Ptgs2 activation by endotoxin mediates the decrease in *Igf1*, but not in *Igfbp3*, gene expression in the liver

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Abstract

The aim of this work was to analyse the role of cyclooxygenase-2 (*Ptgs2*) in endotoxin-induced decrease in *Igf1* and Igf binding protein-3 (*Igfbp3*). For this purpose, male Wistar rats were injected with lipopolysaccharide (LPS) and/or the *Ptgs2* inhibitor meloxicam. LPS induced a significant decrease ($P < 0.01$) in serum concentrations of *Igf1* and *Igfbp3* and their mRNAs in the liver. Meloxicam administration prevented the inhibitory effect of LPS injection on serum *Igf1* and its liver mRNA. By contrast, meloxicam administration was unable to modify the inhibitory effect of LPS on *Igfbp3*. LPS injection also induced a decrease in GH receptor (*Ghr*) mRNA in the liver, and meloxicam attenuated this effect. In order to elucidate a direct action of the *Ptgs2* inhibitor on the liver cells, the effect of LPS

and/or meloxicam was studied in primary cultures of hepatocytes with non-parenchymal cells. LPS decreased *Igf1* and *Ghr* but not *Igfbp3* gene expression in liver cells in culture. Meloxicam administration attenuated the inhibitory effect of LPS on *Igf1* mRNA, whereas it did not modify the decrease in *Ghr* mRNA after LPS. The effect of meloxicam on the LPS response does not seem to be mediated by changes in nitric oxide or tumour necrosis factor (Tnf) production, since meloxicam did not modify the stimulatory effect of LPS on nitric oxide or *Tnfα* gene expression both *in vivo* and *in vitro*. All these data suggest that LPS-induced *Ptgs2* activation decreases *Igf1* gene expression in liver cells.

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Introduction

One of the endocrine alterations induced by sepsis and inflammation is the inhibition of growth hormone (GH)–insulin-like growth factor I (*Igf1*) system. The decrease in these anabolic hormones contributes to the negative nitrogen balance, growth inhibition and cachexia observed in chronic inflammation (Delano & Moldawer 2006, Thissen 2007). All these alterations lead to a slower recovery from the illness, and increased mortality.

Sepsis can be experimentally induced in rats by endotoxin (lipopolysaccharide, LPS) administration, since it reproduces most of the physiological responses triggered by sepsis. One of the mechanisms by which endotoxin inhibits serum concentrations of *Igf1* is by inducing GH resistance and decreasing its gene expression in the liver (Defalque *et al.* 1999, Priego *et al.* 2003a). The inhibitory effect of LPS can be exerted directly on the hepatocyte, since LPS is able to decrease *Igf1* gene expression in hepatocyte cultures (Priego *et al.* 2006).

We have previously reported that LPS-induced decrease in serum and liver *Igf1* seems to be due to inducible nitric oxide synthase (Nos2) activation, since Nos2 inhibitors are able to prevent the inhibitory effect of LPS on liver *Igf1* gene expression both *in vivo* and *in vitro* (Priego *et al.* 2004, 2006). However, the Nos2 inhibition was not able to prevent the LPS-induced decrease in *Igf1* mRNA when the hepatocytes

were cultured with Kupffer cells (Priego *et al.* 2006). These data indicate that nitric oxide release is not the only factor responsible for the inhibition of *Igf1* gene expression after endotoxin exposure.

The role of Kupffer cells on LPS-induced *Igf1* inhibition was also evidenced by the fact that Kupffer cell depletion by gadolinium administration prevents the inhibitory effect of LPS on GH receptor (*Ghr*), *Igf1* and Igf binding protein-3 (*Igfbp3*) gene expression (Granado *et al.* 2006). Kupffer cells (resident liver macrophages) have a pivotal role in liver inflammatory response; they release cytokines and prostanoids after LPS exposure, which might decrease *Igf1* gene expression. Tumour necrosis factor- α (*Tnfα*) has been reported to inhibit *Igf1* mRNA induction after GH stimulation both *in vivo* (Yumet *et al.* 2002) and *in vitro* (Ahmed *et al.* 2006). However, it has been demonstrated that suppression of *Tnfα* by pentoxifylline fails to restore both circulating *Igf1* levels and GH sensitivity in rats injected with LPS (Colson *et al.* 2003).

