Adiponectin reduces connective tissue growth factor in human hepatocytes which is already induced in non-fibrotic non-alcoholic steatohepatitis

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ABSTRACT

Connective tissue growth factor (CTGF) is induced in liver fibrosis and enhances the activity of transforming growth factor β (TGFβ). Recently we have shown that the hepatoprotective adipokine adiponectin downregulates CTGF in primary human hepatocytes (PHH). In the current study, the mechanisms mediating suppression of CTGF by adiponectin and the well described downstream effector of adiponectin receptor 2 (AdipoR2), peroxisome proliferator activated receptor α (PPARα), were analyzed in more detail. Adiponectin downregulated CTGF mRNA and protein in primary human hepatocytes (PHH) and suppression was blocked by a PPARα antagonist indicating that AdipoR2 is involved. The PPARα agonists fenofibrate and WY14643 also reduced CTGF protein in these cells. Adiponectin further impaired TGFβ-mediated upregulation of CTGF. Phosphorylation of the TGFβ downstream effectors SMAD2 and -3 was reduced in PHH incubated with adiponectin or PPARα agonists suggesting that early steps in TGFβ signal transduction are impaired. CTGF and TGFβ mRNA levels were increased in human non-fibrotic non-alcoholic steatohepatitis (NASH), and here AdipoR2 expression was significantly reduced. Current data show that CTGF and TGFβ are already induced in non-fibrotic NASH and this may be partly explained by low adiponectin bioactivity which interferes with TGFβ signaling by reducing phosphorylation of SMAD2/3 and by downregulating CTGF.

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Introduction

Hepatic steatosis is a common finding in westernized countries and its higher prevalence in obesity suggests that accompanying metabolic abnormalities contribute to increased fat storage in the liver. Fatty liver is more susceptible to the development of non-alcoholic steatohepatitis (NASH) characterized by hepatic inflammation which may progress to liver fibrosis and even cirrhosis (Tilg and Moschen, 2010). Adiponectin reduces hepatocyte fat storage, inflammation and enhances fibrinolysis, and thereby may protect from NASH and fibrotic liver disease (Schäffler et al., 2005; Wanninger et al., 2011c).

Activation of hepatic stellate cells (HSC) by transforming growth factor β (TGFβ) is the central process in liver fibrosis and TGFβ strongly stimulates synthesis of extracellular matrix proteins (Schäffler et al., 2005). Adiponectin completely blocks TGFβ mediated nuclear translocation of SMAD2 in HSC, and subsequent production of TGFβ and connective tissue growth factor (CTGF) (Kamada et al., 2003).

CTGF, however, is predominantly synthesized by hepatocytes (Gressner et al., 2009) and TGFβ strongly stimulates CTGF synthesis in these cells while having no or only marginal effects in HSC (Gressner et al., 2007). This indicates that reduced CTGF synthesis in HSC may be a direct effect of adiponectin rather than being a secondary consequence of impaired TGFβ activity (Kamada et al., 2003). CTGF is strongly upregulated in fibrotic liver tissues, (Hayashi et al., 2002; Paradis et al., 1995) and its crucial role in liver fibrosis has been demonstrated in recent studies. Intraportal administration of CTGF and TGFβ leads to multiorgan fibrosis whereas administration of a single cytokine has no effect (Wang et al., 2011). Further, knock-down of CTGF using siRNA prevents experimental liver fibrosis (George and Tsutsumi, 2007; Li et al., 2006). Furthermore, mice expressing a hepatocyte CTGF transgene are more susceptible to liver insults (Tong et al., 2009). These data demonstrate a central function of CTGF in fibrotic liver disease.

Recently our group has shown that adiponectin impairs TGFβ-mediated induction of CTGF in primary human hepatocytes (Wanninger et al., 2011b). Adiponectin upregulates the TGFβ decoy receptor BAMBI overexpression in hepatoma cell lines impairs induction of CTGF by TGFβ. Adiponectin also lowers CTGF in PHH not treated with TGFβ suggesting that additional mechanisms mediate reduced CTGF expression (Wanninger et al., 2011b).
Adiponectin signals through two receptors, AdipoR1 and AdipoR2, with the latter being thought to be more relevant for the hepatic effects of this adipokine at least in mice (Yamauchi et al., 2007). Overexpression of hepatic Adipor2 improves methionine–choline deficient diet (MCD) induced liver injury by activating PPARα which is a well described downstream effector of this receptor (Tomita et al., 2008; Yamauchi et al., 2007). In-vitro studies have shown that the PPARα agonists WY14643 and bezafibrate inhibit TGFβ mediated activation of CTGF in human hepatoma cells (Suk et al., 2009) indicating that adiponectin mediated activation of PPARαx may contribute to lower CTGF in PHH. Therefore, in the current study the effect of adiponectin and PPARαx agonists on hepatocyte CTGF and TGFα was analyzed.

