British Journal of Pharmacology (1998) 125, 1313–1319
http://www.stockton-press.co.uk/bjp

Expression of cyclooxygenase-2 in foetal rat hepatocytes stimulated with lipopolysaccharide and pro-inflammatory cytokines

[1,2]Paloma Martín-Sanz, [1]Nuria A. Callejas, [1]Marta Casado, [1]María J.M. Díaz-Guerra & [1]Lisardo Boscá

[1]Instituto de Bioquímica (CSIC-UCM). Facultad de Farmacia. Universidad Complutense. 28040 Madrid, Spain

1 Cyclooxygenase-2 (COX-2) is involved in the biosynthesis of prostanoids in the course of inflammatory reactions. This isoenzyme is regulated at the transcription level and many cells express COX-2 upon challenge with lipopolysaccharide (LPS) or pro-inflammatory cytokines.

2 Since hepatocytes respond to LPS and pro-inflammatory stimuli, we investigated the expression of COX-2 in foetal and adult hepatocytes upon challenge with these substances.

3 COX-2 was expressed in foetal hepatocytes incubated with LPS, tumour necrosis factor-α and interleukin-1β. This response rapidly decreased after birth and was absent in hepatocytes from animals aged 2 days or more and treated under identical conditions. The expression of COX-2 was determined at the mRNA, protein and enzyme activity levels using Northern and Western blot, and following the synthesis of prostaglandin E_2, respectively. The use of NS 398, a specific pharmacological inhibitor of COX-2, confirmed the expression of this isoenzyme in activated foetal hepatocytes.

4 Synergism in COX-2 expression was observed between LPS, tumour necrosis factor-α and interleukin-1β. Interleukin-6 and permeant analogues of cyclic AMP failed to induce COX-2 or to synergize with LPS. Also, transforming growth factor-β inhibited the LPS- and pro-inflammatory cytokines-dependent expression of COX-2.

5 These results indicate that foetal hepatocytes are competent to express COX-2 upon challenge with pro-inflammatory stimuli, a process lost completely in hepatocytes isolated from animals aged 2 days.

Keywords: Cyclooxygenase-2; foetal hepatocyte; inflammation; lipopolysaccharide

Introduction

Prostaglandin H synthase, also known as cyclooxygenase (COX), catalyzes the synthesis of prostaglandin H-2 from arachidonate, and constitutes a key regulatory step in the biosynthesis of prostanoids (Xie *et al*., 1991; DeWitt, 1991). Two COX isoenzymes have been identified in mammals and they are referred to as COX-1 and COX-2. The two isoenzymes are encoded by different genes and the control of its expression, regulation of enzyme activity and physiological functions are very different (Xie *et al*., 1991; DeWitt 1991; Pilbeam *et al*., 1993; Crofford, 1997). COX-1 is constitutively present in many tissues and is the responsible of the low prostaglandin synthesis involved in cell homeostasis (Pilbeam *et al*., 1993). The expression of this isoenzyme is scarcely regulated in response to mitogens, hormones or growth factors (Crofford, 1997; DeWitt & Smith, 1988). COX-2 is a highly-inducible enzyme that is expressed in the course of inflammation or other cellular stresses, and accounts for the important synthesis of prostanoids that occurs in several physiopathological situations such as endotoxemia, septic shock and local inflammation of target tissues (DeWitt, 1991; Kujubu *et al*., 1991; Feng *et al*., 1995).

Hepatocytes well respond both *in vivo* and *in vitro* to most of the stimuli that positively regulate COX-2 expression in other cells, including LPS, IL-1β, TNF-α, and reactive oxygen intermediates (DeWitt, 1991; Feng *et al*., 1995; Herschman *et al*., 1995; Williams & DuBois, 1996). For example, iNOS, another enzyme involved in inflammatory responses and which activity is controlled at the transcriptional level, is expressed upon challenge of hepatocytes with these factors (Curran *et al*., 1989; Geller *et al*., 1993; Casado *et al*., 1997). However, except for the expression of COX-2 in Kupffer cells, other liver cells, including hepatocytes, failed to induce COX-2 transcription regardless of the stimuli used (Nanji *et al*., 1997; Zhang *et al*., 1995). This segregation of COX-2 from the inflammatory response of activated hepatocytes is rather paradoxical in view of the widely observed cross-regulation of COX-2 with other genes that participate in these processes (Williams & DuBois, 1996; Sweirkosz *et al*., 1995; Salvemini *et al*., 1995). When the expression of COX-2 was investigated in foetal hepatocytes of 21 days of gestation we observed that these cells, but not the adult counterparts, induced this enzyme. Our results show that foetal hepatocytes exhibited an important COX-2 activity upon challenge with LPS and pro-inflammatory cytokines, whereas this response was impaired after birth.

Methods

Chemicals

Cytokines, permeant cyclic AMP analogues and LPS from *Salmonella typhimorium* were obtained from Boehringer (Mannheim, Germany) or Sigma (St. Louis, MO, U.S.A.). Antibodies were from Santa Cruz Laboratories (Santa Cruz, CA, U.S.A.). Tissue culture dishes were from Falcon (Lincoln Park, NJ, U.S.A.). Tissue culture media were from Biowhittaker (Walkersville, MD, U.S.A.). The endotoxin content of the IL-1β, TNF-α, TGF-β, 8Br-cyclic AMP and solutions was negligible using the *Limulus polyphemus* test (Sigma). NS 398 was from Universal Biological Ltd (London, U.K.).

[2] Author for correspondence.

