Celecoxib activates Stat5 and restores or increases the expression of growth hormone-regulated genes in hepatocarcinogenesis

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We have previously evaluated the chemopreventive effect of celecoxib on preneoplastic lesions in rat liver. However, though the effects of celecoxib have been tested in a variety of carcinomas, there has not been a study on the modulation of gene expression in response to this drug. Here, we evaluated the effect of celecoxib on the gene expression profile associated with hepatocarcinogenesis. Male Sprague–Dawley rats underwent the modified resistant hepatocyte model and were fed a diet containing 1500 ppm of celecoxib. Gene expression profiles were evaluated using DNA microarrays and further validations were performed using quantitative PCR, western blotting and immunohistochemical staining. Celecoxib modulated the expression of 46 genes, and those regulated by growth hormone were selected for further analysis. Celecoxib significantly upregulated the expression of the *Cyp2b1/2*, *Cyp3a1*, and alpha2-urinary globulin (*α2uG*) genes and restored the expression of *Cyp2b3* to normal. The protein expression of Cyp2b1/2 was increased, but the expressions of Cyp3a1 and α2uG were only restored to normal levels. The increased Cyp2b1/2 expression in response to celecoxib was mainly confined to preneoplastic lesions. A search for the upstream mediator of these genetic alterations found that carcinogenesis inactivated by 87% the signal transducer and activator of transcription 5 (Stat5), a transcription factor that is activated by growth hormone signaling, but celecoxib treatment restored its activation. In conclusion, these results suggest that celecoxib exerts anticancer effects on altered hepatic cells by restoring mRNA and the protein expression levels of specific genes, in part through the reactivation of Stat5. *Anti-Cancer Drugs* 21:411–422 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Hepatocellular carcinoma (HCC) is a common form of cancer that could be largely preventable because of its slow progression [1,2]. Currently, the most efficient treatment strategy is early detection and tumor resection. Thus, prevention strategies administered during the initial stages of disease represent a promising strategy for the treatment of HCC. The field of cancer prevention focuses on identifying agents that modulate molecular targets in preclinical models and on applying these agents to high-risk groups [2].

Celecoxib, a nonsteroidal anti-inflammatory drug, inhibits proliferation, arrests the cell cycle, activates apoptosis, and inhibits angiogenesis and metastasis [3]. Recently, we showed that celecoxib prevents the formation of γ-glutamyltranspeptidase (GGT) by 80%, and mediates the regression of GGT and glutathione S-transferase placental (GST-p)-positive preneoplastic liver lesions by 60 and 70%, respectively, through its antiproliferative activity [4,5]. However, there have been no studies thus far that have evaluated the effect of celecoxib on the gene expression profiles (GEP) of hepatocarcinogenesis. DNA microarray analysis enables the exploration of GEP changes and their protein products, and facilitates the identification of upstream mechanisms that can be targeted by anticancer agents.

On the basis of our earlier research and using the same protocols [4,5], here we analyzed the GEP in early hepatocarcinogenesis and detected that celecoxib affected the gene expression normally regulated by growth hormone (GH) signaling, which was related to Stat5 participation. GH regulates a large number of metabolic and other processes in the liver, primarily through its effects on gene transcription. GH initiates its biological actions by the binding of GH to its cell surface receptor; hormone binding induces GH receptor dimerization; and the

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complex binds to and activates Janus kinase (JAK) 2, a GH receptor-associated tyrosine kinase. JAK2, in turn, phosphorylates the GH receptor on multiple cytoplasmic domain tyrosine residues; this event activates several signaling pathways, including members of the Stat transcription factors family. Activation requires tyrosine phosphorylation of Stat, which leads to Stat homodimer and heterodimer formation, translocation to the nucleus, binding to Stat DNA response elements, and the stimulation of gene transcription. Stat5 is directly activated in male rat liver in response to each incoming plasma GH pulse [6,7]. In addition, it has been reported that the deficiency of Stat5 affects the expression of several genes including members of the *cytochrome P450* (*Cyp*) superfamily, which participate in the detoxification and metabolic activation of xenobiotics, and metabolize endogenous substrates. It also affects the expression of the *α2uG* gene family, which encodes proteins for pheromone transport [8–10].

In this study, using microarray analysis we assessed the potential of celecoxib to regulate GEP in early hepatocarcinogenesis. We found that, among others, celecoxib modulated the expression of genes that belong to the *Cyp* and *α2uG* families, both regulated under a hormonal control and related to Stat5 participation [10–12]. This prompted us to search for the upstream mechanism, which revealed that Stat5 is involved in gene expression modulation induced by celecoxib.

Materials and methods

Reagents and antibodies

Celecoxib was prepared from commercial Celebrex capsules by solvent extraction. The identity and purity of the molecule was more than 99.9%, verified by NMR at the Chemistry Department (CINVESTAV-IPN, Mexico City, Mexico). Diethylnitrosamine (DEN) and 2-acetylaminofluorene (2AAF) were obtained from Sigma (St Louis, Missouri, USA). SS II Reverse transcriptase and Platinum SYBR Green kit were obtained from Invitrogen (Carlsbad, California, USA). Anti-α2uG was obtained from SSI, Denmark; anti-Stat5 and anti-p-Stat5 from Cell Signaling (Danvers, Massachusetts, USA); anticytochrome P450 (Cyp) 2b1/2 and anti-Cyp3a1 from Chemicon (Temecula, California, USA); anti-glutathione S-transferase placental (GST-p) from DAKO (Carpinteria, California, USA); anti-Lamin B from Santa-Cruz (Santa Cruz, California, USA); anti-actin from Cinvestav, Mexico City; and human GH from Pfizer of Mexico.

Animals

Six-week-old male Sprague–Dawley rats from Harlan Industries (D.F., Mexico) were fed *ad libitum* and housed in a controlled environment. The experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

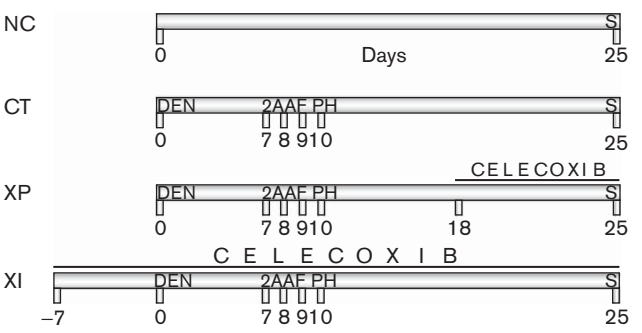
Experimental design

For carcinogenic treatment (CT), the resistant hepatocyte modified model was used [5]. The rats were initiated intraperitoneally with a necrogenic dose of DEN (200 mg/kg of body weight). Seven days later, 2AAF was orally administered at 20 mg/kg for 3 consecutive days before partial hepatectomy. The rats were randomized into four groups (Fig. 1). One group included five rats that did not receive treatment and served as the normal control (NC) group. The other three groups of 10 rats underwent CT. The CT group served as the positive control for carcinogenesis. The other groups received celecoxib in their diet at 1500 ppm according to the following two experimental protocols: (i) the XP protocol consisting of celecoxib administration for a total of 7 days, starting 18 days after DEN administration, and (ii) the XI protocol consisting of celecoxib administration 7 days before and 25 days after DEN administration. All animals were killed at 25 days after DEN administration. At the end of the experiment, the final number of rats was five for the NC group and six each for the CT, XP, and XI groups. For immunoblotting and immunostaining analyses, all rats in each group were used. For the microarray and quantitative PCR (qPCR) analyses, four randomly chosen rats from each group were used.

