Nitric oxide production by hepatocytes contributes to the inhibitory effect of endotoxin on insulin-like growth factor I gene expression

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Abstract

We tested whether endotoxin (lipopolysaccharide, LPS) inhibits IGF-I gene expression in hepatocytes and the possible role of Kupffer cells and nitric oxide (NO) in this effect. LPS decreased IGF-I mRNA in hepatocyte cultures and increased the nitrite + nitrate levels in the culture medium. Furthermore, there was a negative correlation between the IGF-I mRNA and the nitrite + nitrate levels. When hepatocytes were cocultured with Kupffer cells, the inhibitory effect of LPS on IGF-I mRNA was higher than in hepatocyte cultures, but the stimulatory effect on nitrite + nitrate was similar in both conditions. The exogenous NO donated by S-nitroso-n-acetyl-l-penicillamide also decreased the IGF-I gene expression in hepatocyte cultures. In addition, two specific inducible NO synthase (iNOS) inhibitors, l-N6-(1-iminoethyl)lysine (l-NIL) and aminoguanidine, prevented the effect of LPS on nitrite + nitrate levels and on IGF-I gene expression in hepatocyte cultures. These data indicate that iNOS-derived NO may cause downregulation of IGF-I gene expression in hepatocytes. However, in cocultures, the iNOS inhibitor l-NIL prevented the effect of LPS on nitrite + nitrate levels, but only attenuated the LPS-induced decrease in IGF-I gene expression. We conclude that in hepatocytes, LPS-induced decrease in IGF-I is mainly due to induction of iNOS, whereas in the presence of Kupffer cells LPS inhibits IGF-I through NO release and through other inhibitory pathways.

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Introduction

Inflammation can induce a catabolic state associated with hypercatabolism, negative nitrogen balance, and cachexia. Inflammatory cachexia cannot be solely explained by the decrease in food intake (Douglas & Shaw 1989), because it is related to modifications in the neuroendocrine system. One of these modifications is the decrease in anabolic hormones, such as insulin-like growth factor-I (IGF-I; Ibáñez de Cáceres et al. 2000).

The inflammatory response can be experimentally induced by lipopolysaccharide (LPS) administration. LPS is a component of Gram-negative bacteria and its administration triggers the sepsis response (Karima et al. 1999). A decrease in serum concentrations of IGF-I has been described after LPS administration in experimental animals and humans (Fan et al. 1994, Lang et al. 1997). Although LPS or inflammation also modifies pituitary growth hormone (GH) secretion (Kasting & Martin 1982, Soto et al. 1998), LPS administration is able to decrease liver IGF-I gene expression, acting independently of pituitary GH and liver GH receptors (GHR; Priego et al. 2003).

In the liver, the endotoxin response in vivo is mediated by complex cellular interplay between the different cell types. LPS activates Kupffer cells by increasing the release of nitric oxide (NO) and various cytokines (Decker 1990). Furthermore, Kupffer cell inactivation prevents the inhibitory effect of LPS injection in vivo on IGF-I gene expression in the liver (Granado et al. 2006). Taking into account that a direct inhibitory effect of cytokines on IGF-I gene expression in hepatocyte cultures has been reported (Wolf et al. 1996, Thissen & Verniers 1997), the inhibitory effect of LPS on liver IGF-I can be mediated by the release of mediators from Kupffer cells.

Another possibility is that LPS directly decreases IGF-I gene expression in hepatocytes. LPS receptors have been reported in hepatocytes and they respond to LPS through the toll-like receptor pathway (Liu et al. 2002). Recent experimental observations from our laboratory (Priego et al. 2004) suggest that overproduction of NO by inducible NO synthase (iNOS) is one of the mechanisms responsible for LPS-induced decrease in circulating IGF-I and its gene expression in the liver. One of the major features in endotoxic shock is the induction of iNOS in the liver. iNOS is induced by LPS or inflammatory stimuli in macrophages and hepatocytes, and it is responsible for the production of most NO release during endotoxemia. Inhibition of NO from iNOS protects from liver injuries induced by both mycobacterial infection and endotoxin administration (Guler et al. 2004). iNOS is expressed by hepatocytes, and LPS is able to induce iNOS gene expression and NO release.
in hepatocyte cultures (Geller et al. 1993). In addition, microarray studies revealed that iNOS in hepatocytes suppresses proliferation and protein synthesis (Zamora et al. 2002), effects which are IGF-I dependent.

