EXPERIMENTAL STUDY

The decrease in hepatic IGF-I gene expression in arthritic rats is not associated with modifications in hepatic GH receptor mRNA

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Abstract

Objective: Adjuvant-induced arthritis induces a catabolic response, and a decrease in circulating IGF-I. Hypermetabolism and GH insensitivity have been described in acute inflammation. The aim of this study was to analyze whether impaired IGF-I secretion in arthritic rats can be attributed to hepatic GH resistance.

Design and methods: Male Wistar rats were injected with complete Freund's adjuvant, and 14 days afterwards arthritic and control rats were injected daily with recombinant human GH (rhGH) (3 IU/kg) or saline for 8 days. GH receptor (GHR) gene expression in the liver and the effect of rhGH on hepatic IGF-I synthesis in arthritic rats were examined.

Results: There was a significant decrease in hepatic concentrations of IGF-I ($P < 0.01$) as well as in the IGF-I gene expression in arthritic but not in pair-fed rats. In contrast, arthritis did not modify GHR mRNA levels in the liver. The 8 day administration of rhGH resulted in an increase in body weight gain in arthritic but not in control rats. There was an increase in hepatic IGF-I synthesis and in GHR mRNA levels after rhGH treatment, both in control and in arthritic rats. Two endotoxin lipopolysaccharide (LPS) (1 mg/kg) injections decreased hepatic concentrations of IGF-I and IGF-I mRNA ($P < 0.01$). Contrary to the results obtained in arthritic rats, mRNA expression of GHR in the liver was lower in LPS- than in saline-treated rats ($P < 0.01$).

Conclusion: These data suggest that the decrease in IGF-I synthesis induced by chronic arthritis is not secondary to GH resistance.

European Journal of Endocrinology 144 529–534

Introduction

Chronic inflammation, as well as sepsis, decreases body weight (BW) by increasing protein catabolism (1), and this catabolic response can be due to neuroendocrine modifications. An increase in the hypothalamic–pituitary–adrenal axis secretion (2), together with a decrease in the somatotrophic axis, has been previously described in rats after lipopolysaccharide (LPS) administration (3, 4) and in adjuvant-induced arthritis (5, 6). These data suggest that the weight loss during chronic inflammation may result, at least in part, from a lack of anabolic hormones such as insulin-like growth factor-I (IGF-I) and growth hormone (GH).

The mechanism by which chronic arthritis inhibits the somatotrophic axis is not well known. A significant decrease in serum concentrations of IGF-I as well as in pituitary GH mRNA content has been described in arthritic rats (5, 6), indicating a decrease in pituitary GH secretion. In humans with rheumatoid arthritis, a significant decrease in GH and IGF-I secretion has also

been described (7, 8). We have recently observed that recombinant human GH (rhGH) administration to arthritic rats can ameliorate the decrease in IGF-I and BW and the increase in circulating IGF-binding proteins (9). Similarly, in children with juvenile chronic arthritis (JCA), rhGH administration partially counteracts the adverse effects of this disease on growth and metabolism (10, 11). These data suggest that chronic arthritis is not associated with GH insensitivity, and the inhibition of pituitary GH secretion can be responsible for the IGF-I decrease and BW loss induced by chronic inflammation. However, there are also data supporting the idea that the inflammatory response induces GH resistance. GH administration to septic patients failed to increase circulating IGF-I levels (12). In rats, endotoxin injection induces a state of GH insensitivity associated with decreased liver GH receptor (GHR), and the decrease in IGF-I synthesis cannot be prevented by GH administration (13).

The aim of this study was to analyze the effect of chronic arthritis and rhGH administration on the

expression of hepatic GHR and IGF-I mRNA. In addition, we compared the effect of arthritis and LPS administration on the somatotrophic axis.