Isolation and incubation of foetal and neonatal hepatocytes

Hepatocytes from 21 and 22 day-old foetuses were prepared from pregnant albino Wistar rats (300–350 g) aged 3 months. Animals were cared following the Institutional Animal Care Instructions. Neonatal hepatocytes were prepared from animals aged 0, 1 or 2 days. Animals were fed on a standard laboratory diet and killed between 9:00 and 10:00 h. Gestational age was assessed by standard criteria and foetuses were delivered by Caesarean section (Martin-Sanz *et al*., 1989). A suspension of foetal or neonatal hepatocytes was prepared by a non-perfusion collagenase dispersion method that involved incubation (3 g per flask) of chopped foetal liver for 30 min at 37°C with 15 ml of Ca^{2+}-free Krebs-bicarbonate buffer containing 0.5 mM EGTA, under continuous gassing with carbogen (O_2/CO_2, 19/1) (Martin-Sanz *et al*., 1989). The cell suspension was centrifuged (35 g for 2 min) and the cell pellet was resuspended and incubated for 60 min in the presence of 2.5 mM Ca^{2+} and 0.5 mg ml^{-1} of collagenase A (Boehringer). At the end of the incubation period, the cells were centrifuged at $50 \times g$ for 5 min and the resuspended cell pellet was progressively filtered through nylon membranes of 500, 100 and 50 μm mesh. Cell viability was assessed by Trypan blue exclusion and was always higher than 90%. The hepatocyte suspension was washed twice with sterile DMEM medium and then resuspended in this medium supplemented with 50 μg ml^{-1} of gentamicin, 50 μg ml^{-1} of penicillin G and 50 μg ml^{-1} of streptomycin. Hepatocytes from adult male rats aged 3 months were prepared by perfusion with collagenase in Krebs-bicarbonate buffer under continuous gassing with carbogen (O_2/CO_2, 19/1), and following the classic perfusion/recirculation protocol (Martin-Sanz *et al*., 1989; Hortelano *et al*., 1995). The hepatocyte suspension was washed twice with sterile DMEM medium and then resuspended as indicated for the foetal hepatocytes. Foetal, neonatal and adult hepatocytes were maintained in the absence of serum for 30 min in a 10 cm dish at $2-3 \times 10^7$ cells to remove adherent cells, and then the suspension was plated at $3-4 \times 10^6$ cells in 6 cm tissue-culture dishes, in a culture medium containing 2.5 ml of DMEM supplemented with 10% of heat inactivated foetal calf serum. Four hours after seeding the cells, the medium was aspirated and the plates were washed twice with PBS to remove the non-adherent cells. The hepatocytes were maintained in 2 ml of phenol red-free DMEM supplemented with 2% of heat-inactivated foetal calf serum (Casado *et al*., 1997; Martin-Sanz *et al*., 1989). The amount of foetal hepatocytes in the culture was evaluated by immunocytochemistry using an anti-α-foetoprotein Ab and was higher than 84%. The amount of cells expressing CD11b/c was also evaluated by immunocytochemistry and was below 5% in these cultures.

Preparation of microsomal fractions

Cultured cells were washed twice with ice-cold PBS and homogenized with 1 ml of ice-cold extraction buffer (100 mM Tris-HCl, pH 7.4; 2 mM EDTA, 10 μg ml^{-1} leupeptin, 20 μg ml^{-1} aprotinin, 0.5 mM phenylmethylsulfonylfluoride) followed by three cycles of 15 s of sonication at 4°C. The homogenates were centrifuged at $10{,}000 \times g$ for 15 min at 4°C. The resulting supernatants were centrifuged at $105{,}000 \times g$ for 1 h at 4°C and the microsomal pellets were shared in buffer (20 mM Tris-HCl, pH 7.4; 0.2 mM DTT, 0.5% Nonidet P-40). An aliquot was removed for protein determination (Bio-Rad protein reagent). Microsomes were boiled in Laemmli sample buffer (Laemmli, 1970) and equal amounts of microsomal protein (20 μg) were loaded to a 10% SDS–PAGE, followed by Western blotting analysis (see below).

RNA extraction and analysis

Total RNA ($3-4 \times 10^6$ cells) was extracted following the guanidinium thiocyanate method (Chomczynski & Sacchi, 1987). After electrophoresis in a 0.9% agarose gel containing 2% formaldehyde the RNA was transferred to a Nytran membrane (NY 13-N; Schleicher & Schüell, FRG) with $10 \times$SSC ($10 \times$SSC is 1.5 M NaCl, 0.3 M sodium citrate, pH 7.4). The membrane was prehybridized and the level of COX-2, α-foetoprotein and albumin mRNA was determined using the corresponding full length cDNAs as probes (Gil *et al*., 1996), labelled with [α-^{32}P]dCTP using the Rediprime labelling kit (Amersham). The membrane was washed with $2 \times$SSC and 0.5% SDS at room temperature for 10 min and twice at 42°C for 30 min. Quantification of the radioactive emission was performed in a FUJI BAS1000 detector, avoiding saturation of the bands, and followed by exposure to X-ray film (Kodak X-OMAT). Normalization of the blots for RNA lane charge was performed using the hybridization with a probe specific for the 18S-ribosomal RNA inserted into a PBR322 plasmid and labelled by nick translation (Casado *et al*., 1997).

Western blot analysis

The amount of COX-1 and COX-2 was determined in enriched microsomal preparations. After determining the protein content, samples were boiled in Laemmli sample buffer and equal amounts of protein were size-fractionated in a 10% acrylamide gel, transferred to a PVDF membrane (Amersham) and, after blocking with 5% non-fat dry milk, incubated with anti-COX-1 (1:1000) or anti-COX-2 (1:1000) from Santa Cruz Laboratories (Santa Cruz, CA). The blot was revealed after incubation with horse-radish peroxidase-conjugated IgG (1:2000) and following the ECL protocol (Amersham) as recommended. Different exposition times were performed in each blot to ensure the linearity in the band intensities. Densitometric analysis of the bands was carried out using a laser densitometer (Molecular Dynamics, Sunnyvale, CA).