When activated by LPS, Kupffer cells release the cyclooxygenase-2 (*Ptgs2*) products, prostaglandin E₂ (Ptges2) and thromboxane A₂ (Tbxa2r) (Keller *et al.* 2005, Bezugla *et al.* 2006). We have shown that *Ptgs2* inhibition is able to prevent chronic arthritis-induced decrease in serum concentration of *Igf1* and its gene expression in the liver (Granado *et al.* 2007). Taking into account that *Ptgs2* is

expressed in Kupffer cells, and not in hepatocytes, incubated with LPS (Martín-Sanz *et al.* 1998), it is possible that *Ptgs2* activation can contribute to the LPS-induced decrease in *Igf1* gene expression in the liver.

The present study was designed in order to elucidate the possible inhibitory effect of *Ptgs2* activation on LPS-induced inhibition of *Igf1* and *Igfbp3* gene expression in the liver. For this purpose, the effect of meloxicam on the LPS-induced decrease in *Ghr*, *Igf1* and *Igfbp3* has been studied. In addition, studies have been performed using primary cultures of hepatocytes with non-parenchymal cells in order to analyse the possible interaction between *Nos2* and *Ptgs2* induction by LPS stimulation.

Materials and Methods

Male Wistar rats weighing 200–225 g were used in all experiments; they were obtained from Harlan (Barcelona, Spain). Rats were housed 3–4 per cage, under controlled conditions of temperature (22 °C) and light (lights on from 0730 to 1930 h). Food and water were made available *ad libitum*. Rats were quarantined for 1 week before any experimental use. All procedures on animals were carried out according to the guidelines recommended by the EU for the care and use of laboratory animals, and were approved by the Complutense University Animal Care Committee.

In vivo experiment

Rats were randomly assigned to the treatment groups. Twenty rats were i.p. injected with 250 µg/kg LPS (serotype 055:B5, Sigma Chemical Co.) in 250 µl sterile saline. Another 20 rats were injected with saline. Half of the rats in each group were simultaneously i.p. injected with the selective *Ptgs2* inhibitor, meloxicam (1 mg/kg, Sigma), while the other half were injected with 250 µl sterile saline. Meloxicam at this dosage decreased serum concentrations of *Ptgs2* and prevented arthritis-induced decrease in liver *Igf1* gene expression (Granado *et al.* 2007).

Rats received the treatments at 1700 h and at 0800 h the following day. This LPS administration protocol was shown to decrease levels of serum *Igf1* and its mRNA in the liver (Priego *et al.* 2003a). Meloxicam administration was not able to modify the LPS-induced decrease in food and water intake. All animals were killed by decapitation at 1200 h, 19 h after the first, and 4 h after the second LPS and/or meloxicam injection. Liver was removed, frozen immediately in liquid nitrogen, and stored at –80 °C for isolation of liver RNA. Blood was allowed to clot, and the serum was stored at –20 °C for *Igf1*, *Igfbp3* and nitrite/nitrate assays.

In vitro experiments

The role of *Ptgs2* and *Nos2* induction on the inhibitory effect of LPS on *Ghr* and *Igf1* gene expression was studied in hepatocytes

and non-parenchymal cell cultures, as previously reported (Granado *et al.* 2008). Both hepatocytes and non-parenchymal cells from one culture were isolated from the same rat liver. Rats were anaesthetized with pentobarbital (Sigma, Chemical Co). The liver was perfused *in situ* through the portal vein at a flow rate of 40 ml/min with calcium-free buffer at 37 °C 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 the cells were obtained by gentle raking with a comb and filtered through a 100 µm mesh to remove cell aggregates and tissue debris. Hepatocytes were separated from non-parenchymal cells by differential centrifugation at 25 g (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 were kept on ice while the non-parenchymal cells were isolated.