The aim of this study was to analyze whether LPS is able to decrease IGF-I gene expression directly in rat hepatocytes or if it is mediated by the activation of Kupffer cells, and also to study the role of NO in this effect.

Materials and Methods

Male Wistar rats (200–250 g) were purchased from Harlan (Barcelona, Spain). They were housed four per cage under controlled conditions of temperature (20–22 °C) and light (lights on from 0730 to 1930 h). Food and water were freely available. The procedures followed the guidelines recommended by the European Union for the care and use of laboratory animals.

Hepatocyte cultures

Hepatocytes were isolated by modification of the in situ collagenase perfusion technique as previously described (Seglen 1976). Rats were anesthetized with pentobarbital (Sigma, Chemical Co, St Louis, MO, USA). The portal vein was cannulated after opening the abdomen, perfused with calcium-free buffer for 15 min, and then the liver was digested with a 0.04% collagenase (Roche) for another 5–10 min at 37 °C. The liver was transferred to a Petri dish and cells were obtained by gentle teasing with a comb and filtered through a 100 μm mesh. Hepatocytes were separated from non-parenchymal cells by differential centrifugation at 400 r.p.m. (three times, 5 min each). Hepatocyte purity was assessed by microscopy and was >90% viability was measured by Trypan Blue exclusion and was >80%. Hepatocytes (5 × 10⁶ in 5 ml medium) were plated on 100 mm gelatin-coated Petri dishes. Medium consisted of Williams’ medium E (GIBCO, Cedex, France) with l-glutamine (2 mM), insulin (1 μM), HEPES (15 mM), penicillin + streptomycin (100 units/ml + 100 g/ml), and 10% low endotoxin calf serum.

Hepatocyte and Kupffer cell cocultures

Hepatocytes were isolated as described earlier. Kupffer cells were isolated from supernatants of the differential centrifugations and centrifuged at 2200 r.p.m. for 7 min. Kupffer cells were finally suspended and plated with hepatocytes in a proportion of 3:2 (3 × 10⁶ hepatocytes/2 × 10⁶ Kupffer cells in 5 ml medium).

Experimental design

After 24-h incubation (37 °C in 95% air/5%CO₂), the culture medium was removed and different stimuli were added in serum-free medium. Cells were incubated with the stimuli for another 24 h. On the following day, the medium was removed and stored at −80 °C for nitrite+nitrate determination. Total RNA from cells was isolated to measure IGF-I mRNA by northern blot hybridization.

The LPS response was analyzed after adding LPS (serotype 055:B5, Sigma) at different concentrations (0, 0.1, 1, 10, and 50 μg/ml) to the culture medium. In order to elucidate the effect of NO on hepatocytes, different concentrations (10, 100, and 1000 μg/ml) of a NO donor, S-nitroso-n-acetyl-dl-penicillamine (SNAP; Alexis Corporation, Lausanne, Switzerland), were used. Finally, two selective iNOS inhibitors, L-N6-(1-iminoethyl)lysine (L-NIL), 0.1 mM and aminoguanidine hemisulfate, 0.1 mM (Alexis Corporation), were used in order to test their ability to prevent the effect of 50 μg/ml LPS on hepatic cells IGF-I mRNA. Experiments were performed at least twice.

Nitrite + nitrate determination

Nitrite+nitrate concentration in the culture medium was measured by a modified method of the Griess assay, described by Miranda et al. (2001). One hundred microliters were mixed with 100 μl vanadium chloride, rapidly followed by the addition of the Griess reagents. The determination was performed at 37 °C for 30 min. The absorbance was measured at 540 nm. Nitrite + nitrate concentration was calculated using a NaNO₂ standard curve and expressed as micromolar.

RNA extraction

Total RNA was extracted using a commercial kit (Real Total RNA, C.E. Durviz S.L., Valencia, Spain) according to the protocol supplied by the manufacturer. Total RNA was dissolved in 0.1% SDS diethylpyrocarbonate-treated water and quantified at 260 nm. The integrity and the concentration of the RNA were confirmed using agarose gel electrophoresis.