Determination of metabolites

PGE_2 levels were determined in the culture medium using a specific enzymeimmunoassay (EIA) system and following the indications of the manufacturer (Amersham). To determine the amount of NO release nitrate was reduced to nitrite, and this was measured spectrophotometrically using Griess reagent as described (Casado *et al*., 1997).

Immunocytochemistry of COX-2

Cultured foetal (21 days of gestation) hepatocytes were stimulated for 24 h with 1 μg ml^{-1} of LPS or 10 ng ml^{-1} of IL-1β and the cell layers were washed with ice-cold PBS and fixed for 2 min with methanol (−20°C). Fixed cells were blocked for 1 h at room temperature with blocking solution (3% gelatin in PBS) and then treated for 1 h with a 1:50 dilution of anti-rat-COX-2 antibody (Santa Cruz). After two washes with PBS, the cells were incubated for 30 min at RT with fluorescein-labelled goat-anti-rabbit IgG Ab (Cy3, Amersham) diluted 1:150 in PBS. The cell layers were washed three times with PBS and analysed in a MRC-100 confocal microscope (Bio-Rad). The fluorescence of the cell was

digitalized using the Cosmos software (Bio-Rad). Cells expressing α-foetoprotein were characterized by using a specific antibody and following an identical protocol. Results were expressed as the total fluorescence intensity associated to the cytoplasmic compartment (Diaz-Guerra *et al*., 1997).

Data analysis

The number of experiments is indicated in the corresponding figure. Statistical differences ($P<0.05$) between mean values were determined by one-way analysis of the variance followed by Student's *t*-test.

Results

Foetal hepatocytes express COX-2 in response to LPS and pro-inflammatory cytokines

Primary cultures of hepatocytes prepared from foetal (21-days of gestation) or adult liver contain constitutively COX-1 as deduced by the presence of immunoreactive enzyme and a basal synthesis of PGE_2 (Figure 1), although with important quantitative differences between both types of cells (Table 1). When foetal hepatocytes were incubated with LPS an important expression of COX-2 was observed as evidenced by the detection of both the immunoreactive protein and the corresponding mRNA (Figure 1A). However, COX-2 was not expressed in adult cells under these conditions (Figure 1A). Indeed, the COX-2 expressed in foetal hepatocytes was enzymatically active and a large synthesis of PGE_2 was measured (Figure 1B). In addition to these biochemical criteria, the nature of the COX isoenzymes present in control and LPS-activated foetal hepatocytes was assessed using pharmacological inhibitors of COX-1 and COX-2 enzymes. As Figure 2 shows, when control or LPS-treated cells were incubated with increasing amounts of indomethacin, a general COX inhibitor (Salvemini *et al*., 1995; Smith *et al*., 1996), or with the COX-2-specific inhibitor NS 398, the release of PGE_2 behaved differently depending on the stimulation of the hepatocytes. NS 398 failed to inhibit PGE_2 release in unstimulated cells, and blocked the synthesis in LPS-activated foetal hepatocytes. The apparent K_i values were 0.1 and 0.2 μM for indomethacin in control and LPS-treated cells, and 0.08 μM for NS 398 in cells treated with LPS. These K_i values were in the range of those previously published (Salvemini *et al*., 1995).

To ensure that the absence of COX-2 expression in adult hepatocytes treated with LPS was not due to a differential kinetic response between foetal and adult hepatocytes, the time-course of COX-2 protein and PGE_2 accumulation was analysed over a 36 h period. As Figure 3 shows, only foetal hepatocytes expressed COX-2 with a peak at 24 h in the protein levels. Indeed, the absence of COX-2 expression in adult hepatocytes was not due to a defect in the LPS-signalling pathway, since LPS-dependent expression of iNOS was measured as determined by the accumulation of nitrite plus nitrate in the culture medium (Figure 3).

The effect of pro-inflammatory cytokines, growth factors, liver-acute phase response modulators and second messengers was tested in an attempt to define conditions favouring COX-2 inducibility in foetal and adult hepatocytes. As Table 1 shows, good agreement was observed between the immunodetected COX-2 protein and the accumulation of PGE_2 in the culture medium. Only foetal hepatocytes responded to IL-1β, TNF-α

