Endotoxin at low doses stimulates pituitary GH whereas it decreases IGF-I and IGF-binding protein-3 in rats

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Abstract

While it is well known that sepsis inhibits serum IGF-I and its gene expression in the liver, the effect on pituitary GH and IGF-binding protein-3 (IGFBP-3) is poorly understood. The GH-IGF-I-IGFBP-3 response to different doses of lipopolysaccharide (LPS) administration has been investigated in adult male rats. Two experiments were performed, administration of low doses of LPS (5, 10, 50 and 100 µg/kg) and high doses of LPS (100, 250, 500 and 1000 µg/kg). Rats received two i.p. injections of LPS (at 1730 h and 0830 h the following day) and were killed 4 h after the second injection. LPS administration induced a biphasic response in serum concentrations of GH, with an increase at the 10 µg/kg dose, followed by a decrease at higher doses (100 µg/kg on up). Pituitary GH mRNA was also increased by the administration of 10 and 50 µg/kg LPS, whereas at higher doses LPS did not modify pituitary GH mRNA. We also analyzed the GH response to LPS in primary pituitary cell cultures. When exposed to LPS, in the culture medium, there was an increase in GH release at the concentration of 0.1 and 10 ng/ml, whereas more

concentrated LPS did not modify GH release. Serum concentrations of IGF-I declined in a dose-dependent fashion after LPS administration in the rats injected with 10 µg/kg LPS on up. This decrease is secondary to modifications in its synthesis in the liver, since endotoxin injection decreased both IGF-I and its mRNA in the liver. The liver GH receptor mRNA was also decreased by LPS administration, but only in the animals injected with high LPS doses. There was a decrease in both the IGFBP-3 serum levels and its gene expression in the liver with all LPS doses studied. These data suggest a biphasic LPS effect on pituitary GH, a stimulatory effect at low doses and an inhibitory effect at higher doses, whereas it has a clear inhibitory effect on IGF-I and IGFBP-3 synthesis in the liver. The decrease in liver IGFBP-3 mRNA and in serum concentrations of IGFBP-3 in the rats injected with LPS may contribute to the decrease in serum concentrations of IGF-I.

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Introduction

Sepsis, inflammation and critical illnesses are catabolic states that result in a series of changes characterized by protein hypercatabolism and negative nitrogen balance. These situations are associated with several neuro-endocrine changes that include an increase in serum concentrations of glucocorticoids together with a decrease in anabolic hormones. A decrease in serum concentrations of insulin-like growth factor-I (IGF-I) has been described in different inflammatory conditions in humans and experimental animals (Dahn *et al.* 1988, Fan *et al.* 1994). However, the precise mechanism responsible for this inhibition is not well known.

Lipopolysaccharide (LPS) is a component of Gram-negative bacteria, and its administration triggers the sepsis response (Karima *et al.* 1999). LPS administration

decreases the serum concentrations of IGF-I in humans (Lang *et al.* 1997) and experimental animals (Fan *et al.* 1994). We have previously observed a decrease in serum concentrations of IGF-I 3 h after a low dose (25 µg/kg) LPS injection (Soto *et al.* 1998). Although this effect seems to be due to an inhibition of its synthesis in the liver, it is necessary to administer a much higher LPS dose (2500 or 7500 µg/kg), and to measure liver IGF-I mRNA 10 h after LPS administration in order to find a decrease in liver IGF-I mRNA (Defalque *et al.* 1999).

Decreased growth hormone (GH) secretion has been reported to be responsible for the LPS-induced decrease in IGF-I in rodents, but GH resistance has been described in LPS-injected rats mediated by the loss of liver GH receptor (GHR) (Defalque *et al.* 1999). However, an increase in the number of GHRs in the liver has also been reported in septic rats (O'Leary *et al.* 2000).