Materials and methods

Adjuvant-induced arthritis

The procedures followed the guidelines recommended by the EU for the care and use of laboratory animals. Rats were housed three or four per cage under controlled conditions of light (lights on from 0730 to 1930 h) and temperature ($22 \pm 2^\circ\text{C}$). Food and water were freely available. Male Wistar rats were purchased from Charles River (Barcelona, Spain). Arthritis was induced by an intradermal injection of complete Freund's adjuvant (1 mg heat-inactivated *Mycobacterium butyricum*; Difco Laboratories, Detroit, MI, USA) at the base of the tail. Control animals were injected with vehicle (paraffin oil). Arthritic and control rats were divided into two groups: one group was injected daily with 3 IU/kg s.c. of rhGH (Saizen, Serono, Italy) from day 14 to 22, and the second group received 250 μl saline. All rats were weighed and the arthritis index score was examined daily. The arthritis index of each animal was scored by grading each paw from 0 to 4, determined as: 0, no erythema or swelling; 1, slight erythema or swelling of one or more digits; 2, entire paw swollen; 3, erythema and swelling of the ankle; 4, ankylosis, incapacity to bend the ankle. The severity score was the sum of the clinical scores of each limb, the maximum value being 16 (14). Food intake per cage was calculated daily by measuring the difference between the initial and the remaining amount of pellets in the feeder, and expressed as grams per rat per 100 g BW. The same amount of food consumed by the arthritic rats was given to the pair-fed group the following day.

Both 20 control and 20 arthritic rats were killed by decapitation 22 days after adjuvant or vehicle injection and after 8 days of GH treatment (2.5 h after injection). Pair-fed ($n = 10$) and their respective control group ($n = 10$) were killed 1 day later. Immediately after decapitation the liver was removed, dissected, frozen in liquid nitrogen and stored at -80°C until RNA extraction and RIA were performed. Hind paws were amputated and paw volume measured by water displacement with an accuracy of 0.05 ml.

LPS administration

The experiment was performed after a 7 day adaptation period. Male Wistar rats ($n = 7$) weighing 250 g were injected i.p. with LPS (*Escherichia coli*, serotype O55:B5; Sigma, St Louis, MO, USA) at 1 mg/kg BW at 1700 h and on the following day at 0830 h. The control group ($n = 7$) received 250 μl sterile saline. Four hours after the last injection, at 1230 h, all rats were killed by

decapitation and livers were collected. This procedure was chosen as a result of a previous experiment carried out in order to find a significant decrease in hepatic concentrations of IGF-I mRNA.

RIA

Liver IGF-I was extracted as described by Torres-Aleman *et al.* (15). Samples were homogenized in 1 M acetic acid, boiled for 20 min, and lyophilized. The IGF-I antiserum (UB2-495) was a gift from Dr Underwood and Dr Van Wyk, and is distributed by the Hormone Distribution Program of the National Institute of Diabetes, Digestive and Kidney Diseases, Bethesda, MD, USA through the National Hormone and Pituitary Program. Levels of IGF-I were expressed in terms of IGF-I A52-EPD-186 standard, kindly provided by Eli Lilly & Company (Madrid, Spain). The intra-assay coefficient of variation was 8%. Samples from one experiment were run in the same assay.

Protein content was measured by Bradford's method (16).

RNA extraction and Northern blot analysis

Total liver RNA was extracted by the guanidinium thiocyanate method using a commercial kit (Ultraspec RNA; Biotecx Laboratories, Houston, TX, USA). The extracted total RNA was dissolved in 0.1% SDS in diethylpyrocarbonate-treated water and quantified at 260 nm. The integrity and the concentration of the RNA were confirmed using agarose gel electrophoresis. For Northern blot analysis, each 20–30 μg sample of total RNA was denatured, separated by formaldehyde-agarose gel electrophoresis, transferred to nylon membranes (Hybond-N+; Amersham International, Amersham, Bucks, UK) by overnight capillary blotting and fixed by UV crosslinking (Fotodyne, Hartland, WI, USA). Homogeneity of gel loading was confirmed by the intensity of the ribosomal 28S RNA bands in the transferred membranes stained with ethidium bromide. A rat IGF-I cDNA (17) was generously supplied by Dr LeRoith, Bethesda, MD, USA. IGF-I mRNA transcripts, as visualized by Northern blot analysis, consist of a group of transcripts ranging from 7.5 to 0.8 kb. Because all these transcripts may potentially be translated to IGF-I, the densitometric results corresponded to the sum of all IGF-I transcripts. The rat GHR cDNA probe was kindly provided by Dr Baumbach (Princeton, NJ, USA) (18), and encodes the GHR and the GH-binding protein (GHBP) mRNA of 4.5 and 1.2 kb; both transcripts were quantified by densitometric analysis and results refer to the total GHR mRNA. The rat IGF-I and GHR probes were derived from a HindIII fragment of the pGEM-3 plasmid vector (Promega, Madison, WI, USA). ^{32}P -labeled RNA antisense probes were generated from linearized plasmid with $[\alpha\text{-}^{32}\text{P}]$ cytidine triphosphate (Nuclear Ibérica,

