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Effects of Lipopolysaccharide Endotoxin on the Insulin-like Growth Factor I System in Rats with Cirrhosis

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Abstract. *Background: Patients with liver cirrhosis have disturbances in the insulin-like growth factor I (IGF-I) system that favour insulin resistance and catabolism. High morbidity and mortality of such patients from infections may be related to further aggravation of this problem but human data are controversial. Here, the effect of lipopolysaccharide (LPS) endotoxin on the IGF system was studied in rats with cirrhosis. Materials and Methods: One month after induction of cirrhosis by bile duct ligation, LPS was administered (0.5 mg/kg) and the IGF system assessed 24 h later. Sham-operated animals acted as controls. Circulating and liver mRNA of IGF-I and its binding proteins (IGFBPs) were measured. Results: LPS reduced IGF-I and IGFBP-3 by 20% in the cirrhosis group. LPS induced insulin resistance (HOMA) in both groups. Conclusion: Our results show that LPS administered to cirrhotic rats induced changes in the IGF system that facilitate catabolism. This may be of importance for the accelerated tissue loss during infection and the acute phase response in liver cirrhosis.*

Patients with liver cirrhosis are often characterized by muscle wasting and negative nitrogen (N) balance (1). Such patients are at increased risk of infections especially with Gram-negative bacteria (2) and infections may further induce catabolism in a vicious circle. In addition, insulin resistance is a characteristic feature in liver cirrhosis (3) and infections *per se* may further induce insulin resistance.

The liver plays a central role in insulin-like growth factor (IGF) homeostasis as a major source of circulating IGF-I and the majority of its binding proteins (IGFBP-1 to -6). In patients with cirrhosis, the IGF system is disturbed, with reduced total and free IGF-I, IGFBP-3 and IGF-II levels (4-6). Similar changes in the IGF system are found in rat models of liver cirrhosis, with reduced liver *IGF-I* mRNA expression, reduced serum IGF-I levels and variable alterations in the levels of circulating IGFBPs and liver IGFBPs mRNA (7-9).

Injection of lipopolysaccharide (LPS), an endotoxin from bacterial cell walls, is a standardized method for inducing the systemic component of the acute phase response in clinical and experimental models of inflammation. In normal healthy rats, LPS administration is followed by reduced plasma and liver tissue IGF-I levels and elevated plasma levels of IGFBP-1, while IGFBP-2 and -3 levels are unchanged (10). However, in another study, serum IGFBP-3 decreased (11). Acute endotoxemia causes insulin resistance in rats and humans (12, 13). In a rat model of experimental cirrhosis, administration of LPS markedly augmented liver injury and mortality (14), but it is unknown how LPS affects the IGF system in cirrhosis.

We tested the hypothesis of whether inflammation induced by LPS further aggravates the aberrations in the IGF system and insulin resistance in a cirrhotic rat model. The aim of the present study was to study the effect of LPS on the IGF system in bile duct-ligated (BDL) and sham-operated control rats. After injection of LPS in cirrhotic and sham-operated rats, the serum levels of IGF-I, IGFBPs and insulin, and liver gene expression of IGF/IGFBPs were examined.

Materials and Methods

Animals. Female Wistar rats (body weight 200-210 g; Taconic M&B, Ejby, Denmark) were housed at 19±3˚C, with a 12-h artificial light cycle, 2-3 animals in each cage and with free access to tap water and standard chow (Altromin, Lage, Germany) during the whole experiment. The study was undertaken in accordance with prevailing local and national guidelines for animal welfare.

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Design. Four groups were studied: two groups of sham-operated animals injected with saline or LPS, and two groups of BDL animals injected with saline or LPS 24 h before experimental examination as follows:

Sham-operated animals injected with saline (Sham) (n=9), sham-operated animals injected with LPS (Sham+LPS) (n=11), BDL-operated animals injected with saline (Cirrhosis) (n=11), BDL-operated animals injected with LPS (Cirrhosis+LPS) (n=9).