Materials and methods

Culture media and reagents

Dulbecco’s modified eagle medium (DMEM) was from PAA (Karlsruhe, Germany). RNeasy Mini Kit was from Qiagen (Hilden, Germany) and oligonucleotides were synthesized by Metabion (Planegg-Martinsried, Germany). LightCycler FastStart DNA Master SYBR Green I was purchased from Roche (Mannheim, Germany). Palmitic acid, oleic acid, RU486, and fenofibrate were ordered from Sigma (Deisenhofen, Germany). Fatty acids were complexed to fatty acid-free bovine serum albumin (Roche) with a molar ratio of 1:1. Equal amounts of bovine serum albumin were added to control cells. The PPARα agonist WY14643 was from Calbiochem (Darmstadt, Germany). GAPDH antibody was from New England Biolabs GmbH (Frankfurt, Germany). Recombinant full-length human adiponectin, and recombinant TGFβ were from R&D Systems (Wiesbaden-Nordenstadt, Germany). The monoclonal CTGF antibody was from Abnova (Heidelberg, Germany). SMAD2/3, SMAD3, phospho-SMAD2 (Ser465/467) and phospho-SMAD3 (Ser423/425) antibodies were from New England Biolabs GmbH (Frankfurt, Germany).

Human liver tissue and primary liver cells

Human liver tissue used for immunoblot or cell isolation was obtained from liver resections of patients undergoing partial hepatectomy for metastatic liver tumors of colorectal cancers. Experimental procedures were performed according to the guidelines of the charitable state controlled foundation HTCR (Human Tissue and Cell Research), with the informed patient’s consent approved by the local ethical committee of the University of Regensburg (Thasler et al., 2007). Human liver tissue used for immunoblot or cell isolation was obtained from liver resections of patients undergoing partial hepatectomy for metastatic liver tumors of colorectal cancers. Experimental procedures were performed according to the guidelines of the charitable state controlled foundation HTCR (Human Tissue and Cell Research), with the informed patient’s consent approved by the local ethical committee of the University of Regensburg (Thasler et al., 2003). Primary human hepatocytes were isolated and cultivated in serum-free medium (DMEM supplemented with 4.5 g/l glucose, 0.4 ng/ml hydrocortisone, 0.415 mU/ml insulin, 2 mM glutamine, and 100 U/ml penicillin/streptomycin) for 3 days as previously described (Weiss et al., 2003). Liver tissues for mRNA expression analysis were obtained of 9 patients without fatty liver, 9 patients with simple liver steatosis, and 8 patients with non-alcoholic steatohepatitis (NASH). Histological examination revealed that NASH liver was steatotic, and had increased infiltration of inflammatory cells whereas liver fibrosis was not identified. Anamnesis excluded alcohol intake, drugs and viral infections as cause for non-alcoholic fatty liver disease. Surgery was done because of hepatic metastases of extrahepatic tumors and only healthy tissue was used. Details of the study group are given in Table 1.

Monitoring of gene expression by real-time RT-PCR

Real-time PCR was performed as recently described (Bauer et al., 2011; Neumeier et al., 2007). The primers for CTGF were: CTGF uni: 5′-CTC CTG CAG CCT AGA GAA GAC GGC ACT TGA ACT C-3′ and CTGF rev: 5′-CGT CAG GGC ACT CAC TCC TGG AAA-3′. The primers for PPARα were: PPARαx uni: 5′-GCA CTG CAA CTG GAC AG-3′ and PPARαx rev: 5′-TCG CTT CAG AAC TCC TGG AAA-3′. The primers for α-SMA were: SMA uni: 5′-GCT TGG TAT TCC TTC TTC GTG AC-3′ and SMA rev: 5′-TGC CAG CAG ACT CTA CCA CCC CTT A-3′ and TGFβ1 uni: 5′-TTC CTT CTA GGC CCT TCT A-3′ and TGFβ1 rev: 5′-GGCCGACCTTGGACAGGATC-3′. The primers for Actin were: Actin uni: 5′-CTC CTG CAG GCT AGA GAA GC-3′ and Actin rev: 5′-GGCCACACCTGAGCAAGATC-3′. Adipor2 mRNA was amplified as described (Bauer et al., 2010).

SDS-PAGE and immunoblotting

Proteins (10–20 µg) were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Bio-Rad, Munich, Germany). Incubations with antibodies were performed in 1.5% BSA in PBS, 0.1% Tween. Detection of the immune complexes was carried out with the ECL Western blot detection system (Amersham Pharmacia, Deisenhofen, Germany).

Statistical analysis

Data are presented as box plots indicating median, lower and upper quartiles and range of the values. Statistical differences were analyzed by two-tailed Mann–Whitney U Test or paired Student’s t-test, and a value of p<0.05 was regarded as statistically significant. The Pearson’s correlation was calculated using the PASW statistics 17.0 program.