Growth hormone administration

To determine whether the GH signaling had participation, another nine rat groups were included ($n = 5$ per group). The first group, a nontreated group (NC), served as normal control. A second group was administered DEN (DEN24), a third group 2AAF (2AAF24), and a fourth group received PH (PH24). All the rats in these three last groups were killed 24 h after their respective interventions. A fifth group was treated with DEN, 2AAF, and PH, and killed after 24 h (CT24), after PH intervention. The sixth, seventh, and eighth groups underwent the same

Fig. 1



Schematic representation of carcinogen and celecoxib treatments. 2AAF, 2-acetylaminofluorene; CT, carcinogenic treatment; DEN, diethylnitrosamine; NC, normal control; PH, partial hepatectomy; S, sacrifice; XI, celecoxib administered before and during carcinogenic treatment; XP, celecoxib administered after carcinogenic treatment.

procedure as the second to fourth groups, but 1 h before killing they were administrated hGH (12.5 μg/100 g of body weight), DEN24 + GH, 2AAF24 + GH, and PH24 + GH. The ninth group received DEN, 2AAF, and PH (CT24 + GH) and 24 h after PH they received a dose of GH, and were killed 1 h later.

Total RNA extraction

Total RNA was extracted from liver tissues according to the RNAeasy kit protocol (Qiagen, Valencia, California, USA). The quality and quantity of RNA were determined by capillary electrophoresis (RNA 6000 Nano Assay, Agilent, Nassy, France). Samples with ratios of 260/280 nm greater than 1.9 and 28S/18S rRNA greater than 1.7 underwent DNA microarray analysis.

Microarray analysis

GEP were evaluated using cDNA synthesized from total RNA. Four DNA microarray slides were used for each group. Preparation of labeled cDNA, array hybridization, and scanning were carried out as reported earlier, using the dye-switch scheme [13]. Data were analyzed with BioPlot and Bioclust software (*http://biopuce.insa-toulouse.fr/*; Genopole-INSA, Toulouse, France). The intensity of each spot was corrected by subtracting the background with the lowest allowed value of 10, and the dataset was log-transformed and normalized using the Lowess method [14]. Slides containing 28 000 spots, corresponding to 27 004 transcripts and 22 012 annotated genes, were made at Biochips Platform of Toulouse Genopole (Toulouse, France) using oligos from the Operon-V3-rat oligo set (Operon biotechnologies, Cologne, Germany). Gene expression changes were determined by averaging the normalized log-ratios of each spot ($n = 4$) from five separate competitive hybridization experiments (CT/NC, XP/NC, XI/NC, XP/CT, and XI/CT), and were evaluated as a ratio to either NC or CT. For statistical analysis, the genes were first filtered by a ratio cut-off of 1.5, followed by a paired bi-tail Student's *t*-test. Genes that showed significant changes ($P < 0.05$) and had expression ratios of less than 0.667 or greater than 1.5 were considered to be downregulated or upregulated, respectively. The dataset generated in this study has been deposited in NCBI's Gene Expression Omnibus and is accessible through GEO Series accession number GSE12112 (*http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE12112)*.

Quantitative PCR

To validate the microarray results, qPCR was performed using a Rotor Gene 3000 cycler (Corbett Research, Australia). The primers were synthesized by Invitrogen (Carlsbad, California, USA) (Table 1). cDNA was synthesized using SuperScript II Reverse Transcriptase. cDNAs were amplified using a Platinum SYBR qPCR Green SuperMix Kit. The cycle conditions were as follows: 1 cycle of 2 min at 50°C; 1 cycle of 2 min at 94°C; and 45 cycles of 30 s at 95°C, 30 s at 57–59°C and 35 s at 72°C.

Table 1 Quantitative PCR primers

Gene	Sequence	Product size (bp)
Cyp2b1/2	5′-TAGTCAGGGGACACCCAAAG-3′ 5′-CGACCAGAGAAATCCTCAGC-3′	238
Cyp2b3	5′-TGCTTCAGCCAATTATGCAG-3′ 5′-GGAACTGGCGGTCTCTGTAG-3′	279
Cyp3a1	5′-AGTGGGGATTATGGGGAAAG-3′ 5′-CTCCAAATGATGTGCTGGTG-3′	242
α2uG	5′-GCTCTACGGCAGAACAAAGG-3′ 5′-ACTCCATGGACAGGAAATGG-3′	213

Fluorescence was measured in each elongation step, using standard curves of known quantities. The nontemplate negative control consisted of mastermix solution and water instead of cDNA. The experiments were performed in duplicate. The threshold cycle (Ct) was used to calculate the relative amount of cDNA synthesized, using the $2^{-\Delta C't}$ method described earlier [15].

Immunohistochemical staining

The serial sections were deparaffinized and gradually hydrated. The antigens were unmasked by immersing the sections in 0.1 mol/l of sodium citrate buffer (pH 6) in a heated water bath for 15 min. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol, followed by incubation for 1 h with the primary antibodies. The specimens were incubated with antibodies against GST-p and Cyp2b1/2 for 1 h at room temperature. After a standard staining protocol using the LSAB Plus-kit and chromogen development, the sections were lightly counterstained with hematoxylin, dehydrated, and mounted. Representative images were captured by optical microscopy (Olympus 1 × 70, Olympus Europa GmbH, Hamburg, Germany).

Western blot analysis

Microsomal proteins were prepared as reported earlier [16]. The tissues were homogenized in four volumes of TKE buffer and briefly centrifuged. The supernatants were centrifuged at 100 000*g* for 60 min. The resultant supernatants were stored at −80°C and used as cytosolic extracts; the pellets were resuspended in TKM buffer and centrifuged at 100 000*g* for 60 min. Microsomal pellets were resuspended in TKM buffer plus 5% glycerol and stored at −80°C. Nuclear proteins were extracted using ice-cold 0.25 and 2.3 mol/l of sucrose in TKM buffer, as described earlier [17]. Buffers were supplied with protease inhibitors, and all procedures were performed at 4°C to reduce protein degradation. Thirty micrograms of nuclear and microsomal protein and 100 μg of cytosolic protein were separated by SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The protein of interest was visualized by western blotting using the indicated antibody and a chemiluminescence system. Anti-actin and anti-lamin B levels were used as loading controls for cytosolic and nuclear proteins, respectively.