BDL, sham-operation and endotoxemia induction. BDL and sham-operation were performed under anesthesia with a subcutaneous injection of fentanyl/fluanisone (Hypnorm®; Jansen Pharma, Birkeroed, Denmark) 0.5 ml × kg^{-1} and midazolam (Dormicum®; La Roche, Basel, Schwitzerland) 2.5 mg × kg^{-1}. Following a midline abdominal incision, the common bile duct was isolated, triple ligated with 3-0 monofil polyamide and sectioned between the ligatures. The sham operation consisted of isolation and gentle manipulation of the common bile duct. Twenty-five to 30 days after operation, the animals were injected *i.p.* with either 0.5 mg/kg LPS (from *Ecsherichia coli* obtained from Sigma (0111:B4) (catalogue no. L2630), Vallensbæk, Denmark) dissolved in 0.5 ml isotonic NaCl, or with the vehicle alone.

Cirrhosis verification. Liver tissue from all BDL-operated animals was fixed overnight in formalin, embedded in paraffin and stained with hematoxylin-eosin and Masson's trichrome for histological examination. Inclusion required macroscopic cirrhosis (micro-nodular surface) and microscopic diffuse architectural changes with proliferation of bile duct-like structures with fibrosis and solid porto-portal septa formation. Nearly all BDL-operated animals, *i.e.* 99% , fulfilled these criteria.

mRNA levels. Following anesthesia as used for BDL and sham operation, approximately 200 mg of liver tissue from the left lobe was snap frozen in liquid nitrogen and stored at –80˚C. mRNA levels of IGF-I, and the binding proteins IGFBP-1, -2 and -4 were determined.

Total RNA was isolated with RNeasy® Midi Kit (Quiagen, Hilden, Germany) and mRNA levels were measured by slot blot hybridisation as described in detail elsewhere (15). Hybridization was performed with QuickHyb® hybridisation solution (Stratagene, La Jolla, CA, USA) at 68˚C for 1 h, followed by stringent wash. Hybridisation signals were analysed in a Fujix Bioimaging Analyzer System BAS2000 (Fuji Photo Film, Tokyo, Japan).

cDNA probes for IGF-I and IGFBPs have been given elsewhere (16, 17). DNA fragments were separated by agar gel electrophoresis and eluted on Spin Bind DNA Extraction Units (FMC) (Medinova Scientific A/S, Glostrup, Denmark).

Analyses. Total serum IGF-I was measured after acid-ethanol extraction by an IGF-I radioimmunoassay as described elsewhere (18). IGFBPs were measured by SDS-PAGE and Western ligand blot (WLB) analysis as described elsewhere (18, 19).

Serum insulin levels were measured by ultrasensitive Rat Insulin ELISA (DRG Diagnostics, Marburg, Germany) and plasma glucose by a routine analytical method. All assays had intra- and interassay coefficients of variance (CV) below 5% and 10% , respectively. The HOMA index was calculated in accordance with the model given in (20).

Statistical methods. Data are presented as mean + SEM. Data were analysed by one-way ANOVA followed by least significant difference method for multiple comparisons and adjusted with a Bonferroni correction for multiple testing. Differences were considered significant when $p<0.05$ in a two-sided test.

Results

Total IGF-I, IGFBP-3 and IGF-I/IGFBP-3 ratio. LPS reduced the total circulating IGF-I level by 20% in cirrhotic rats (950±30 *vs*. 766±45 μg/l, $p<0.05$), but did not change the level in sham-operated rats (680±41 *vs*. 641±22 μg/l). Cirrhosis increased total circulating IGF-I level by 40% ($p<0.05$) (Figure 1A).

Similar changes were observed for IGFBP-3, with a trend towards reduction in cirrhotic rats following LPS (2039±176 *vs*. 1517±157 AU/mm^2) but no effect of LPS was seen in sham-operated controls (1292±303 *vs*. 876±79 AU/mm^2) (Figure 1B). Cirrhosis increased IGFBP-3 levels by 130% ($p<0.05$).

The IGF-I/IGFBP-3 ratio, which may be an indicator of bioactive IGF-I, was not changed by LPS in either group (0.64±0.08 and 0.53±0.10, respectively), whereas cirrhosis reduced the IGF-I/IGFBP-3 ratio (0.76±0.05 *vs*. 0.50±0.03, $p<0.05$) (Figure 1C).

