CLINICAL STUDIES

Elevated concentrations of 15-deoxy-\(\Delta^{12,14}\)-prostaglandin \(J_2\) in chronic liver disease propose therapeutic trials with peroxisome proliferator activated receptor \(\gamma\)-inducing drugs

Olav A. Gressner\(^1\), Chunfang Gao\(^2\), Katharina Rehbein\(^1\), Birgit Lahme\(^1\), Monika Siluschek\(^1\), Thomas Berg\(^3\), Tobias Müller\(^3\) and Axel M. Gressner\(^1\)

1 Institute of Clinical Chemistry and Pathobiology, RWTH-University Hospital, Aachen, Germany
2 Department of Laboratory Medicine, Eastern Hepatobiliary Hospital (EHBH), Second Military Medical University, Shanghai, China
3 Department of Gastroenterology and Hepatology, Charité University Hospital Berlin, Campus Virchow-Hospital, Berlin, Germany

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Abbreviations
BMP, bone morphogenetic protein; CTGF, connective tissue growth factor; 15-d-PGJ\(_2\), 15-deoxy-\(\Delta^{12,14}\)-prostaglandin \(J_2\); HCC, hepatocellular carcinoma; HCV, hepatitis \(C\) virus; NLD, non-liver disease; PPAR\(\gamma\), peroxisome proliferator activated receptor \(\gamma\); TGF-\(\beta\), transforming growth factor-\(\beta\)

Correspondence
Olav A. Gressner, Institute of Clinical Chemistry and Pathobiology, RWTH-University Hospital, Pauwelsstraße 30, 52074 Aachen, Germany
Tel: +49 241 808 8671
Fax: +49 241 808 2512
e-mail: ogressner@ukaachen.de

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Prostaglandin \(D_2\) is produced by a variety of tissues including the bone marrow, skin, brain and spleen (1). In vivo, prostaglandin \(D_2\) is dehydrated into the \(J\)-series of prostaglandins with the key end product metabolite of prostaglandin \(D_2\), 15-deoxy-\(\Delta^{12,14}\)-prostaglandin \(J_2\) (referred to herein as 15-d-PGJ\(_2\)) (2).

Of great importance, several investigators have shown that 15-d-PGJ\(_2\) is a potent activator of the nuclear hormone receptor peroxisome proliferator activated receptor \(\gamma\) (PPAR\(\gamma\)), belonging to the PPAR family of ligand-activated nuclear transcription factors (3, 4). To date, PPAR\(\gamma\) has been found primarily in the adipose tissue, where it plays a key role in the regulation of adipogenesis (3, 5−7).

We and others have reported previously that hepatocytes are likely to be the major cellular source of connective tissue growth factor (CTGF/CCN2) in the liver and that CTGF is sensitively upregulated by transforming growth factor (TGF)-\(\beta\) (8−10). By changing the activity ratio of TGF-\(\beta\) to its antagonist bone morphogenetic protein (BMP)-7, also belonging to the TGF-\(\beta\) superfamily, CTGF is proposed as a fibrogenic master switch for epithelial–mesenchymal transition of hepatocytes to fibroblast-like cells (11, 12). This process is well known in the context of embryonic development (13) and carcinogenesis (13, 14), but is now also discussed as an important mechanism in the generation of fibroblasts during fibrogenesis in adult tissues such as the liver (13, 15). This suggests a crucial role of CTGF in hepatic fibrogenesis and carcinogenesis, which is supported by the demonstration of increased CTGF expression in various non-hepatic tumour tissues (16−20), by a strong upregulation in fibrotic liver tissue (21−23), and, even more importantly, by recent studies, in which knock-down of CTGF by siRNA almost prevented experimental liver fibrosis (24, 25).

Thus, pharmacological modulators of CTGF expression may be expected to have a great pathogenetic relevance for fibrosis and tumour growth.