Madrid, Spain) and T7 RNA polymerase (Roche Molecular Biochemicals, Barcelona, Spain). Prehybridization was performed for 30 min at 68 °C in ULTRAhyb buffer (Ambion, Austin, TX, USA) followed by hybridization for 16 h at the same temperature with 1×10^6 c.p.m./ml IGF-I labeled riboprobe or 2×10^6 c.p.m./ml GHR labeled riboprobe. The membranes were washed twice with $2 \times \text{SSC}$, 0.1% SDS at 68 °C for 10 min, and twice with $0.1 \times \text{SSC}$, 0.1% SDS at 68 °C also for 10 min. Exposure to X-ray film was performed at -80 °C. The intensities of autoradiograph signal levels and bromide-stained ribosomal 28S RNA in the nylon filters were analyzed by densitometric scanning using a PC-Image VGA24 program for Windows (Foster Findlay Associated Ltd, Newcastle, UK).

Statistics

Data are expressed as means $\pm$ S.E.M. and were analyzed by one-way ANOVA followed by multiple comparisons of the means by Tukey's test. Student's *t*-test was used when comparing only two groups (i.e. the LPS experiment). Statistical significance was set at $P < 0.05$.

Results

As shown in Fig. 1, arthritis decreased BW gain, and rhGH administration increased BW gain in arthritic ($P < 0.05$) but not in control rats. Arthritic rats ate 10% less than control animals (9.82 ± 0.32 vs 10.88 ± 0.16 g/100 g BW, $P < 0.01$) during the 8 day treatment. The BW in pair-fed rats was lower than

Figure 1 Effect of adjuvant-induced arthritis and administration of rhGH (3 IU/kg) on BW gain. PF = pair-fed rats injected with saline, C = control rats injected with saline, C-GH = control rats injected with rhGH, AA = arthritic rats injected with saline, and AA-GH = arthritic rats receiving rhGH. Each point represents the mean $\pm$ S.E.M. for at least nine rats per group. * $P < 0.05$, ** $P < 0.01$ vs arthritic rats injected with saline (Tukey's multiple range test).

Table 1 Paw volume and arthritis score index, before and at the end of rhGH treatment (days 14 and 22 after adjuvant injection), in arthritic rats treated with saline or rhGH. Data are expressed as means $\pm$ S.E.M. for at least nine rats per group.

	Arthritis score		Paw volume (ml)
	Day 14	Day 22	
Control+saline	—	—	1.88 ± 0.12
Control+rhGH	—	—	1.86 ± 0.05
Arthritic+saline	6.1 ± 0.4	12.5 ± 0.6	$3.52 \pm 0.25^{**}$
Arthritic+rhGH	6.2 ± 0.6	11.2 ± 0.9	$3.14 \pm 0.21^{**}$

** $P < 0.01$ vs control saline group, one-way ANOVA followed by Tukey's test.

in the control group and similar to that in the arthritic group during the first 3 days of 10% food restriction (Fig. 1). Afterwards, the increment in BW was parallel to that of the control group (37.7 ± 1.16 g between day 3 and day 8 of food-restriction in pair-fed vs 37.6 ± 2.07 g in controls). GH administration to arthritic rats did not modify the arthritis index scores or the paw volume on day 22, 8 days after the rhGH treatment had begun (Table 1).