IGFBP-1, IGFBP-2 and IGFBP-4. LPS increased both IGFBP-1, -2 and -4 in rats with cirrhosis (270±25 *vs*. 117±13 AU/mm^2, $p<0.05$; 135±20 *vs*. 70±6 AU/mm^2, $p<0.05$; and 283±25 *vs*. 171±11 AU/mm^2, $p<0.05$), but only IGFBP-4 in sham-operated rats (249±26 *vs*. 134±20 AU/mm^2, $p<0.05$), (Figure 2). Cirrhosis increased IGFBP-2 (44±3 *vs*. 70±6 AU/mm^2, $p<0.05$), while IGFBP-1 (80±8 *vs*. 117±13 AU/mm^2) and IGFBP-4 (134±20 *vs*. 171±11 AU/mm^2) were unchanged.

Insulin, glucose and HOMA index. LPS increased insulin in both cirrhotic (47±5 *vs*. 27±2 μU/ml, $p<0.05$) and control rats (83±16 *vs*. 36±5 μU/ml, $p<0.05$). There was no difference in basal insulin levels between cirrhotic and control rats (Table I). There was no effect of LPS on blood glucose in sham-operated (6.6±0.3 *vs*. 7.3±0.2 mmol/l) or cirrhotic rats (6.3±0.7 *vs*. 5.1±0.3 mmol/l), however, glucose was reduced in cirrhotic rats compared to controls ($p<0.05$). LPS increased the HOMA index in both sham-operated (24.2±6.1 *vs*. 11.9±1.9, $p<0.05$) and cirrhotic rats (12.5±1.9 *vs*. 6.1±0.4, $p<0.05$). Cirrhosis did not change the HOMA index.

Liver mRNA levels of IGF-I. There was no effect of LPS on the liver mRNA levels of IGF-I in sham-operated (110% ±5% *vs*. 100% ±5%) or cirrhotic rats (66% ±6% *vs*. 69% ±5%). In cirrhotic rats a decrease in the liver mRNA level of IGF-I compared to controls was observed ($p<0.05$) (Figure 3A).