Earlier reports showed that the natural PPAR\(\gamma\) agonist 15-d-PGJ\(_2\), after binding to its receptor, has a potent inhibitory effect on TGF-\(\beta\)-induced CTGF expression in the liver (26−28).
However, receptor expression seems to be the limiting factor. Recently, we could show that the methylxanthine derivative caffeine leads to an upregulation of PPARγ expression in hepatocytes, thus sensitizing these cells to the inhibitory effect of 15-d-PGJ2 on CTGF expression (26).

The aim of the current study was to compare serum concentrations of 15-d-PGJ2 in patients with fibrotic liver diseases or with hepatocellular carcinoma (HCC) compared with controls and non-liver disease (NLD) sick, in order to test these patients for their suitability for therapeutic approaches with PPARγ-inducing (i.e. CTGF inhibitory) drugs such as caffeine.

Materials and methods

Patients and study design

Five groups with a total number of 863 probands were enrolled in the investigation: group 1 – Caucasian patients with chronic hepatitis C and different stages of liver fibrosis [thereafter denominated HCV; \( n = 289 \); mean age 41 years, range 25–74 years; 154 males (age 25–51 years) and 135 non-pregnant females (age 39–74 years)]. Results of carbohydrate-deficient transferrin in serum did not indicate current alcohol abuse; group 2 – Caucasian patients with NLDs [hereafter denominated NLD; \( n = 307 \); mean age 44 years, range 24–68 years; 172 males (age 24–64 years) and 134 non-pregnant females (age 40–68 years)]; group 3 – Caucasian healthy controls [blood donors, \( n = 136 \); mean age 42 years, range 27–58 years; 82 males (age 28–47 years) and 54 non-pregnant females (age 27–58 years)]; group 4 – Chinese patients with HCC [thereafter denominated HCC; \( n = 43 \); mean age 47 years, range 24–69 years; 37 males (age 35–69 years) and six non-pregnant females (age 24–61 years)]; and group 5 – Chinese healthy controls [blood donors, \( n = 63 \); mean age 27 years, range 24–53 years; 40 males (age 24–53 years) and 23 non-pregnant females (age 24–42 years)] were included in the present study in order to avoid the ethnic difference as a confounder when looking at Chinese HCC patients.

Where possible, Caucasian patients with liver fibrosis were further divided into subgroups depending on the stage (F1 = 95 patients, F2 = 98 patients, F3 = 49 patients and F4 = 47 patients) or grade (A1 = 55 patients, A2 = 172 patients and A3 = 38 patients) of liver fibrosis as well as hepatic steatosis (no steatosis = 45 patients, S1 = 79 patients, S2 = 22 patients and S3 = 16 patients). As serum samples of Caucasian HCV patients derive from three major hospitals in Germany (University hospitals of Aachen, Cologne and Berlin), data on grading and steatosis were not available for all samples as indicated by the different final total. Chinese patients with HCC were divided into subgroups depending on the TNM Classification of Malignant Tumours (tTNM) (stage 1 = 20 patients, stage 2 = six patients and stage 3 = 16 patients) or Edmondson–Steiner’s grading system (I = one patient, II = six patients, III = seven patients and IV = 27 patients) as well as the grade of tumour differentiation (stage 1 = no patient, stage 2 = four patients and stage 3 = 36 patients).

Serological investigations

Written informed consent was obtained from each participant or his/her spouse, and the study was approved by the local ethics committee. Peripheral venous blood samples [serum as well as ethylenediaminetetraacetic acid (EDTA)- and citrate-anticoagulated whole blood] were taken at the time of diagnosis of liver fibrosis or HCC. Serum was separated at 4000g after clot retraction and stored at \(-70^\circ\)C.