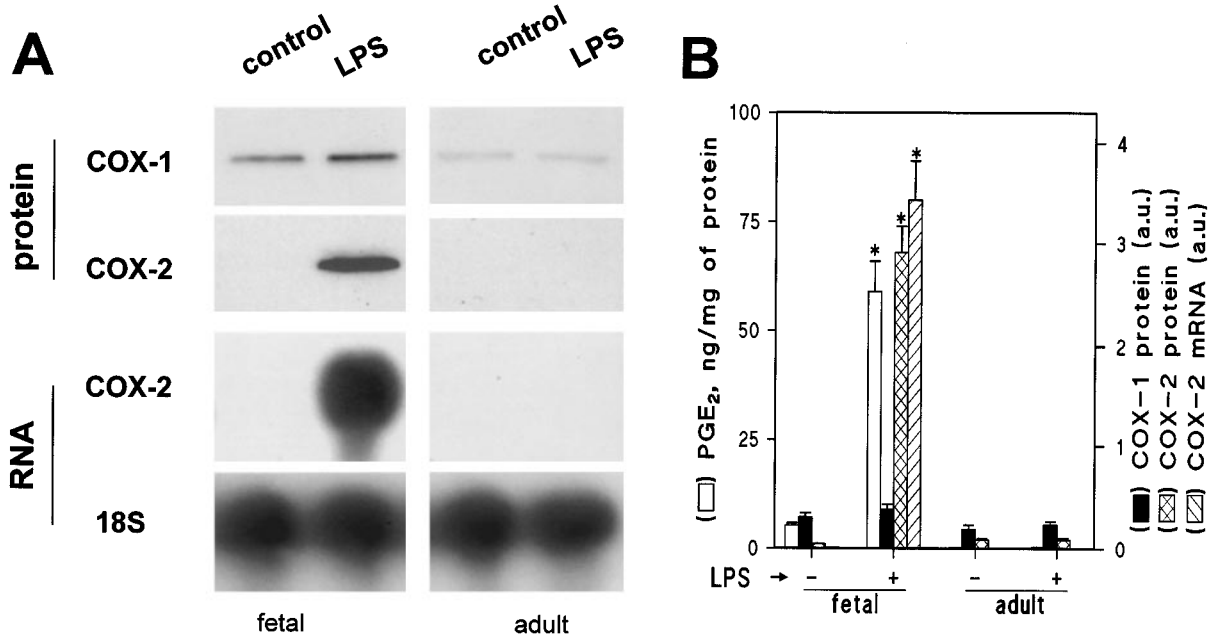

Figure 1 COX-2 is expressed in cultured foetal hepatocytes stimulated with LPS. Hepatocytes ($3-4\times10^6$ cells) were prepared from livers of animals of 21 days of gestation or from adult animals and were incubated for 24 h in the absence or presence of 1 μg ml^{-1} of LPS. Microsomal extracts (20 μg of protein) and RNA (15 μg of total RNA, prepared at 8 h after stimulation) were analysed for the content of COX-1 and COX-2 by Western and Northern blot (COX-2), respectively (A). The accumulation of PGE_2 in the culture medium was measured (note that the levels in adult hepatocytes are very low; see the values in the text (B). The densitometry of the immunodetected COX-1 and COX-2 protein bands is shown in (B). The amount of COX-2 mRNA was determined after normalization for the content of ribosomal 18S RNA (B). Results show a representative experiment out of four or the mean ± s.e.mean ($n=4$) of the PGE_2 and band intensities of the Western and Northern blots. *, Denotes $P<0.001$ vs the corresponding value in the absence of LPS treatment.

Table 1 Effect of extracellular stimuli and permeant cyclic AMP analogues on COX-2 expression in cultured foetal and adult hepatocytes

Hepatocytes	*Foetal*		*Adult*	
Treatment	*Protein, band intensity (a.u.)*	*PGE_2 ng mg^{-1} of protein*	*Protein, band intensity (a.u.)*	*PGE_2 ng mg^{-1} of protein*
None	<1	6±1	<1	0.05±0.01
LPS, 1 μg ml^{-1}	100	52±4**	<1	0.15±0.03
IL-1β, 10 ng ml^{-1}	67	35±3**	<1	0.09±0.01
TNF-α, 10 ng ml^{-1}	53	24±4**	<1	0.16±0.01
IL-1β+TNF-α	135	75±6**	<1	0.16±0.02
LPS+IL-1β+TNF-α	145	84±9**	<1	0.10±0.01
IL-6, 10 ng ml^{-1}	2	7±1	<1	0.05±0.01
8Br-cAMP, 0.2 mM	<1	5±1	<1	0.04±0.01
TGF-β, 1 ng ml^{-1}	<1	3±1	<1	0.04±0.01
LPS+8Br-cyclic AMP	87	44±4**	<1	0.07±0.01
LPS+TGF-β	68	35±2**	<1	0.05±0.01
TNF-α+IL-1β+TGFβ	14	11±1*	<1	0.04±0.01

Hepatocytes were treated for 24 h with the indicated stimuli and the amount of COX-2 protein associated with the microsomal fraction (analysed by Western blot) and the accumulation of PGE_2 in the culture medium were measured. Results show the mean of the COX-2 band intensities of two experiments (protein detected by Western blot), in which the content of cells treated with LPS was considered as 100. The levels of PGE_2 (mean±s.e.mean) were determined in three experiments, *$P<0.05$, **$P<0.01$ versus the control condition.

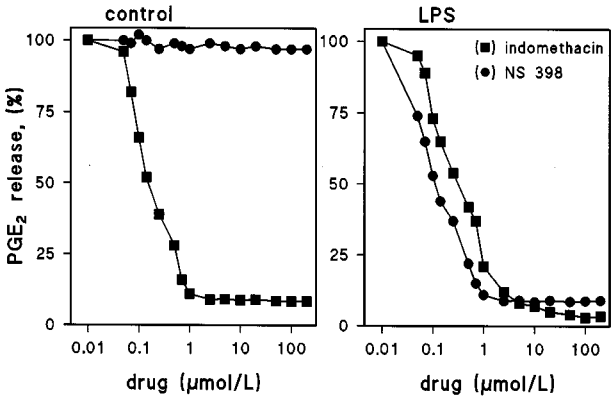

Figure 2 Dose-dependent inhibition of PGE_2 release by COX inhibitors in cultured foetal hepatocytes. Cells were incubated for 24 h in the absence (left panel) or in the presence of 1 μg ml^{-1} of LPS (right panel) and the indicated amounts of indomethacin or NS 398 and the PGE_2 accumulation was determined. The amount PGE_2 released in control and LPS stimulated cells was 5.4±0.3 and 63.4±5.3 ng mg^{-1} of protein, respectively. Results show the mean of two experiments assayed per duplicate.