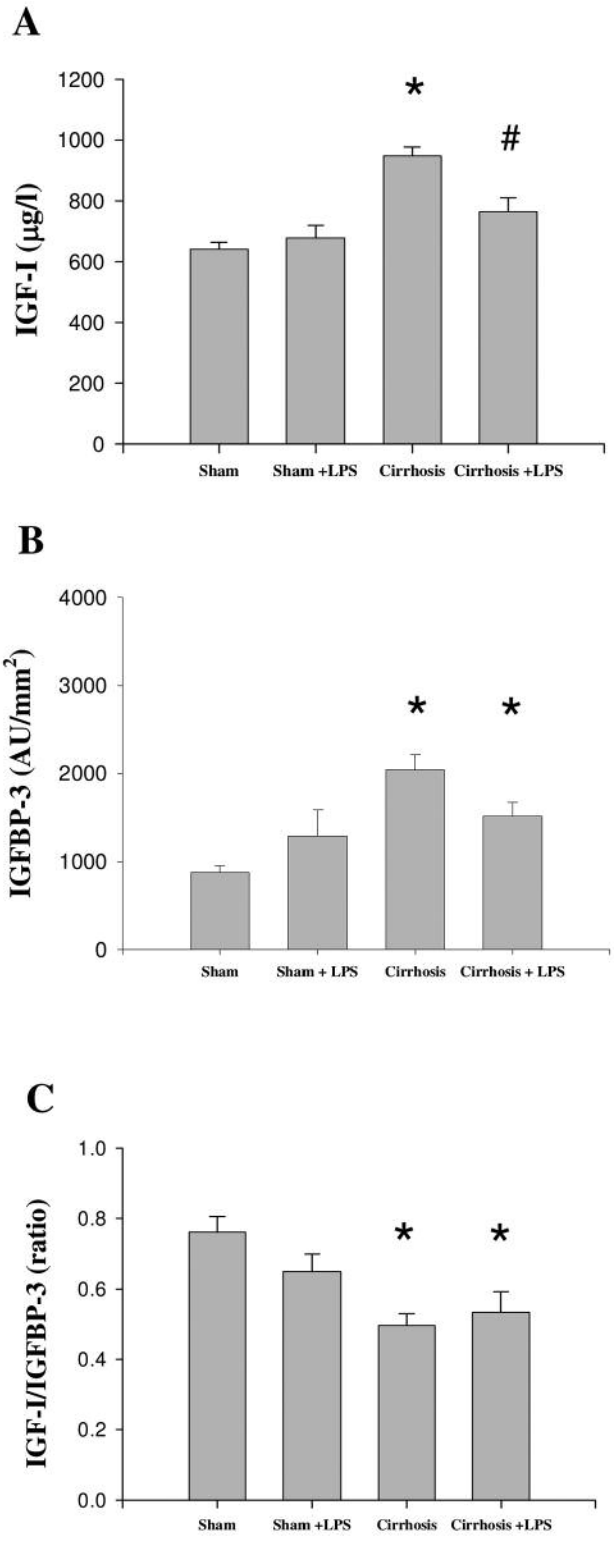

Figure 1. *Total IGF-I (A), IGFBP-3 (B) and IGF-I/IGFBP-3 ratio (C) in sham-operated (n=9), sham-operated injected with LPS (n=11), cirrhotic (n=11) and cirrhotic animals injected with LPS (n=9). Bars represent mean+SEM. *p<0.05 Compared with sham, #p<0.05 for cirrhotic animals + LPS compared with cirrhotic animals.*