Non-parenchymal cells were isolated from supernatant of the differential centrifugations and were then centrifuged at 650 g for 6 min at 4 °C. The pellet was suspended in a 50 ml culture medium and centrifuged at 650 g for 7 min. Medium consisted of Williams' medium E (Gibco) with L-glutamine (2 mM), insulin (1 µM), HEPES (15 mM), penicillin + streptomycin (100 units/ml + 100 µg/ml) and 10% low-endotoxin calf serum. Non-parenchymal cells were finally suspended and plated with hepatocytes in a proportion of 3:2 (3·10⁶ hepatocytes/2·10⁶ non-parenchymal cells in 5 ml medium).

The culture medium was replaced with fresh medium 24 h before starting all experiments. A concentration of 100 ng/ml LPS was used; in earlier experiments we used a higher dose to stimulate hepatocytes, but when hepatocytes were cultured with non-parenchymal cells the effect of LPS on *Igf1* can be observed at a lower LPS dose (Priego *et al.* 2006), since the effect of LPS was enhanced.

Real-time PCR

RNA was extracted by the guanidine thiocyanate method using a commercial kit (Ultraspec RNA, Biotecx Laboratories Inc., Houston, TX, USA). The integrity and the concentration of the RNA were confirmed using agarose gel electrophoresis. For RT-PCR analysis, 1 µg liver or cocultures cell total RNA was reverse-transcribed with Quantiscript Reverse Transcription kit (Qiagen Comb Hilden). Primers for PCR (Table 1) were obtained from Roche by using the Exiqon Universal Probe Library (*Igfbp3* and *Ghr*) or from previously published sequences of *Igf1*, *Tnfα* (Dehoux *et al.* 2004), *Ptgs2* (Bianchi *et al.* 2005) and hypoxanthine-guanine phosphoribosyl transferase (*Hprt1*) (Peinnequin *et al.* 2004).

Each real-time PCR consisted of 10 ng total RNA equivalents, 1× Takara SYBR Green Premix Ex Taq (Takara BIO INC, Otsu, Shiga, Japan) and 300 nM forward and reverse primers in a reaction volume of 25.5 µl. Reactions were carried out on a SmartCycler (Cepheid, Sunnyvale, CA, USA). Parameters included an initial activation of hotStarTaq DNA polymerase at 95 °C for 15 s, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C and extension

Table 1 Primers for real-time PCR

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Product (bp)
<i>Igf1</i>	GCTATGGCTCCAGCATTCG	TCCGGAAGCAACTCATCC	62
<i>Ghr</i>	TCCCGGGATCTATTCAGCTA	TGTTTGCTCCAGCAGAGACAT	99
<i>Igfbp3</i>	GGAAAGACGACGTGCATTG	GCGTATTTGAGCTCCACGTT	78
<i>Ptgs2</i>	TACAAGCAGTGGCAAAGGCC	CAGTATTGAGGAGAACAGATGGG	303
<i>Tnfα</i>	GCCACCAGCTCTTCTGTCT	GTCTGGGCCATGGAACTGAT	100
<i>Hprt1</i>	CTCATGGACTGATTATGGACAGGAC	GCAGGTCAGCAAAGAACTTATAGCC	122

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 analysed by agarose gel electrophoresis. Results were calculated as the percentage of control rats, using the cycle threshold 2 (C_t) methods (Livak & Schmittgen 2001) with the *Hprt1* as the reference gene.

IGF1, PTGES2 and nitrite + nitrate determinations

Serum IGF1 concentrations were measured by a double-antibody RIA. The IGF1 antiserum (UB2-495) was a gift from Dr Underwood and Dr Van Wik, and it is distributed by the Hormone Distribution Program of NIDDK through the National Hormone and Pituitary Program. Levels of Igf1 were expressed in terms of IGF1 from Gropep Ltd (Adelaide, Australia). The intra-assay coefficient of variation was 8%. All samples were run in the same assay.

Serum concentrations of PTGES2 were measured by an enzymeimmunoassay system using a commercial kit (Amersham Biosciences) following the manufacturer's instructions.

Nitrite + nitrate concentration in the serum and culture medium was measured by a modified method of Griess assay, described by Miranda *et al.* (2001). Serum was deproteinized to reduce turbidity by centrifugation through a 30 kDa molecular mass filter using a Centrifree micropartition device with a YM-30 ultrafiltration membrane (Amicon Division, Millipore Corporation, Bedford, TX, USA) at 300 g for 1 h at 37 °C for 300 μ l samples. To 100 μ l vanadium chloride, 25 μ l serum or 100 μ l medium were mixed; the Griess reagents were added very soon after. 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 μ M.

