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Effects of growth hormone treatment on arginine to asymmetric dimethylarginine ratio and endothelial function in patients with growth hormone deficiency

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Abstract

Patients with growth hormone deficiency (GHD) are known to have reduced life expectancy due to increased cardiovascular and cerebrovascular events. An increase in asymmetric dimethylarginine (ADMA) levels previously found in GHD patients could promote premature atherosclerosis. The aim of this study was to determine whether 6-month growth hormone (GH) replacement therapy was able to decrease ADMA levels and ameliorate endothelial dysfunction. Thirty-one GHD patients were studied before and after 6 months of GH (4 μg/[kg d], daily) replacement therapy. Reduced pretreatment levels of serum insulin-like growth factor (IGF) 1 were normalized during GH treatment (88.2 ± 62.5 to 191.7 ± 80.3 ng/mL, $P < .0001$). After 6 months of GH replacement, plasma cyclic guanosine monophosphate levels significantly increased (2.14 ± 0.52 to 3.54 ± 1.2 ng/mL, $P < .0001$), serum ADMA levels were significantly decreased (0.65 ± 0.1 vs 0.59 ± 0.11 μmol/L, $P < .05$), and arganine (Arg) to ADMA ratio was significantly higher (155 ± 53 vs 193 ± 61, $P < .01$). No changes were observed for plasma nitric oxide end products (nitrite and nitrate) levels after GH treatment (21.9 ± 14.9 vs 24.1 ± 19.0 μmol/L, not significant). Basal forearm blood flow remained unchanged, whereas reactive hyperemia increased from 7.30 ± 5.31 mL/100 mL forearm per minute before GH therapy to 13.18 ± 7.30 mL/100 mL forearm per minute after 6 months of therapy ($P < .001$). There was a positive correlation between IGF-1 and cyclic guanosine monophosphate ($r = 0.73$, $P < .0001$), IGF-1 and reactive hyperemia ($r = 0.63$, $P < .0001$), and IGF-1 and Arg/ADMA ratio ($r = 0.44$, $P < .01$). Conversely, a negative correlation was found between IGF-1 and ADMA levels ($r = -0.41$, $P < .02$). At the end of the study period, fat-free mass, plasma glucose, and hemoglobin A_{1c} levels significantly increased, even if they were still in the reference range, suggesting moderate alteration of glucose metabolism. In conclusion, in GHD patients, GH replacement contributes to decreased, to some extent, cardiovascular risk, reducing ADMA levels and improving Arg/ADMA ratio and endothelial dysfunction.
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1. Introduction

The relationship between growth hormone deficiency (GHD) and reduced life expectancy due to cardiovascular and cerebrovascular events has already been demonstrated [1,2]. Growth hormone deficiency determines a premature atherosclerosis throughout a cluster of vascular risk factors such as abnormal lipid profile, abdominal obesity, insulin resistance, hypertension, reduced exercise capacity [3], and increased intima media thickness of carotid arteries [4]. It is known that GHD is characterized by impaired vascular reactivity, associated with a reduced bioavailability of nitric oxide (NO) [5]. On the contrary, it was demonstrated that treatment with human growth hormone (GH) may restore urinary NO and cyclic guanosine monophosphate (cGMP) excretion through an insulin-like growth factor (IGF) 1 stimulation [6]. Recently, these data were reinforced by Ren et al [7] who demonstrated in diabetic hearts that IGF-1

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increases endothelial nitric oxide synthase (eNOS) phosphorylation and eNOS recoupling, with a final result of increasing NO levels.

Asymmetric dimethylarginine (ADMA) is a naturally occurring L-arginine analogue found in plasma and various types of tissues, acting as an endogenous NO synthase inhibitor in vivo [8,9]. Elevated ADMA concentration has been reported in patients with hypercholesterolemia, hypertension, coronary artery disease, diabetes mellitus, chronic renal failure, and hypopituitarism [10]. It was demonstrated that high ADMA concentrations are a crucial factor for the development of endothelial dysfunction and oxidative stress, being considered a key player in the process of chronic vascular disease and a novel predictor of cardiovascular disease [8,11,12]. Recently, a strong relationship was demonstrated between ADMA and coronary and peripheral endothelial dysfunction [13].

