Sixteen Weeks of Hexarelin Therapy in Aged Dogs: Effects on the Somatotropic Axis, Muscle Morphology, and Bone Metabolism

Silvano G. Cella,1 Cesare G. Cerri,2 Sergio Daniel,3 Valeria Sibilia,1 Antonello Rigamonti,1 Lorena Cattaneo,1 Romano Deghenghi,4 and Eugenio E. Müller 1

1Department of Pharmacology, University of Milan, Italy.
2Institute of Biomedical Sciences, “San Gerardo Hospital,” Monza, Italy.
3Institute of Neurology, “C. Besta,” Milan, Italy.
4Europeptides, Argenteuil, France.

Hexarelin (HEXA; 500 μg/kg/die, s.c.) was administered for 16 weeks to six old beagle dogs. The treatment consisted of three on-drug periods spaced by two off-drug periods. During each on-period, the growth hormone (GH) peak response to HEXA initially increased and then dropped to pretreatment values. Each time, a wash-out interval restored the same pattern of GH responsiveness. HEXA significantly augmented the indices of spontaneous pulsatility of GH, but plasma insulin-like growth factor I levels did not change during treatment. HEXA apparently reduced bone resorption since it significantly decreased the urinary concentration of lysylpyridinoline, a bone matrix component. Bone formation apparently was not affected since unchanged levels of alkaline phosphatase were recorded. In three of six old dogs, HEXA induced an improvement of some morphological and biochemical muscular indices, evaluated in muscle specimens that, instead, remained unchanged in a group of young untreated controls. These findings indicate that HEXA effectively releases GH and primes the pituitary of old dogs, and strengthen the view that in aging, GH secretion may be restored by pharmacological means. It would also appear that HEXA-induced GH release improves some indices of body composition in old dogs.

A REDUCED function of the somatotropic axis is a characteristic feature of normal aging. In most individuals of different animal species and humans, circulating concentrations of growth hormone (GH) decline with advancing age (Finkelstein et al., 1972; Sonntag et al., 1980; Rudman et al., 1981; Kahler et al., 1986; Cella et al., 1989), as do the pituitary GH responsiveness to a variety of provocative stimuli (Shibasaki et al., 1984; Ceda et al., 1986, 1990) and the plasma levels of somatomedin-C [insulin-like growth factor I (IGF-I); Rudman et al., 1981; Vermeulen, 1987]. These alterations are paralleled by changes in body composition, including a decrease of lean body mass, muscle mass, and strength and loss of bone mineral (Hoffman et al., 1993). Hence the proposition has been made that the somatic changes may be related, at least in part, to GH deficiency (Rudman, 1985), and preliminary attempts have been made to substitute for the declining somatotropic function in the elderly by administration of exogenous GH (Rudman et al., 1990).

Caution, however, should be exercised in initiating a long-term therapy with GH in aged subjects, in view of the potential adverse effects of such treatment, which mainly consist of carbohydrate intolerance, sodium and water retention, symptoms of carpal tunnel compression, hypertension, and arthralgias (Underwood, 1988). Moreover, based on the ability of the aged pituitary to respond normally to pharmacological stimuli acting at the hypothalamus and/or the pituitary (Sonntag et al., 1980; Franchimont et al., 1989; Jovino et al., 1989; Panzeri et al., 1989; Corpas et al., 1992; Cella et al., 1993), it may be hypothesized that stimulation of endogenous GH secretion may help better than exogenous GH administration to counter structural and functional alterations related to somatroph deficiency in the elderly. Thus, it would be of interest to explore alternative therapeutic strategies aimed at activating endogenous GH secretion.