Western ligand blot

Serum concentrations of IGFBP3 were measured by western blot. Two microliters of serum were diluted in sample buffer and boiled for 2 min at 90 °C, loaded onto 1% SDS-12.5% polyacrylamide gels, and electrophoresed under non-reducing conditions. Proteins were transferred onto nitrocellulose sheets (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 ¹²⁵I-labelled Igf1 (1.5×10^6 c.p.m./ml). The nitro-cellulose sheets were then washed, dried and exposed at –80 °C to X-ray film (Kodak X-Omat AR, Eastman Kodak) and to two intensifying screens for 1–4 days according to the signal obtained. The signals of the film were quantified by densitometry using a PC-Image VGA24 program for Windows. The density of the IGFBP3 band in each lane was expressed as the percentage of the mean density of sera from control rats injected with saline.

Statistical analysis

Statistics were computed using the statistics program STATGRAPHICS plus for Windows (Statpoint Inc., Madrid, Spain). Data are presented as means $\pm$ S.E.M. and differences among experimental groups were analysed by two-way ANOVA. *Post hoc* comparisons were made by using a subsequent LSD multiple range test. Serum concentrations of nitrites + nitrates and liver *Tnf α* mRNA data in the *in vivo* experiment were subjected to log transformation, since variances showed a log-normal distribution. Statistical significance was set at $P < 0.05$.

Results

In vivo experiment

The serum concentrations of IGF1 and its gene expression in the liver, in rats injected with saline, LPS, meloxicam or LPS and meloxicam are shown in Fig. 1. LPS injection induced a significant ($P < 0.01$) decrease in serum concentrations of IGF1 and mRNA in the liver ($P < 0.01$). The *Ptgs2* inhibitor meloxicam prevented the inhibitory effect of LPS on IGF1, since the rats injected with LPS and meloxicam had higher serum IGF1 ($P < 0.01$) and *Igf1* mRNA in the liver ($P < 0.05$) than the control rats injected with LPS. In the control rats, meloxicam decreased serum concentrations of Igf1 ($P < 0.01$), whereas the *Igf1* mRNA in the liver was similar in the rats injected with saline and those injected with meloxicam. In the control rats, LPS injection decreased *Ghr* mRNA in the liver ($P < 0.01$, Fig. 1C). LPS also decreased *Ghr* mRNA in the rats injected with meloxicam ($P < 0.01$). However, this decrease was lower in the rats injected with meloxicam than in the control rats ($36.6 \pm 7\%$ of the control rats injected with

meloxicam versus $58 \pm 3.4\%$ of the control rats injected with saline, $P < 0.05$).

In the rats injected with LPS there was a significant decrease in the serum concentrations of *Igf1* and gene expression in the liver ($P < 0.01$, Fig. 2). In contrast to the *Igf1* data, meloxicam administration was not able to modify

the inhibitory effect of LPS on IGFBP3, since the rats injected with LPS and meloxicam had similar serum IGFBP3 and liver *Igf1* mRNA to the levels in rats injected with LPS alone (Fig. 2). Meloxicam administration did not modify the serum concentrations of IGFBP3 or the liver *Igf1* mRNA in control rats.

In control rats, LPS increased *Ptgs2* mRNA in the liver ($193 \pm 33\%$ vs $100 \pm 16\%$ in control rats injected with saline, $P < 0.05$). The effect of LPS and meloxicam administration on serum concentrations of nitrites+nitrates is shown in Fig. 3A. As expected, LPS injection increased the serum nitrite+nitrate levels ($P < 0.01$). Meloxicam increased the serum concentration of nitrites+nitrates in control rats ($P < 0.05$), but not in the rats injected with LPS. The effect of LPS and meloxicam administration on liver *Tnfα* mRNA was similar to the effect on serum nitrites+nitrates (Fig. 3B). Both LPS and meloxicam increased the *Tnfα* mRNA in the liver, but the increase was only significant in the control rats injected with LPS ($P < 0.05$). In the rats injected with meloxicam, LPS administration did not modify *Tnfα* mRNA.