To understand the role of GH on systemic NO bioavailability, Thum et al [14] demonstrated that a 10-day supplementation with GH in healthy middle-aged volunteers significantly decreased ADMA levels while increasing cGMP levels. However, to our knowledge, no data are available on the long-term effects of GH therapy in GHD patients on NO availability and endothelial dysfunction. Therefore, the aim of the present study was to evaluate whether a long-term GH replacement might decrease ADMA levels, increase arganine (Arg) to ADMA ratio, and ameliorate endothelial dysfunction in GHD patients.

2. Methods

2.1. Patients and study design

Thirty-one patients (24 male and 7 female) with partial or complete hypopituitarism were recruited from the Endocrine Unit at San Raffaele Hospital. Their mean age was 28 ± 2 years. Sixteen patients were affected by childhood-onset GHD; and 15 patients, by adulthood-onset GHD. Table 1 shows the anthropometric, hormonal, and metabolic characteristics of patients divided in the 2 groups. All but age, total cholesterol levels, and low-density lipoprotein (LDL) cholesterol levels were similar in the 2 groups. Thus, the 2 groups were pooled together; and data about the effects of GH treatment are showed in the whole group.

All patients presented severe *GHD*, defined as a peak GH response less than 9 mU/L after a GH-releasing hormone/Arg test according to the conventional guidelines [15]. All patients were fully stabilized on hormone replacement therapy with corticosteroids, thyroxin, and sex steroids, where appropriate, for at least 6 months; but none was on GH treatment in the preceding 12 months. These treatments remained stable throughout the study period, and no patients were assuming any other drugs. All patients presented a normal kidney function, and no changes were shown during the study period (creatinine levels before: 0.73 ± 0.13 vs after: 0.72 ± 0.12 mg/dL, $P = .6$). None of the patients was known to have a history of chronic or acute inflammatory disorder, acromegaly, or Cushing disease at the time of the study.

The design of the study was an open treatment trial of GH replacement over a 6-month period. All subjects recruited were nonsmokers; and none have a history or evidence of type 2 diabetes mellitus, impaired glucose tolerance, hypertension, or renal failure. Patients were instructed in self-administration of GH using a pen device and in injecting GH subcutaneously at bedtime for 6 months. The dose of GH was at the beginning titrated to get IGF-1 into the reference range. Mean stable GH dose was 4 μg/(kg d).

Informed consent to participate into the study was obtained after the purpose, nature, and potential risk were explained to the subjects. The experimental protocol was reviewed and approved by the local ethics committee.

Before starting GH replacement and at the end of the study period, they were submitted to dual-energy x-ray absorptiometry to measure free fat and fat mass; and body mass index and waist circumference were measured. In the morning after an overnight fast, a blood sample was drawn to measure glucose, insulin, hemoglobin A_{1c} (HbA_{1c}), IGF-1, total cholesterol, high-density lipoprotein (HDL) cholesterol, LDL cholesterol, triglycerides, and endothelial parameters (NO measured as nitrate/nitrite, cGMP, L-arginine, ADMA, and symmetric dimethylarginine [SDMA]).

All subjects kept a diary of food intake for 3 days before measurement of NO and cGMP to control for differences in food intake, measured using Nutritionist Pro 2.5 (Axxya Systems, Stafford, TX). Because L-arginine intake could influence the measurement of nitrate/nitrite levels, the amount of L-arginine consumed was calculated. No differences were found in the daily energy intake of the subjects (2498 ± 180 vs 2515 ± 250 kcal/d, before vs after GH; not significant [NS]) or the proportion of calories as carbohydrate (350 ± 25 vs 360 ± 45 g/d, before vs after GH; NS), lipid (78 ± 15 vs 75 ± 16 g/d, before vs after GH; NS), and protein (99 ± 19 vs 100 ± 11 g/d, before vs after GH; NS). Furthermore, because animal and vegetable L-arginine daily intakes were identical before and throughout the study for all subjects (4500 ± 350 vs 4700 ± 270 mg/d, before vs after GH; NS), it is unlikely that variations in any of these variables would influence the measurement of nitrate/nitrite levels [16].