The recent availability of a new class of small peptides, GHRPs (GH-releasing peptides), which consist of 6 or 7 amino acids, whose prototype compound is the hexapeptide GHRP-6 (His-D-Trp-Ala-Trp-D-Phe-Lys-NH2) (Momany et al., 1981), seems very promising in this regard. The novel GHRP-6 analog His-D-2-methyl-Trp-Ala-Trp-D-Phe-Lys-NH2 [hexarelin (HEXA)] shows a superimposable but more sustained activity than the parent compound (Deghenghi et al., 1994). Recently, we have described the GH-releasing effect of single HEXA administration in the dog (Cella et al., 1995), a species that behaves like humans in many aspects of GH regulation (Cocola et al., 1976). HEXA was greatly effective as GH releaser also in the aged animals, an observation that dictated this study of the effects of a long-term treatment with the peptide in old dogs. Major end points of this study were the function of the GH/IGF-I axis and some indices of bone and muscle metabolism.

MATERIALS AND METHODS

Animals. — Six old (age, 11–17 years; weight, 10–15 kg; two male and four female), well-trained beagle dogs were used in this study. An additional group of four young (age,
untreated dogs was used exclusively as controls for the muscle biopsies. Animals were fed normal dry food (Diete Standard, Charles River, Calco, Italy) with water available ad libitum. They were on a 12-h light/12-h dark regimen, with light on at 0700. Body weights of all dogs were stable and they had no observable disease. All the experimental procedures were in accordance with the protocol previously authorized by the Committee on Animal Care and Use of the University of Milan.

Treatment schedule. — HEXA was administered once daily at the dose of 500 μg/kg, s.c. The whole treatment period lasted 16 weeks and was constituted by three on-drug periods (7, 4, and 1 weeks, respectively) spaced by two wash-out periods. The schedule of treatment was determined by monitoring at intervals (0, 1, 2, 4, 6, 9, 10, 11, 12, 15, and 16 weeks) the peak GH response to an acute challenge with HEXA: when the responsiveness to the peptide dropped, the treatment was interrupted by a 2-week wash-out period and then resumed.

Acute HEXA testing. — Starting at 0900 of the established day (see Results) and after two baseline blood samples had been taken (−30 and −15 min), dogs received the daily dose of acid-extracted (Daughaday et al., 1987) basal (−15 min) blood was collected into tubes containing EDTA and immediately chilled. Plasma was obtained through an indwelling, nonthrombogenic intravenous catheter (Venosystem Butterfly-10; Abbott Ireland Ltd, Sligo, Republic of Ireland). The needle was positioned in the cephalic vein and fixed with an adhesive bandage 1 h before starting the experiment; the cannula was kept open by slow infusion of normal saline. In total, less than 40 ml blood was removed.

GH secretory pattern. — Before treatment and 24 h after its end, blood samples (1 ml) were taken from dogs which had fasted since 1600 of the preceding day, every 10 min for 6 h between 0900 and 1500, to study the basal GH secretory pattern, as described (Cella et al., 1989). Samples were obtained through an indwelling, nonthrombogenic intravenous catheter (Venosystem Butterfly-10; Abbott Ireland Ltd, Sligo, Republic of Ireland). The needle was positioned in the cephalic vein and fixed with an adhesive bandage 1 h before starting the experiment; the cannula was kept open by slow infusion of normal saline. In total, less than 40 ml blood was removed.

GH radioimmunoassay. — Blood was collected into tubes containing EDTA and immediately chilled. Plasma was frozen until assayed for GH by a double-antibody radioimmunoassay. Highly purified canine GH (batch AFP 1983b; Pituitary Hormones and Antisera Center, Torrance, CA), obtained through the courtesy of Dr. A. F. Parlow, was used for iodination and as a standard. The intraassay coefficients of variation were 3.8% and 4.1% at concentrations of 12.5 and 3.1 ng/ml, respectively. To avoid possible interassay variation, all samples of a given experiment were assayed in the same assay run period high pressure liquid chromatography, following a preliminary hydrolysis with 12 M HCl and fractionation of the samples using CFI partition chromatography, according to the method of Black et al. (1988). The fluorescence was monitored by a spectrofluorimeter with excitation at 295 nm and an emission at 400 nm. The intra- and interassay variations were 2.58% and 9.27% for HP and 4.73% and 10.4% for LP, respectively. Analytical recovery ranged from 90% to 96% for both analytes. The amounts of HP and LP were related to creatinine excretion and are expressed as pmol/mol creatinine. Before hydrolysis, urinary creatinine was measured colorimetrically by the Jaffe’s method using a commercial kit provided by Biochemia (Boehringer, Mannheim, Germany).