In vitro experiment: liver cell cultures

LPS at the concentration of 100 ng/ml induced a significant increase in *Ptgs2* mRNA in liver cells (463 ± 57 vs 100 ± 10 in the cells incubated with medium alone, $P < 0.01$) and in the PTGES2 in the culture medium ($P < 0.01$, Fig. 4). The *Ptgs2* inhibitor, meloxicam, prevented the stimulatory effect of LPS on *Ptgs2* release (Fig. 4).

As in the *in vivo* experiment, 100 ng/ml of LPS decreased *Igf1* and *Ghr* gene expression in liver cell cultures ($P < 0.01$, Fig. 5). Meloxicam attenuated the inhibitory effect of LPS on *Igf1* gene expression, since the *Igf1* mRNA in the cells incubated with LPS and meloxicam was higher ($P < 0.05$) than in the cells incubated with LPS alone (Fig. 5A). By contrast, meloxicam was not able to modify the LPS-induced decrease in *Ghr* mRNA (Fig. 5B). Meloxicam alone did not modify *Igf1* or *Ghr* mRNA in the cultured cells.

Unsuspectingly, the addition of LPS to the culture medium did not significantly decrease *Igf1* gene expression

Figure 1 Serum concentrations of (A) *Igf1*, (B) *Igf1* mRNA in the liver and (C) *Ghr* mRNA in the liver in male rats i.p. injected with saline, meloxicam (1 mg/kg), LPS (0.25 mg/kg) or with meloxicam and LPS. *Igf1* mRNA was measured by real-time PCR as described in the material and methods, and is presented as percentage of the mean value in control rats treated with saline by analysing the CT numbers corrected by CT readings of corresponding internal *Hprt1* mRNA controls. There was an interaction between the effect of meloxicam and LPS on serum concentration of *Igf1* ($F_{1,37} = 39$, $P < 0.01$) and on liver *Igf1* mRNA ($F_{1,38} = 6$, $P < 0.05$), since LPS decreased the serum concentrations of *Igf1* and the *Igf1* mRNA in the liver in the rats injected with saline, but not in those that were injected with meloxicam. LPS decreased *Ghr* mRNA ($F_{1,37} = 27$, $P < 0.01$) in the liver. Results are expressed as mean $\pm$ S.E.M. ($n = 9$ –11 rats). Values without the same letter are significantly different, $P < 0.05$.

Figure 2 Effect of LPS (0.25 mg/kg) and meloxicam (1 mg/kg) administration on serum concentrations of (A) IGFBP3 and (B) *Igfbp3* mRNA in the liver. Serum concentrations of IGFBP3 were determined by ligand blot using ^{125}I -Igf1. *Igfbp3* mRNA was measured by real-time PCR by analysing the CT numbers corrected by CT readings of corresponding internal *Hprt1* mRNA controls. Results are expressed as percentage of the control rats injected with saline, for at least eight rats per group. LPS injection decreased serum concentration of IGFBP3 ($F_{1,37}=33$, $P<0.01$) and liver *Igfbp3* mRNA ($F_{1,39}=31$, $P<0.01$). Data are the mean $\pm$ S.E.M. Values without the same letter are significantly different, $P<0.05$.

in the cells, although LPS tended to decrease it (Fig. 5C). Meloxicam did not modify *Igfbp3* mRNA.

The nitrite + nitrate concentration in the culture medium was higher in the cells cultured with meloxicam than in the cells cultured with medium alone ($P<0.01$, Fig. 6). LPS induced a similar increase in the concentrations of nitrite + nitrate in control cells and in the cells incubated with meloxicam (Fig. 6A). *Tnfa* mRNA in the liver cells was also increased by LPS ($P<0.01$). Meloxicam addition to the culture medium increased *Tnfa* gene expression ($P<0.05$), and LPS was not able to further increase *Tnfa* mRNA in the liver cells cultured with meloxicam (Fig. 6B).