IGF-I northern blot

For northern blotting, 10 μg denatured RNA were separated by formaldehyde–agarose gel electrophoresis, transferred to nylon membranes (Hybond-N+, Amersham) by overnight capillary blotting and fixed by UV crosslinking (Fotodyne, Chicago, IL) and transferred to a Hybond-N+ membrane (Amersham, UK) via free access.
Figure 1 Nitrite + nitrate levels in incubation media (A), northern blot hybridization and quantification of liver IGF-I mRNA in hepatocyte cultures (B), after incubation with different LPS concentrations for 24 h. A representative northern blot of IGF-I mRNA hybridization is shown on the right. Ten micrograms total RNA were hybridized with an RNA probe for rat IGF-I mRNA; each lane corresponds to the indicated LPS concentration; the 28 S ribosomal RNA is shown below. Detectable IGF-I mRNA species are indicated on the right and consist of a group of transcripts ranging from 7·5 to 0·9 kb. Since all these transcripts may potentially be translated to IGF-I, the densitometric results corresponded to the sum of all IGF-I transcripts. LPS induced an increase in the nitrite + nitrate values in the media (F1,39 = 26, P < 0·01), whereas it decreased the IGF-I mRNA at all tested concentrations (F1,38 = 25, P < 0·01). (C) There was a significant correlation (r1,22 = 11, P < 0·01) between medium concentrations of nitrites + nitrates and liver IGF-I mRNA in the hepatocytes incubated with LPS (solid circles) but not in those incubated with medium (open circles). Values are expressed as the mean ± S.E.M. (n = 5–12). **P < 0·01, *P < 0·05 versus medium alone. One-way ANOVA and Student’s t-test.
The rat IGF-I cDNA (Roberts et al. 1987) was generously supplied by Dr LeRoith. To generate radiolabeled cRNA, the plasmid vector (pGEM-3, Promega) was linearized with HindIII. 32P-labeled RNA antisense probes were generated from linearized plasmid with [α-32P]CTP (Nuclear Ibérica, Madrid, Spain) and T7 RNA polymerase (Roche Molecular Biochemicals). 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. The membranes were washed twice with 2×SSC, 0.1% SDS at 68°C for 10 min, and twice with 0.1×SSC, 0.1% SDS at 68°C also for 10 min. To verify loading, control hybridization was performed with a 28 S DNA probe labeled with32P-dCTP by random primer amplification. The membranes were exposed at −80°C for 1–4 days to X-ray film (Kodak X-Omat AR, Eastman Kodak) and with two intensifying screens. Autoradiographs were analyzed by densitometric scanning using a Gengenius (Syngene, Cambridge, UK). The intensities of autoradiograph signal levels were measured and expressed as the percentage of the mean intensity of the control cultures.

**Real-time PCR**

For reverse transcriptase (RT)-PCR analysis, 2 μg mRNA were reverse-transcribed using the instructions in the commercial kit Quantitec Reverse Transcription Kit (Qiagen). Primers for PCR were obtained from previously published sequences (Peinnequin et al. 2004), suppressor of cytokine signaling-3 (SOCS-3) forward: CCTCCAGCATCTTTGTGGAAAGGC; SOCS-3 reverse: TACTGCGCTGGGAACTCCCGAATG; hypoxanthine–guanine phosphoribosyl transferase (Hprt) forward: CTCATGGACTGATTATGGACAGGAC; Hprt reverse: GCAGGTCAGCAAAGAACTTATAGCC. They were designed from

![Graph](image-url)