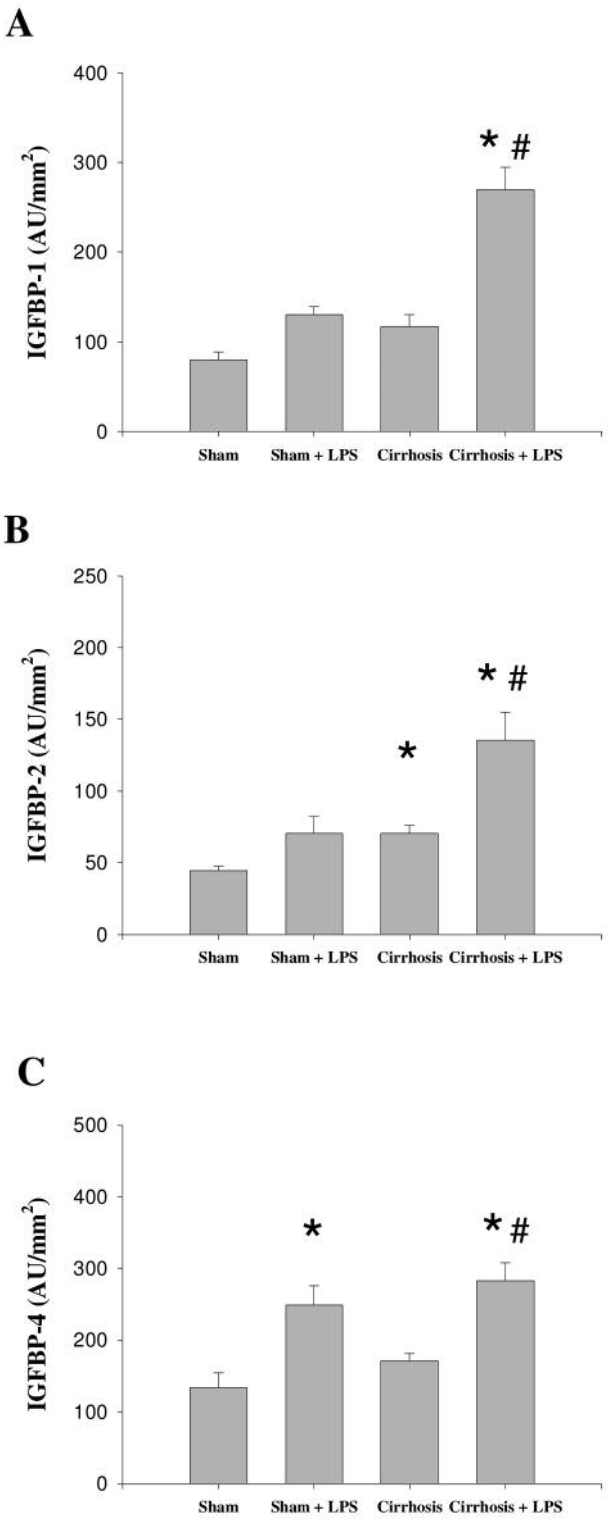

Figure 2. *IGFBP-1 (A), IGFBP-2 (B) and IGFBP-4 (C) in sham-operated (n=9), sham-operated injected with LPS (n=11), cirrhotic (n=11) and cirrhotic animals injected with LPS (n=9). Bars represent mean+SEM. *p<0.05 Compared with sham, #p<0.05 for cirrhotic animals + LPS compared with cirrhotic animals.*