Figure 3 Effect of meloxicam administration (1 mg/kg) on (A) the serum concentrations of nitrites + nitrates and (B) *Tnfa* mRNA in the liver in rats injected with LPS or saline. *Tnfa* mRNA was determined by real-time PCR, and is presented as percentage of the mean value in control rats treated with saline by analysing the CT numbers corrected by CT readings of corresponding internal *Hprt1* mRNA controls. There was an interaction between the effect of meloxicam and LPS ($F_{1,39}=3.8$, $P<0.05$), since meloxicam increased serum nitrites + nitrates in the rats injected with saline, but not in those that received LPS. Data are the mean $\pm$ S.E.M. for 8–10 rats per group. Values without the same letter are significantly different $P<0.05$.

Discussion

Our data show that the inhibitory effect of LPS on IGF1 is prevented by administration of the Ptgs2 inhibitor meloxicam. By contrast, the LPS-induced decrease in IGFBP3 *in vivo* does not seem to be significantly affected by meloxicam. LPS does not decrease *Igfbp3* gene expression in the liver cells in culture, whereas it exerts a potent inhibitory effect on *Igf1* and *Ghr* gene expression. These data suggest that *Igf1* and *Igfbp3* are regulated by different mechanisms during the inflammatory response induced by endotoxin. We have

Figure 4 Effect of incubation with meloxicam (10^{-5} M) on the LPS (100 ng/ml)-induced *Ptgs2* release in liver cell cultures. There was an interaction between the effect of meloxicam and LPS ($F_{1,30}=10$, $P<0.01$), since LPS increased PTGES2 concentration in the culture medium in control cells but not in those incubated with meloxicam. Results are expressed as mean $\pm$ S.E.M. ($n=7-9$ wells). Values without the same letter are significantly different, $P<0.05$.

previously suggested this hypothesis (Priego *et al.* 2003b) since, in Lewis rats, LPS decreased *Igfbp3* (Priego *et al.* 2003b), whereas both *Igf1* and *Ghr* were not modified by endotoxin (Priego *et al.* 2002). Although *Igf1* and *Igfbp3* genes in the liver are positively regulated by GH, other stimuli differentially affect them. For example, in cocultures of rat hepatocytes and Kupffer cells cAMP decreases *Igfbp3*, whereas it increases *Igf1* biosynthesis (Scharf *et al.* 2001). Furthermore, glucocorticoids inhibit *Igf1*, whereas they increase *Igfbp3* mRNA in the liver (Luo & Murphy 1990, Koedam *et al.* 2000, Priego *et al.* 2005b). It has been reported that diabetes-induced decrease in *Igf1* can be secondary to the increased release of glucocorticoids and the decrease in *Igfbp3* due to other factors (Unterman *et al.* 1993). Taking into account that insulin increases *Igfbp3* gene transcription (Villafuerte *et al.* 1997), the lack of insulin can be the cause of the decrease in *Igfbp3* gene expression in the liver. In diabetic rats, corticosterone at a high dose decreases IGF1, whereas at a lower dose increases IGFBP3 (Rodgers *et al.* 1995). All these data indicate that IGF1 and IGFBP3 are not always modified in a parallel fashion. Furthermore, in a model of chronic inflammation, such as adjuvant-induced arthritis, serum concentrations of IGF1 are decreased, whereas those of IGFBP3 are increased (Ibáñez de Cáceres *et al.* 1990).

The lack of a significant effect of LPS on *Igfbp3* gene expression in the cultured liver cells suggests that the inhibitory effect of LPS on *Igfbp3* *in vivo* seems to be mediated by a non-hepatic factor. The data indicate that LPS or cytokines do not directly inhibit *Igfbp3* gene expression. It has been reported that IL6, IL1b and TNF increase the biosynthesis of IGFBP3 in primary cocultures of hepatocytes and Kupffer cells, whereas the biosynthesis of IGF1 is

Figure 5 Effect of meloxicam (10^{-5} M) and LPS (100 ng/ml) on (A) *Igf1*, (B) *Ghr* and (C) *Igfbp3* mRNA in liver cell cultures. *Igf1*, *Ghr* and *Igfbp3* mRNA were measured by real-time PCR, and results are expressed as percentage of the control cells incubated with medium alone by analysing the CT numbers corrected by CT readings of corresponding internal *Hprt1* mRNA controls. LPS decreased *Igf1* ($F_{1,55}=40$, $P<0.01$) and *Ghr* ($F_{1,57}=35$, $P<0.01$) mRNA. Data are expressed as mean $\pm$ S.E.M. ($n=13-15$ wells). Values without the same letter are significantly different, $P<0.05$.

