghrelin-(13–28) was radiiodinated by the lactoperoxidase method. A moniodinated ligand was purified by reverse-phase HPLC on a µBondasphere C18 column (3.9 mm × 150 mm; Waters). The tracer was stable for 3 months, stored at −20 °C in 0.1 % BSA. The RIA incubation mixture consisted of 100 µl of standard ghrelin...
or unknown sample, normal rabbit serum and 200 µl of antiserum at a dilution of 1:10 000. After a 12 h incubation at 4 °C, 100 µl of 125I-labelled ligand (15 000 c.p.m.) was added to the mixture. After a 36 h incubation at 4 °C, 100 µl of goat anti-(rabbit IgG) serum was added. Free and bound tracers were separated by centrifugation at 1000 g for 30 min after incubation for 24 h at 4 °C. After aspiration of the supernatant, radioactivity in the pellet was quantified using a gamma counter (ARC-600; Aloka).

Other biochemical measurements
Serum GH was measured using an Ab Bead HGH Eiken immunoradiometric assay kit (Eiken Chemical Co., Tokyo, Japan). Serum prolactin was measured using a SPAC-S Prolactin immunoradiometric assay kit (Daichi Radioisotope Laboratories, Tokyo, Japan). Plasma ACTH was measured with an ACTH IRMA Mitsubishi immunoradiometric assay kit (Mitsubishi Chemical Co., Tokyo, Japan). Plasma cortisol was measured using a Gamma-Coat Cortisol RIA kit (Date Behring Inc.). Serum IGF-1 was determined using a SOMATOMEDIN CII immunoradiometric assay kit (Bayer Medical Ltd., Tokyo, Japan). Plasma noradrenaline and adrenaline were measured by HPLC.

Statistical analysis
All data were expressed as means ± S.E.M. unless otherwise indicated. Comparisons of the time course of parameters among the four groups were performed by two-way ANOVA for repeated measures, followed by the Newman–Keuls test. A P value of < 0.05 was considered statistically significant.

RESULTS
All subjects completed this study protocol, although three subjects complained of a warm sensation and a feeling of hunger.

Time course of plasma ghrelin level after subcutaneous administration of ghrelin
Subcutaneous administration of ghrelin dose-dependently increased the plasma ghrelin level in study subjects. The level at 30 min after administration was increased by approx. 2-fold (1 µg/kg), 8-fold (5 µg/kg) and 12-fold (10 µg/kg) compared with the baseline value (Figure 1). The plasma ghrelin level peaked at 30 min and remained elevated for longer than 60 min after subcutaneous injection of 10 µg/kg ghrelin.

Hormonal responses to subcutaneous ghrelin
Subcutaneous ghrelin dose-dependently increased the serum GH level from 0.5 ± 0.4 to 3.6 ± 2.1 ng/ml (1 µg/kg ghrelin; P = 0.99 compared with baseline), 27.1 ± 12.0 ng/ml (5 µg/kg; P < 0.01 compared with baseline) and 45.4 ± 12.8 ng/ml (10 µg/kg; P < 0.01 compared with baseline) 30 min after administration (Figure 2). The serum GH level peaked at 30 min and remained elevated for longer than 60 min after the subcutaneous injection of 10 µg/kg ghrelin; it tended to be still increased at 90 min (18 times baseline) and 120 min (8 times baseline) after subcutaneous injection, although these changes did not reach statistical significance. Subcutaneous administration of ghrelin did not significantly increase circulating levels of ACTH, cortisol, IGF-1, noradrenaline or adrenaline, although 10 µg/kg ghrelin slightly increased the prolactin level (Table 1).