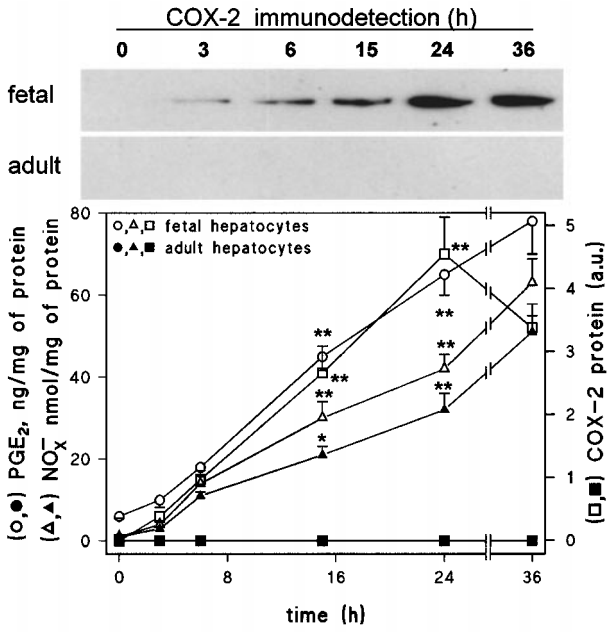

Figure 3 Time course of COX-2 expression in foetal and adult hepatocytes. Cultured foetal and adult hepatocytes were challenged with 1 μg ml^{-1} of LPS and the amount of COX-2 protein and PGE_2 accumulation were analysed at the indicated times. The amount of nitrate plus nitrite in the culture medium was determined to ensure that adult hepatocytes responded to LPS challenge. Results show the means±s.e.mean of three experiments in which the band intensities of the Western blots (upper part of the panel) was measured. The PGE_2 and COX-2 protein values corresponding to adult hepatocytes are overlapped (●, ■). *, and **represent $P<0.01$ and $P<0.001$ vs the corresponding value at time 0 h.

or combinations of both. Permeant cyclic AMP analogues did not induce COX-2 expression, but rather exerted a moderate inhibition on the effect of LPS. Stimulation of foetal hepatocytes with LPS or IL-1β plus TNF-α in the presence of TGF-β resulted in an important decrease of COX-2 protein levels and activity, indicating an inhibitory effect of TGF-β in the regulation of COX-2 levels in these cells. These results confirmed that adult hepatocytes failed to express COX-2 upon challenge with factors related with inflammatory processes.

COX-2 expression is immunolocalized in foetal hepatocytes challenged with LPS or IL-1β

To further characterize the cells expressing COX-2 in response to LPS or IL-β, as a model of pro-inflammatory cytokine, immunocytochemical experiments were carried out in foetal hepatocyte cultures using confocal microscopy. As Figure 4 shows, an important cell-associated fluorescence was observed in foetal hepatocytes treated with LPS or IL-1β, but not in the corresponding control. The COX-2-dependent immunofluorescence was detected in the cytoplasmic compartment, and associated to the Golgi/endoplasmic reticulum and the perinuclear envelope. Results of the quantitative analysis of the distribution of the fluorescence are given in Table 2. Moreover, treatment of the cells with anti-α-foetoprotein antibodies stained 88% of the cells analysed in Figure 4, therefore representing foetal hepatocytes the main cell population in the culture.

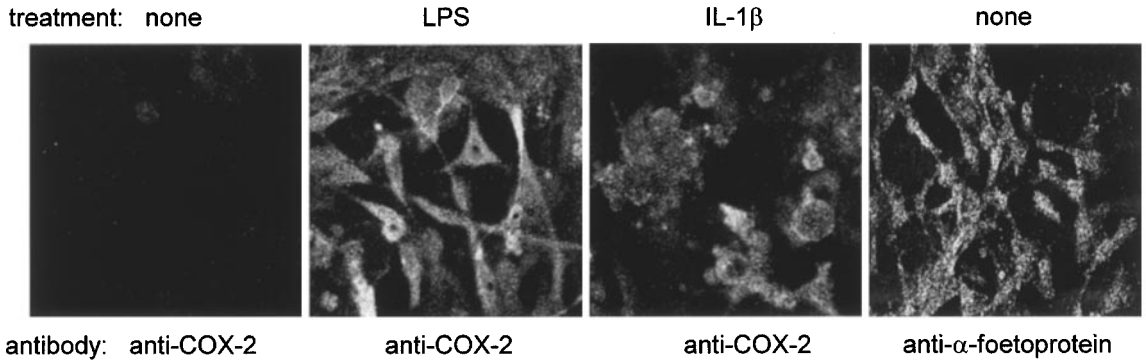

Figure 4 COX-2-dependent immunofluorescence of hepatocytes. Cultured foetal hepatocytes were treated for 24 h with 1 μg ml^{-1} of LPS or 20 ng ml^{-1} of IL-1β. After fixing with cold methanol (-20°C), the cells were incubated with an anti-rat-COX-2 or anti-rat-α-foetoprotein antibodies (1 : 50 dilution) and revealed with a Cy3-labelled secondary antibody. The cells were analysed by confocal microscopy and the fluorescence intensity was digitalized and measured. The quantitative analysis of the average fluorescence intensity in the cytoplasm is shown in Table 2.

Table 2 Measurement of COX-2 expression in primary cultures of foetal hepatocytes by confocal microscopy

Treatment	*Antibody*	*Fluorescence (a.u.)*	*% of stained cells*
None	COX-2	<0.8 (23)	<2
LPS, 1 μg ml^{-1}	COX-2	29 ± 3.4 (25)	69
IL-1β, 20 ng ml^{-1}	COX-2	24 ± 2.5 (20)	61
None	α-foetoprotein	33 ± 4 (15)	89
LPS, 1 μg ml^{-1}	α-foetoprotein	29 ± 4 (20)	83
IL-1β, 20 ng ml^{-1}	α-foetoprotein	30 ± 3 (27)	87

Hepatocytes were treated for 24 h with the indicated stimuli and after fixing and permeabilization with cold methanol the amount of COX-2 and α-foetoprotein was determined using 1 : 50 dilutions of anti-rat COX-2 and α-foetoprotein antibodies. The fluorescence was digitalized and the emission corresponding to 20–25 cells was evaluated. Results show the average fluorescence $\pm$ s.e.mean for the number of cells analysed given in parenthesis.