Figure 6 (A) Nitrites + nitrates in the culture medium and (B) *Tnfa* mRNA in liver cells cultured with meloxicam (10^{-5} M) and LPS (100 ng/ml). *Tnfa* mRNA was measured by real-time PCR, and results are expressed as percentage of the control cells incubated with medium alone by analysing the CT numbers corrected by CT readings of corresponding internal *Hprt1* mRNA controls. There was an interaction between the effect of meloxicam and LPS on the concentration of nitrites + nitrates in the culture medium ($F_{1,59} = 5.6$, $P < 0.05$), since meloxicam increased nitrites + nitrates in the cells incubated with medium alone, but not in the cells incubated with LPS. Both meloxicam ($F_{1,57} = 4.2$, $P < 0.05$) and LPS ($F_{1,57} = 8.6$, $P < 0.01$) increased *Tnfa* mRNA. Data are expressed as mean $\pm$ S.E.M. ($n = 14$ –15 wells). Values without the same letter are significantly different, $P < 0.05$.

inhibited (Lelbach *et al.* 2001). The stimulatory *in vitro* effect of cytokines on *Igfbp3* has been described in other cell types such as salivary gland tumour (Katz *et al.* 1999) and human monocytic cell line GLI1 (Agnese *et al.* 2002). Similar to our data, no effect of LPS on *Igfbp3* has been observed in primary rata microglia cultures, whereas LPS decreased *Igfbp5* and -6

(Chesik *et al.* 2004). All these data indicate that the inhibitory effect *in vivo* of LPS on *Igfbp3* is not exerted directly by endotoxin or cytokines on a hepatic level.

The fact that LPS decreased *Igf1* gene expression directly in the liver cell cultures indicates that LPS has an inhibitory effect on *Igf1* gene expression *per se*. However, we cannot exclude that part of the inhibitory effect of LPS administration *in vivo* can be secondary to a decrease in food intake. Nevertheless, we have previously reported that LPS administration is able to decrease both serum IGF1 and *Igf1* mRNA in fasted rats (Granado *et al.* 2008).

The *Ptgs2* activation seems to directly suppress liver *Igf1* gene expression, since meloxicam attenuated the inhibitory effect of LPS on liver cells in culture. However, meloxicam *in vivo* prevented the inhibitory effect of LPS on *Igf1* gene expression in the liver, whereas *in vitro* it only attenuated the LPS-induced decrease in *Igf1* mRNA. This difference cannot be explained by the fact that meloxicam was not able to block *Ptgs2* activity, since LPS-induced increase in *Ptgs2* release to the culture medium was prevented by meloxicam. One possible explanation could be that part of the *in vivo* meloxicam effect is an indirect effect, exerted outside the liver. In this sense, we have reported that meloxicam prevents the inhibitory effect of chronic arthritis on both pituitary GH and liver *Igf1* gene expression (Granado *et al.* 2007). In the present data we did not evaluate pituitary GH, since LPS at the dose of 0.25 mg does not significantly decrease GH mRNA (Priego *et al.* 2003a).

In contrast to the *Igf1* response, meloxicam did not modify the LPS-induced decrease in *Ghr* mRNA in the liver cells *in vitro*, although the decrease was attenuated *in vivo*. Taking into account that one of the factors that controls *Ghr* gene expression is GH (Flores-Morales *et al.* 2006), and that LPS is able to inhibit pituitary GH secretion (Kasting & Martin 1982), by increasing hypothalamic somatostatin (Spangelo *et al.* 1990, Priego *et al.* 2005a), it is logical to think that pituitary GH can mediate the LPS-induced decrease in *Ghr* mRNA. In fact, LPS at a low dose (10 μ g/kg) increases pituitary GH release, does not modify liver *Ghr* mRNA and decreases *Igf1* mRNA (Priego *et al.* 2003a). However, at higher doses, LPS decreases pituitary GH, liver *Ghr* and *Igf1* (Priego *et al.* 2003a). These data support the hypothesis that some of the changes in liver *Ghr* and *Igf1* genes during endotoxin administration are secondary to modifications in pituitary GH, whereas others are exerted directly on liver cells. In the liver LPS very rapidly induces a GH resistance state, since 4 h after LPS injection there is a decrease in GH post-receptor signalling (Jak2–Stat5a phosphorylation), in spite of the fact that the GHR protein levels are still unchanged (Chen *et al.* 2007). It has been recently reported that LPS can also directly suppress *Ghr* expression *in vitro* by inhibiting *Ghr* promoter activity (Dejckhamron *et al.* 2007). However, these authors find that LPS *in vivo* inhibits *Ghr* gene expression by a cytokine-dependent mechanism (Denson *et al.* 2001). It is also possible that the effect of meloxicam in