**Figure 2** Effect of LPS on the nitrite+nitrate levels in the culture medium (upper panel) and IGF-I mRNA levels (lower panel) in hepatocyte cultures (HC) or in hepatocyte and Kupffer cell cocultures (HC+KP). A representative northern blot analysis showing the 7.5, 1.7 and 0.9 kb IGF-I transcripts and the 28 S ribosomal RNA in each sample is shown on the right. LPS induced an increase in the nitrite+nitrate release (F1,49 = 92, P < 0.01), and there were no differences between hepatocyte and hepatocyte+Kupffer cell cocultures. IGF-I mRNA was significantly decreased by LPS (F1,46 = 19, P < 0.01), but this decrease was higher in cocultures than in hepatocyte cultures (F1,46 = 6.2, P < 0.05). Results are expressed as means±S.E.M. (n=6–10 per group); **P<0.01 versus their respective control group incubated without LPS; †P<0.05, ††P<0.01 versus hepatocytes cultured with the same LPS concentration. Two-way ANOVA and Student’s t-test.
spanning a single sequence derived from two exons (i.e., separated by an intron in genomic DNA and primary RNA transcripts to minimize amplification). All primers were obtained from Qiagen. Each real-time PCR consisted of 10 ng total RNA equivalents, 1× Takara SYBR Green Premix Ex Taq (Takara Bio, Inc., Shiga, Japan), and 300 nM forward and reverse primers in a reaction volume of 25 μl. Reactions were carried out on a SmartCycler (Cepheid, Sunnyvale, CA, USA).

Parameters included 40 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Specific amplification was confirmed by the presence of one single peak in the melting curve plots. In addition, the PCR products were analyzed by agarose gel electrophoresis. Results were calculated as percent of control cultures, using the cycle threshold method (Livak et al. 2001) with the Hprt as reference gene.

Statistical analysis
All data are presented as the mean±S.E.M. Simple linear regression was used to determine correlations between variables. Differences among experimental groups were analyzed by one- or two-way ANOVA. Where there were differences among groups, the individual means were compared by Student’s t-test. Significance was assumed when P<0.05.

Results
Figure 1 shows the IGF-I mRNA and nitrite+nitrate levels in hepatocytes incubated with different LPS concentrations (from 0·1 to 50 μg/ml LPS). The nitrite+nitrate concentrations in the culture medium increased in parallel with the increase in the LPS concentration. The IGF-I mRNA in the cells also showed a dose-related response to LPS, being
significantly decreased by all LPS concentrations employed. In the groups of cells incubated with LPS, there was a negative correlation between the nitrite+nitrate concentrations in the culture medium and the IGF-I mRNA ($r = -0.61$, $P < 0.01$), but not in the cells cultured with medium alone (Fig. 1).

We compared the effect of two LPS concentrations (low 0.01 μg/ml, and intermediate 1 μg/ml) in hepatocyte cultures or in hepatocytes cocultured with Kupffer cells. The stimulatory effect of the two LPS concentrations, 0.01 and 1 μg/ml, on the nitrite+nitrate levels was similar in hepatocyte cultures and in cocultures (Fig. 2). Incubation of cells with LPS decreased the IGF-I mRNA levels in both types of cultures, but the inhibitory effect of LPS was higher in cocultures than in hepatocytes alone ($P < 0.01$). When the hepatocytes were cocultured with Kupffer cells, both LPS concentrations induced a significant decrease in IGF-I gene expression ($P < 0.01$). However, in hepatocyte cultures only the higher LPS concentration tested (1 μg/ml) induced a significant decrease in IGF-I gene expression ($P < 0.01$). In order to elucidate whether SNAP, a NO donor, treatment decreased hepatocyte IGF-I gene expression, we exposed hepatocytes to increasing concentrations of SNAP. Figure 3 shows that SNAP treatment resulted in an increase in nitrite+nitrate levels in the culture medium, which was only significant at the concentration of 1000 μM ($P < 0.01$). In addition, exogenous NO donated by SNAP also decreased IGF-I gene expression ($P < 0.01$) at the SNAP concentration of 1000 μM, whereas it was not modified by the other SNAP concentrations (Fig. 3).

We studied the role of iNOS induction in the inhibitory effect of LPS on IGF-I gene expression in hepatocyte cultures. For that purpose, the effect of the two iNOS inhibitors,
t-NIL, and aminoguanidine, on the hepatocyte response to 50 μg/ml LPS was analyzed. Nitrite + nitrate concentrations in the culture medium were increased by 50 μg/ml LPS (P<0.01). Both iNOS inhibitors decreased the nitrite + nitrate concentrations in basal conditions (P<0.01), and totally prevented the stimulatory effect of LPS on nitrite + nitrate release to the culture medium (Fig. 4). The IGF-I mRNA was decreased by LPS (P<0.01), but it was not modified by either incubation with t-NIL or aminoguanidine. The addition of t-NIL to the incubation medium prevented LPS-induced decrease in IGF-I mRNA (P<0.01). Aminoguanidine attenuated the decrease in IGF-I mRNA induced by LPS; the cells incubated with LPS + aminoguanidine had higher IGF-I mRNA levels (P<0.01) than the cells incubated with LPS alone.