LPS-dependent COX-2 expression is impaired after birth

To establish the moment in perinatal development at which hepatocytes fail to induce COX-2 expression in response to challenge with LPS or pro-inflammatory cytokines, hepatocytes from animals of different ages were prepared. As Figure 5 shows, the immunodetection of COX-2 in foetal hepatocytes at day 21 and 22 of gestation was similar. However, the expression of COX-2 was reduced to 50% in hepatocytes prepared from animals at birth, and was negligible (1.2% of the value at 21 days of gestation) 2 days after birth.

The mRNA levels of α-foetoprotein and albumin were measured in these preparations and an important switch in the transcription of both genes was observed around birth. However, the changes in the kinetic profile of α-foetoprotein/albumin transcription were more extended in time than the fall in COX-2 inducibility.

Discussion

Prostaglandins have been recognized as important mediators of hepatic, renal, cardiovascular and pulmonary functions and their synthesis is altered in the course of several physiopathological situations (DeWitt, 1991; Otto & Smith, 1995; Kurumbail *et al*., 1996). The basal synthesis of PGE_2 in foetal hepatocytes is two-orders of magnitude higher than in the corresponding adult counterparts, and COX-1 is the enzyme responsible for this activity. However, biochemical and genetic studies point to COX-2 expression as the main contributor of prostanoid synthesis under pathological circumstances (DeWitt, 1991; Pilbeam *et al*., 1993; Tsuji & DuBois, 1995; Morham *et al*., 1995; Feng *et al*., 1993; DeWitt & Smith, 1995), and therefore, the study of the mechanisms controlling the expression of COX-2 has constituted an area of intense research in the past years (DeWitt & Smith, 1995). Since both foetal and adult hepatocytes respond to pro-inflammatory stimuli we focused our attention on the response of these cells in terms of COX-2 expression. Indeed, COX-2 expression has not been described previously in hepatocytes, and only a contribution by Kupffer cells is well documented (Nanji *et al*., 1997; Zhang *et al*., 1995).

Our data show that adult hepatocytes failed to induce COX-2 in response to a wide array of stimuli, including LPS and pro-inflammatory cytokines. However, primary cultures of foetal hepatocytes expressed COX-2 upon challenge with LPS or pro-inflammatory cytokines, as assessed by the detection of the corresponding mRNA, protein levels, and PGE_2 synthesis. Selective inhibition of COX-2 with NS 398 (Salvemini *et al*., 1995) reinforced the identification of this isoenzyme in activated foetal hepatocytes. To our knowledge, this is the first description of COX-2 expression in foetal hepatocytes, at the time that our results suggest the existence of a process that impairs COX-2 inducibility in the hepatocyte immediately after birth.

The absence of COX-2 expression in adult hepatocytes cannot be attributed to a lack of signalling of the stimuli used since, for instance, the cells well responded in terms of NO synthesis. However, some cases have been reported in which a restricted expression of COX-2 has been observed. For example, in microglial cells LPS, but not pro-inflammatory

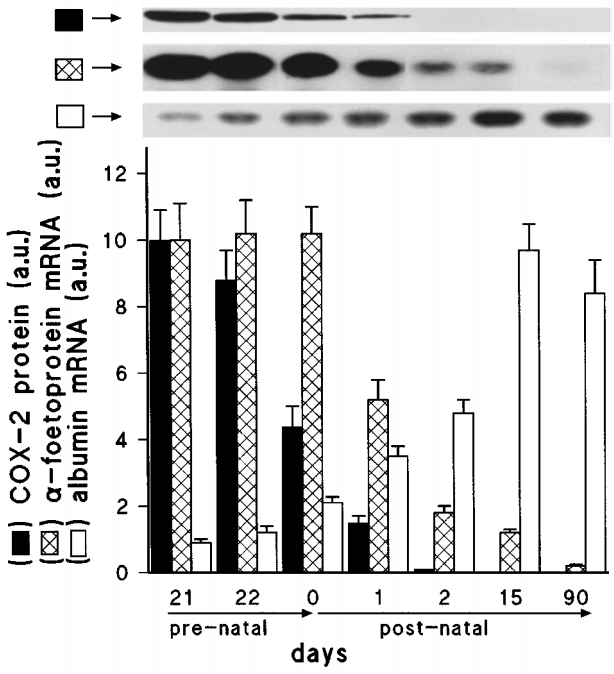

Figure 5 COX-2 expression is abrogated after birth. Cultured hepatocytes from foetal, neonatal and adult animals were stimulated with 1 μg ml^{-1} of LPS and the amount of COX-2 protein was measured. In parallel, the levels of α-foetoprotein and albumin mRNA were determined. Results show the means$\pm$s.e.mean ($n=3$) of the densitometries of the bands, upon normalization for the content of 18S ribosomal RNA in the case of Northern blot analysis. *denotes $P<0.001$ vs the COX-2 protein levels at day 22 of gestation.

cytokines, induced COX-2 (Bauer *et al*., 1997). For this reason we used several combinations of stimuli, although all of them failed to induce COX-2 in adult hepatocytes.