Effects of subcutaneous ghrelin on cardiac function
Subcutaneous administration of ghrelin dose-dependently increased the LV ejection fraction from 63.5 ± 0.6 % to 65.1 ± 0.9 % (1 µg/kg ghrelin; P = 0.97 compared with baseline), 69.6 ± 1.3 % (5 µg/kg; P < 0.01 compared with baseline) and 71.5 ± 0.9 % (10 µg/kg;
Table 1  Hormonal responses to subcutaneous injection of 10 µg/kg ghrelin
Data are means ± S.E.M. Significance: *P < 0.01 compared with baseline.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Baseline</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
</tr>
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<tbody>
<tr>
<td>Prolactin (pg/ml)</td>
<td>4.3 ± 0.5</td>
<td>6.7 ± 0.8*</td>
<td>5.0 ± 0.5</td>
<td>3.5 ± 0.3</td>
<td>3.2 ± 0.4</td>
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<tr>
<td>ACTH (pg/ml)</td>
<td>30.9 ± 20.5</td>
<td>32.3 ± 12.4</td>
<td>25.3 ± 7.3</td>
<td>19.0 ± 8.2</td>
<td>19.9 ± 8.3</td>
</tr>
<tr>
<td>Cortisol (µg/ml)</td>
<td>9.8 ± 1.6</td>
<td>9.5 ± 0.9</td>
<td>8.0 ± 1.1</td>
<td>5.4 ± 0.5</td>
<td>5.3 ± 1.0</td>
</tr>
<tr>
<td>IGF-1 (ng/ml)</td>
<td>212 ± 17</td>
<td>212 ± 16</td>
<td>198 ± 15</td>
<td>206 ± 22</td>
<td>210 ± 16</td>
</tr>
<tr>
<td>Noradrenaline (pg/ml)</td>
<td>73.7 ± 15.4</td>
<td>83.2 ± 23.7</td>
<td>68.8 ± 12.3</td>
<td>91.2 ± 22.6</td>
<td>105.5 ± 25.6</td>
</tr>
<tr>
<td>Adrenaline (pg/ml)</td>
<td>18.3 ± 5.3</td>
<td>28.3 ± 7.4</td>
<td>192 ± 8.7</td>
<td>16.2 ± 5.2</td>
<td>22.7 ± 4.5</td>
</tr>
</tbody>
</table>

Table 2  Changes in haemodynamic parameters after subcutaneous injection of 10 µg/kg ghrelin
HR, heart rate; MAP, mean arterial pressure; LVDd, LV diastolic dimension; LVDs, LV systolic dimension; LVEDV, LV end-diastolic volume; LVESV, LV end-systolic volume; SV, stroke volume. Data are means ± S.E.M. Significance: *P < 0.05, †P < 0.01 compared with baseline.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (beats/min)</td>
<td>68 ± 2</td>
<td>63 ± 3</td>
<td>62 ± 3</td>
<td>60 ± 2</td>
<td>61 ± 3</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>80 ± 2</td>
<td>76 ± 3</td>
<td>78 ± 3</td>
<td>77 ± 4</td>
<td>78 ± 4</td>
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<tr>
<td>LVDd (mm)</td>
<td>50 ± 1</td>
<td>51 ± 1</td>
<td>52 ± 1</td>
<td>51 ± 1</td>
<td>51 ± 1</td>
</tr>
<tr>
<td>LVDs (mm)</td>
<td>33 ± 0.4</td>
<td>30 ± 0.4</td>
<td>31 ± 1</td>
<td>32 ± 1</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>LVEDV (ml)</td>
<td>122 ± 6</td>
<td>129 ± 5</td>
<td>131 ± 8</td>
<td>125 ± 5</td>
<td>125 ± 6</td>
</tr>
<tr>
<td>LVESV (ml)</td>
<td>44 ± 2</td>
<td>34 ± 1*</td>
<td>37 ± 2</td>
<td>41 ± 2</td>
<td>43 ± 2</td>
</tr>
<tr>
<td>SV (ml)</td>
<td>78 ± 4</td>
<td>95 ± 5*</td>
<td>95 ± 6*</td>
<td>84 ± 4</td>
<td>81 ± 4</td>
</tr>
</tbody>
</table>

P < 0.01 compared with baseline) 30 min after administration (Figure 3). Ghrelin significantly decreased LV end-systolic volume, although it did not significantly alter LV end-diastolic volume (Table 2). Consequently, stroke volume increased significantly after injection of 10 µg/kg ghrelin. Subcutaneous injection of ghrelin did not significantly alter mean arterial pressure or heart rate.