Total serum alkaline phosphatase (AP) levels were determined using a commercial kit (Granustest 3) provided by Merk Diagnostica (Darmstadt, Germany).

Muscle study. — Three months before and 1 week after the end of treatment, old dogs and a group of four young untreated dogs which served as controls underwent muscle biopsy of the vastus lateralis muscle. Biopsy specimens (about 4 × 4 × 10 mm) were obtained under general anesthesia induced with xylazine (Rompun, Bayer Italia, Milan, Italy; 0.8 ml/10 kg BW, i.m.) and ketamine (Inoketam 500, Virbac, Milan, Italy; 0.3 ml/10 kg BW, i.m.). The samples were immediately frozen and stored in liquid nitrogen. Cryostat sections were prepared, and the following hystological and histochemical reactions performed: hematoxylin and eosin, modified Gomori’s trichrome, NADH, succinic dehydrogenase, Schiff’s periodic acid reaction, phosphomyrase, acid phosphatase, oil Red O, and ATase at pH 9.4, 4.6, and 4.3.

Two slices from each dog were examined by two independent observers, and a complete report was given following the usual guidelines for human muscle biopsy reports (Dubowitz and Brooke, 1982). In the second biopsy, performed after the treatment, the observations were rated as improved, unmodified, or worsened in comparison to the first biopsy. Scoring (mean of the subjective scores by the two independent observers) was based on (1) conservation of the normal...
hystoenzymatic structure, (2) amount of perimisial connective tissue, (3) presence of clustered lipid droplets, (4) alteration of the normal distribution of cellular enzymes, and (5) presence of atrophic fibers.

For quantitative studies (fiber diameter, percentage of connective tissue, number of lipid droplets), a computerized image analyzer with a camera directly mounted on the microscope and an acquisition system driven by a Macintosh computer and IMAGE software (obtained from the National Institutes of Health, and written by Wayne Rasband) were used. Each slice was firstly seen at lower magnification to set the center of the analysis field to coincide with the center of the slice. Then, the magnification was increased to ×400 to get the first image. Once the microscope image was acquired from the computer, a rectangle with the lower left corner at 100,100 and the upper right corner at 300,400 was selected for analysis. Then the microscope field was moved up vertically and then leftward and downward and each time the acquisition procedure was repeated to get the second, third, and fourth samples. Quantitative studies were done on ATPase slices and hematoxylin-eosin slices. On ATPase slices, the density of staining, the area, the perimeter, and the major and minor axes of the single fibers were measured. On hematoxylin-eosin slices, the relative amounts of connective and muscular tissues were measured.

Data analysis. — The patterns of GH secretion were analyzed by the “cluster analysis” method, directly derived from Veldhuis and Johnson (1986), to search for significant increases and significant decreases within the data series. A significant increase was judged in relation to a specified nadir width by using a moving nadir that began at onset of the experimental series. The individual values making up the nadir cluster were compared against the values making up a possible peak, defined as a second set of consecutive samples of specified number immediately after the test nadir (cluster size: two nadir values against two peak values). Nadir and peak cluster were compared by a pooled $t$-test using the actual experimental replicates present in the test nadir and peak (3 replicates/point). A peak was considered to have occurred only when there was significant increase followed by a significant decrease. The areas under the GH plasma concentration vs time curve (24-h integrated mean growth hormone levels [IC-GH], peak area) were calculated by the linear trapezoidal method.

Statistical comparisons were performed with the distribution free methods Mann-Whitney U-test and Kendall S-test (Kendall, 1955). Longitudinal data were evaluated by Tukey test. Proportion comparison was done with $\chi^2$ test.