The following biochemical parameters were determined in serum: alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, \( \gamma \)-glutamyltransferase, pseudocholinesterase, total protein, pre-albumin, albumin, direct bilirubin, total bilirubin, total cholesterol, triglycerides, iron, ferritin, immunoglobulin (Ig) A, IgG and IgM. All analyses were performed on the Roche Modular Analytics System (Roche, Mannheim, Germany). \( \alpha \)-futcosidase was quantified manually using a colorimetric immunoassay (BioQuant, Heidelberg, Germany). Total bile acids were determined manually using a colorimetric test with endpoint determination (DiaSys, Holzheim, Germany). The following biochemical parameters were determined in EDTA plasma: haemoglobin and mean corpuscular erythrocyte volume (MCV) were determined by routine colourimetric test with endpoint determination (DiaSys, Holzheim, Germany). HCV detection, quantification and genotypization were performed on the Cobas Amplicor (Roche).

Quantification of serum levels of 15-deoxy-\( \Delta^{12,14} \)-prostaglandin \( \text{J}_2 \)

Serum concentrations of 15-d-PGJ2 were determined using the Correlate-EIA\textsuperscript{TM} 15-deoxy-\( \Delta^{12,14} \)-Prostaglandin \( \text{J}_2 \) kit (Assay Designs, Ann Arbor, MI, USA). The principle is based on the competitive immunoassay technique using an alkaline phosphatase-labelled polyclonal antibody against 15-d-PGJ2. Colorimetric changes were detected using a microplate reader at 405 nm, and the measured optical density was used to calculate the concentration of 15-d-PGJ2. The intra-assay (interassay) coefficient of variation (CV) was 5.6–7.4% \((n=16)\) [13.0–15.7% \((n=14)\)].

Pathological investigations

Biopsy specimens were fixed in formalin, and the size of liver biopsy specimens was assessed by the number of portal areas in it: 0–1 portal areas as insufficient, 2–5 as limited and 6 or more as sufficient. Insufficient biopsies were excluded from this study. If several biopsies were performed, the first one for each patient was selected for analysis.

Scoring of liver histology for fibrosis (stage) and inflammatory activity (grade) was performed according to the META VIR classification (29). In META VIR, fibrosis is classified into four stages (F1–F4) and inflammatory activity into three grades (A1–A3). Experienced pathologists examined all the specimens. Liver steatosis was classified into four degrees based on the proportion of hepatocytes with macrovesicular steatosis: no steatosis (no or \(<5\% \) of hepatocytes involved), S1 (mild steatosis; 6–33%), S2 (moderate steatosis; 34–66%) and S3 (severe; \(>66\% \)) (30).

HCC considered in this study was exclusively originating from non-fibrotic liver, which represents a rare, ill-defined subgroup of HCC (31). A final diagnosis of HCC was based on histological findings in resected hepatic tumours or biopsy specimens or on the radiological findings of hepatic arteriography. After pathological examination of the resected liver.
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specimen, tumours were staged according to the conventional TNM scoring system (pTNM, stage 1–3), the modified Edmondson–Steiner grade (I–IV) (32) and the histopathological grade of differentiation [well, moderate, poor (1–3)] based on the recommendations of the Liver Cancer Study Group of Japan (33, 34).

Detection of transforming growth factor-β1 gene polymorphisms in Chinese patients with hepatocellular carcinoma

Analyses for genetic polymorphisms of the TGF-β1 gene at positions −509 (C or T) and +869 (C or T, codon 10), both of which are associated with a reduced risk of HCC in patients with chronic hepatitis B virus infection (35), were carried out in genomic DNA obtained from EDTA blood samples. The TGF-β1 gene −509 C/T polymorphism typing was performed by polymerase chain reaction restriction fragment length polymorphism, with standard amplification protocols. The primer sets used were as follows: sense primer 5′-GGT CCC AGG ACA GGC TTC GTG G-3′ and antisense 5′-AGA CGA GGA GAG TCA GG-3′. The TGF-β1 polymorphism at position +869 (codon 10) was determined as described previously (36).