Statistical analysis

The statistical differences between the celecoxib-treated and control groups were compared using one-way analysis of variance and the Bonferroni post-hoc test. Data were expressed as mean ± SE. Differences were considered significant when P value was less than 0.05.

Results

General observations

The averages of the body and liver weights of the animals that underwent CT and administered a standard diet or celecoxib remained similar throughout the experiment (data not shown).

GEP is modulated by celecoxib treatment

We investigated the effect of celecoxib on the GEP of the CT, XP, and XI groups versus the NC group. A total of 46 genes were differentially expressed. Of these genes, 17 were rat genes, 20 were predicted genes, and nine were unknown. Hierarchical clustering identified two distinct gene expression patterns for the known rat genes (Fig. 2): (i) genes downregulated by the CT protocol that returned to normal in response to celecoxib treatment, including members of the *α2uG* family, and (ii) genes that were unaffected by the CT protocol but were upregulated in response to celecoxib; these included members of the *Cyp* superfamily (Table 2). We also compared the GEP of the NC, XP, and XI groups versus the CT group (Table 3). The *α2uG* genes showed similar upregulation for all three groups, and the *Cyp* genes were also upregulated. These results indicated that celecoxib restored *α2uG* expression and upregulated *Cyp* gene expression.

Validation of gene expression

The regulation of several genes belonging to the *Cyp* and *α2uG* families involves the participation of Stat5, which is a mediator of GH signaling [11,18]. We selected these genes for further validation. qPCR analyses revealed that *Cyp2b1/2* and *Cyp3a1* were upregulated by 27.1-fold and 1.6-fold, respectively, in the XP group, and upregulated 60.8-fold and 3.7-fold ($P < 0.0001$), respectively, in the XI group (Fig. 3a and c). In contrast, *Cyp2b3* expression did not differ from that of the NC group, but was downregulated 0.4-fold ($P < 0.01$) in the XP group (Fig. 3b). The expression of *α2uG* was downregulated in the CT group (0.7-fold, $P < 0.002$), but celecoxib treatment upregulated its expression by 2.0-fold ($P < 0.01$) in the XI group (Fig. 3d).

The effect of celecoxib at the protein level

To determine whether the changes in gene expression were reflected at the protein levels, we evaluated only the protein expression of the *Cyp2b1/2*, *Cyp3a1*, and *α2uG* genes, which were upregulated, but not the expression of the *Cyp2b3* gene, as it was only restored to normal levels by celecoxib as confirmed by qPCR analysis. Cyp2b1/2 expression was downregulated by 0.5-fold in the CT group ($P < 0.05$) but was upregulated by 3.6-fold in the groups treated with celecoxib ($P < 0.0001$). CT also downregulated Cyp3a1 and α2uG expression by 0.3-fold ($P < 0.04$) and 0.7-fold ($P < 0.0001$), respectively, but celecoxib treatment returned the expressions to basal levels (Fig. 4). There was a discrepancy in the CT group between the behavior of gene and protein expression; however, it is clear that the increase in Cyp2b1/2 protein expression correlates with its gene expression after celecoxib treatments. Although gene expression was variably increased, its protein expression was consistent in both celecoxib treatment groups. These results indicate that the anticancer effect of celecoxib is not only limited to gene expression, but also modifies protein expression.

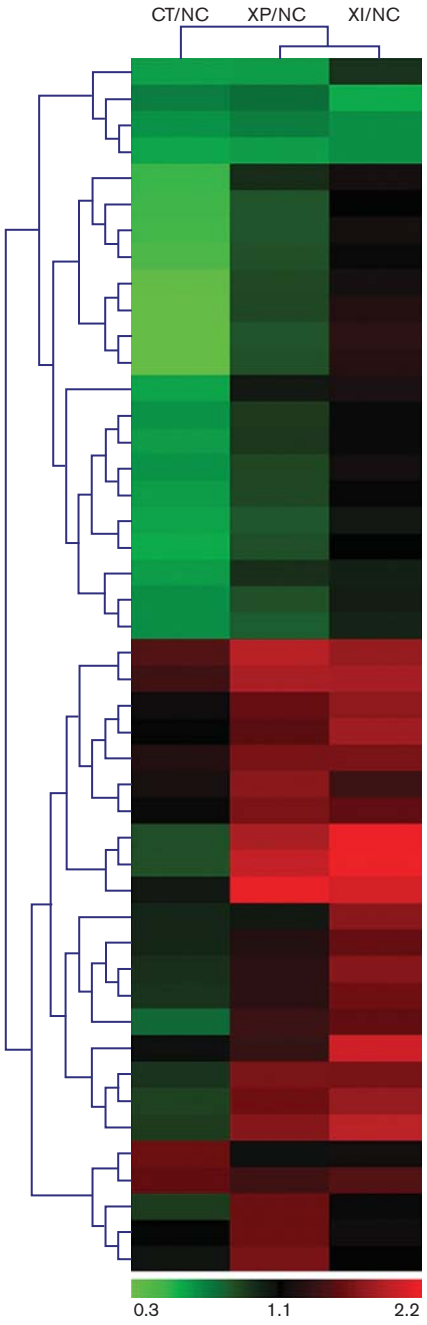
Cyp2b1/2 expression in preneoplastic lesions is associated with decreased proliferation

As there is an association between decreased Cyp2b1/2 protein expression and increased hepatocyte proliferation [19], we evaluated the localization of this protein in liver tissue. The expression levels of GST-p, a commonly used marker of preneoplastic lesions [5] (Fig. 5c and e), and Cyp2b1/2 were analyzed in serial tissue sections. Cyp2b1/2 was weakly expressed within lesions that had undergone the CT protocol (Fig. 5d), but it was strongly expressed within lesions and in the periportal and pericentral zones, with bridges of expression connecting them, in the livers that underwent celecoxib treatment (Fig. 5f). It is striking that Cyp2b1/2 expression was strongly observed within the lesions of livers treated with celecoxib but not in those that underwent only CT, confirming that the antiproliferative effect of celecoxib is associated with the activation of Cyp2b1/2 expression.