In rodents, the GH response to LPS or sepsis is more variable than the IGF-I response, since both a decrease (Kasting & Martin 1982, Fan *et al.* 1994, Soto *et al.* 1998) and the absence of modifications (Defalque *et al.* 1999, O'Leary *et al.* 2000) in circulating levels of GH have been described in septic rats. The difference may reside in pulsatile secretion of this hormone. However, in other species such as in humans (Lang *et al.* 1997), sheep (Daniel *et al.* 2002) and pigs (Hevener *et al.* 1997), LPS administration stimulates GH release, albeit it inhibits serum concentrations of IGF-I. Discrepancies can be due to the LPS dose; in humans and sheep the most frequently used LPS dose is less than 1 µg/kg (Coleman *et al.* 1993, Lang *et al.* 1997, Briard *et al.* 2000). In contrast, in rodents the effect of LPS on the somatotrophic axis has been studied employing high LPS doses (1–15 mg/kg), which also induce endotoxic shock.

LPS administration decreases serum concentration of the main IGF-I binding protein, IGF binding protein-3 (IGFBP-3), by a mechanism independent of GH and IGF-I (Priego *et al.* 2003). We have previously reported that LPS administration to Lewis rats decreased serum concentrations of IGF-I, but liver IGF-I or its mRNA in the liver was not modified (Priego *et al.* 2002); these data suggest that LPS may increase IGF-I turnover in serum.

In this study we analyzed the effect of different LPS doses on pituitary GH gene expression and on serum concentrations of GH, IGF-I and IGFBP-3, as well as the GHR, IGF-I and IGFBP-3 gene expression in the liver. Since we previously found a significant decrease in liver GHR, IGF-I and IGFBP-3 mRNA after two LPS injections of 1 mg/kg (Priego *et al.* 2002), in this study we have analyzed the GH-IGF-I-IGFBP-3 response to two LPS injections, at different doses, in order to identify the primary site of inhibition of the somatotrophic axis during endotoxin administration. As the pituitary GH response to LPS can be influenced by the LPS effect on circulating levels of IGF-I, the pituitary GH release *in vitro* after LPS administration was also tested. We describe the lack of correlation between pituitary GH and the IGF-I-IGFBP-3 system in rats injected with a low LPS dose, and the possible role of LPS-induced decrease in circulating IGFBP-3 in decreasing the serum concentrations of IGF-I.

Materials and Methods

Animals

Adult male Wistar rats (200–250 g body weight) were used for the *in vivo* and *in vitro* studies. They were purchased from Harlan (Barcelona, Spain), and maintained under standardized conditions of temperature (20–22 °C) and light (lights on from 0730 to 1930 h), with free access to food and water. The procedures followed the guidelines recommended by the European Union for the care and use of laboratory animals.

LPS administration

Rats were injected with different low doses (0, 5, 10, 50 and 100 µg/kg) and high doses (100, 250, 500 and 1000 µg/kg) of LPS (serotype 055:B5; Sigma Chemical Co, St Louis, MO, USA) in 250 µl sterile saline i.p. at 1730 h and at 0830 h the following day. All animals were killed by decapitation at 1230 h in a separated room, within 30 s after having been removed from their cages. Trunk blood was collected in cooled tubes, allowed to clot, centrifuged and the serum stored at –20 °C until GH, IGF-I and IGFBP-3 analyses were performed. Immediately after decapitation the neurointermediate lobe of the pituitary was removed and the anterior pituitary lobe was frozen in liquid nitrogen and stored at –80 °C until RNA extraction was performed. The liver was removed, dissected, frozen in liquid nitrogen and stored at –80 °C until RNA extraction was performed.

