Identification of Paraxanthine as the Most Potent Inhibitor of TGF-\(\beta\) Dependent Connective Tissue Growth Factor Expression Among the Three Primary Caffeine Metabolites – A New Approach in the Pharmacological Management of Chronic Fibrogenic Diseases?

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Abstract: Several epidemiological studies suggest that coffee drinking is associated with a slower progression of fibrogenesis in patients with chronic, particularly alcoholic, liver disease. However, a causal, mechanistic explanation was pending. New results indicate that the methylxanthine caffeine, major component of coffee and the most widely consumed pharmacologically active substance in the world, might be responsible for this phenomenon as it inhibits the synthesis of Connective Tissue Growth Factor (CTGF/CCN2) in liver parenchymal and non-parenchymal cells, primarily by inducing degradation of Smad2 (and to a much lesser extent Smad3). In particular paraxanthine has been identified as the most potent inhibitor of CTGF synthesis among the three primary metabolites of caffeine, i.e. paraxanthine, theophylline, and theobromine. CTGF plays a crucial role in the fibrotic remodeling of various organs which has therefore frequently been proposed as therapeutic target in the management of fibrogenic disorders. This article summarizes the clinical-epidemiological observations as well as the pathophysiological background and provides suggestions for the therapeutic use of methylxanthine derivatives in the management of fibrotic liver diseases.

Keywords: Methylxanthines, caffeine, paraxanthine, TGF-beta, Connective Tissue Growth Factor, Fibrosis.

INTRODUCTION

“A coffee with your brandy, Sir?”. This citation does not only reflect English club traditions – much more, recent scientific reports propose a medical and molecular-biological rationale behind such cultural habits.

Liver fibrosis, most commonly caused by alcoholism and hepatitis C, and characterized by replacement of functional liver tissue by fibrotic scar tissue as well as regenerative nodules, was the 11th leading cause of death in the United States in 2001, killing about 27,000 people each year with a 10-year mortality of 34-66% [1]. Disease progression and fibrogenic activity show significant inter-individual variability, allowing discrimination between slow, intermediate and rapid fibrosers. Both, environmental and host genetic factors are suspected to modify disease susceptibility and progression rate [2].

Data on 5,994 adult patients with chronic liver disease, collected by US-American scientists during the third National Health And Nutrition Examination Survey (NHANES III) [3, 4] of the National Centers of Health Statistics, Disease Control and Prevention (CDC; Atlanta/ GA, USA) proposed a hepatoprotective effect of increased coffee consumption. Similar results were obtained previously by NHANES I as well as during a recent study by the National Institute of Diabetes and Digestive and Kidney Disease/National Institutes of Health (NIDDK/NIH; Bethesda/MD, USA) [5].

In summary, these studies gave evidence that patients with higher coffee consumption displayed a milder course of fibrosis [4, 5], especially in alcoholic liver disease [3, 4, 6] and lower serum activities of alanin-aminotransferase (ALT) and \(\gamma\)-glutamyltransferase (GGT) [3, 6]. According to Ruhl et al., two cups of coffee daily were sufficient, to markedly reduce the risk of fibrosis progression [4].

Scientists from Tohoku University Hospital in Sendai/Japan who evaluated 9-year data of coffee consumption of 60,107 subjects for the association of coffee intake and the risk of developing primary liver cancer (hepatocellular carcinoma; HCC) found that regular coffee drinkers had a risk for suffering from HCC, which was significantly reduced compared to those who remained abstinent towards coffee consumption [7].

The Japan Collaborative Cohort Study for Evaluation of Cancer Risk (JACC Study) investigated 110,688 cohort members aged 40-79 years in respect of their average coffee intake and calculated a hazard ratio of 0.50 for death due to HCC for drinkers of one and more cups of coffee per day. In contrast, the ratio for drinkers of less than one cup per day was 0.83, which therefore confirmed an inverse association between coffee consumption and HCC mortality [8].

These findings were supplemented with a Swedish meta-analysis of the Karolinska Institute, Stockholm which evaluated the data of 9 cohort and case-control studies involving a total of 2,260 cases and 239,146 non-cases. All epidemiological studies that were considered reported an inverse relation between coffee consumption and risk of liver cancer, and in 6 studies the association was statistically significant. Overall, this meta-analysis revealed an
association of an increase in consumption of 2 cups of coffee per day and a 43% reduced risk of developing HCC [9].