Stimulation of the SOCS-3 expression has been involved as a mechanism of sepsis-induced decrease in IGF-I gene expression. Therefore, we determined whether NO may modulate the SOCS-3 in hepatocyte cultures. The effect of t-NIL and aminoguanidine alone or with LPS on SOCS-3 expression in hepatocytes is shown in Fig. 5. In the cultures incubated with LPS, there was a notable increase in SOC-3 mRNA (P<0.01, Fig. 5). Neither t-NIL nor aminoguanidine modified the SOCS-3 gene expression basally or after LPS stimulation.

In order to elucidate the role of iNOS induction in Kupffer cells in the LPS-induced decrease in IGF-I mRNA, cocultures were incubated with LPS and t-NIL. As shown in Fig. 6, LPS increased the nitrite + nitrate levels (P<0.01), and t-NIL blocked this stimulatory effect. LPS induced a significant decrease in IGF-I mRNA levels (P<0.01) in hepatocytes cultured with Kupffer cells (Fig. 6). t-NIL alone did not modify the IGF-I gene expression, but it attenuated the inhibitory effect of LPS on IGF-I.

Discussion

Our data show that LPS is able to decrease basal IGF-I gene expression in isolated hepatocytes. The inhibitory effect of LPS administration in vivo on IGF-I gene expression is well known. In contrast, to our knowledge, this is the first study that shows an inhibitory effect of LPS on basal IGF-I gene expression in hepatocyte cultures. Similarly, other authors have shown that LPS alone is able to decrease IGF-I mRNA in myoblast cultures (Frost et al. 2003).

The fact that the inhibitory effect of LPS on IGF-I is higher in cocultures than in hepatocytes alone is not unexpected, since stimulated Kupffer cells release many inflammatory mediators that can potentially inhibit IGF-I gene expression. The release of these mediators, tumour necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6), after LPS stimulation is higher in cocultures than in Kupffer or hepatocyte cultures (Hoibe et al. 2001, Yao et al. 2004). In addition, suppression of Kupffer cell function in vivo by gadolinium chloride injection blocks Kupffer cell phagocytosis and the subsequent release of its mediators, such as cytokines and NO after LPS administration, as well as the decrease in IGF-I gene expression in the liver (Hardonk et al. 1992, Granado et al. 2006). The increased inhibitory effect of LPS in vitro when incubated with Kupffer cells does not seem to be due to NO release, since nitrite + nitrate levels after LPS were similar in the medium of hepatocyte cultures and in cocultures. In addition, the blockade of LPS-induced NO release by t-NIL only partially prevents LPS-induced decrease in IGF-I mRNA when Kupffer cells were present in the cultures. All these data indicate that Kupffer cells increase the inhibitory effect of LPS on IGF-I mRNA by a pathway that is different to NO release. One of these pathways can be the release of cytokines, since an inhibitory effect of cytokines on in vitro IGF-I gene expression has been reported. IL-6 inhibits IGF-I gene expression both in hepatocyte and in hepatocyte with Kupffer cell cocultures (Lelbach et al. 2001). IL-1 is also able to decrease basal IGF-I mRNA levels and IGF-I release to the culture medium in isolated hepatocytes (Wolf et al. 1996). However, this effect is weak, and other authors were not able to find a decrease in IGF-I mRNA levels after IL-1 addition, basally in the absence of GH (Thissen & Verniers 1997).