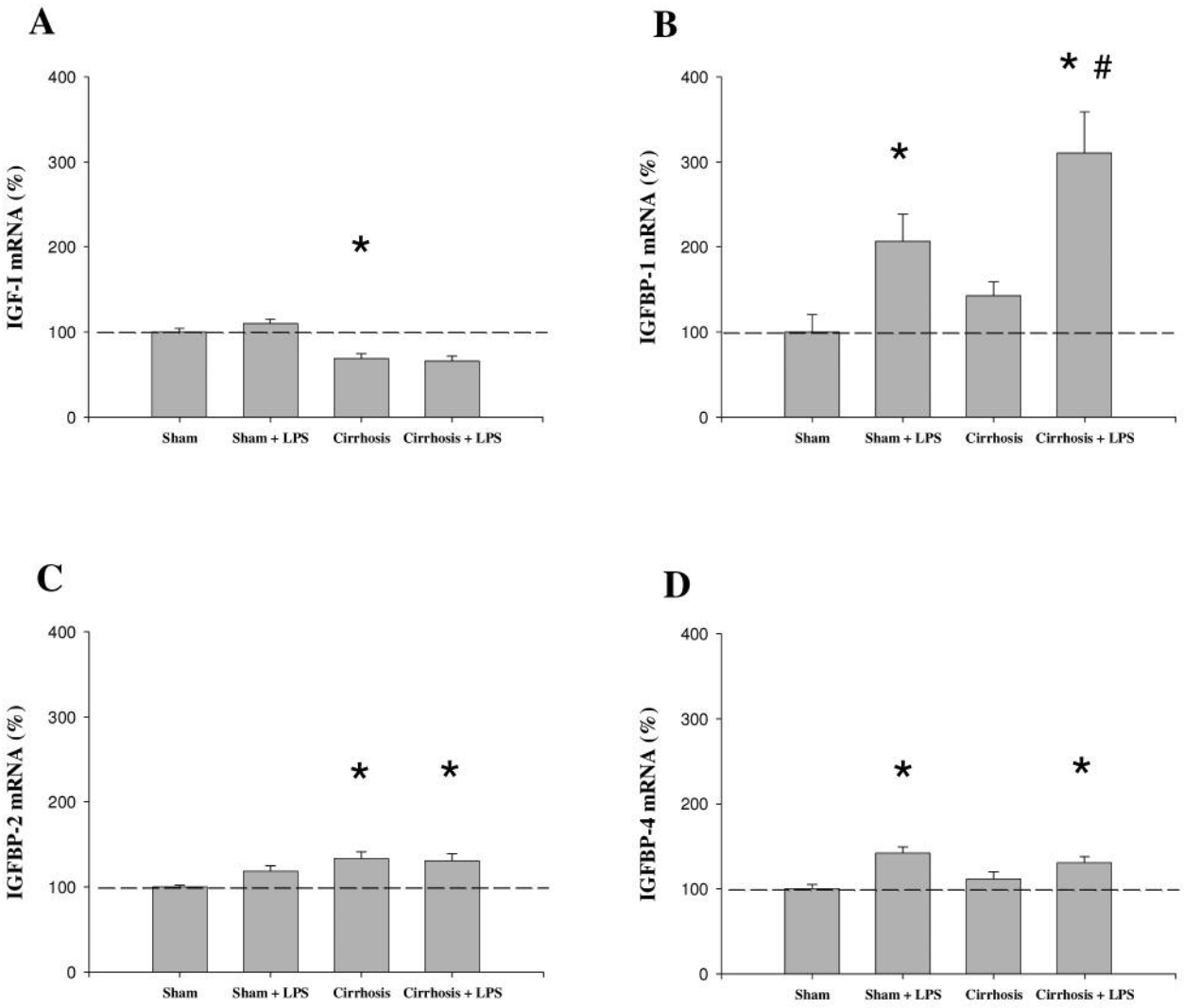

Figure 3. *Liver mRNA levels of IGF-I (A), IGFBP-1 (B), IGFBP-2 (C) and IGFBP-4 (D) in sham-operated (n=9), sham-operated injected with LPS (n=11), cirrhotic (n=11) and cirrhotic animals injected with LPS (n=9). Bars represent mean+SEM. *p<0.05 Compared with sham, #p<0.05 for cirrhotic animals + LPS compared with cirrhotic animals.*

Liver mRNA levels of IGFBP-1, IGFBP-2 and IGFBP-4. The liver mRNA levels of IGFBP-1 and -4 were increased by LPS in sham-operated rats (207%±32% *vs.* 100%±21%, $p<0.05$) and (142%±8% *vs.* 100%±5%, $p<0.05$), while no change was observed in the liver mRNA levels of IGFBP-2 (119%±6% *vs.* 100%±3%) (Figure 3B-D). In cirrhotic rats only the liver mRNA level of IGFBP-1 was increased by LPS (310%±48% *vs.* 143%±16%, $p<0.05$), while no change was observed in the liver mRNA level of IGFBP-2 (131%±9% *vs.* 134%±7%) or IGFBP-4 (131%±7% *vs.* 112%±8%). In the cirrhotic rats, there were no changes in the liver mRNA levels of IGFBP-1, -2 or -4 compared to sham-operated controls.

Table I. *Glucose, insulin and HOMA index in sham-operated (n=9), sham-operated injected with LPS (n=11), cirrhotic (n=11) and cirrhotic animals injected with LPS (n=9).*

	Sham	Sham + LPS	Cirrhosis	Cirrhosis + LPS
Glucose (mmol/l)	7.3±0.2	6.6±0.3	5.1±0.3**	6.3±0.7
Insulin (mU/l)	36±5	83±16*	27±2	47±5*
HOMA index	11.9±1.9	24.2±6.1*	6.1±0.4	12.5±1.9*

*Significantly increased by LPS in both sham-operated and cirrhotic rats ($p<0.05$). **Significantly reduced in cirrhotic rats compared to controls ($p<0.05$).

Discussion

The main findings of our study were that LPS significantly reduced total circulating IGF-I and IGFBP-3 and increased IGFBP-1 in rats with cirrhosis and had no such effects in control rats. In addition, LPS increased the HOMA index, indicating insulin resistance, both in sham-operated animals and animals with cirrhosis.

Administration of LPS is an established experimental method for activating the innate immune system and inducing an acute phase response. Rats with cirrhosis due to BDL are known to be highly sensitive to LPS (21, 22), and therefore we used a low dose (0.5 mg/kg) of LPS in this study. The acute weight loss of the treated animals confirmed that this dose was effective and the dose caused a 25% mortality in the rats with cirrhosis as previously described (22). Still, we earlier showed that this dose of LPS does not compromise general metabolic liver function, as assessed by the galactose elimination capacity (GEC), in either normal or cirrhotic rats (23).