However, despite of these striking epidemiological data, the cellular and molecular mechanisms underlying the antifibrotic and tumor-suppressive effects of coffee consumption remained obscure.

CAFFEINE: WHAT IS IT ALL ABOUT?

At the behest of the German writer Johann Wolfgang von Goethe, Friedlieb Ferdinand Runge, a chemist and pharmacist from Wroclaw, was the first to investigate coffee beans with the objective of finding the psychoactive substance in coffee [10]. In 1820, he finally extracted chemically pure caffeine, from which the German scientists Christoph Heinrich Pfaff and Justus von Liebig successfully deduced the structural formula C_{8}H_{10}N_{4}O_{2} by burning analysis [10]. In his 1875 professorial dissertation, the Würzburg chemist and pharmacist Ludwig Medicus transferred this structural formula into the chemical structure 1,3,7-trimethylxanthine [10]. However, after major disputes with Hermann Emil Fischer, based in Berlin and nobel prize winner in 1902, Ludwig Medicus’ chemical structure only received public acceptance after the first chemical synthesis of caffeine by Fischer in 1895 [10].

Today, global consumption of caffeine has been estimated at 120,000 tonnes per annum, making it the most widely consumed pharmacologically active substance in the world [10]. In North America, 90% of adults consume caffeine daily [10]. It is completely absorbed by the stomach and small intestine within 45 minutes of ingestion, and is eliminated by first-order kinetics [11].

Caffeine is metabolized in the liver, particularly in liver parenchymal cells (hepatocytes), by the cytochrome P450 oxidase enzyme system (CYP1A2) into the three metabolic dimethylxanthines paraxanthine (1,7-dimethylxanthine; 84%), theobromine (3, 7-dimethylxanthine; 12%), and theophylline (1, 3-dimethylxanthine; 4%) [12-15], (Fig. (1)). Further demethylation and oxidation form urates and uracil derivatives. About a dozen metabolites can be recovered in the urine of regular coffee consumers [12, 14].

Caffeine and its metabolites act through multiple mechanisms involving both action on receptors and channels on the cell membrane, as well as intracellular action on calcium and cAMP pathways [16]. Even though the major caffeine derivatives, i.e. paraxanthine, theobromine, and theophylline, have common mechanisms of action, the fraction, by which any of the pathways is affected, differs between them.

By virtue of its purine structure, caffeine can act on some of the same targets as adenosine related nucleosides and nucleotides, i.e. activation of intracellular Ryanodine receptors [which are the physiological target of cADPR (cyclic ADP ribose)] in vitro as well as interaction with adenosine receptors and competitive inhibition of the cyclic adenosine monophosphate phosphodiesterase (cAMP-PDE) in vitro and in vivo [17-20]. Physiologically, caffeine action is unlikely due to increased Ryanodine receptor opening, as it requires plasma concentrations above lethal dosage [20].

Caffeine furthermore is a non-selective adenosine receptor antagonist, with reported similar in vitro affinities for A1 (whose activation leads to a reduction of intracellular cAMP levels), and A2A receptors (elevating cAMP levels) and with lower affinity for A3 receptors [21]. However, A1 and A2A receptors are the adenosine receptors predominantly expressed in the brain. Because of this and because of their opposite roles in regulating cAMP dependent pathways, the contribution of blockade of adenosine A1 and A2A receptors to the non-central effects of caffeine is still a matter of debate. Therefore, accumulation of cAMP within the cell through direct
Identification of Paraxanthine as the Most Potent Inhibitor of TGF-β

The Open Conference Proceedings Journal, 2010, Volume 1

inhibition of the cAMP-PDE is currently regarded as the central pharmacological effect of caffeine in peripheral tissues [21].

cAMP was one of the first identified second messengers transmitting signals via G-protein coupled receptors and protein kinase A (PKA) from the cell surface to the nucleus [22]. In the unactivated state, PKA resides in the cytoplasm. Induction by cAMP liberates the catalytic subunits of PKA, which then are capable of diffusing into the nucleus where they phosphorylate transcription factors, i.e. cAMP response element binding protein (CREB) [23]. PKA phosphorylates CREB at serine 133, which then transactivates cAMP-responsive genes by binding as a dimer to a conserved, 8 bp, palindromic cAMP response element (CRE), TGACGTCA. Over 100 genes with functional CREs have been identified so far and a modulation of various cell signaling proteins by cAMP has been reported [24].