There was a significant decrease ($P < 0.05$) in hepatic concentrations of both IGF-I and IGF-I mRNA in arthritic rats treated with saline, but the decrease did not seem to be due to lower food intake, since pair-fed rats had levels similar to those of control rats (Fig. 2). GH injections in control and arthritic rats increased hepatic concentrations of both IGF-I and IGF-I mRNA, but these increases had no statistical significance. However, arthritic rats injected with rhGH had IGF-I mRNA levels ($77 \pm 11\%$) intermediate between control ($100 \pm 7\%$) and arthritic rats injected with saline ($55 \pm 7\%$). Arthritis did not modify the liver expression of the GHR gene, since only control and arthritic rats injected with rhGH showed a significant increase in GHR gene expression (Fig. 3).

Two injections of LPS decreased hepatic concentrations of IGF-I (0.97 ± 0.25 vs 2.3 ± 0.25 ng/mg protein, $P < 0.01$). Fig. 4 illustrates IGF-I and GHR expression in LPS- or saline-treated rats. LPS-injected rats showed a marked decrease in IGF-I mRNA ($63 \pm 5.5\%$ over control values vs $100 \pm 7.8\%$ in controls, $P < 0.01$) and in GHR mRNA in the liver ($58 \pm 6.0\%$ over control values vs $100 \pm 2.3\%$, $P < 0.01$).

Discussion

As we have previously reported (9), adjuvant-induced arthritis decreases BW, whereas GH administration to arthritic rats is able to stimulate BW gain. Our results show that arthritic rats had a reduction in hepatic IGF-I as well as in IGF-I mRNA concentrations, which suggests a decrease in IGF-I synthesis. We found that arthritis did not significantly modify hepatic GHR mRNA. Furthermore, the fact that rhGH is able to

Figure 2 Effect of adjuvant-induced arthritis and rhGH administration on hepatic IGF-I and IGF-I mRNA. Upper panel: liver IGF-I concentrations, values represents means $\pm$ S.E.M. of eight rats per group. Middle panel: liver IGF-I mRNA levels, data from four to eight individual rats were quantified by densitometry and expressed as a percentage of the mean value in control rats treated with saline. PF = pair-fed rats injected with saline, C = control rats injected with saline, C + GH = control rats injected with rhGH, AA = arthritic rats injected with saline, and AA + GH = arthritic rats receiving GH. * $P < 0.05$ vs control group (Tukey's multiple range test). Lower panel: a representative Northern blot analysis showing the 7.5, 1.7 and 0.9 kb IGF-I transcripts and an ethidium bromide stained ribosomal 28S RNA in each sample.

increase hepatic IGF-I synthesis in arthritic rats is in accord with the lack of modifications in GHR gene expression in arthritic rats treated with saline. All these results suggest that there is no GH resistance in the liver of the arthritic rat.

Fasting is a well-known inhibitory stimulus on IGF-I synthesis and insensitivity to GH has been reported in rats subjected to prolonged protein-calorie malnutrition (19). In the present study, the decrease in food intake in arthritic rats does not seem to be responsible for the decrease in hepatic synthesis of IGF-I, since pair-fed rats had IGF-I levels similar to those of control rats. A possible explanation of this discrepancy could be the degree of food restriction. Arthritic rats ate 90% of the food intake of controls, whereas most of the studies performed to evaluate the effect of food restriction

Figure 3 Hepatic GHR mRNA levels in: pair-fed rats injected with saline (PF), control rats injected with saline (C), control rats injected with rhGH (C + GH), arthritic rats injected with saline (AA), and arthritic rats receiving rhGH (AA + GH). The upper panel shows GHR mRNA abundance determined by densitometry and expressed as a percentage of the mean value in control rats treated with saline. Data represent the means $\pm$ S.E.M. for at least four rats per group. * $P < 0.05$ vs control group, ** $P < 0.01$ vs control group (Tukey's multiple range test). The lower panel shows a representative Northern blot analysis of total RNA (30 μ g) hybridized with a 32 P-labeled cRNA probe encoding the GHR/GHBP common extracellular domain, and an ethidium bromide stained ribosomal 28S RNA in each sample.