To evaluate fasting insulin resistance, the homeostasis model assessment (HOMA) index was used. The following formula was applied: glucose (in millimoles per liter) × insulin (in microunits per milliliter)/22.5.

After 30 minutes of rest in supine position, basal forearm blood flow (FBF) and reactive hyperemia of FBF were evaluated using venous occlusion plethysmographic technique. Briefly, the strain gauge was secured to the forearm of the left arm and connected to the plethysmographic device. Two

Table 1
Anthropometrics and hormonal characteristics of GHD patients before GH replacement

	Childhood onset before (n = 16)	Adulthood onset before (n = 15)	*P*	Total population before GH therapy	Total population after GH therapy	*P*
Age (y)	22 ± 4	38 ± 12	.0001	30 ± 7	–	–
BMI (kg/m^2)	25.8 ± 1.5	26.4 ± 1.2	.8	26.1 ± 0.9	26.5 ± 1.1	.7
Waist circumference (cm)	87.8 ± 13.4	91.2 ± 10.6	.5	89.6 ± 11.9	90.9 ± 15.5	.7
Total body weight (kg)	75.6 ± 22.2	77.8 ± 16.7	.7	73 ± 19	75 ± 20	.8
Fat mass (kg)	22.9 ± 11.4	22.5 ± 7.0	.9	22.8 ± 9.4	22.0 ± 11.1	.7
Fat-free mass (kg)	48.9 ± 12.7	52.0 ± 10.0	.5	50.3 ± 11.4	53.0 ± 12.3	.0001
Fasting glucose (mg/dL)	79.6 ± 4.8	84.1 ± 9.4	.1	81.7 ± 7.6	83.9 ± 8.7	.05
Fasting insulin (μU/mL)	8.1 ± 3.6	10.1 ± 6.7	.3	9.0 ± 5.2	7.1 ± 5.1	.1
HbA_{1c} (%)	4.9 ± 0.45	4.9 ± 0.56	.9	4.9 ± 0.4	5.3 ± 0.4	.03
HOMA index	1.58 ± 0.8	2.09 ± 1.6	.6	1.8 ± 1.1	1.7 ± 1.0	.2
Total cholesterol (mg/dL)	203 ± 32	234 ± 50	.05	217 ± 43	205 ± 39	.05
HDL cholesterol (mg/dL)	56 ± 19	50 ± 21	.5	53 ± 20	53 ± 19	.9
LDL cholesterol (mg/dL)	125 ± 10	159 ± 14	.05	137 ± 15	123 ± 22	.003
Triglycerides (mg/dL)	109 ± 61	125 ± 47	.5	116 ± 55	144 ± 70	.1
IGF-1 (ng/mL)	73.6 ± 63.0	104.9 ± 59.7	.2	88.2 ± 62.5	191.7 ± 80.3	.0001
GH peak after GHRH + Arg	1.6 ± 2.2	2.0 ± 1.9	.6	–	–	–
Nitrate/nitrite (μmol/L)	23.0 ± 18.0	20.7 ± 10.9	.7	21.9 ± 14.9	24.1 ± 19.0	.6
cGMP (ng/mL)	2.24 ± 0.57	2.04 ± 0.44	.9	2.14 ± 0.52	3.54 ± 1.2	.0001
L-arginine (μmol/L)	99.2 ± 34.7	96.7 ± 27.9	.8	97.9 ± 31.1	112.9 ± 31.8	.06
ADMA (μmol/L)	0.65 ± 0.09	0.65 ± 0.12	.9	0.65 ± 0.10	0.59 ± 0.11	.05
SDMA (μmol/L)	0.52 ± 0.13	0.49 ± 0.12	.6	0.51 ± 0.13	0.52 ± 0.10	.7
Arg/ADMA	154.9 ± 56.0	154.5 ± 51.1	.9	155 ± 53	193 ± 61	.01
Basal FBF (mL/[100 mL min])	2.03 ± 0.6	2.23 ± 1.1	.8	2.5 ± 1.0	2.6 ± 0.8	.7
Reactive hyperemia (mL/[100 mL min])	3.9 ± 2.6	5.7 ± 3.4	.3	7.30 ± 5.31	13.18 ± 7.30	.001
SBP (mm Hg)	118.6 ± 14.6	112.5 ± 9.6	.5	116.3 ± 12.9	120.0 ± 12.5	.5
DBP (mm Hg)	70.7 ± 9.3	72.5 ± 9.6	.7	71.3 ± 9.0	76.0 ± 5.2	.2