CONNECTIVE TISSUE GROWTH FACTOR (CTGF/CCN2): A CENTRAL PLAYER IN FIBROGENESIS

As mentioned previously, caffeine and its primary metabolites act as competitive intracellular inhibitors of cAMP-PDE, which converts cAMP to its non-cyclic form [25], thus allowing cAMP to build up in cells. This aspect is of particular relevance, as cAMP was shown to inhibit Transforming Growth Factor (TGF)-β induced Connective Tissue Growth Factor (CTGF/CCN2) expression [26, 27].

CTGF is a 36-38 kDa cysteine-rich, heparin-binding, and secreted protein synthesized by various cell types. It is now classified as the second of six members of the CCN gene family containing CTGF itself, cyt61, NOV, and others [28], which share approximately 40 to 60% sequence similarity and are characterized as mosaic proteins that comprise four conserved structural modules [29]. These modules are important for the pleiotropic functions of CTGF including among others matrix production, cell migration, cell adhesion, and cellular differentiation [30, 31].

As may be deduced from these effects, CTGF has reached considerable pathophysiological relevance because of its involvement in the pathogenesis of fibrotic diseases, carcinogenesis, atherosclerosis, skin scarring, and other conditions with excess production of connective tissue [32].

REGULATION OF CTGF EXPRESSION BY THE TRANSFORMING GROWTH FACTOR (TGF)-β SUPERFAMILY

The competence of hepatocytes for the synthesis of CTGF was recently shown by detailed cell culture studies, which clearly demonstrate CTGF expression in parenchymal liver cells, which is sensitively up-regulated by exogenous Transforming Growth Factor (TGF)-β [33, 34] but occurs also spontaneously in TGF-β-free culture conditions due to intracellular activation of latent TGF-β [35]. Thus, hepatocytes are now recognized as a quantitatively important source of CTGF, which responds to TGF-β.

TGF-β belongs to a superfamily of cytokines, which comprises further ligands, such as bone morphogenetic proteins (BMPs), and Activin A. All TGFβ superfamily ligands bind to a type II receptor dimer, which recruits a type I receptor dimer forming a hetero-tetrameric complex with the ligand [36], resulting in the phosphorylation of the Type

![Fig. (2)](image)

*Fig. (2).* Immunocytochemical demonstration of CTGF in normal and bile-duct ligated fibrotic rat liver. Localization of CTGF in cytokeratin 18 positive hepatocytes and in few desmin-positive (myo-)fibroblasts is shown.
I receptor. The activated type I receptor then phosphorylates receptor-regulated SMADs (R-SMADs), which can now bind the coSMAD SMAD4. R-SMAD/coSMAD complexes accumulate in the nucleus where they act as transcription factors and participate in the regulation of target gene expression [36].

CTGF gene activation by TGF-β is mediated by a functional Smad-binding element, which resides within the CCN2 promoter [31]. In hepatocytes TGF-β-driven CTGF gene expression is dependent primarily on Smad2 and, to a much lesser extent, Smad3 [32]. However, recent works point to an increasing importance of Smad2 in hepatocellular CTGF expression [35, 37]. A large number of Smad2/3-associated transcriptional co-activators, including CREB binding protein (CBP) and p300, has been identified to possess intrinsic acetyltransferase activities that are important for their abilities to enhance transcription [38-45]. In particular DNA binding activity and association with target promoters of Smad2 and Smad3 are tightly regulated by CBP/p300-mediated acetylation of these Smads in response to TGF-β signaling [46].