Cell cultures

Pituitaries were obtained within 1 min of killing by decapitation, the neurointermediate lobes were removed, and the anterior lobes were dissected into small pieces and enzymatically dispersed in dissociating buffer containing 0.4% collagenase type I (Sigma), 0.01% DNase I (Boehringer Mannheim, Barcelona, Spain) and 50 µg/ml gentamicin sulfate (ICN Biomedicals, Inc., Aurora, OH, USA) for 45 min at 37 °C and centrifuged at 260 g. Cell suspension was filtered through a nylon mesh (100 µm), centrifuged and washed firstly in sterile cold Ca²⁺-free buffer (119.4 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄ and 25.1 mM NaHCO₃, 1 mM EDTA, 20 mM HEPES pH 7.3), and secondly in sterile cold buffer containing 2.6 mM CaCl₂ and without EDTA. Cellular viability was estimated by the trypan blue test, and was greater than 96%. Cells were resuspended in culture medium RPMI 1640 supplemented with 10% fetal bovine serum (Bio-Whittaker Europe, Verviers, Belgium), 50 µg/ml gentamicin sulfate and 2 mM glutamine (Sigma). Cells (10⁵/well in 500 µl culture medium) were distributed in 24-well tissue culture plates (Nunc, Roskilde, Denmark) and grown at 37 °C with 5% CO₂. After 3 days, cells were washed and incubated for 4 h in 250 µl serum-free RPMI 1640 containing 0.01% BSA, 50 µg/ml gentamicin sulfate and 2 mM glutamine. LPS was dissolved in the assay medium at a 0.1 mg/ml stock solution. The experiments were performed twice.

Hormone determination

Concentrations of GH in serum and in culture medium were measured by an RIA using reagents provided by Dr Parlow of the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK) National Hormone and Pituitary Program, NIH, Bethesda, MD, USA. Levels of

GH were expressed in terms of NIDDK rat-RP-2 standard. The GH detection level was 10 pg, and the intra-assay coefficient of variation was 3%. IGF-I concentrations were measured by a double-antibody RIA using the antibody NIDDK UB2-495, a gift from Drs Underwood and Van Wik, and it is distributed by the Hormone Distribution Program of NIDDK through the National Hormone and Pituitary Program. Serum IGF-I binding proteins (IGFBPs) were removed by an acid-ethanol extraction, liver samples were homogenized in 1 M acetic acid, boiled for 20 min, and lyophilized (López-Calderón *et al.* 1999). Levels of IGF-I were expressed in terms of human IGF-I from Gropep Ltd (Adelaide, Australia). The intra-assay coefficient of variation was 8%. All necessary comparisons between test and control animals were carried out within the same assay.

Western ligand blot

Serum samples (2.5 µl) were subject to 1% SDS-12.5% acrylamide gel non-reducing electrophoresis, and electrotransferred to nitrocellulose membranes (Hybond-C extra; Amersham). The membranes were dried and blocked for 1 h with 5% non-fat dry milk, 0.1% Tween (Sigma) in Tris-buffered saline. Membranes were probed overnight at 4 °C with ^{125}I -IGF-I (5×10^5 c.p.m./ml). The nitrocellulose sheets were then washed, dried and blots were exposed at -80 °C to X-ray film (Kodak X-Omat AR; Eastman Kodak, Rochester, NY, USA) and two intensifying screens for 1–5 days according to the signal obtained. Autoradiographs were analyzed by densitometric scanning using a PC-Image VGA24 (Foster Findlay Associates Ltd, Newcastle upon Tyne, Tyne and Wear, UK) program for Windows. The density of the IGFBP bands in each lane was expressed as the percentage of the mean density of sera from control rats injected with saline.

RNA extraction and Northern blot analysis

Total RNA extraction was performed with a kit (Ultraspec RNA; Biotecx Laboratories, Houston, TX, USA) following the manufacturer's instructions. The integrity and the concentration of the RNA were confirmed using agarose gel electrophoresis. For Northern blotting, 30 µg denatured RNA from livers and 5 µg from pituitaries were separated by formaldehyde-agarose gel electrophoresis, transferred to nylon membranes (Hybond-N+; Amersham) by overnight capillary blotting and fixed by UV crosslinking (Fotodyne, Hartland, WI, USA).

IGF-I and GHR mRNA hepatic levels were measured by Northern blot hybridization using riboprobes (Roberts *et al.* 1987, Baumbach *et al.* 1989). 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 [α - ^{32}P]CTP (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 3×10^6 c.p.m./ml GHR labeled riboprobe, in the same buffer. 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.