Data are means as mean ± SD. BMI indicates body mass index; GHRH, GH-releasing hormone; SBP, systolic blood pressure; DBP, diastolic blood pressure.

minutes before each measurement and throughout measurement of blood flow, the wrist cuff was inflated to a pressure of 50 mm Hg greater than systolic blood pressure to exclude the hand circulation from the measurement. At the end of this period, the upper arm cuff was inflated to 60 mm Hg; and FBF was immediately measured. To obtain reactive hyperemia, FBF was occluded by inflating the cuff over the left wrist to a pressure of 250 mm Hg for 5 minutes. After release of the cuff occlusion, FBF was measured for 2 minutes; and maximal increase was detected. Three plethysmographic measurements were averaged to obtain FBF at baseline and during reactive hyperemia [17]. This method has been recently validated, and results are reported in Alomari et al [18]. Moreover, it was demonstrated that the measurement of endothelial function by strain gauge plethysmography has a high correlation with brachial artery ultrasound [19].

2.2. Assays

Plasma glucose, total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides were measured with spectrophotometric methods adapted to Cobas MIRA using commercial kits (ABX, Rome, Italy), whereas serum insulin was assayed with a microparticle enzyme immunoassay (IMX; Abbot Laboratories, Rome, Italy). Glycated hemoglobin was assayed using a commercial kit (Unimate; Roche, Basel, Switzerland); serum GH, with a radioimmunoassay (RIA) kit (Sorin; Biomedica, Saluggia, Italy); and serum IGF-1 levels, with an RIA kit (Bioclone IGF-1 kit; Bioclone, Sydney, Australia).

Plasma NO levels were evaluated through the measurement of metabolic end products, that is, nitrite and nitrate. Specifically, NO_3^- was reduced to NO_2^- by 0.1 U nitrate reductase, 5×10^{-5} mol/L flavin adenine dinucleotide, and 250×10^{-6} mol/L nicotinamide adenine dinucleotide phosphate (NADPH). Samples were incubated at 37°C for 3 hours, 8.8 U lactate dehydrogenase and 1022 mol/L pyruvate were added to each well, and the sample was incubated for another 90 minutes at 37°C. Finally, Griess reactives were added to each well; and the sample was read at 540 nm.

Plasma cGMP levels were measured with RIA kits (NEN Life Science Products, Boston, MA).

Serum ADMA and serum SDMA were extracted from samples by cation exchange Strata SCX 100-mg columns (Phenomenex, Bologna, Italy) and assayed by high-performance liquid chromatography as described by Pi et al [20]. The detection limit of the assay for ADMA was 0.05 μmol/L, and the recovery rate was 93%. For ADMA, the intraassay variation was 5% and the interassay variation was 6.5%, whereas for SDMA, the intraassay variation was 7% and the interassay variation was 8.5%. Serum L-arginine levels were assayed by high-performance liquid chromatography after extraction of plasma samples by cation exchange Strata SCX 100-mg columns (Phenomenex).

2.3. Statistical analysis

All values are expressed as mean ± SD at each time interval. To evaluate possible difference at baseline between patients with childhood onset and adulthood onset, a Student *t* test for unpaired data was performed, whereas a Student *t* test for paired data was used to evaluate the effects of GH therapy. A simple Pearson regression analysis was also done. A 2-tailed probability level of less than .05 was considered statistically significant.

3. Results

Because Student *t* test for unpaired data did not show any difference in the baseline parameters of the 2 groups of patients, except for age and total and LDL cholesterol levels, we decided to pool the 2 groups together to evaluate the effects of GH replacement.

Total body weight slightly but not significantly increased, and this increment was due to a significant increase in fat-free mass. The fat mass and waist circumference remained unchanged after GH therapy.