Statistical analysis

Because of skewed distributions of most variables, the median and range are given. Differences between two groups were assessed by the Mann–Whitney U-test or, between more than two groups, by the Kruskal–Wallis analysis of variance. Comparisons between subgroups are illustrated with parallel boxplot graphics. The boundaries of the box are Tukey’s hinges. The length of the box is the interquartile range (IQR) computed from Tukey’s hinges. Values more than three IQRs from the end of a box are labelled as extreme, denoted with an asterisk (*). Values > 1.5 IQRs but < 3 IQRs from the end of the box are labelled as outliers (○). The correlations between variables were analysed with the Spearman correlation tests. Values of P < 0.05 were considered statistically significant. Associations between parameters were calculated by multiple regression analysis, which is presented as a range diagram with regression line and individual 95% confidence interval.

Results

15-deoxy-Δ12,14-prostaglandin J2 concentrations in healthy probands and non-liver disease sick

The mean 15-d-PGJ2 serum concentrations in healthy Caucasian controls were 2.3 ± 1.0 μg/L (estimated 95% reference range 2.1–2.4 μg/L), with no significant difference between the genders. Also, age did not significantly influence 15-d-PGJ2 concentrations. NLD patients had mean 15-d-PGJ2 serum concentrations of 2.7 ± 1.4 μg/L (estimated 95% reference range 2.5–2.9 μg/L), which were not significantly different from those obtained for the healthy controls. Neither age nor gender was a relevant influencing variable (Fig. 1).

15-deoxy-Δ12,14-prostaglandin J2 concentrations in healthy probands and in hepatitis C patients

The mean serum levels of 15-d-PGJ2 in the HCV group were 6.2 ± 5.9 μg/L (estimated 95% reference range 5.5–6.9 μg/L) and thus significantly elevated compared with healthy controls (P < 0.0001) and NLD patients (P < 0.0001) (Fig. 1). No correlation between 15-PGJ2 serum levels and the extent of inflammatory response (grading), the fibrotic (staging) or steatotic (steatosis) remodelling of the liver was observed (Fig. 2). Also, there was no correlation of 15-d-PGJ2 concentrations with any of the biochemical parameters listed in ‘Materials and Methods’.

15-deoxy-Δ12,14-prostaglandin J2 concentrations in healthy Chinese probands and in Chinese hepatocellular carcinoma

Mean 15-d-PGJ2 serum concentrations in Chinese controls were 0.4 ± 0.2 μg/L (estimated 95% reference range 0.3–0.4 μg/L), and thus significantly lower than those of Caucasian controls (P < 0.0001). Concentrations were independent of gender and age.

The estimated 95% reference range for 15-d-PGJ2 serum concentrations in Chinese HCC patients was between 1.1 and 1.5 μg/L, with a mean value of 1.3 μg/L, and thus differed significantly from those obtained in the control group (P < 0.0001) (Fig. 3). As for controls, concentrations in the HCC group were independent of gender and age. Severity of neoplastic disease, as determined according to the conventional TNM scoring system (pTNM, stages 1–3), the modified Edmondson–Steiner grade (I–IV) (32) and the histopathological grade of differentiation (1–3), was no significantly relevant influencing variable on 15-d-PGJ2 serum concentrations (Fig. 4).

As in Caucasian HCV patients, no correlation between 15-d-PGJ2 concentrations and concentrations of the determined biochemical parameters or virological sero lest results was
observed. Also, TGF-β1 gene polymorphisms [−509 (C or T) and +869 (C or T, codon 10), both of which were associated with a reduced risk of HCC in patients with chronic hepatitis B infection (35)], had no relevant influence on 15-d-PGJ2 serum levels.

**Discussion**

By changing the activity ratio of TGF-β to its antagonist BMP-7, CTGF is proposed as a profibrogenic master switch for epithelial–mesenchymal transition of hepatocytes to extracellular matrix synthesizing fibroblast-like cells, a mechanism, which, during the last years, has attracted tremendous attention in the pathophysiological understanding of hepatic fibro- and carcinogenesis (13–15). Consequently, a largely beneficial effect of CTGF knockdown by gene silencing through siRNA has been shown independently in two toxic models of rat liver fibrosis (24, 25), whereas a role of CTGF in tumour growth has been described in other organ systems (16–20).