The rat GH cDNA probe was the HindIII fragment of the pRGH-1 (Peter *et al.* 1977). The rat IGFBP-3 cDNA probe encodes the IGFBP-3 protein mRNA (Albiston & Herington 1990); the probe was obtained by cutting the PEGEM 4Z plasmid vector using EcoRI and HindIII. Prehybridization was performed for 30 min in ULTRAhyb buffer, followed by hybridization at 42 °C for 16 h with 0.5×10^6 c.p.m./ml GH cDNA or 3×10^6 c.p.m./ml IGFBP-3 labeled with a random priming DNA labeling kit (DECAprime™ II; Ambion). The membranes were washed twice with $2 \times \text{SSC}$, 0.1% SDS at 42 °C for 10 min, and twice with $0.1 \times \text{SSC}$, 0.1% SDS, also for 10 min at 42 °C.

To verify loading, control hybridization was performed with a 28S DNA probe labeled with [^{32}P]dCTP by random primer. The membranes were exposed at -80 °C for 1–4 days. The intensities of autoradiograph signal levels were measured and expressed as the percentage of the mean intensity of the control group injected with saline.

Statistical analysis

All data are presented as the means $\pm$ S.E.M. Serum GH data were subject to log transformation since variances showed a log-normal distribution. Simple linear regression was used to determine correlations between variables. Differences among experimental groups were analyzed by one-way ANOVA. Where there were differences among groups, these were subjected to post-hoc comparisons by using the unpaired Student's *t*-test. Significance was assumed when $P < 0.05$.

Results

Figure 1 shows the effect of LPS administration on GH gene expression in the pituitary and on serum concentrations of GH in rats injected with low LPS doses. LPS induced a significant ($P < 0.05$) increase in pituitary GH gene expression at the doses of 10 and 50 µg/kg LPS (Fig. 1B). Similarly, serum concentrations of GH was increased ($P < 0.05$) in the group of rats injected with 10 µg/kg LPS (Fig. 1A). However, this stimulatory effect disappeared when the LPS dose was increased, since LPS administration decreased serum concentrations of GH in the

Figure 1 (A) Serum concentrations of GH; there was an increase in serum concentrations of GH in the rats injected with 10 µg/kg, and a decrease in the group of rats injected with 100 µg/kg LPS. Each point represents the mean $\pm$ S.E.M. for 9–12 rats per group. * $P < 0.05$ vs control rats injected with saline. (B) Northern blot hybridization and quantification of pituitary GH mRNA in rats injected with low LPS doses (5–100 µg/kg). Pituitary GH mRNA was expressed as the percentage in relation to control values $\pm$ S.E.M. for 6–8 samples. Autoradiographs of a representative GH Northern blot hybridization of 5 µg pituitary RNA and the 28S ribosomal RNA as load control are shown below. LPS administration increased GH mRNA in rats injected with 10 and 50 µg/kg LPS. Each point represents the mean $\pm$ S.E.M. for 6–8 rats per group. * $P < 0.05$ vs control rats injected with saline.

Figure 2 (A) Serum concentrations of GH in rats injected with high LPS doses. There was a decrease in circulating GH in all groups of rats injected with LPS. Each point represents the mean $\pm$ S.E.M. for 9–10 rats per group. ** $P < 0.01$ vs control rats injected with saline. (B) Northern blot hybridization and quantification of pituitary GH mRNA in rats injected with high LPS doses (100–1000 µg/kg). Pituitary GH mRNA was expressed as the percentage in relation to control values $\pm$ S.E.M. for 7–8 samples. Autoradiographs of a representative GH Northern blot hybridization of 5 µg RNA and the 28S ribosomal RNA as load control are shown below.

groups of rats injected with 100–1000 µg/kg LPS (Figs 1A and 2A). The GH gene expression in the pituitary was not modified by LPS administration at high doses (Fig. 2B), and there was no correlation between the serum concentrations of GH and its gene expression in the pituitary.

When LPS was added to the pituitary cell cultures, a significant increase in GH release was also observed at the two lowest concentrations studied (0.1 and 10 ng/ml), whereas no modifications in GH release could be observed at higher concentrations (Fig. 3).

The liver GHR mRNA response to LPS administration was not modified by low LPS doses (Fig. 4A), but

when administered at higher doses, LPS administration decreased liver GHR mRNA (Fig. 4B).