**IMPACT OF CTGF ON EPITHELIAL TO MESENCHYMAL TRANSITION**

The fibrogenic mechanisms in the liver are dependent on an interplay of many pro- and anti-fibrotic/inflammatory cytokines [47, 48]. The hierarchy of pro-fibrogenic growth factors most importantly includes TGF-β, designated as "fibrogenic master cytokine" with multiple effects on extracellular matrix turnover [36, 49], hepatoacellular apoptosis [50-53], proliferation and liver regeneration [49, 54, 55], inflammation and immunosuppression [56], and cancerogenesis [52, 57]. The natural antagonist of many actions of TGF-β is bone-morphogenetic protein 7 (BMP-7), a member of the TGF-β superfamily [58]. Thus, the balance of both growth factors, i.e. TGF-β and BMP-7, will be crucial for development of fibrosis and outcome of (chronic) liver disease, i.e. risk for the development of HCC.

Even though the molecular mechanism of action of CTGF is still not known in detail yet, a modulator role in the epithelial to mesenchymal transition (EMT) of adhering hepatocytes into cells with reduced intercellular adhesion, increased motility and mesenchymal, fibroblast-like properties, is discussed [59]. This process is gaining more and more importance in the pathogenetic understanding of hepatic fibrogenesis [60, 61], but accumulating evidence also points to a critical role of EMT-like events during tumor progression and malignant transformation, endowing the incipient cancer cell with invasive and metastatic properties [62].

The prototype of the currently most powerful inducer of EMT is TGF-β [63], activating this pathway via induction of Smad2/3 phosphorylation and the Snail transcription factor [63]. In contrast, BMP-7, the most important molecular counterpart of TGF-β, not only inhibits EMT, but can even induce a mesenchymal-epithelial transition (reverse EMT = MET) [60]. Recent reports gave evidence that up-regulation of CTGF inhibits BMP-7 signal transduction in the diabetic kidney [64]. Abreu et al. furthermore presented data describing CTGF as extracellular trapping protein for BMP and TGF-β [59]. According to their functional studies on Xenopus laevis, CTGF directly binds BMP and TGF-β through their cysteine-rich (CR) domain, thus antagonizing BMP activity by preventing its binding to BMP receptors. Of note, the opposite effect, enhancement of receptor binding, was observed for TGF-β [59]. These results suggest that CTGF inhibits BMP and activates TGF-β signals by direct binding in the extracellular space. From this, CTGF would act pro-fibrogenic.

A central role of CTGF in liver fibrogenesis and tumor growth, which may thus be expected, is documented by reports on increased CTGF expression in various tumor tissues [65-70] as well as in fibrotic liver tissue, (Fig. (2)) [28, 71, 72], and, even more important, by recent studies, in which knock-down of CTGF by siRNA leads to substantial attenuation of experimental liver fibrosis [73, 74]. Thus, modulators of CTGF-expression will have a great pathogenetic relevance for fibrosis.

**CAFFEINE REDUCES TGF-β-DEPENDENT CTGF SYNTHESIS OF THE HEPATOCYTE THROUGH PROTEASOME MEDIATED DEGRADATION OF THE TGF-β EFFECTOR SMAD 2**

We previously talked about Smad2 (and, to a lesser extent, Smad3) as key mediator of TGF-β-induced CTGF expression in hepatocytes [32].

Very recent observations gave evidence, that caffeine is able to enforce proteasomal Smad2 degradation by enhancing the activity of SMURF2, a member of the family of E3 ubiquitin ligases, (Fig. (3)) [75-77], with the consequence that Smad2 is increasingly bound to ubiquitin and proteasomally degraded [76, 77]. This finding seems to be of particular relevance for clinical situations of TGF-β activation such as viral hepatitis and tumor growth [36, 57, 79, 80], as degradation of Smad2 in response to TGF-β requires receptor-mediated phosphorylation of the C-terminal serines [76-78], which would suggest a normal or even stimulated TGF-β type 1 / ALK5 receptor-dependent phosphorylation, i.e. intra- or extracellular presence of TGF-β [35]. Enhanced degradation of Smad3 by caffeine was much less pronounced, but its phosphorylation by the TGF-β type 1 / ALK5 receptor (TβRI) was clearly impaired, (Fig. (3)). This indicates a stimulation of proteasome-mediated degradation which is largely specific for Smad2. Such a high degree of specificity of SMURF2 to preferentially degrade Smad1 and Smad2 but to a much lesser extent Smad3, was previously described by Lo et al. [76]. The caffeine-induced inhibition of phosphorylation (and much less pronounced degradation) of Smad3, however, may still be secondary to enhancement of SMURF2 activity, as this ubiquitin ligase is also able to bind the activated TGF-β receptor complex, leading to TβRI degradation and thus, inhibition of phosphorylation of Smad3 and degradation of total Smad3 protein allosterically bound to the type 1 receptor in the Smad3/SARA (Smad anchor for receptor activation)/receptor kinase complex [77-81]. Still, both receptor-phosphorylated Smads do not seem to be interchangeable and each one seems to follow specific metabolic routes.