Figure 4 Northern blot analysis of IGF-I (upper panel) and GHR (lower panel) mRNAs in livers of control (C) and LPS-treated rats. Rats were injected i.p. twice with LPS (1 mg/kg) or saline and killed 4 h after the second injection. The sizes of transcripts are indicated on the left and an ethidium bromide stained ribosomal 28S RNA in each sample is depicted below.

utilized 50–60% of the food intake of controls (20, 21). Food restriction could also be a stressful stimuli *per se*, since in our experiment pair-fed rats did not gain BW during the first 2 days of restriction, and afterwards rats seemed to adapt to the restriction and started to gain weight in a way similar to control rats.

The data show that hepatic GHR expression is differentially regulated by LPS or by adjuvant-induced arthritis, since hepatic GHR mRNA is decreased in LPS-injected rats. It has been demonstrated that sepsis induces hepatic resistance to endogenous GH (22) by decreasing hepatic GHR (13). However, a different study indicated that LPS did not modify hepatic GHR abundance, suggesting a postreceptor resistance (23). The discrepancy between these two studies could be due to different LPS dosages and administrations. In the first study the analysis was performed 10 h after 7.5 mg/kg i.p. LPS, and in the second 4 h after i.p. injection of 1 mg/kg LPS. We administered two injections of 1 mg/kg and analyzed the liver 18 h after the first injection. In these conditions we found that LPS decreases GHR expression in the liver.

Although both LPS administration and adjuvant-induced arthritis are experimental models of inflammation, and both induce an inhibition of the GH–IGF axis, the immune and endocrine responses should not be exactly the same. The different effect of sepsis and chronic arthritis on hepatic GHR can explain the response to exogenous GH administration in both states. GH administration to children with rheumatoid arthritis increases height velocity and circulating IGF-I (24), and in arthritic rats rhGH stimulates BW gain and IGF-I synthesis in the liver (our present data). In contrast, GH partially normalized GHR mRNA, but it did not prevent the endotoxin-induced decrease in hepatic IGF-I mRNA (13).

GH insensitivity in childhood chronic arthritis has been described (25). This effect can be due to the glucocorticoid treatment, since glucocorticoids induce GH resistance by diminishing GHR synthesis (26, 27). However, as mentioned above, GH administration to children with rheumatoid arthritis increases height velocity and circulating IGF-I (24, 28).

GH-primed rats become extremely sick after endotoxin injection, having much more severe organ damage and drastic metabolic disturbances (29). The potentiating effect of GH on the endotoxin response, which leads to an increased lethality, is IGF-I-independent (22). In contrast, in adjuvant-induced arthritis GH administration did not potentiate the inflammatory response. Similar data have been reported in patients with critical illnesses, where high doses of GH are associated with increased morbidity and mortality (30). However, in JCA, in accord with our data, GH administration had no detected effect on disease activity (10, 11). Differences in GH responsiveness with time have also been reported in humans during severe illness (for reviews see (31)). The acute phase

has low IGF-I levels that have been interpreted as GH resistance, which may be related to decreased GHR expression (32). On the contrary, in prolonged critically ill patients the elevated serum levels of GHBP (33), and the rise in serum GH and IGF-I after GH secretagogues (31, 34), indicate a peripheral GH responsiveness.

In summary, our study indicates that the decline in hepatic IGF-I caused by chronic arthritis does not results from decreased liver GHR or GH response. Therefore, rhGH therapy may counteract the harmful effects of chronic inflammation on BW and metabolism.

Acknowledgements

We thank Dr F Santos from the Department of Pediatrics, Faculty of Medicine, University of Oviedo for his help in the setting-up of the IGF-I and GHR Northern blot. We are indebted to A Carmona for technical assistance and to Christina Bickart for correction of the manuscript. This work was supported by grants from Dirección General de Investigación Científica y Técnica (DGICYT PM95-0068) and Fondo de Investigaciones Santitarias de la Seguridad Social (FIS 00/0949) and by a fellowship from FIS to IIC.

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Received 5 October 2000

Accepted 9 January 2001