the LPS-induced decrease in *Ghr* and *Igf1* *in vivo* are partly mediated by changes at the pituitary GH.

TNF and nitric oxide were measured as possible mediators of the meloxicam effect, since they inhibit *Igf1* gene expression in hepatocytes (Thissen & Verniers 1997, Ahmed *et al.* 2006, Priego *et al.* 2006). Although meloxicam prevented the inhibitory effect of LPS on *Igf1* gene expression in the liver, it was not able to prevent the stimulatory effect of LPS on the release of nitrites + nitrates *in vivo* and *in vitro*. On the contrary, meloxicam increased nitrites + nitrates in basal conditions both *in vivo* and *in vitro*. These paradoxical results are in accordance with those recently reported (Razzak *et al.* 2008), in which *Ptgs2* inhibition increases NO production in macrophages. *Ptgs2* inhibitors have also been reported to increase nitric oxide production in oestrogenized rat uterus (Cella *et al.* 2006). These data suggest that PTGS2 products decrease nitric oxide synthesis.

Prostaglandins also have an inhibitory effect on *Tnfα* gene expression and secretion in macrophages (Kunkel *et al.* 1988, Treffkorn *et al.* 2004) and Kupffer cells (Karck *et al.* 1988), whereas prostaglandin inhibition by indomethacin increases the *Tnfα* response to LPS stimulation in the rat glial cells (Shemi *et al.* 2001).

The fact that meloxicam did not prevent *Tnfα* and nitric oxide response to LPS suggests a direct effect of *Ptgs2* products on *Igf1* gene expression, rather than being mediated by nitric oxide or *Tnfα*. In hepatocyte cultures it has been reported that *Tnfα* was not able to alter basal *Igf1* mRNA, but TNF- α inhibits the induction of *Igf1* mRNA by GH (Ahmed *et al.* 2006). Inhibition of *Tnfα* release by pentoxifylline administration prevents LPS-induced IL-1, whereas it is not able to prevent the increased release of PTGES2 (Shemi *et al.* 2001), or the decrease in serum IGF1 and liver *Igf1* mRNA (Colson *et al.* 2003). These data support the hypothesis that *Tnfα* does not seem to be implicated in the LPS-induced decrease in basal *Igf1* gene expression in the liver.

We have previously reported that *Nos2* activation plays an important role in LPS-induced inhibition of *GH-Igf1*. Aminoguanidine, an *Nos2* inhibitor, prevents the LPS-induced activation of hypothalamic somatostatin (Priego *et al.* 2005a), and the decrease in *Igf1* in the liver (Priego *et al.* 2004). It has been reported that nitric oxide or iNOS activation positively regulates *Ptgs2* gene expression (Salvemini *et al.* 1993, Yang *et al.* 2006). In addition, the *Nos2* inhibitor aminoguanidine decreases LPS-induced *Ptgs2* activation in Kupffer cells (Ahmad *et al.* 2002), and suppresses prostaglandin production in the LPS-treated animals (Salvemini *et al.* 1995). Accordingly, it is possible that part of the inhibitory effect of nitric oxide on the *GH-Igf1* system can be due to the activation of *Ptgs2* gene expression.

In summary, our data suggest that *Ptgs2* activation plays an important role in the inhibitory effect of LPS on the *Igf1* system by directly decreasing *Igf1* gene expression in liver cells. On the contrary, *Ptgs2* activation does not seem to be involved in the LPS-induced decrease in *Igf1bp3* gene expression in the liver.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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