It is not known yet, how caffeine triggers proteasomal degradation, however, earlier works already gave evidence that the cAMP/PKA-dependent pathway can directly regulate the activity of the ubiquitin-proteasome system [82,
Identification of Paraxanthine as the Most Potent Inhibitor of TGF-β

The Open Conference Proceedings Journal, 2010, Volume 1 243

and that phosphorylation by PKA can alter the proteasomal degradation rate of the phosphorylated protein [84, 85], so a link between cAMP accumulation and ubiquitin ligase activity may be presumed.

CAFFEINE PROMOTES THE INHIBITION OF TGF-β-INDUCED CTGF SYNTHESIS IN THE HEPATOCYTE THROUGH UPREGULATION OF THE NUCLEAR PEROxisome PROLIFERATOR-activated receptor γ

Peroxisome proliferator-activated receptors (PPARs) comprise a group of nuclear receptor isoforms intimately connected to cellular lipid metabolism and cell differentiation. Three types of PPARs have been identified: α, γ and δ (β). Without going into pathophysiological detail, earlier reports demonstrated that the prostaglandin analogon and PPARγ ligand 15-deoxy-Δ12,14-prostaglandin J2 (15-d-PGJ2) has a potent inhibitory effect TGF-β1-induced CTGF expression in the liver [86, 87], suggesting that hepatic CTGF is a PPARγ-regulated gene. However, in human aortic smooth muscle cells it was shown that activation of PPARγ abrogates TGF-β-induced CTGF expression by directly interfering with the Smad3 signaling pathway [88] and the p300 system, which are necessary cofactors for target gene activation by the Smad2/3 transcriptional complex [88-90].

The expression of type 2 PPARγ (PPARγ2) was previously described as being directly regulated by cAMP/CREB, in that phosphorylated CREB binds to the promoter of PPARγ2 together with activating transcription factor 1 (ATF1), initiating gene transcription [91, 92]. Thus, it is not surprising that upregulation of PPARγ expression by caffeine, in particular in combination with TGF-β, was identified as a further mechanism of the inhibitory effects of caffeine on CTGF expression in hepatocytes [75]. As previously observed for aortic smooth muscle cells [88], binding of 15-d-PGJ2 to PPARγ also leads to a dissociation of Smad2 (and Smad3) transcriptional complex, that involves the cofactors p300 and CREB binding protein (CBP) within the hepatocyte, thus preventing the transcriptional activation of TGF-β target genes such as CTGF, (Fig. (3)) [46, 75]. However, it has to be considered that controversial data on the role of CBP/p300 in CTGF promoter activation are communicated, depending on the cell type [88-90, 93].

Still, this finding is of interest insofar as patients with fibrogenic liver disease or with HCC originating from nonfibrotic livers display strikingly higher serum concentrations of 15-d-PGJ2 compared to healthy controls and non-liver disease sick, (Fig. (4)) [94]. Thus, it may be suggested that these patients display particular sensitivity towards antifibrotic therapy approaches with PPARγ inducing drugs such as caffeine.

INTRAPERITONEAL APPLICATION OF CAFFEINE PREVENTS N-ACETYL-D-GALACTOSAMINE-6-SULFATE INDUCED HEPATIC EXPRESSION OF CTGF

All the findings discussed above were based exclusively on results of in vitro experiments. However, results obtained from in vitro studies are often not directly applicable to the in vivo situation. Even more exciting were the results of very recent investigations, aiming at more closely mimicking the

Fig. (3). Simplified and schematic overview of the proposed signaling pathway of caffeine-mediated CTGF suppression in hepatocytes.