Modulation of GEP by celecoxib is associated with Stat5 reactivation

It has been reported that the regulation of some genes found in this investigation depends on Stat5 activity [7,8]. We found that celecoxib modulated the expression of *Cyp3a1*, *Cyp2b3*, and *Cyp2b1*, that the latter contains several binding Stat sites in its promoter sequence [20] and that *α2uG* variants have binding sites for HNF6, which is stimulated by GH in a mechanism involving Stat5 participation [21,22]. To show the involvement of Stat5 in celecoxib-induced gene expression, we investigated the effect of celecoxib on Stat5 activity. CT reduced phosphorylated Stat5 (p-Stat5) levels by 87% in the nuclear fraction, but celecoxib treatment restored its phosphorylation to normal levels (Fig. 6a and b) ($P < 0.0001$). Total Stat5 levels were similarly decreased with CT, but celecoxib did not restore expression to normal levels. In the cytosolic fraction (Fig. 6c and d), p-Stat5 was only observed in the NC and XI groups. The difference in p-Stat5 levels between these groups was significant ($P < 0.002$), but there was no significant

Fig. 2



Ensembl ID	Gene name	Gen Bank
ENSRNOG00000024202	Unknown	–
ENSRNOG00000012020	Unknown	–
ENSRNOG00000012473	*Casp8 and FADD-Like apoptosis regulator*	AF244366
ENSRNOG00000028598	*Predicted: similar to Ectodysplasin-A receptor**	XM_345109
ENSRNOG00000005769	*Predicted: similar to RIKEN cDNA 1200011M11**	XM_213430
ENSRNOG00000013821	*Predicted: similar to KIAA0825 protein**	XM_001059496
ENSRNOG00000017547	*CC Chemokine CCL28*	AF361490
ENSRNOG00000017562	*Alpha-2u globulin PGCL4*	X14552
ENSRNOG00000013275	*Major urinary protein precursor (MUP)*	LOC259246
ENSRNOG00000009273	*Alpha-2u globulin PGCL5 (Fragment)*	AB039827
ENSRNOG00000005764	*Alpha-2u globulin PGCL5*	AB039827
ENSRNOG00000009142	*Alpha-2u globulin*	AB039829
ENSRNOG00000022781	*Predicted: similar to KIAA1128 protein**	XM_224672
ENSRNOG00000024531	Unknown	–
ENSRNOG00000022875	*Similar to triggering receptor on myeloid cells-like 2**	XM_001059150
ENSRNOG00000021788	Unknown	–
ENSRNOG00000027311	*Similar to family with sequence similarty 22, member F**	XM_225188
ENSRNOG00000003005	Unknown	–
ENSRNOG00000004070	Unknown	–
ENSRNOG00000009585	*Transcription factor 20 (mapped)**	NM_001130574
ENSRNOG00000020330	*Predicted: WD repeat domain 47**	XM_001081957
ENSRNOG00000023093	*Predicted: similar to expressed sequence C79407**	XR_009346
ENSRNOG00000001814	*T-Kininigen II precursor (ALPHA-1-MAP)*	CO571108
ENSRNOG00000011589	*Calcium/calmodulin-DPK II subtype delta-5*	J05072
ENSRNOG00000026277	*Zinc finger CCCH type contatining 6**	NM_001107772
ENSRNOG00000007219	*Heat shock protein 86*	AJ428213
ENSRNOG00000026128	*Similar to copine VIII isoform 1**	NM_001108750
ENSRNOG00000004041	*Similar to putative p-inositide 5-phosphatase II**	BC083572
ENSRNOG00000027706	*Receptor tyrosine kinase (ERBB4)**	AF041838
ENSRNOG00000020790	*Rat (Sprague–Dawley) cytochrome P450 2B3*	BC088103
ENSRNOG00000015083	Unknown	–
ENSRNOG00000024832	*Predicted: G protein-coupled receptor 158**	XM_225625
ENSRNOG00000014409	*Predicted: myosin, light polypeptide 7, regulatory**	BC161995
ENSRNOG00000020775	*Cytochrome P450 2B1*	J00719
ENSRNOG00000001002	*Dynein-like protein 10*	D26502
ENSRNOG00000028409	*Predicted: testis-specific protein TSA806**	XM_001059438
ENSRNOG00000001466	*Cytochrome 2C24*	CK481568
ENSRNOG00000000196	*Cytochrome P450 19A1 (aromatase)*	M33986
ENSRNOG00000022313	Unknown	–
ENSRNOG00000024016	*Predicted: cytochrome P450 2c37**	XM_001063361
ENSRNOG00000000978	*Cytochrome P450 3A1*	X96721
ENSRNOG00000027674	*Similar to protein tyrosine phosphatase 4a1**	BC081772
ENSRNOG00000019004	*Predicted: metallothionein 4**	NM_001126084
ENSRNOG00000017876	*Putative regulation protein GS3*	BC086587
ENSRNOG00000023205	Unknown	–
ENSRNOG00000012174	*Predicted: similar to ribosomal protein S10**	XM_573067

Hierarchical clustering analysis of gene expression profiles of carcinogenic treatment (CT), celecoxib administered after carcinogenic treatment (XP), and celecoxib administered before and during carcinogenic treatment (XI) protocols with respect to the normal control (NC) group. The color gradient represents gene expression ratios. Green and red represent downregulation and upregulation, respectively, as indicated in the ratio scale bar. The dendogram indicates the degree of similarity between gene expression signatures. The clusters indicate the behaviors of gene subsets induced by each treatment strategy. The clustering was generated by TIGR-MeV software (*http://www.tigr.org/software/microarray.shtml*). *Predicted genes.

difference in the expression of total Stat5. As Stat5 regulates the expression of the *Cyp* and *α2uG* genes and is activated through GH [7,8,11,12,18], these results suggest that celecoxib induced the reactivation and nuclear translocation of Stat5 to restore the expression of genes regulated by GH.

GH signaling seems to be altered by CT in rat liver and restored by celecoxib. To determine whether this effect is directly related to the hormone effect, in each of the three interventions of the complete carcinogenic model GH was administered to rat groups treated with DEN, 2AAF, HP, and TC 1 h before they were killed, and its

Table 2 Expression ratios of known rat genes with respect to the NC group

		Ratio vs. NC		
GenBank	Gene name	CT	XP	XI
CO571108	*T-Kininigen II precursor (ALPHA-1-MAP)*	1.47	1.85*	1.72
J05072	*Calcium/calmodulin-DPK II subtype delta-5*	1.39	1.81*	1.79
BC088103	*Rat (Sprague–Dawley) cytochrome P450 2B3*	0.86	1.81*	2.10*
X96721	*Cytochrome P450 3A1*	0.91	1.65*	1.87
AJ428213	*Heat shock protein 86*	1.09	1.49	1.75*
M33986	*Cytochrome P450 19A1 (aromatase)*	1.08	1.36	1.95*
J00719	*Cytochrome P450 2B1*	0.99	1.24*	1.53*
D26502	*Dynein-like protein 10*	0.95	1.29*	1.65*
CK481568	*Cytochrome 2C24*	0.78	1.37*	1.52*
BC086587	*Putative regulation protein GS3*	0.91	1.55*	1.14
AF244366	*Casp8 and FADD-like apoptosis regulator*	0.64*	0.71*	0.65
AF361490	*CC chemokine CCL28*	0.46*	0.84	1.17
X14552	*Alpha-2u globulin PGCL4*	0.43*	0.85	1.14
LOC259246	*Major urinary protein precursor (MUP)*	0.34*	0.87	1.18
AB039827	*Alpha-2u globulin PGCL5 (Fragment)*	0.32*	0.88	1.23
AB039827	*Alpha-2u globulin PGCL5*	0.32*	0.84	1.30
AB039829	*Alpha-2u globulin*	0.32*	0.85	1.27

Ratio <0.667, downregulated and >1.5, upregulated.
CT, carcinogenic treatment; NC, normal control; XI, celecoxib administered before and during carcinogenic treatm; XP, celecoxib administered after carcinogenic treatmentent.
*Statistically different from NC, $P<0.05$.