In healthy humans, injection of endotoxin reduces total IGF-I levels, whereas free IGF-I and IGFBP-3 levels remain unchanged. Moreover, endotoxin increases IGFBP-1 and -2 levels (24). It is reported that in normal rats, LPS reduces total IGF-I, whereas its effects on IGFBP-3 vary considerably (10, 11). We found that LPS caused a marked reduction in total circulating IGF-I and IGFBP-3 in cirrhotic rats and no change in control rats. The mechanism of this suppression of IGF-I by LPS is not known. It has been suggested to be secondary to the effect of LPS in decreasing growth hormone (GH) secretion and inducing GH resistance in rodents (10, 11, 25). Moreover, endotoxin stimulates production of interleukin IL-1β and tumour necrosis α (TNF-α) (24), for example, both decreasing IGF-I in normal rats (26, 27) and this effect is blunted by neutralization of both interleukins (27, 28). In cultured rat hepatocytes, both interleukins furthermore directly inhibit GH stimulation of IGF-I (29,30), but this effect has not been confirmed *in vivo*. LPS also induces release of interleukin 6 (IL-6) (31), but evidently this interleukin does not have the aforementioned effect (30). In the present study, IL-6 levels in the cirrhotic rats injected with LPS were unchanged compared to controls (data not shown). In any case, the rats were studied 24 h after the single injection of LPS, so due to their short half-life, any circulating cytokines would probably have been cleared at that time.

LPS increased circulating IGFBP-1 and also liver IGFBP-1 mRNA in rats with cirrhosis and only liver IGFBP-1 mRNA levels in the sham-operated controls. This protein fluctuates inversely with insulin levels and at the same time binds and releases circulating IGF-I (32). Its key role is thought to regulate free IGF-I (4). Cirrhosis and the acute phase response both increase serum IGFBP-1 levels, which, in combination with reduced circulating IGF-I, may further limit the effect of IGF-I (6, 10, 33). The physiological regulators that increase IGFBP-1 under stress conditions have not been fully elucidated and vary depending on the trauma (33), however, cytokines as described above may be involved.

LPS increased the HOMA index in both sham-operated and cirrhotic rats and this is in line with earlier studies on acute endotoxemia causing insulin resistance in normal rats (12). Our animals had free access to food and drink for the entire duration of the experiment and LPS injection is known to acutely reduce the animals' food intake (34). Differences in insulin and glucose levels between the sham-operated and cirrhotic animals may be explained by the non-fasting condition, however, the effect of LPS was similar, with 100% increase in both groups. If the animals had been kept fasting after LPS injection, the difference in the HOMA index between the two groups is likely to have been even more pronounced. We observed no change in the HOMA index in the cirrhotic rats compared to the sham-operated ones. In previous studies, cirrhotic rats were insulin resistant, with elevated plasma insulin levels after an overnight fast, after oral glucose tolerance test (OGTT), or during euglycemic hyperinsulinaemic clamp studies, respectively (35, 36). In both studies only moderate fasting hyperinsulinaemia and a degree of insulin resistance were found.

In a previous rat study, cirrhosis reduced total plasma IGF-I levels and liver IGFBP-3 mRNA (7), however, conflicting data on IGFBP-3 have been published (9). In humans with advanced liver cirrhosis, circulating total and free IGF-I and IGFBP-3 levels are reduced (4). The BDL model is not a model of clinical end-stage liver disease (22, 23) and this may explain why the production of total circulating IGF-I and IGFBP-3 was preserved in the present study. However, IGF-I mRNA levels were reduced and we also found a reduction in the IGF-I/IGFBP-3 ratio, indicating reduced IGF-I bioactivity.

In conclusion, injection of LPS caused a reduction in total circulating IGF-I and IGFBP-3 levels in cirrhotic rats whereas no change was observed in sham-operated rats. We found a diminished IGF-I/IGFBP-3 ratio in the cirrhotic rats compared to controls after LPS injection, indicating diminished IGF-I bioactivity. Furthermore, LPS increased circulating IGFBP-1 levels in cirrhotic rats and the HOMA index in both sham-operated and cirrhotic rats. The results show that LPS administered to cirrhotic rats induced changes in the IGF system and insulin sensitivity, which is known to promote catabolism. This observation may be involved in the accelerated catabolism seen during infection in patients with severe liver cirrhosis and the classical pronounced acute phase response and high mortality observed in these patients.

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