Caffeine inhibits hepatocellular CTGF expression through an elevation of intracellular cAMP levels leading to enhanced ubiquitination/proteasomal degradation of the TGF-β effector Smad 2 by the ubiquitin-ligase SMURF2, which displays high sensitivity towards this particular Smad as well as to the TGF-β receptor complex. Also observed is an inhibition of Smad3 phosphorylation but only little degradation of the total Smad3 protein, which is in contrast to Smad2. This may very likely be the result of enhanced SMURF2 dependent degradation of the TGF-β type 1/ALK5 receptor complex to which Smad3 is allosterically bound. Last, caffeine (cAMP) stimulates PPARγ expression, thus sensitizing hepatocytes towards the natural PPARγ ligand 15-d-PGJ2, which, after binding to PPARγ, prevents the activation of TGF-β target genes such as CTGF expression by inducing a dissociation of the cofactors CBP/p300 from the Smad2/3 transcriptional complex.
The situation of therapeutic caffeine application, that impressively demonstrated the capability of caffeine to suppress hepatocellular CTGF expression not only in vitro, but also in the experimental rat model of toxic hepatitis induced by N-Acetyl-D-galactosamine-6-sulfate (D-GalN; 500 mg / kg body weight) in vivo, (Fig. 5) [unpublished data by the author]. These data, even though only obtained in the small number of totally 4 rats, show that concomitant application of 6 intraperitoneal injections of each 50 mg / kg body weight caffeine every 4 h over 24 h markedly reduced D-GalN induced CTGF expression in the damaged liver, and that it raised intrahepatic cAMP levels approximately 2.2-fold compared to the control rat [unpublished data by the author]. Furthermore, caffeine markedly reduced the spill-over of hepatic derived CTGF into the circulation when compared to the rat treated with D-GalN alone, which displayed significant higher CTGF serum concentrations than the control animal [unpublished data by the author]. The two caffeine treated animals were alive and in good condition [unpublished data by the author].

COMPARISON OF THE INHIBITORY CAPACITIES OF THE PRIMARY CAFFEINE-DERIVED METABOLITES ON HEPATOCELLULAR CTGF SYNTHESIS

We previously discussed the capacity of caffeine to almost entirely inhibit spontaneous CTGF synthesis at a calculated 50% inhibitory dose (ID50) of 4.42mM), (Fig. (6)) [95]. Based on this, further studies were initiated to investigate the repressive capacities of the primary demethylated caffeine metabolites paraxanthine, theobromine, and theophylline on hepatocellular CTGF expression.

The data suggest paraxanthine as the most potent caffeine-derived pharmacological repressor of hepatocellular CTGF expression with an ID50 of 1.15mM, i.e. 3.84-fold lower than what is observed for caffeine Fig. (6), by simultaneously displaying least cytotoxicity of all tested metabolites [95]. At the toxicological threshold concentration of 1mM for paraxanthine, defined by the US-American Hazardous Materials Information System® III (HMIS® III) [96] and the National Fire Protection Association (NFPA) [97], an inhibition of hepatocellular CTGF synthesis by still 44% was observed [95]. As for caffeine, this effect was strongly reverted in the presence of a specific competitive cAMP inhibitor, indicating that also paraxanthine mediates its inhibitory effect on CTGF synthesis through an elevation of intracellular cAMP concentrations [95]. Furthermore, paraxanthine (1.25mM and 2.5mM) also reduced TGF-\(\beta\)-induced hepatocellular CTGF synthesis by in average 27% and 45%, respectively, and thus, next to caffeine itself, proved to be the strongest inhibitor also of CTGF expression caused by exogenous TGF-\(\beta\) [95].

TRANSDIFFERENTIATION DEPENDENT INHIBITION OF CTGF, AND COLLAGEN A1 TYPE 1 SYNTHESIS IN RAT HEPATIC STELLATE CELLS BY PARAXANTHINE

We now learned in detail how caffeine and its primary metabolite paraxanthine suppress TGF-\(\beta\)-dependent and -independent CTGF expression in hepatocytes. However, the question of a possible repressive effect of caffeine or, its even more potent primary metabolite paraxanthine, on CTGF synthesis also in hepatic stellate cells (HSC) was not yet addressed, even though the contribution of HSC to the pathogenesis of hepatic fibrosis is unequivocally substantial.
Identification of Paraxanthine as the Most Potent Inhibitor of TGF-β

The Open Conference Proceedings Journal, 2010, Volume 1

Fig. (5). Intraperitoneal caffeine injection reduces hepatic CTGF expression following toxic liver injury by D-galactosamine (D-GalN).