Table 3 Expression ratios of known rat genes with respect to the CT group

		Ratio vs. CT		
GenBank	Gene name	NC	XP	XI
X14552	*Alpha-2u globulin PGCL4*	2.309*	1.988*	3.304*
AF361490	*CC chemokine CCL28*	2.16*	1.75*	2.788*
AF326113	*Testis-specific transporter TST-2*	1.769*	1.98*	3.016*
AB039827	*Alpha 2u-globulin PGCL5 (Fragment)*	3.227*	2.584*	3.083*
AB039829	*Alpha-2u globulin*	3.228*	2.906*	3.294*
AB039827	*Alpha-2u globulin PGCL5*	3.126*	2.757*	3.467*
LOC259246	*Major urinary protein precursor (MUP)*	2.929*	2.48*	3.493*
AB039824	*Alpha-2u globulin PGCL3*	2.455	2.709	3.23*
BC088103	*Rat (Sprague–Dawley) cytochrome P450 2B3*	1.167*	2.017*	2.756*
BC061756	*Ribophorin I*	1.462*	1.314	1.922*
BAA25372	*Sterol O-acyltransferase 1*	1.407	1.197	1.911*
X96721	*Cytochrome P450 3A1*	1.101	1.717*	2.025*
CK481568	*Cytochrome P450 2C24*	1.268	1.640	1.951*
D26502	*Dynein-like protein 10*	1.049	1.392*	2.25*
AF244366	*Casp8 and FADD-like apoptosis regulator*	1.563*	1.139	1.158
BC083546	*DNA cross-link repair 1C*	1.199	0.983	1.587*
BC089222	*Leucine-rich glioma-inactivated protein 1*	1.178*	1.091	1.581*
AJ428213	*Heat shock protein 86*	0.914	1.426	1.579*
J03606	*Fc-epsilon RI-alpha*	1.092	1.145	1.517*
AAH85688	*Annexin A4*	1.054	1.290	1.539*
J00719	*Cytochrome P450 2B1*	1.014	1.295*	1.535*
Q64614	*Cytochrome P450, subfamily IIC6*	0.991	1.283*	1.517*

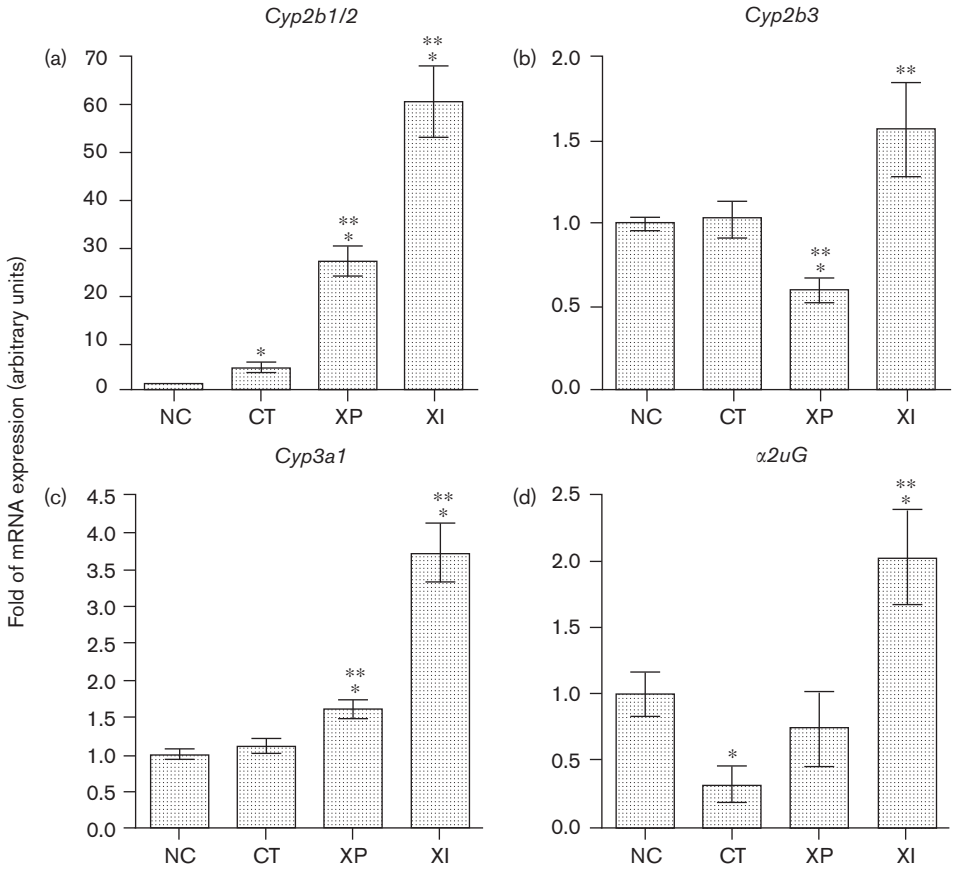
Ratio <0.667, downregulated and >1.5, upregulated.
CT, carcinogenic treatment; NC, normal control; XI, celecoxib administered before and during carcinogenic treatment; XP, celecoxib administered after carcinogenic treatment.
*Statistically different from CT, $P<0.05$.

effect on Stat5, Cyp2b1/2, and α2uG status was evaluated. As shown in Fig. 7, GH strongly restored the presence of nuclear p-Stat5 after DEN, 2AAF, PH, and CT groups ($P = 0.037$, $P < 0.001$, $P = 0.041$ and 0.003, respectively). Moreover, GH administration induced the expression of Cyp2b1/2 in the DEN and 2AAF groups ($P = 0.017$ and $P < 0.001$, respectively) whereas the expression of α2uG was only induced in the 2AAF group ($P < 0.001$). This last induction coincided with the greater activation of Stat5 induced by GH. These results suggest that celecoxib could affect Stat5 phosphorylation and the Cyp2b1/2 and α2uG expressions through a mechanism similar to that of GH signaling.