After 6 months of GH therapy, serum IGF-1 levels were normalized (from 88.2 ± 62.5 to 191.7 ± 80.3 ng/mL, $P < .0001$; Table 1). Plasma total and LDL cholesterol levels significantly decreased, whereas plasma glucose and HbA_{1c} levels significantly increased, although these were still in the reference range. Fasting plasma HDL cholesterol, triglycerides, serum insulin, and HOMA levels remained unchanged (Table 1).

After 6 months of GH replacement, plasma cGMP levels significantly increased (2.14 ± 0.52 to 3.54 ± 1.20 ng/mL, $P < .0001$) and serum ADMA levels significantly decreased (0.65 ± 0.1 vs 0.59 ± 0.11 μmol/L, $P < .05$). Serum SDMA remained unchanged, whereas serum L-arginine levels slightly but not significantly increased (97.9 ± 31.1 vs 112.9 ± 31.9 μmol/L, $P < .06$). In addition, Arg/ADMA ratio showed a significant increase (155 ± 53 vs 193 ± 61, $P < .01$). No changes were observed for plasma nitric oxide end products (nitrite and nitrate) levels. Basal FBF remained unchanged, whereas reactive hyperemia was significantly higher, after GH treatment (7.30 ± 5.31 vs 13.18 ± 7.30 mL/100 mL forearm per minute, $P< .001$). No changes were observed for both systolic blood pressure and diastolic blood pressure (Table 1).

The increment of serum IGF-1 levels after GH therapy significantly correlated with plasma cGMP increment ($r = 0.73$, $P < .0001$), reactive hyperemia increment ($r = 0.63$, $P < .0001$), Arg/ADMA increment ($r = 0.44$, $P < .01$), and serum ADMA decrement ($r = -0.41$, $P < .02$) (Fig. 1).

4. Discussion

We demonstrated that GH treatment restores IGF-1 levels, decreases ADMA levels, and increases Arg/ADMA ratio in patients affected by GHD, while also improving endothelial function.

Because ADMA inhibits vascular NO production, previous studies have found that even small modifications of ADMA levels could significantly change vascular tone and systemic vascular resistance [21]. The presence of enhanced

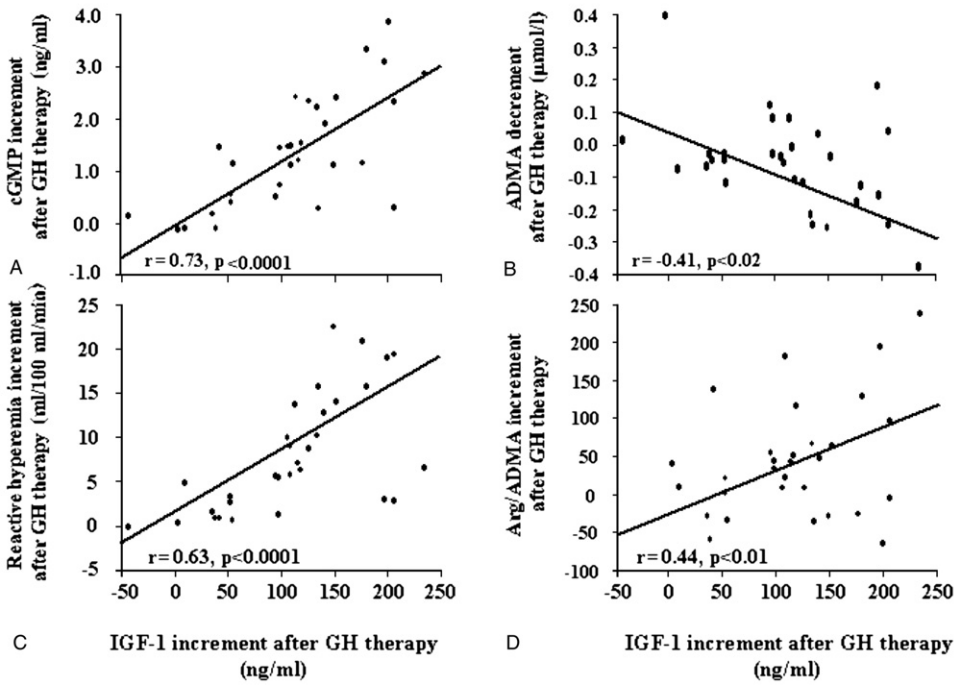

Fig. 1. Relationship between IGF-1 increment after GH therapy and cGMP increment (A), ADMA increment (B), Arg/ADMA increment (C), and reactive hyperemia increment (D) at the end of 6 months of GH therapy.