DISCUSSION

In the present study, we have demonstrated that (1) subcutaneous administration of ghrelin dose-dependently increased GH release without significant adverse effects in healthy humans, and that (2) subcutaneous ghrelin significantly increased LV ejection fraction and stroke volume, and decreased LV end-systolic volume.

Ghrelin is a novel GH-releasing peptide, isolated from the stomach, which acts through a mechanism that is different from that of GH-releasing hormone [1]. Studies have shown that intravenous injection of ghrelin dose-dependently stimulates GH release in healthy humans, associated with significant increases in the levels of prolactin, ACTH, cortisol and adrenaline [3]. To our knowledge, however, the biological activities of subcutaneously administered ghrelin remain unknown. Like intravenous injection, subcutaneous injection of ghrelin dose-dependently increased the serum GH level, which peaked at 30 min, remained elevated for longer than 60 min, and tended to be higher than the basal level at 90 and 120 min. The peak plasma ghrelin level after subcutaneous administration of ghrelin (10 µg/kg) was not as high as that after intravenous administration at the same dose reported previously (12-fold compared with 61-fold of baseline value) [6]. Nevertheless, the GH-releasing effect of subcutaneous ghrelin lasted for approximately the same duration as that following intravenous ghrelin administration. Thus subcutaneous injection of ghrelin elicits potent, long-lasting GH release in humans. On the other hand, unlike intravenous injection, subcutaneous injection of ghrelin did not significantly increase levels of ACTH, cortisol or adrenaline, although the maximum dose of ghrelin (10 µg/kg) slightly increased the prolactin level. Subcutaneous administration of ghrelin induced a modest and long-lasting increase in plasma ghrelin, which may contribute to the apparent specificity of ghrelin in selectively releasing GH after subcutaneous administration. Thus subcutaneous delivery of ghrelin may eliminate non-specific hormonal responses, but may be sufficient to induce dose-dependent GH release in humans.

In the present study, the subcutaneous administration of ghrelin increased the LV ejection fraction and stroke
volume without significant changes in mean arterial pressure or catecholamine levels. These results suggest that subcutaneous ghrelin may increase LV myocardial contractility. Because GH up-regulates sarcoplasmic reticulum Ca\(^{2+}\)-ATPase activity and thereby enhances myocardial contractility \([10]\), some of the cardiac effects of ghrelin may be mediated by GH. On the other hand, ghrelin may have direct cardiac actions. GHS-R mRNA is detected not only in the hypothalamus and pituitary but also in the heart \([4–6]\). Stimulation of GHS-R has been shown to prevent cardiac damage after ischaemia/reperfusion in hypophysectomized rats \([11]\). Recently, Imazio et al. \([12]\) have shown that intravenous administration of hexarelin, a synthetic GH secretagogue, increased the LV ejection fraction not only in patients with dilated cardiomyopathy but also in those with GH deficiency. These findings suggest that ghrelin may enhance cardiac performance partly through GH-independent mechanisms. Further studies are necessary to elucidate the underlying mechanisms responsible for the cardiac actions of ghrelin.

The subcutaneous administration of ghrelin induced relatively specific GH release and enhanced cardiac performance in humans without significant adverse effects. A considerable amount of ghrelin is known to circulate together with its stimulation of GH make ghrelin of interest for the treatment of GH deficiency, aging or heart failure. The potential therapeutic use of ghrelin, however, will be determined by its chronic effects after repeated subcutaneous injection.

In conclusion, the subcutaneous administration of ghrelin dose-dependently induced GH release and enhanced cardiac performance in healthy humans. This novel route of ghrelin administration may provide a potential new therapeutic strategy that allows the chronic administration of this peptide.

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