RESULTS

Acute HEXA testing. — The mean of individual GH maximal responses (peaks) to the acute HEXA challenge were $32.0 \pm 13.8$ ng/ml before treatment, $66.6 \pm 23.3$ ng/ml after the first off-period and $88.9 \pm 30.8$ ng/ml after the second off-period (Figure 1). In all dogs, plasma GH levels attained the peak value between 15 and 30 min after the injection of the peptide and, in all but one dog, they were still elevated after 60 min.

The mean peak GH response to HEXA progressively increased during the first month of treatment, reached its maximum value after 4 weeks ($79.7 \pm 22.7$ ng/ml, $p < .01$ vs baseline), and then, at the 6th week, dropped to values lower than before treatment ($15.9 \pm 5.4$ ng/ml). After the first off-period (9th week), the treatment was resumed and the mean peak GH response to HEXA, already elevated, remained unchanged during the subsequent week (maximum value at the 10th week: $67.1 \pm 19.4$ ng/ml, $p < .01$ vs baseline), then declined more rapidly than during the first treatment cycle (nadir value: $23.7 \pm 11.2$ ng/ml at the 12th week). At the last treatment reinstitution (15th week), GH responsiveness to acute HEXA was superimposable on that occurring during the first two cycles of treatment ($88.9 \pm 30.8$ ng/ml, $p < .01$ vs baseline) and remained unaltered during the subsequent week (Figure 1).

GH secretory pattern. — Under basal conditions, in only three out of six dogs was the GH secretory pattern pulsatile (one spontaneous burst of GH secretion each, during the entire 6-h period). After treatment all dogs had a pulsatile GH secretion and at least two spontaneous peaks were recorded for each dog during the sampling period (Figure 2). Chronic administration of HEXA also increased the mean amplitude and area of the spontaneous GH bursts (Table 1). A trend toward an increased IC-GH was observed following the treatment, but due to the high interindividual variability, it did not reach statistical significance from the baseline value (Table 1).

Plasma IGF-I levels. — During treatment, baseline plasma levels of IGF-I did not increase significantly from pretreatment values at each time interval considered (Figure 3).

Markers of bone resorption and bone formation. — Chronic administration of HEXA did not modify HP but
Figure 2. Spontaneous GH secretory pattern in old dogs before (closed circles) or after (open circles) 16-week treatment with HEXA. Blood samples were taken every 10 min for 6 h (from 0900 to 1500). Asterisks indicate the GH peaks detected by cluster analysis.

Table 1. Growth Hormone Secretory Pattern During a 6-h Period in Six Old Dogs Under Baseline Conditions and After 16 Weeks of Treatment With HEXA

<table>
<thead>
<tr>
<th>IC-GH (ng/ml)/6 h</th>
<th>Mean Peak Frequency (n/6 h)</th>
<th>Mean Peak Amplitude (ng/ml)</th>
<th>Mean Peak Area (ng/ml)/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>184.6 ± 13.8</td>
<td>0.5 ± 0.2</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td>HEXA</td>
<td>340.5 ± 78.2</td>
<td>3.0 ± 0.4**</td>
<td>4.5 ± 0.9***</td>
</tr>
</tbody>
</table>

Note: HEXA was administered at the daily dose of 500 μg/kg, s.c. Each point is the mean ± SEM of six determinations made in duplicate. 
*p < .05 vs baseline; **p < .01 vs baseline.
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Figure 3. Plasma-free IGF-I concentrations in old dogs evaluated before and during treatment with HEXA. Shown is the mean ± SEM of six determinations made in duplicate.

caused a significant decrease of LP (Figure 4), thus suggesting a protective effect of the peptide on bone mass through a reduction of bone resorption. The inhibition of bone resorption seemed uncoupled to any effect on bone formation, since AP was not modified by treatment (Figure 4).

Muscle study. — Before treatment, no structural alterations were found in young dogs, whereas in four out of six old dogs angulated fibers, fiber type disproportion (with predominance of type 2 fibers), and fiber grouping were present. The percent amount of connective tissue in young dogs was similar to that of human normal skeletal muscle. Three old dogs showed a moderate increase and two showed hypertrophy of the perimisial connective tissue. In contrast to young dogs, all aged animals showed an increase in size and number of lipid droplets, which were irregularly distributed and showed a tendency to cluster.