As it has been described (Saad et al. 1995), LPS alone is able to increase NO production in cultured hepatocytes. The fact that there was an inverse correlation between nitrites and IGF-I mRNA in the hepatocytes incubated with LPS, but not in controls, suggests an inhibitory effect of NO on IGF-I gene expression. Moreover, when a NO donor, SNAP, was added to hepatocytes, a decrease in IGF-I mRNA was also observed, once again emphasizing the involvement of NO
in the inhibition of IGF-I gene expression. The effect of NO donors on hepatocyte function is not well known. It has been reported that SNAP pretreatment protects hepatocytes from TNF-induced apoptosis by inducing heat shock protein (Kim et al. 1997). However, in another study (Liang et al. 1997) NO from NO donors induced cell death by inhibiting mitochondrial respiration, but at a higher concentration than those used in the present study. An inhibitory effect of SNAP on IGF-I gene expression has previously been described in myoblasts (Frost et al. 2003). In those cells, TNF-α induces iNOS expression and decreases IGF-I mRNA, but the NOS inhibitors (L-NMMA or 1400W) are not able to prevent the inhibitory effect of TNF-α on IGF-I mRNA (Frost et al. 2003). Differences can be due to the fact that IGF-I gene regulation is different in myoblasts than in hepatocytes, or to the inhibitors employed; in our data, both inhibitors totally prevent the effect of LPS on nitrite release to the culture medium.

The fact that the inhibitory effect of LPS can be prevented by two specific iNOS inhibitors suggests that the effect of LPS on IGF-I is related to the generation of NO through induction of iNOS. All these data suggest that during sepsis, the overproduction of NO during septic shock has been demonstrated to contribute to circulatory failure, myocardial dysfunction, organ injury and multiple organ failure. Taking into account the effect of IGF-I in

Figure 6 Effect of the iNOS inhibitor, L-NIL, on the response of nitrite+nitrate levels (upper panel) and IGF-I mRNA levels (lower panel) to LPS (50 μg/ml) in hepatocytes cocultured with Kupffer cells. A representative northern blot of IGF-I mRNA hybridization is shown on the right; the size of the hybridization band is indicated on the right, 28 S, 28 S ribosomal RNA. Quantitative analysis is expressed as percentage of control group incubated with medium. There was an interaction between the effect of LPS and L-NIL (F1,33 = 851, P < 0.01) on nitrite+nitrate levels, since LPS increased the nitrite+nitrate levels in the cocultures incubated with medium, but not in those incubated with L-NIL. LPS decreased the IGF-I mRNA (F1,30 = 72, P < 0.01), whereas L-NIL increases the IGF-I mRNA (F1,30 = 10, P < 0.01). Results are expressed as means ± S.E.M. for n = 8–10 per group; **P < 0.01 versus control group incubated with medium; ††P < 0.01 vs LPS group incubated with medium; §§P < 0.01 versus control group incubated with L-NIL. Two-way ANOVA and Student's t-test.
Specific information on the effects of iNOS-derived NO on hepatocyte IGF-I gene expression is limited. We have previously reported that iNOS inactivation by aminoguanidine administration prevents the LPS-induced decrease in serum concentrations of IGF-I as well as in its mRNA in the liver (Priego et al. 2004). Aminoguanidine also has additional pharmacological properties unrelated to the NO pathway (Nilsson 1999), and therefore, its beneficial effects could arise from a mechanism different to iNOS inhibition. This does not seem to be the case, since in the present data l-NIL was even more potent than aminoguanidine in preventing LPS-induced decrease in IGF-I gene expression.

SOCS-3 is an acute-phase protein gene that is upregulated in the liver by LPS and inflammatory cytokines. It has been suggested that upregulation of SOCS-3 is involved in LPS-induced inhibition of STAT5b and IGF-I in vivo (Denson et al. 2003). This hypothesis is supported by previous studies in rodents showing that impairment of GH signaling in the liver induced by LPS, IL–6, chronic renal failure, and sepsis is accompanied by elevated expression of SOCS-2 and SOCS-3 (Mao et al. 1999, Denson et al. 2003). Our data do not support this hypothesis, since inhibition of NO release prevents LPS-induced decrease in IGF-I gene expression, but not the increase in SOCS-3. Similarly, it has been reported that the inhibitory effect of IL–1 on IGF-I mRNA, following GH induction, is not caused by JAK/STAT signaling or increased SOCS-3 expression (Shumate et al. 2005). Additional studies will be required to determine the mechanism by which NO inhibits IGF-I gene expression in the hepatocyte.

In conclusion, our data suggest that LPS decreases IGF-I gene expression in hepatocytes through the induction of iNOS and through the release of other inhibitory mediators in Kupffer cells.

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