The effect of LPS on serum concentrations of IGF-I is shown in Figs 5A and 6A. There was a significant decrease in serum IGF-I levels from the dose of 10 µg/kg until the highest of 1000 µg/kg, and this decrease showed a dose-dependent effect. LPS-induced decrease in serum concentrations of IGF-I seems to be secondary to its hepatic synthesis, since the liver IGF-I was decreased starting at the dose of 10 µg/kg (Figs 5B and 6B). There was a positive correlation between the serum concentrations of IGF-I and the liver IGF-I in both experiments

Figure 3 GH concentrations in incubation media from dispersed adenohypophyseal cells, after incubation with different LPS concentrations for 4 h. Values are expressed as the mean $\pm$ S.E.M. ($n=9-12$). ** $P<0.01$, * $P<0.05$ vs medium alone.

($r=0.638$, $F_{1,40}=31$, $P<0.01$, in the low doses, and $r=0.676$, $F_{1,45}=35$, $P<0.01$, in the high doses). The liver IGF-I mRNA was also decreased in the rats injected with LPS, but this decrease was not statistically significant until the 50 $\mu\text{g/kg}$ LPS dose on up (Figs 5D and 6D). Nevertheless, similarly to the liver IGF-I, the liver IGF-I mRNA correlates with the serum concentrations of IGF-I ($r=0.65$, $F_{1,32}=23$, $P<0.01$ in the low doses, and $r=0.461$, $F_{1,33}=8.64$, $P<0.01$ in the high doses). At all doses studied the LPS-induced decrease in serum concentrations of IGF-I is more dramatic than in liver IGF-I or in

liver IGF-I mRNA (e.g. in the rats injected with 1 mg/kg LPS the decreases were 76.3, 44 and 42% respectively).

LPS administration decreased both the serum concentrations of IGFBP-3 and the liver IGFBP-3 mRNA in all doses studies (Figs 7 and 8). In contrast to the IGF-I data, the LPS-induced decrease was more dramatic in the liver IGFBP-3 mRNA than in the serum concentrations of IGFBP-3 (81 and 46% decrease in rats injected with 1 mg/kg LPS). There was a positive correlation between these two variables ($r=0.59$, $F_{1,34}=18.1$, $P<0.01$) in the rats injected with high LPS doses.

Discussion

Our data show that LPS, at low doses, not only not decreases but stimulates pituitary GH synthesis and secretion. Nevertheless, LPS decreases circulating IGF-I and IGFBP-3 as well as their gene expression in the liver. A direct inhibitory effect of LPS on liver IGF-I and IGFBP-3 gene expression which is independent of pituitary GH is not surprising, since the liver is the first organ that the circulating blood reaches after draining the peritoneum. Then, it seems logical that the liver would be more sensitive than the pituitary to the effect of LPS injected i.p. In addition, a direct inhibitory effect of cytokines on IGF-I and GHR gene expression in liver cell cultures has also been described (Wolf *et al.* 1996).

Figure 4 Northern blot hybridization and quantification of liver GHR mRNA in (A) rats injected with low LPS doses (5–100 $\mu\text{g/kg}$) and in (B) rats injected with high LPS doses (100–1000 $\mu\text{g/kg}$). Liver GHR mRNA consists of two mRNAs of 4.5 and 1.2 kb; both transcripts were quantified by densitometric analysis and results were expressed as the percentage in relation to control values $\pm$ S.E.M. for 5–8 samples. Autoradiographs of a representative GHR Northern blot hybridization of 30 μg RNA and the 28S ribosomal RNA as load control are shown below. There was a decrease in liver GHR mRNA in rats injected with high LPS doses. * $P<0.05$, ** $P<0.01$ vs control rats injected with saline.