Immunocytochemical analysis of foetal cells expressing COX-2 revealed that this was a general response of hepatocytes treated with LPS or IL-1β. The intracellular localization of the fluorescence, cytosolic and associated to the membranes of the endoplasmic reticulum and around the perinuclear envelope, was in agreement with previous reports (Morita *et al*., 1996). However, the staining was completely negative in adult hepatocytes (not shown), confirming previous studies (Ledwith *et al*., 1997). In agreement with these results it should be mentioned that immortalized liver cells, that presumably have lost most of the characteristics of the hepatocyte phenotype, express COX-2 upon challenge with different bacterial and pro-inflammatory factors or tumour promoters (Ledwith *et al*., 1997), suggesting that the specific differentiation of hepatocytes in the adult animal contributes to abolish COX-2 expression. Regarding the mechanism responsible of the suppression of COX-2 inducibility in adult hepatocytes we can only speculate that it might be related to transcription factors that are expressed, repressed or modified following birth. However, it is difficult to propose a likely candidate since the expression of most of these tissue-specific transcription factors occurs earlier in development (Cereghini, 1996; Bisgaard *et al*., 1996). Further analysis of the structure and activity of the COX-2 promoter during this period should provide the basis of the loss of inducibility.

The biological relevance of COX-2 expression in foetal hepatocytes remains to be established. However, it has been proposed that COX-2 has an important role in early stages of pregnancy (Majerus, 1998), in the response against pathogens invading the foetal territory (Reisenberger *et al*., 1998), or controlling the growth of foetal cells.

Finally, the identification of the mechanism responsible of the lack of COX-2 inducibility could contribute to a better understanding of those situations in which a progressive de-differentiation of liver occurs and presumably a gain in COX-2 responsiveness appears. In this regard, abrogation of COX-2 activity both at the pharmacological and genetic levels suppresses intestinal polyposis, and suggests a role for COX-2 in tumorigenesis (Oshima *et al*., 1996; Williams *et al*., 1996).

The authors thank Dr A. Alvarez from Centro de Citometría de Flujo de la Universidad Complutense for help in the experiments of confocal microscopy. This work was supported by grants FIS92/0267 and PM95-007 from CICYT (Spain).

References

BAUER, M.K., LIEB, K., SCHULZE-OSTHOFF, K., BERGER, M., GEBICKE-HAERTER, P.J., BAUER, J. & FIEBICH, B.L. (1997). Expression and regulation of cyclooxygenase-2 in rat microglia. *Eur. J. Biochem.,* **243,** 726–731.

BISGAARD, H.C., NAGY, P., SANTONI-RUGIU, E. & THORGEIRSSON, S.S. (1996). Proliferation, apoptosis, and induction of hepatic transcription factors are characteristics of the early response of biliary epithelial (oval) cells to chemical carcinogens. *Hepatology,* **23,** 62–70.

CASADO, M., VELASCO, M., BOSCA, L. & MARTIN-SANZ, P. (1997). Absence of NO synthase type II expression in fetal liver from pregnant rats under septic shock conditions. *Hepatology,* **25,** 1406–1411.

CEREGHINI, S. (1996). Liver-enriched transcription factors and hepatocyte differentiation. *FASEB J.,* **10,** 267–282.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidium thiociante-phenol-chloroform extraction. *Anal. Biochem.,* **162,** 156–159.

CROFFORD, L.J. (1997). COX-1 and COX-2 tissue expression: implications and predictions. *J. Rheumatol.,* **24,** 15–19.

CURRAN, R.D., BILLIAR, T.R., STUEHR, D.J., HOFMANN, K. & SIMMONS, R.L. (1989). Hepatocytes produce nitrogen oxides from L-arginine in response to inflammatory products of Kupffer cells. *J. Exp. Med.,* **170,** 1769–1774.

DEWITT, D.L. & SMITH, W.L. (1988). Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from the complementary DNA sequence. *Proc. Natl. Acad. Sci. U.S.A.,* **85,** 1412–1416.

DEWITT, D.L. (1991). Prostaglandin endoperoxide synthase: regulation of enzyme expression. *Biochem. Biophys. Acta.,* **1083,** 121–134.

DEWITT, D. & SMITH, W.L. (1995). Yes, but do they still get headaches? *Cell,* **83,** 345–348.

DIAZ-GUERRA, M.J.M., VELASCO, M., MARTIN-SANZ, P., ALVAREZ, A. & BOSCA, L. (1997). Rapid up-regulation of IκBβ and abrogation of NF-κB activity in peritoneal macrophages stimulated with lipopolysaccharide. *J. Biol. Chem.,* **272,** 23025–23030.

FENG, L., SUN, W., XIA, Y., TANG, W.W., CHAMMUGAN, P., SOYOOLA, E., WILSON, C.B. & HWANG, D. (1993). Cloning of two isoforms of rat cyclooxygenase: differential regulation of their expression. *Arch. Biochem. Biophys.,* **307,** 361–368.

FENG, L., XIA, Y., GARCÍA, G.E., HWANG, D. & WILSON, C.B. (1995). Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-α and lipopolysaccharide. *J. Clin. Invest.,* **95,** 1669–1675.

GELLER, D.A., NUSSLER, A.K., DI SILVIO, M., LOWENSTEIN, C.J., SHAPIRO, R.A., WANG, S.C., SIMMONS, R.L. & BILLIAR, T.R. (1993). Cytokines, endotoxin and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 522–526.

GIL, B., CASADO, M., PAJARES, M.A., BOSCA, L., MATO, J.M., MARTIN-SANZ, P. & ALVAREZ, L. (1996). Differential expression pattern of S-adenosylmethionine synthetase isoenzymes during rat liver development. *Hepatology,* **24,** 876–881.

HERSCHMAN, H.R., GILBERT, R.S., XIE, W., LUNER, S. & REDDY, S.T. (1995). The regulation and role of TIS10 prostaglandin synthase-2. Adv. Prostaglandin. *Thromboxane. Leukot. Res.,* **23,** 23–28.

HORTELANO, S., DEWEZ, B., GENARO, A.M., DIAZ-GUERRA, M.J.M. & BOSCA, L. (1995). Nitric oxide is released in regenerating liver after partial hepatectomy. *Hepatology,* **21,** 776–786.