ADMA levels are frequently associated with critical atherosclerotic processes in which oxidative stress is enhanced, and its levels have been strongly correlated with intima media thickness and considered predictive of cardiovascular events and/or mortality [22]. Clinical studies documented a strong correlation between increased ADMA levels and cardiovascular morbility and mortality [11,23,24]. Thum et al [14] found a significant increase of cGMP accompanied by a decrease of plasma ADMA levels in 16 healthy middle-aged volunteers treated for 10 days with recombinant human GH. The present study is confirmatory of the previous one on the role of IGF-1 on NO bioavailability. The novelty is that, in the present study, GH treatment was prolonged for 6 months in GHD patients, with a decrement of ADMA levels and a significant correlation between IGF-1 and both cGMP and ADMA levels.

Recent studies have suggested that Arg/ADMA ratio is a good predictor of endothelial function and cardiovascular risk [25,26]. The levels of Arg/ADMA ratio found in GHD patients are comparable with those found in patients with cardiovascular, cerebrovascular, and metabolic diseases as reported by Bode-Boger et al [27]. In contrast with the previous study in which ADMA did not decrease but only arginine increased [28], in the present study, ADMA decreased significantly, whereas L-arginine levels were slightly but not significantly higher. The positive correlations between IGF-1 and cGMP and between IGF-1 and Arg/ADMA ratio strongly support a role of long-term GH treatment in ameliorating endothelial dysfunction in GHD patients. This is in line with previous studies demonstrating that GH supplementation in GHD patients resulted in significant reduction in total peripheral resistance via increased NO bioavailability [5,6,29], whereas in patients with dilated cardiomyopathy, GH substitution significantly improved NO production and vascular dysfunction [30].

The fact that NO did not increase whereas a significant increment of cGMP was observed after GH treatment is in agreement with Boger et al [6] who demonstrated that a treatment with recombinant human GH was able to normalize urinary cGMP excretion, through an IGF-1 stimulation of endothelial NO production. Moreover, Evans et al [31] demonstrated that GH replacement could improve endothelial function and oxidative stress in GHD patients. Indeed, both GH and IGF-1 induce an up-regulation of eNOS, increasing NO bioavailability in coronary arteries in rats [32]. The results of the present study of a 2-fold increase in reactive hyperemia after GH replacement are in line with those of Napoli et al [33] in which GH determined an acute effect on FBF, inducing vasodilation and enhancing endothelial sensitivity to vasodilating agents in vivo. All in all, these data support an improvement of endothelial dysfunction by GH treatment. However, some caution has to be taken because the present study is a nonblinded trial without placebo control.

Growth hormone treatment improved lean body mass in agreement with several previous studies [3] but significantly increased fasting glucose levels and HbA_{1c} in the presence of unchanged insulin levels. The elevations in glucose and in HbA_{1c} levels, although of statistical evidence, were not of clinical relevance because glucose and HbA_{1c} levels remained in the reference range. Recently Gotherstrom et al [34] demonstrated an increase in blood glucose and HbA_{1c} in the range reported by the present study after 12 months of GH therapy. Interestingly, these metabolic variables did not further deteriorate during a 10-year prospective study. Furthermore, it has been reported that GHD patients without GH treatment showed a significant increment in glucose levels and insulin resistance over a 60-month follow-up, suggesting that it is the natural history of GHD patients to develop impaired glucose tolerance independent of GH replacement [35].

In conclusion, long-term GH treatment in GHD patients improves endothelial function and has a potential vasoprotective effect, decreasing ADMA levels and increasing Arg/ADMA ratio, through its effect on IGF-1, suggesting a potential beneficial influence on cardiovascular risk factors. The Arg/ADMA ratio might be a new index in the evaluation of the beneficial cardiovascular effects of GH, but long-term double-blind clinical trials are necessary.

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