Discussion

One feature of cancer is its continuous growth, associated with increase in cell proliferation, loss of apoptotic

Fig. 3



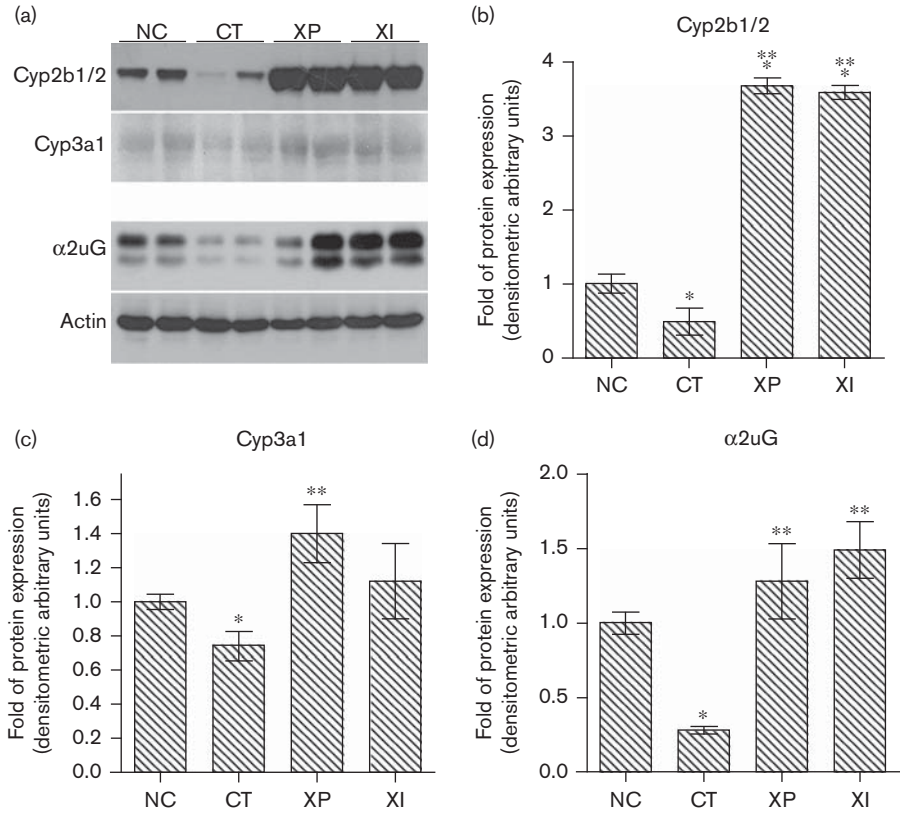
Validation of gene expression by quantitative PCR. (a) Cyp2b1/2, (b) Cyp2b3, (c) Cyp3a1, and (d) a2uG gene expressions. cDNA amplification was detected by incorporation of SYBR Green fluorescence into double-stranded DNA. Each reaction was performed in duplicate. Statistically different from *normal control (NC) and **carcinogenic treatment (CT), $P<0.05$. Data are expressed as mean ± SE. XI, celecoxib administered before and during carcinogenic treatment; XP, celecoxib administered after carcinogenic treatment.

mechanisms, and cell cycle continuity. Several studies have shown that celecoxib inhibits cell proliferation and induces apoptosis in cancer cell lines [3]. We have found that this drug prevents and regresses existing preneoplastic liver lesions through its antiproliferative activity; however, we found that celecoxib does not induce apoptosis, even though this phenomenon was evaluated at different stages of this model using different procedures [4,5]. Recently, this was reported in a similar study in a model of human medullary thyroid cancer in nude mice [23]. These data underline the fact that biological celecoxib effects on cells grown in culture do not necessarily predict its effects *in vivo* as reported earlier [24].

Cancer also involves the deregulation of a growing list of hundreds of genes that play a role in tumor initiation and progression. Microarray analysis is a tool that has contributed to the identification of genes that are deregulated in cancer. However, it remains unclear which transcriptionally deregulated genes play a causative role in tumor initiation and maintenance and which do not represent a selective advantage [25]. In the liver, GH regulates the expression of a variety of genes through the activation of Stat5. In Stat5-deficient rodents, at least 1500 genes are affected, including members of the *Cyp* and *α2uG* families [8,11]. Here, we report that members of these families are altered during hepatocarcinogenesis and are modulated by celecoxib.

Cyps constitute a superfamily of monooxygenases that participate in the detoxification and metabolic activation of xenobiotics, and metabolize endogenous substrates [9]. Earlier, we reported that celecoxib diminishes the hepatic toxicity induced by DEN through a mechanism of denitrosation through Cyp2b1/2 activity [26]. Our study shows that Cyp3a1 and Cyp2b1/2 expression was increased by celecoxib. Similarly, the prevention of liver carcinogenesis by indole-3-carbinol treatment was associated with the induction of Cyp3a and Cyp2b1/2 [27]. In addition, it was reported that bicyclol prevents the development of liver pre-neoplastic lesions; this