Figure 5 Effect of LPS administration at low doses (5, 10, 50 and 100 $\mu\text{g/kg}$) on IGF-I concentrations in serum (A) and in the liver (B) and on liver IGF-I mRNA (D). (C) A representative Northern blot of IGF-I mRNA hybridization; 30 μg total RNA were hybridized with an RNA probe for rat IGF-I mRNA, each lane corresponds to an individual animal from the indicated group and the 28S ribosomal RNA is shown below. Detectable IGF-I mRNA species are indicated on the left and consist of a group of transcripts ranging of 7.5, 1.7 and 0.9 kb. Because all these transcripts may potentially be translated to IGF-I, the densitometric results corresponded to the sum of the three IGF-I transcripts. Quantitative analyses of Northern blots are expressed as percentages of control rats injected with saline (D). Each bar represents the mean $\pm$ S.E.M. for at least 7–10 rats. * $P < 0.05$, ** $P < 0.01$ vs control rats injected with saline.

Nevertheless, brain cytokines are also activated by peripheral LPS administration, even if the LPS dose is relatively low (100 $\mu\text{g/kg}$), showing a similar profile to central LPS administration (Turrin *et al.* 2001).

Taking into account that serum concentrations of IGF-I are decreased in rats injected with 10 $\mu\text{g/kg}$ LPS, the possibility exists that the increase in pituitary GH synthesis and secretion would be secondary to the decrease in circulating IGF-I levels. However, the fact that somatotrope cells in culture respond to LPS by increasing GH release suggests that LPS *in vivo* has a stimulatory effect on the pituitary somatotrope cells independently of its effect on circulating IGF-I.

A stimulatory effect of LPS on GH synthesis and release has been described in pituitary cultures from animal species, different from rodents, such as sheep and pigs (Fry *et al.* 1998, Mainardi *et al.* 2002). In the pituitary, the folliculo-stellate cells are the source of cytokines, and

LPS-stimulated cells release interleukin (IL)-6 in the pituitary (Spangelo *et al.* 1990). As IL-1 and IL-6 have been reported to stimulate GH release in the rat pituitary (Bernton *et al.* 1987, Spangelo *et al.* 1989), the effect of LPS on GH secretion can be mediated by a stimulatory effect of one of those cytokines. It has recently been reported that the rodent pituitary can respond to an immune challenge (LPS or IL-1 *i.p.* administration) within a few minutes, and the main pituitary cells that respond to IL-1 are somatotrope and the folliculo-stellate cells (Parnet *et al.* 2003). However, these authors failed to find modifications in serum concentrations of GH or in its pituitary mRNA. One possible explanation could be the LPS dose, since they administered 10 μg to mice of 30 g, and this can be an intermediate dose that does not stimulate or inhibit pituitary GH.

The GH response to different LPS doses is different *in vivo* than *in vitro*. In the pituitary cells *in vitro*, we were

Figure 6 Effect of LPS administration at high doses (100, 250, 500 and 1000 µg/kg) on serum and liver IGF-I concentration and on its gene expression in the liver. (A) Serum concentrations of IGF-I; values represent means $\pm$ S.E.M. of 8–10 rats/group. (B) Liver IGF-I; values represents data from 9–10 rats/group. (C) A representative Northern blot analysis showing the 7.5, 1.7 and 0.9 kb IGF-I transcripts; the 28S ribosomal RNA is shown below. (D) Liver IGF-I mRNA levels; the sum of the three IGF-I transcripts were quantified by densitometry and expressed as a percentage of the mean value in control rats treated with saline, $n=6-8$ rats. * $P<0.05$, ** $P<0.01$ vs control group.

unable to find a decrease in GH release, even with an LPS dose as high as 10 µg/ml, whereas *in vivo*, LPS at high doses decreases serum concentrations of GH. This could be explained by the fact that at low LPS doses, the stimulatory effect on the pituitary GH prevails, whereas at higher LPS doses the increased release of somatostatin, or other hypothalamic modifications, inhibit GH secretion.