The enzymatic activities were similar in both groups, with the exception of a tendency to a peripheral distribution of oxidative activity in old dogs. In addition, they had some atrophic fibers (evenly distributed among the different fiber types), whereas in the young dogs only two had some muscle fibers with reduced diameter, but none had atrophic fibers (Figure 5).

At the second biopsy, one young dog presented with some structural alterations (clustering of fibers according to the type) and a moderate increase of connective tissue, and two other dogs had few atrophic fibers. In the remaining dogs, no alterations were evident. All the other muscular indices were unchanged in young dogs. The same three dogs had a slight increase in the fiber diameter (mean, 5%) between the two biopsies, whereas in the remaining dogs this index was unchanged (Table 2 and Figure 5).

Side effects. — HEXA was well tolerated and did not induce noticeable side effects in any of the dogs studied.

DISCUSSION

These data confirm and broaden previous findings (Cella et al., 1995) by showing that HEXA is a very effective GH releaser in old dogs. Moreover, they reemphasize the concept that the defective GH secretion in aged mammals is not similar to that of young dogs. The same three dogs had a slight increase in the fiber diameter (mean, 5%) between the two biopsies, whereas in the remaining dogs this index was unchanged (Table 2 and Figure 5).
Figure 5. ATPase staining of muscle specimens from a representative young (left column, A and C) or old (right column, B and D) dog, before (upper row, A and B) or after (lower row, C and D) treatment with HEXA. (a) connective atrophic tissue; (b) atrophic fibers. See text for further details.

Table 2. Effect of 16-Week Treatment With HEXA on Indices of Muscular Function in Six Old Dogs Compared to Spontaneous Changes Occurring Over the Same Time Lapse in Four Young Untreated Dogs

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Dogs (sex)</th>
<th>Structure</th>
<th>Connective</th>
<th>Lipids</th>
<th>Enzymes</th>
<th>Atrophy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>1 (M)</td>
<td>-1</td>
<td>-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3 (F)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-1</td>
</tr>
<tr>
<td></td>
<td>5 (F)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-1</td>
</tr>
<tr>
<td></td>
<td>7 (M)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Old</td>
<td>2 (F)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8 (F)</td>
<td>-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>9 (F)</td>
<td>-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10 (M)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+1</td>
</tr>
<tr>
<td></td>
<td>11 (M)</td>
<td>0</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td></td>
<td>12 (F)</td>
<td>0</td>
<td>-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Notes: Biopsy specimens of vastus lateralis muscle were obtained before and after the end of treatment. Scoring (mean of the subjective scores provided by two independent observers) was based on (1) conservation of the normal histoenzymatic structure; (2) amount of perimisial connective tissue; (3) presence of clustered lipid droplets; (4) alteration of the normal distribution of cellular enzymes; and (5) presence of atrophic fibers. See text for further details. Variables are as follows: 0, no variation between biopsies; +1, second biopsy improved; and -1, second biopsy worsened.

an irreversible event (Cella et al., 1989, 1993), but may be restored by a proper stimulation of the pituitary gland.

During the first month of treatment, the GH response to the acute HEXA challenge progressively increased. Moreover, despite the 2-week off-periods, the GH response to HEXA was still higher than pretreatment. These events are likely due to the ability of the GHRPs to prime the pituitary and to stimulate not only GH release, but also GH synthesis (Locatelli et al., 1994). In this context, it is noteworthy that GHRP-6 increased infant GHRH-deprived rat pituitary GH mRNA
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(GHRH) treatment was effective in restoring the amplitude of the spontaneous GHRH bursts (Cella et al., 1993).

After the initial progressive rise in the GHRH responsiveness to HEXA, the GH response to acute HEXA challenge declined to pretreatment values, or even less, in the forthcoming weeks.