Fig. 4



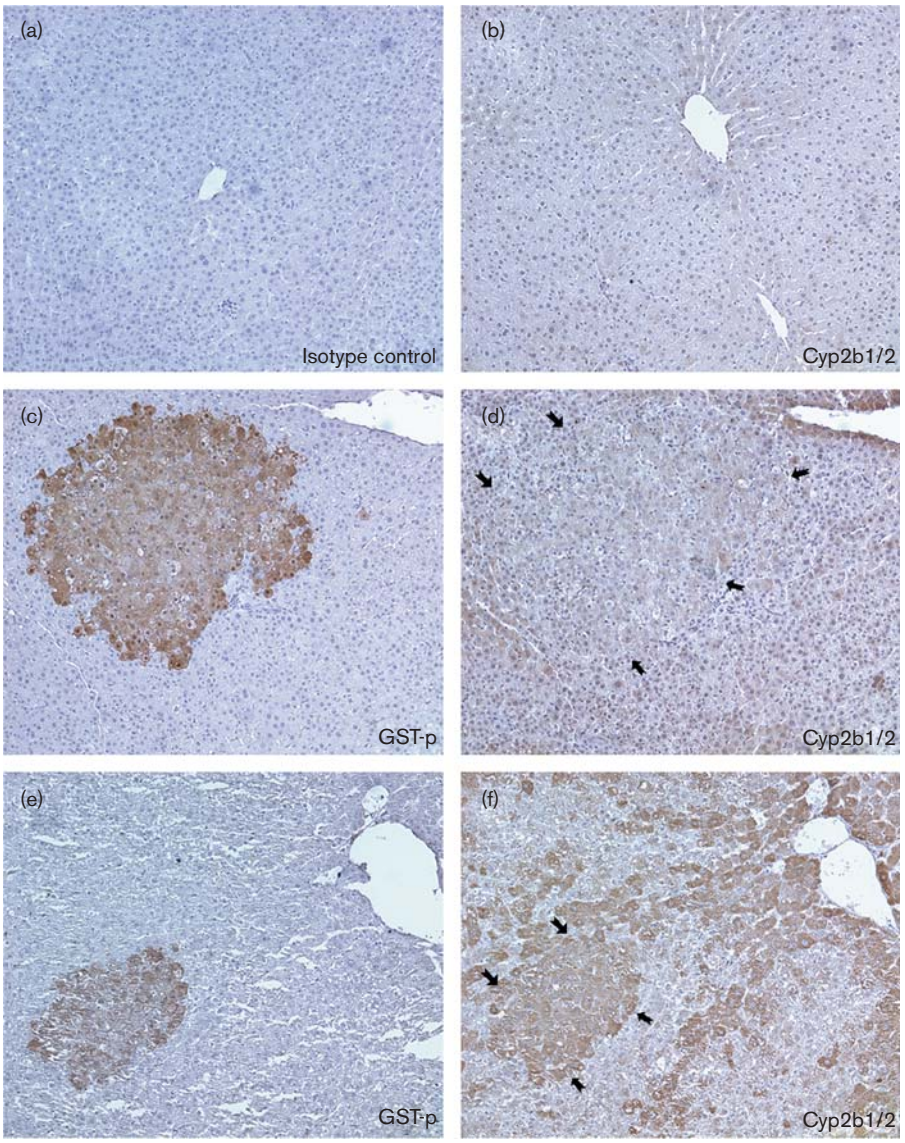
Evaluation of protein expression by western blotting. To assess Cyp and alpha2-urinary globulin (α2uG) expression, microsomal and cytosolic proteins were analyzed, respectively. Actin bands represent the loading control. (a) Western blot analysis of Cyp2b1/2, Cyp3a1, and a2uG. (b–d) Show densitometric analyses. The expression in the normal control (NC) group was adjusted to one in the densitometric units scale. Anti-α2uG recognizes two bands at 18 and 13 kDa. Statistically different from *NC and **carcinogenic treatment (CT), $P<0.05$. Data are expressed as mean ± SE. XI, celecoxib administered before and during carcinogenic treatment; XP, celecoxib administered after carcinogenic treatment.

phenomenon was correlated with an increase in Cyp2b1 enzymatic activity [28]. In contrast, it has been proposed that compounds that inhibit phase-I bioactivating enzymes and/or induce phase-II detoxifying enzymes protect against carcinogenesis [29]. This is because phase-I enzymatic reactions generate reactive oxygen species that may contribute to carcinogenesis [30]. However, our results and those reported earlier [28] suggest that the inhibition of Cyps is not necessary for cancer prevention as has been proposed [29], as carcinogens and chemopreventative agents can increase or decrease Cyp expression and activity [27,31]. Thus, these responses might be related either to the induction or prevention of carcinogenesis. In our case, the effects of celecoxib might be related to the activation of detoxification enzymes.

It has been proposed that preneoplastic lesions with reduced Cyp2b1/2 expression have a greater tendency to proliferate [32], and in cultured hepatocytes undergoing proliferation, Cyp2b1 expression decreases [19]. Earlier, we reported that celecoxib reduces cyclin D1 expression within lesions [5]. In this study, celecoxib increased the weak Cyp2b1/2 expression induced by CT observed in lesions; this expression was also present in the pericentral and periportal zones. A similar correlation was recently observed for cyclin D3 and Cyp2b1 expression in hepatocyte cultures [33]. The association of increased Cyp2b1/2 expression and decreased proliferation within preneoplastic lesions has not yet been determined, but there seems to be a relationship between Cyp2b1/2 expression and cell cycle continuity. Cyp2b1/2 expression in preneoplastic lesions might be associated with inhibited cell cycle progression, suggesting that this cytochrome could negatively regulate proliferation.

Alpha2-urinary globulins are mainly synthesized in rat liver in response to hormonal signaling. α2uG is a male rat-specific protein encoded by a multigene family [18]. It has been reported that *α2uG* gene expression is decreased in areas of hepatic proliferation after DEN administration, indicating that the reduced expression of *α2uG* favors cell growth [34]. This phenomenon has also been observed in other models, including

Fig. 5

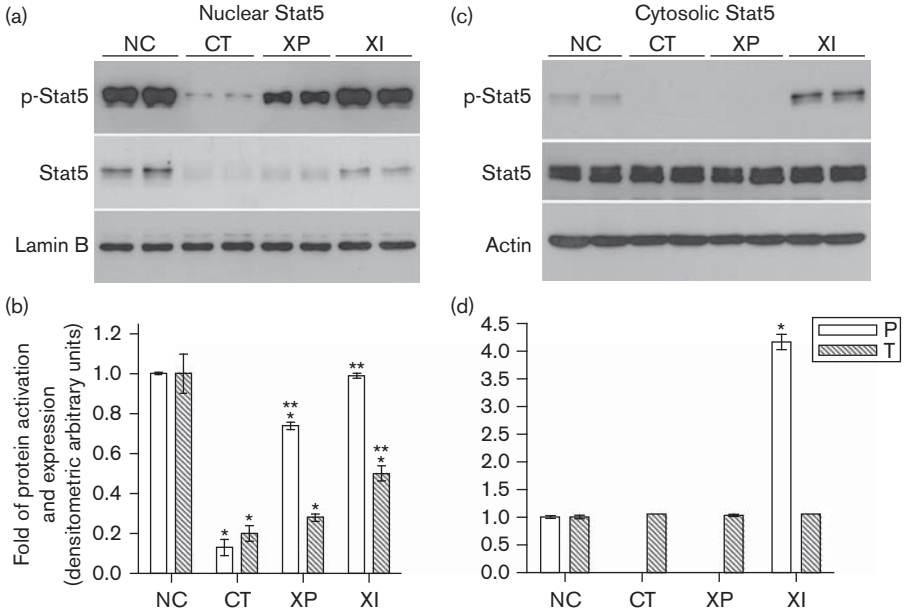


Serial liver sections stained for GST-p and Cyp2b1/2. (a) Isotype control; (b) normal control group; (c and d) carcinogenic treatment protocol; (e and f) XI protocol. Tissues in (c) and (e) show GST-p staining in lesions. Tissues in (b), (d), and (f) were immunostained for Cyp2b1/2. Arrows in (d) and (f) show the limit of the lesions extrapolated from (c) and (e). Magnification is $\times$ 100.

rat liver treated with peroxisome proliferator chemicals, rat cirrhotic liver induced with CCl_4, and advanced rat breast cancer, suggesting that loss of *α2uG* expression might be a marker of rat liver cancer [35–37]. In this study, celecoxib upregulated and restored the carcinogen-induced downregulation of α2uG at both the gene and protein level, and is associated with its antiproliferative effect. Taken together, the Cyps and α2uG results suggest that celecoxib can reverse the altered expression of specific hormonally regulated genes during hepatocarcinogenesis, and that the induction of Cyp2b1/2 and the restoration of α2uG confirm the anticarcinogenic effect of celecoxib.