In rats, the inhibitory effect of LPS administration on GH release *in vivo* can be prevented by a somatostatin antiserum (Kasting & Martin 1982), but this group was unable to find significant changes in somatostatin release in the median eminence after endotoxin administration (Fukata *et al.* 1985). However, chronic LPS administration to rats increased hypothalamic somatostatin content (Soto *et al.* 1998), and IL-1 is able to stimulate somatostatin release in primary neuron cultures (Scarborough *et al.* 1989), and in hypothalamic explants *in vitro* (Honegger *et al.* 1991). These data suggest that LPS stimulates hypothalamic somatostatin release leading to an inhibition of pituitary GH release. The LPS-induced inhibition of

circulating GH does not seem to be mediated by GH-releasing hormone (GHRH), since *in vitro* data suggest that both LPS and IL-1 stimulate hypothalamic GHRH release (Honegger *et al.* 1991, Peisen *et al.* 1995). Nevertheless, the possibility exists that the inhibitory effect of LPS on GH secretion could be also mediated by other central and/or peripheral factors different from somatostatin.

In the present study, the lack of an inhibitory effect of high LPS doses on GH mRNA, in spite of the decreased serum GH levels, suggests that LPS inhibits GH release but not GH synthesis. Another possibility is that we were unable to find modifications in pituitary GH mRNA for the time studied (18 h from the first LPS injection), since we observed that 8 days of LPS administration (250 µg/kg daily) decreased pituitary GH content, indicating a decreased GH synthesis (Soto *et al.* 1998). Moreover, in adjuvant-induced arthritis, an experimental model of chronic inflammation, the decrease in serum concentrations of GH is associated with a decrease in GH gene expression in the pituitary (López-Calderón *et al.* 1999).

Figure 7 Effect of LPS administration at low doses (5, 10, 50 and 100 µg/kg) on serum concentrations of IGFBP-3 and on liver IGFBP-3 mRNA. (A) Western ligand blot analysis and quantification of serum IGFBP-3. Serum IGFBP-3 was separated by SDS-PAGE on a 12.5% acrylamide gel, transferred to nitrocellulose, ligand blotted with ^{125}I -IGF-I, and visualized via autoradiography. Data from 9–11 individual rats were quantified by densitometry and expressed as the percentage of the mean value in control rats injected with saline. (B) A representative Northern blot of IGFBP-3 mRNA hybridization of 30 µg total liver RNA. The size of the hybridization band (in kb) is indicated on the right; 28S, 28S ribosomal RNA. Quantitative analyses are expressed as percentages of control rats injected with saline, for 6–8 rats per group. * $P < 0.05$, ** $P < 0.01$ vs control group.

It has been proposed that the sepsis-induced decrease in IGF-I is associated with GH resistance. LPS administration decreases GHR gene expression *in vitro* and *in vivo* (Wolf *et al.* 1996, Defalque *et al.* 1999), and it is able to block the GH-responsive genes in the liver of hypophysectomized

Figure 8 Effect of LPS administration at high doses (100, 250, 500 and 1000 µg/kg) on serum concentrations of IGFBP-3 and on liver IGFBP-3 mRNA. (A) A representative Western ligand blot of IGFBP-3. Serum IGFBP-3 was separated by SDS-PAGE on a 12.5% acrylamide gel, transferred to nitrocellulose, ligand blotted with ^{125}I -IGF-I, and visualized via autoradiography. Data from 7–10 rats were quantified by densitometry and expressed as the percentage of the mean value in control rats injected with saline. LPS decreased circulating IGFBP-3. (B) A representative Northern blot of IGFBP-3 mRNA hybridization of 30 µg total liver RNA. The size of the hybridization band (in kb) is indicated on the right; 28S, 28S ribosomal RNA. Quantitative analyses are expressed as percentages of control rats injected with saline, for 7 or 8 rats per group. * $P < 0.05$, ** $P < 0.01$ vs control group.

rats (Bergard *et al.* 2000). The effect of LPS on the GHR in the liver was not evident 3 or 6 h after administration of 1 mg/kg LPS, although it inhibits GH activation of Stat5 (Mao *et al.* 1999, Denson *et al.* 2003). The authors concluded that the reduction in liver Stat5 activation was due to induction of postreceptor signaling inhibitors such

as Cis and Socs-3 (Denson *et al.* 2003). However, when using a higher LPS dose (7.5 mg/kg) it was also possible to observe a significant reduction in GHR mRNA (Defalque *et al.* 1999). This is consistent with our data; the GHR mRNA in the liver does not decrease until 18 h after the dose of 100–250 µg/kg, suggesting that at low LPS doses the inhibitory effect of LPS on IGF-I is mediated by a postreceptor GH signaling.