KUJUBU, D.A., FLETCHER, B.S., VARNUN, B.C., LINN, R.W. & HERSCHMAN, H.R. (1991). TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J. Biol. Chem.,* **266,** 12866–12872.

KURUMBAIL, R.G., STEVENS, A.M., GIERSE, J.K., MCDONALD, J.J., STEGEMAN, R.A., PAK, J.Y., GILDEHAUS, D., MIYASHIRO, J.M., PENNING, T.D., SEIBERT, K., ISAKSON, P.C. & STALLINGS, W.C. (1996). Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. *Nature,* **384,** 644–648.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **277,** 680–685.

LEDWITH, B.J., PAULEY, C.J., WAGNER, L.K., ROKOS, C.L., ALBERTS, D.W. & MANAM, S. (1997). Induction of cyclooxygenase-2 expression by peroxisome proliferators and non-tetradecanoylphorbol 12,13-myristate-type tumor promoters in immortalized mouse liver cells. *J. Biol. Chem.,* **272,** 3707–3714.

MAJERUS, P.W. (1998). Critical roles in pregnancy and colon cancer. *Curr. Biol.,* **8,** 87–89.

MARTIN-SANZ, P., CASCALES, M. & BOSCA, L. (1989). Glucagon-induced changes in fructose 2,6-bisphosphate and 6-phosphofructo-2-kinase in cultured rat foetal hepatocytes. *Biochem. J.,* **257,** 795–799.

MORHAM, S.G., LANGENBACH, R., LOFTIN, C.D., TIANO, H.F., VOULOMANOS, N., JENNETTE, J.C., MAHLER, J.F., KLUCKMAN, K.D., LEDFORD, A., LEE, C.A. & SMITHIES, O. (1995). Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell,* **83,** 473–482.

MORITA, I., SCHINDLER, M., BEGIER, M.K., OTTO, J.C., HORI, T., DEWITT, D.L. & SMITH, W.L. (1996). Different intracellular localizations for prostaglandin endoperoxide H synthase-1 and -2. *J. Biol. Chem.,* **270,** 10902–10908.

NANJI, A.A., MIAO, L., THOMAS, P., RAHEMTULLA, A., KHWAJA, S., ZHAO, S., PETERS, D., TAHAN, S.R. & DANNENBERG, A.J. (1997). Enhanced cyclooxygenase-2 gene expression in alcoholic liver disease in the rat. *Gastroenterology,* **112,** 943–951.

OSHIMA, M., DINCHUK, J.E., KARGMAN, S.L., OSHIMA, H., HANCOCK, B., KWONG, E., TRZASKOS, J.M., EVANS, J.F. & TAKETO, M.M. (1996). Suppression of intestinal polyposis in Apc Δ716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell,* **87,** 803–809.

OTTO, J.C. & SMITH, W.L. (1995). Prostaglandin endoperoxide synthases-1 and -2. J. Lipid. Mediat. *Cell Signal.,* **12,** 139–156.

PILBEAM, C.C., KAWEAGUCHI, H., HAKEDA, Y., VOZNESENSKY, O., ALANDER, C.B. & RAISZ, L.G. (1993). Differential regulation of inducible and constitutive prostaglandin endoperoxide synthase in osteoblastic MC3T3-E1 cells. *J. Biol. Chem.,* **268,** 25643–25649.

REISENBERGER, K., EGARTER, C., KNOFLER, M., SCHIEBEL, I., GREGOR, H., HIRSCHL, A.M., HEINZE, G. & HUSSLEIN, P. (1998). Cytokine and prostaglandin production by amnion cells in response to the addition of different bacteria. *Am. J. Obstet. Gynecol.,* **178,** 50–53.

SALVEMINI, D., SETTLE, S.L., MASFERRER, J.L., SEIBERT, K., CURRIE, M.G. & NEEDLEMAN, P. (1995). Regulation of prostaglandin production by nitric oxide; an in vivo analysis. *Br. J. Pharmacol.,* **114,** 1171–1178.

SMITH, W.L., GARAVITO, R.M. & DEWITT, D.L. (1996). Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J. Biol. Chem.,* **271,** 33157–33160.

SWEIRKOSZ, T.A., MITCHELL, J.A., WARNER, T.D., BOTTING, R.M. & VANE, J.R. (1995). Co-induction of nitric oxide synthase and cyclo-oxygenase: interactions between nitric oxide and prostanoids. *Br. J. Pharmacol.,* **114,** 1335–1342.

TSUJI, M. & DUBOIS, R.N. (1995). Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell,* **83,** 493–501.

WILLIAMS, C.S. & DUBOIS, R. (1996). Prostaglandin endoperoxide synthase: Why two isoforms. *Am. J. Physiol.,* **270,** G393–G400.

WILLIAMS, C.S., LUONGO, C., RADHIKA, A., ZHANG, T., LAMPS, L.W., NANNEY, L.B., BEAUCHAMP, R.D. & DUBOIS, R.N. (1996). Elevated cyclooxygenase-2 levels in Min mouse adenomas. *Gastroenterology,* **111,** 1134–1140.

XIE, W., CHIPMAN, J.G., ROBERTSON, D.L., ERIKSON, R.L. & SIMMONS, D.L. (1991). Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by RNA splicing. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 2692–2696.

ZHANG, F., WARSKULAT, U., WETTSTEIN, M., SCHREIBER, R., HENNINGER, H.P., DECKER, K. & HAUSSINGER, D. (1995). Hyperosmolarity stimulates prostaglandin synthesis and cyclooxygenase-2 expression in activated rat liver macrophages. *Biochem. J.,* **312,** 135–143.

(Received April 27, 1998
Revised August 19, 1998
Accepted September 1, 1998)