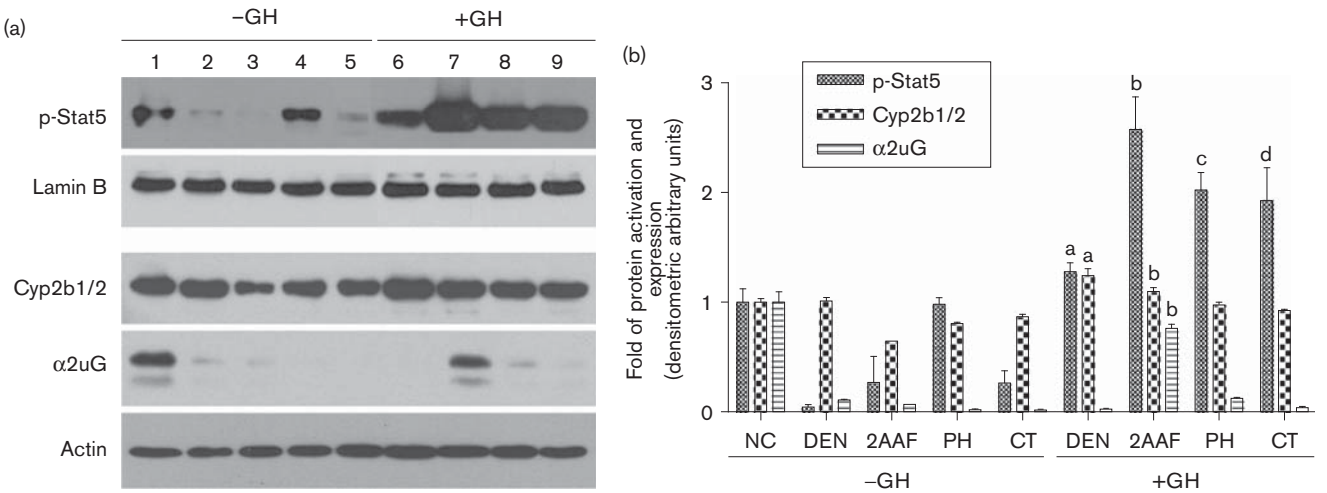
Celecoxib restores the nuclear translocation of Stat5 and the activation of Cyps and α2uG at both the gene and protein level. These alterations seem to be related, and, considering that GH regulates the expression of several genes including members of the Cyp and α2uG families through the Stat5 participation [11], this led us to conclude that the effect of celecoxib was related to GH signaling. In part, we confirmed this correlation because when GH was administered to the rats that had undergone each of the components of the hepatocarcinogenesis model, p-Stat5 was strongly reactivated in all groups treated with GH, the expression of Cyp2b1/2 was increased in the groups treated with carcinogens (DEN

Fig. 6



Expression and phosphorylation of Stat5. (a) Nuclear and (c) cytosolic Stat5. (b) and (d) Densitometric analyses of the western blotting signals. Expression and phosphorylation of normal control (NC) were adjusted to one in the densitometric units scale. P, phosphorylated and T, total Stat5. In each group, two pools of three rats each were used for nuclear evaluations. Statistically different from *NC and **carcinogenic treatment (CT), $P<0.05$. Data are expressed as mean ± SE. XI, celecoxib administered before and during carcinogenic treatment; XP, celecoxib administered after carcinogenic treatment.

Fig. 7



Effect of GH administration on p-Stat5, Cyp2b1/2, and alpha2-urinary globulin (α2uG) status. (a) Western blot analysis of p-Stat5, Cyp2b1/2, and α2uG. (b) Densitometric analyses of the western blotting signals. Proteins from normal control (NC), DEN24, 2AAF24, PH24, and CT24 groups (lanes 1–5) or from DEN24, 2AAF24, PH24, and CT24 groups administered human growth hormone (hGH) at 12.5 μg/100 g of body weight (lanes 6–9) were separated by western blot. To assess p-Stat5, Cyp2b1/2, and α2uG status, nuclear, microsomal, and cytosolic proteins were analyzed, respectively. Lamin B and actin bands represent the loading control. Statistically different from a, diethylnitrosamine (DEN)(–GH), b, 2-acetylaminofluorene (2AAF)(–GH) c, partial hepatectomy (PH)(–GH), and d, carcinogenic treatment (CT)(–GH) groups, $P<0.05$. Data are expressed as mean ± SE.

and 2AAF), and α2uG was increased in the rat group treated with 2AAF. The differences between the effects of celecoxib and GH may be a result of the duration of administration of each of the agents. Thus, although the effect of GH was not exactly equal to that of celecoxib, the administration of GH also modulated the status of

p-Stat5, Cyp2b1/2, and α2uG, which were affected by the carcinogens of the hepatocarcinogenesis model. Although these results do not define a direct mechanism of action by which celecoxib exerts its effect, they allow us to hypothesize that celecoxib acts as an agonist of the GH receptor to activate the downstream signaling pathway. In addition, our study shows that Stat5 restoration is an upstream mechanism used by celecoxib to mediate gene expression. These data support the fact that altered Stat5 activation is a key mechanism in the initiation of liver cancer. The restoration of Stat5 activity has been shown to be an anticancer mechanism mediated by curcumin in tumor-bearing mice [38]. This is congruent with the observation that Stat5 activation is gradually lost during breast cancer progression [39]. In contrast, in prostate cancer Stat5 activation was mainly associated with advanced stages of disease [40]. Stat5 activation correlates with aggressiveness in the metastatic and advanced tumor stages of human HCC, but activation does not occur in primary nonmetastatic HCC cell lines [41].

Our results associate Stat5 alteration with the deregulation of some genes; however, this is not the only alteration possible at the transcription factors level. As the promoter sequence of *Cyp2b1* contains binding sites for HNF3, AP1, NF-κ B and Stat transcription factors [20], the promoter sequences of *α2uG* and of other hepatocyte-specific genes have binding sites for HNF6 [21]. Moreover, GH stimulates the transcription of the *Hnf6* gene through a mechanism involving Stat5 participation [22]. This information suggests that there are other transcription factors that could participate concomitantly with Stat5 in the modulation of altered genes as a result of the celecoxib effect, highlighting the need to study the participation of these molecules.

In summary, we have associated the effect of celecoxib treatment observed in GEP with one of its upstream regulatory mechanisms. We have identified Stat5 alteration as a key mechanism that contributes to the induction of liver cancer. We have also shown that celecoxib acts on genes subject to GH control. Finally, for the first time, we propose Stat5 as a novel target that responds to celecoxib treatment in rat hepatocarcinogenesis.

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