In our data, serum concentrations of IGF-I are more sensitive than the liver IGF-I or IGF-I mRNA to the inhibitory effect of LPS injection. Similarly, 6 h after IL-1 administration to rats, there was a 64% decrease in serum concentrations of IGF-I and a 32% decrease in peptide content in the liver (Fan *et al.* 1996); the authors explained the difference as a rapid clearance of circulating IGF-I. Taking into account that IGFBP-3 is the major IGF-I-binding protein in serum, the decrease in serum concentrations of IGFBP-3 may contribute to increasing IGF-I turnover and then to reducing IGF-I half-life in serum. This hypothesis is consistent with the data showing that IGF-I/IGFBP-3 binary complex administration to septic rats restores gastrocnemius protein synthesis to a rate comparable with that of control rats (Svanberg *et al.* 2000), suggesting that the binary complex administration accelerates protein synthesis by prolonging the availability of IGF-I. The binary complexes form a ternary complex with the acid-labile subunit (ALS), and endotoxin administration also induces a decrease in serum concentrations of ALS (Barreca *et al.* 1998, Kong *et al.* 2002). Then, the decrease in circulating IGFBP-3 and ALS might contribute to the decline in serum IGF-I observed after LPS administration.

In contrast, liver IGFBP-3 mRNA is more sensitive to the inhibitory effect of LPS than liver IGF-I mRNA. Among all variables we have measured, liver IGFBP-3 mRNA is the most affected by LPS administration at low doses. These data can be due to the fact that Kupffer and endothelial cells are the main IGFBP-3-producing cells in the liver, and they would be very quickly activated by LPS stimulation, whereas the other liver cells synthesizing IGF-I, such as hepatocytes, would respond to a higher LPS dose than the Kupffer cells. The present data are in accord with those we have previously reported in Lewis rats (Priego *et al.* 2003), in which the LPS-induced decrease in liver IGFBP-3 mRNA is GH- and IGF-I-independent.

The difference between the decrease in liver IGFBP-3 mRNA and the serum levels of IGFBP-3 after LPS administration is not due to alterations to its proteolysis, since we were unable to find any modifications in the serum IGFBP-3 proteolysis in any of the studied groups (data not shown). These data are in agreement with those we have previously reported using LPS at the dose of 1 mg/kg (Priego *et al.* 2003). Similarly, in another catabolic state, anorexia nervosa, the decrease in serum concentrations of IGFBP-3 is not associated with an increase in its proteolysis (Stoving *et al.* 1999). Taking into account

that IGFBP-3 is also produced by other organs, it is possible that LPS stimulates IGFBP-3 production in other cells than in the liver. Accordingly, an increase in IGFBP-3 mRNA and protein in response to LPS stimulation in a human monocytic cell line has been reported (Agnese *et al.* 2002). Discordant serum IGFBP-3 concentrations and liver mRNA levels have been observed by Lemmey *et al.* (1997), and these authors propose that skin and fibroblast-type cells may be an important additional source of circulating IGFBP-3. All these data suggest that in addition to the liver, other IGFBP-3-synthesizing cells may contribute to the circulating pool of this protein.

In summary, our dose-response study provides new insights into the mechanisms by which LPS affects GH-IGF-I-IGFBP. There is a dissociation between the effect of endotoxin on pituitary GH and the liver production of IGF-I and IGFBP-3. Initially, LPS at low doses may decrease liver IGF-I and IGFBP-3, whereas it potentiates pituitary GH secretion. This acute LPS response is similar to that described in the acute phase of critical illnesses in humans; there is an increased activity of the anterior pituitary and a peripheral decrease of the anabolic hormones (Van den Berghe 2002). At high LPS doses, the decrease in liver and serum IGF-I and IGFBP-3 is concomitant with decreased serum concentrations of GH and GHR mRNA in the liver.

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