Immunohistochemical detection of CTGF in liver (40x magnification). Paraffined rat liver slices were incubated with two different polyclonal antibodies against CTGF and respective non-immune control immunoglobulin factions.

Fig. (6). Dose response of the inhibitory capacities of caffeine and its derived metabolic methylxanthines paraxanthine, theophylline, and theobromine against hepatocellular CTGF protein expression.

Hepatocytes were cultured under serum-free conditions with indicated concentrations of caffeine, paraxanthine, theophylline and theobromine and Western blot analysis performed. Blots were quantified relative to β-Actin using the Lumi Imager System. The diagrams display mean CTGF protein expression (CTGF/β-Actin [BLU]) of triplicate determinations from 4-5 different cell cultures and are described as fraction of the untreated control (%). A representative blot is each demonstrated. Below, range diagrams, regression lines and individual 95% confidence intervals for the correlation of CTGF protein expression and caffeine, paraxanthine, theophylline or theobromine concentrations are given. Circles represent mean values of 3 independent experiments from one cell culture. Green, red and blue lines indicate the concentrations of the metabolites necessary to achieve a 25 (ID25), 50 (ID50) or 75% (ID75) inhibition of CTGF protein expression.
Following liver injury, HSC undergo "activation" which connotes a transition from quiescent vitamin A-rich cells into proliferative, fibrogenic, and contractile myofibroblasts (MFB). This pathway has long been, and probably still is, considered as the "canonical" pathway in the pathogenetic understanding of liver fibrogenesis. The major phenotypic changes after activation include proliferation, contractility, fibrogenesis, matrix degradation, chemotaxis, retinoid loss, and white blood cell chemoattraction [98].

Very recently, emerging early data further elucidated the effect of paraxanthine on HSC activation, as well as on related changes in the synthesis of extracellular matrix components by this cell type (previously unpublished data by Gressner OA et al., 2009).

Results showed a transdifferentiation dependent inhibitory effect of paraxanthine on CTGF protein expression and promoter activity in HSC, being particularly effective in the progressive stage of transdifferentiation. This reduction of CTGF expression was accompanied by a continuous, paraxanthine dependent, inhibition of expression of collagen α1 type 1 (Col1) but not of α smooth muscle actin (αSMA) throughout the entire process of transdifferentiation, (Fig. (7)) (previously unpublished data by Gressner OA et al., 2009).

The observed stronger repression of CTGF (and also Col1) expression by paraxanthine in the later stage of transdifferentiation compared to early stage transdifferentiation may be explained by the previously explained fact that caffeine and its derivatives inhibit CTGF expression primarily through an interruption of TGF-β induced Smad2/3 signaling. All three forms of TGF-β receptors, types I, II, and III (betaglycan), just as TGF-β itself, are synthesized by HSC, however, TGF-β1 binding and responsiveness are greatly enhanced during their activation and transdifferentiation to MFB in vivo and in vitro, facilitating an autocrine stimulation [98]. Interestingly, Smad signaling evolves with stellate cell activation and plays different roles during progressive cellular activation, which could result in such a transdifferentiation dependent increased sensitivity of this mesenchymal cell type towards methylxanthines, as observed [98]. The mode of action of paraxanthine as inhibitor of TGF-β induced Smad signaling also explains the lacking effect on αSMA expression in HSC, as the regulation of αSMA expression is (largely)
TGF-β independent, whereas its re-organization to stress fibers during transdifferentiation is not [99, 100].

Next to its effects in hepatocytes, these results were the first to show a suitability of paraxanthine in also antagonizing transdifferentiation dependent sensitization of HSC towards TGF-β dependent effects, i.e. CTGF and Col1 expression.