Occurrence of a defect in receptor and/or postreceptor mechanisms, due to the prolonged stimulation with a huge dose of the peptide, is the most likely cause of this pattern. A similar event has been already shown in humans for GHRH (Davis et al., 1986) and GHRP-6 (Huhn et al., 1993) and in rats for GHRH, both in vivo (Wehrenberg et al., 1984) and in vitro (Badger et al., 1984; Bilezikjian and Vale, 1984; Ceda et al., 1985). Depletion of pituitary GH stores, decreased GHRH binding capacity of the somatotrophs and decreased sensitivity to GHRH, which was reversible after a 24-h withdrawal, were present in cultured rat pituitary cells exposed for 24 h to GHRH (Bilezikjian et al., 1986).

In our study, a withdrawal of the peptide for 2 weeks restored initial GH responsiveness. It is noteworthy, however, that after the first off-period, reinstitution of HEXA treatment was followed by an earlier blunting of the GH response.

Like humans, dogs have spontaneous bursts of GH secretion, whose amplitude and frequency progressively decrease with aging (Takahashi et al., 1981; Cowan et al., 1984). We have shown previously that combined administration of GHRH and clonidine, an α2-adrenergic agonist, which would also modulate the release of somatostatin (Valcavi et al., 1988; Arce et al., 1990), evoked in old dogs a GH secretory pattern reminiscent of the physiological pattern of secretion (Cella et al., 1993). Reportedly, modulation of somatostatin secretion would be mandatory to establish a GH secretory pattern of increased pulse frequency (Müller, 1987). In our hands, prolonged administration of GHRH alone did not affect GH pulse frequency in old dogs (Cella et al., 1993). Chronic administration of HEXA, instead, increased both GH frequency and amplitude, a result that would indicate that the peptide acts to influence the secretion of both hypothalamic GHRH and somatostatin.

The mechanism(s) through which the GHRPs influence GH secretion in mammals is still the object of debate and will not be considered here. Suffice to say that HEXA, whichever its precise mechanism of action, was greatly effective in “rejuvenating” the GH secretory pattern in old dogs. Such a proposition is likely more valid when considering that the time lapse selected to evaluate the GH secretory pattern (0900–1500h) was unfavorable in detecting significant changes of GH secretion in old dogs. As in humans, GH secretion in dogs also occurs mostly in the night hours (Winer et al., 1990).

Despite the enhanced pulsatile GH release, chronic administration of HEXA did not affect plasma IGF-I levels. Similarly, in elderly humans, sustained administration of GHRH (Blackman et al., 1994) or GHRP-6 (Ghigo et al., 1994a), despite a persistent GH-releasing effect of the peptides, did not significantly increase plasma IGF-I levels. In view of the foregoing, our findings may be due to a defective IGF-I synthesis in and/or secretion from the liver. Supporting this proposition are data showing that exogenous GH induces a lower increase in plasma IGF-I levels in elderly than in young subjects (Liebman et al., 1992).

In spite of the lack of any significant effect in circulating IGF-I levels, HEXA exerted biological effects in the old dogs (see below), suggesting that the enhanced pulsatile secretion of GH augmented the local production of IGF-I, which acted in an autocrine/paracrine fashion to modulate cells activity (D’Ercole et al., 1984). Therefore, it may be hypothesized that in old dogs the action of GH on tissue production of IGF-I is better preserved than that on circulating IGF-I. This hypothesis is supported by the finding that in elderly humans undergoing exercise, plasma GH concentrations were unaltered but the muscular concentrations of mRNAs encoding the IGFs and their receptors were increased (Hoffman et al., 1993).

Old dogs have bone remodeling processes similar in many aspects to those in elderly humans (Frost, 1964; Javorski et al., 1980), and their loss of substantial amounts of bone (Williams and Kelly, 1982) is likely due to the prevailing activity of bone resorption versus bone formation.

In adult dogs GH, administered at the dose of 0.5 mg/kg/day for 84 days, led to marked increases in bone mass (Harris and Heaney, 1969). In elderly humans, however, despite observation that prolonged GH treatment increased the indices of osteoblastic activity, some authors failed to demonstrate any significant changes in bone mineral density (Holloway et al., 1994), whereas others did find this effect (Rudman et al., 1990).