In a recent work, we could show that pharmacological application of the methylxanthine derivative caffeine leads to an upregulation of PPARγ expression in hepatocytes, thus sensitizing these cells to the inhibitory effect of the natural PPARγ ligand 15-d-PGJ2 on TGF-β target gene (i.e. CTGF) expression (26–28). However, upregulation of the receptor alone is not sufficient per se; its physiological ligand 15-d-PGJ2 is required to exert an inhibitory effect on TGF-β target genes such as CTGF by inducing a dissociation of the Smad2/3-CBP/p300 transcriptional complex (26).

Prostaglandins are formed following the oxygenation of arachidonic acid by the cyclooxygenase (COX) pathway (37). There are two COX isoforms: COX-1 is constitutively expressed in a number of cell types and is involved in the homeostatic functions of prostaglandins, whereas COX-2 is inducible by a variety of pro-inflammatory stimuli, such as cytokines and lipopolysaccharides. Therefore, increased COX-2 activity and prostaglandin synthesis have been implicated in hepatic regeneration, liver matrix remodelling and portal hypertension (38). However, data on serum availability of prostaglandin D2 or its dehydrated forms, e.g. 15-d-PGJ2, in chronic liver disease or HCC are sparse (39).

The data presented in this study show that patients with chronic HCV infection and ongoing hepatic fibrogenesis as well as patients with fully developed HCC display remarkably higher serum concentrations of 15-d-PGJ2 compared with healthy probands and patients with extrahepatic manifestation of disease. The reason for the variance of mean 15-d-PGJ2 values in Chinese and Caucasian controls may very likely be a result of genetic or alimentary differences (40). Low concentrations of 15-d-PGJ2 in the control groups furthermore suggest that bioavailability of the natural PPARγ ligand is much more the limiting factor in ligand–receptor interactions of this system, so that these groups are unlikely to experience (un)desired effects from caffeine-induced upregulation of PPARγ and TGF-β target gene repression.

As PPARγ agonists are proposed to inhibit fibrogenic remodelling and carcinogenesis in various organs, such as the
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kidney, lung and liver (28, 41–45), a particular suitability of patients with chronic liver disease or HCC for antifibrotic or tumour-suppressive therapy strategies with methylxanthine derivates, i.e. stimulators of PPAR\(\gamma\) expression, may be proposed, whereas a diagnostic benefit of 15-d-PGJ\(_2\) in the assessment of liver fibrosis or HCC does not appear to be present because of a lack of an association of 15-d-PGJ\(_2\) serum concentrations with the severity of fibrotic or neoplastic disease.

Still, major therapeutic challenges remain. One reason is the difficulty of an organ-specific induction of PPAR\(\gamma\) expression. Caffeine is efficiently metabolized in the liver to its major metabolites paraxanthine, theophylline and theobromine. Thus, absorbed caffeine is concentrated in this organ, in particular in hepatocytes, which makes this therapeutic agent relatively hepatotropic per se. However, caffeine metabolism is impaired in cirrhotic patients (46). Therefore, further targeting of methylxanthine derivates to the site of organ injury or even to the responsible and affected cell (type), e.g. by vesicular carriers, should be emphasized (47).

In conclusion, this work demonstrates strikingly higher serum concentrations of the physiological PPAR\(\gamma\) ligand 15-d-PGJ\(_2\) in patients with ongoing hepatic fibrogenesis and HCC compared with healthy controls or patients with an extrahepatic manifestation of disease. Considering the reports showing an induction of hepatocellular PPAR\(\gamma\) expression by caffeine (26) and a TGF-\(\beta\) target gene (i.e. CTGF) repression following the interaction of this receptor with its physiological ligand 15-d-PGJ\(_2\) (26–28), our data propose an increased suitability of these patient groups for therapeutic approaches with methylxanthine derivates. Our results will hopefully initiate further studies in this direction.

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