**PERSPECTIVES**

Taken together, the studies introduced above point to three major mechanisms how caffeine (and its primary metabolites paraxanthine, theophylline and theobromine) might act on the suppression of hepatocellular CTGF synthesis: (i) reduction of the steady state concentration of total Smad2 (and to a much lesser extent Smad3) protein, (ii) decreased phosphorylation of Smad3, and (iii) upregulation of the PPARγ-receptor resulting in enhanced sensitivity of PC towards natural PPARγ ligands such as 15-d-PGJ2. They all have the common consequence of an interruption of the Smad2/3 signaling pathway, which is also observed in paraxanthine treated HSC undergoing transdifferentiation to MFB.

These findings may partially explain earlier results showing that the activation of the TGF-β response element (TβRE), sharing partial homology with the consensus sequence of the cAMP response element (CRE) [102], is inhibited in the presence of cAMP analogs or agents elevating intracellular cAMP levels [101]. Previously, a direct interaction between cAMP and the TβRE was thus suggested [101]. However, the presented data propose that this phenomenon described by Duncan et al. might not rely on a direct interaction of this promoter sequence with cAMP, but much more on a modulation of (phosphorylated) Smad levels and on a reduction of activity of the Smad2/3 transcriptional complex (via PPARγ) by cAMP elevating substances such as caffeine or paraxanthine.

Without doubt, the data discussed above still have limitations in terms of their immediate therapeutic relevance. For example, the specificity of caffeine and/or paraxanthine in repressing profibrogenic (i.e. CTGF) but not antifibrogenic target genes (i.e. BMPs) has not been assessed yet [102]. Also, the activation of other, non PKA-mediated, hepatocellular signaling pathways by caffeine, such as AMP-activated protein kinases (AMPK) [103] cannot be entirely excluded at present, and need further evaluation.

However, as the overall pivotal role of CTGF in the fibrogenic process of the liver has been convincingly proven in experimental rat liver fibrosis with silenced CTGF [73, 74], as strong overexpression of CTGF is found in both, fibrotic and tumor tissues [28, 65-72], and as caffeine is able to prevent D-GalN induced hepatic expression of CTGF in the rat *in vivo*, the molecular-biological mechanisms summarized above suggest a suppressive effect of caffeine, paraxanthine or cAMP analogs on human liver fibrosis. This suggestion is supported by very recent data showing that blocking adenosine A2A receptors reduces peritoneal fibrosis in two independent experimental models *in vivo* [104]. Therefore, methylxanthines may eventually be proposed as a family of drugs useful in the treatment of chronic fibrogenic (or even carcinogenic) disorders. The presented findings hopefully initiate further studies in this direction. And in the meantime, enjoy your cup of coffee!

**ACKNOWLEDGEMENTS**

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**ABBREVIATIONS**

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<th>ABBREVIATION</th>
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<tr>
<td>15-d-PGJ2</td>
<td>15 deoxy prostaglandin J2</td>
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<td>8-Br-cAMP</td>
<td>8-bromoadenosine-3’, 5’-cyclic adenosine monophosphate</td>
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<tr>
<td>ALK4/ALK5</td>
<td>activin like receptor 4/5</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>cAMP-PDE</td>
<td>cAMP-specific phosphodiesterase</td>
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<td>CBP</td>
<td>CREB binding protein; co = control</td>
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<td>CRE</td>
<td>CREB response element/ cAMP response element</td>
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<td>CREB</td>
<td>cAMP response element binding protein;</td>
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<tr>
<td>CTGF</td>
<td>CTGF response element binding protein;</td>
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<td>CCN2</td>
<td>Connective tissue growth factor</td>
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<td>D-GalN</td>
<td>N-Acetyl-D-galactosamine-6-sulfate</td>
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<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
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<td>NIDDK</td>
<td>National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda/MD, USA)</td>
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<td>PPARγ</td>
<td>peroxisome proliferator-activated receptor γ;</td>
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<td>p-Smad</td>
<td>phospho-Smad</td>
</tr>
<tr>
<td>SMURF</td>
<td>Smad–ubiquitin regulatory factor</td>
</tr>
<tr>
<td>TβRE</td>
<td>TGF-β response element</td>
</tr>
<tr>
<td>TβRI</td>
<td>TGF-β type 1 receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
</tbody>
</table>

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