Our present data would indicate that HEXA treatment did not affect osteoblastic activity, as assessed by plasma concentration of AP, a marker that, though not highly specific, in the dog is nevertheless positively correlated with the histological observation of increased bone formation (Li et al., 1990). A possible reason for the failure of HEXA to influence osteoblastic activity may reside in the age of the animals studied. In fact, it has been reported that, in the rat, signals that are osteogenic in the young-adult skeleton are hardly acknowledged in older bone tissue, likely due to a deterioration of the bone cell population or a failure of their capacity to respond (Rubin et al., 1992).

On the contrary, HEXA significantly decreased the urinary excretion of LP, a more specific marker of bone resorption than HP, since LP is a specific component of bone matrix (Delmas, 1993), whereas HP is also found in other connective tissues (Eyre, 1987). The inhibitory effect of HEXA on bone resorption might be due to an indirect influence of GH on bone metabolism. In fact, it has been reported that GH potentiated the effects of sex steroids on bone and has a positive effect on calcium metabolism (Wüst, 1993).

Furthermore, IGF-I infused continuously into the arterial supply of the hindlimb of rats for up to 14 days reportedly decreases the number of osteoclasts (Spencer et al., 1991). It is possible, therefore, that IGF-I, locally produced, could be responsible for the inhibitory effect of HEXA on bone resorption.

Old dogs, before treatment, presented some features of muscle involution that, likely, may be attributed to pro-
longed GH deprivation; similar alterations were also described in GH-deficient rats (Ayling et al., 1989). These findings would also agree with the observations that GH-deficient humans have a reduced cross-sectional area and strength of the lower limb muscles (Cuneo et al., 1990; Sartorio et al., 1994) that were significantly increased by a 6-month GH treatment (Sartorio et al., 1994). Under our experimental conditions, the small sample size and the different male to female ratio between old treated and young control dogs prohibited evaluation of sex-related differences. HEXA improved the histochemical picture of the hindlimb muscle in three out of six old dogs, making it almost similar to that of control young-adult dogs. In particular, we observed a percent reduction in connective tissue, an improvement of type-1:type-2 fibers ratio (due to an increased proportion of type-1 fibers), and increased fiber diameter. These effects of HEXA are likely mediated by the enhanced pulsatile GH secretion elicited by the peptide and are reminiscent of those seen following GH in hypophysectomized (Ayling et al., 1989) or intact (Ullman and Oldfors, 1989) rats.

In all, these findings demonstrate that, on a long-term basis, HEXA is an extremely effective GH releaser in old dogs and strengthen the view that in aged subjects declining GH secretion and its biological consequences may be restored by pharmacological means. HEXA was administered at a huge dose once daily, so that after about 1 month of treatment its effect on GH release subsided; however, a 2-week period of drug withdrawal was capable of reestablishing full somatotroph sensitivity to the peptide. The effect of the peptide on the GH secretory pattern was divorced from any effect on circulating IGF-I levels, but was coupled with changes in some indices of bone and muscle function, suggesting an action exerted through locally produced IGF-I. Likely, careful selection of HEXA dose(s), route of administration, timing, and/or pharmaceutical form would enhance further GH secretion and its biological consequences without inducing a state of somatotroph refractoriness. It is too premature to know whether a substitutive therapy with HEXA will be beneficial or, instead, whether reduced GH secretion would be desirable and confer survival advantage. However, if these or similar results would be extrapolated to humans (Arvat et al., 1994, 1995; Ghigo et al., 1994b), it may be hypothesized that a restored pulsatile pattern of GH secretion may help to counter structural and functional alterations related to somatotroph deficiency in the elderly.

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Address correspondence to Dr. Silvano G. Cella, Dipartimento di Farmacologia Medica Università di Milano, via Vanvitelli 32, 20129 Milano, Italy.

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Davis, J.R.E.; Sheppard, M.C.; Shakespear, R.A.; Lynch, S.S.; Clayton,


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