Results

Adiponectin lowers CTGF in primary human hepatocytes (PHH)

Recently we demonstrated downregulation of CTGF protein by adiponectin in primary human hepatocytes (PHH) of three different donors (Wanninger et al., 2011b). This result was confirmed using PHH of three additional patients (Figs. 1A, B). To find out whether CTGF mRNA levels are reduced by adiponectin real-time PCR was performed. CTGF mRNA was about 3-fold lower in PHH of four different donors incubated with adiponectin for 24 h (Fig. 1C).

Table 1

<table>
<thead>
<tr>
<th>Anthropometrical and biochemical characteristics of the study group used for analysis of hepatic mRNA expression.</th>
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<tr>
<td>Control</td>
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<tr>
<td>N (females)</td>
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<tr>
<td>Type 2 diabetes</td>
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<td>BMI (kg/m²)</td>
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| *Alanine aminotransferase, ALT; aspartate aminotransferase, AST; body mass index, BMI. Significant differences were only identified between controls and NASH patients and p-values are given in the table. |
The main downstream effectors of adiponectin are PPARα and AMPK (Yamauchi et al., 2007). Preincubation of cells with the PPARα antagonist RU486 blocked adiponectin mediated suppression of CTGF (Fig. 2A). Stimulation of PHH with the PPARα agonist fenofibrate (0.5 and 1 mM for 24 h) also reduced CTGF protein (Fig. 2B). Further, the highly specific PPARα agonist WY14643 dose-dependently reduced CTGF (Fig. 2C). Metformin at a concentration of 0.5 and 1 mM activated AMPK but did not affect CTGF (data not shown). These findings suggest that adiponectin reduces CTGF by a pathway involving PPARG.

Adiponectin lowers TGFβ3 mediated SMAD2/3 phosphorylation

Recently we have shown that adiponectin impairs TGFβ3-mediated upregulation of CTGF (Wanninger et al., 2011b). To evaluate whether adiponectin may directly interfere with TGFβ3 signaling, the effect of adiponectin on the phosphorylation of the TGRβ downstream signaling molecules SMAD2 and SMAD3 was analyzed. PHH were incubated with 10 μg/ml adiponectin for 1 h prior to TGFβ3 stimulation. Adiponectin had no effect on the basal expression of SMAD2 or -3 or their phosphorylated forms (Figs. 3A–D). Phosphorylation of SMAD2 and SMAD3 was enhanced upon TGFβ3 incubation and this effect was markedly diminished by adiponectin (Figs. 3A–D). WY14643 (50 μM) preincubation also reduced TGFβ3-induced SMAD2 and SMAD3 phosphorylation (Figs. 3E, F).

CTGF, TGFβ3, AdipoR2 and PPARG mRNA in human fatty liver and NASH

Liver tissue was obtained of patients without hepatic steatosis and of patients with steatosis with no histological signs of inflammation or fibrosis. NASH livers were steatotic and inflamed but not fibrotic.

CTGF mRNA was significantly higher in NASH compared to control liver compared to control tissues (Fig. 4C). CTGF mRNA was similarly expressed in all the livers analyzed (Fig. 4D). TGFβ3 and αSMA mRNA levels were also determined. Expression of αSMA was similar in all of the livers in accordance with the absence of fibrosis. TGFβ3, however, was significantly higher in NASH (Figs. 4E, F).

The mRNA levels of all of these genes did not correlate with age, BMI, AST and ALT levels nor with each other (data not shown).

Discussion

CTGF in the liver is mainly synthesized by hepatocytes and is strongly induced in liver fibrosis (Gressner and Gressner, 2008; Tong et al., 2009). Hepatocyte produced CTGF is upregulated by TGFβ3 and accelerates fibrogenesis partly by enhancing TGFβ3 activity (Gressner et al., 2007). Adiponectin impairs TGFβ3-mediated upregulation of CTGF suggesting that adiponectin interferes with TGFβ3 signaling.

Binding of TGFβ3 to the TGFβ receptors stimulates phosphorylation of SMAD2 and SMAD3. Precubination with adiponectin or a specific PPARα agonist reduces SMAD2 and SMAD3 phosphorylation indicating impairment of early signaling events. PPARG is activated upon adiponectin mediated activation of AdipoR2 making it likely that this receptor is involved.

Recently we have shown that adiponectin upregulates the TGFβ3 decoy receptor BMP and activin-membrane-bound inhibitor (BAMBI). However, BAMBI only blocks TGFβ3-mediated phosphorylation of SMAD2 (Wanninger et al., 2011b) arguing against enhanced BAMBI expression as the only mechanism responsible for impaired TGFβ3 signaling.

Current data also demonstrate that adiponectin and PPARα agonists reduce basal CTGF levels in primary human hepatocytes. A PPARα antagonist blocks adiponectin mediated suppression of CTGF suggesting that AdipoR2 is also involved herein. Systemic adiponectin is reduced in human fatty liver disease and is further diminished in NASH patients (Bugianesi et al., 2005; Pagano et al., 2005; Schaffer et al., 2005; Vuppalanchi et al., 2005). CTGF mRNA, however, is not increased in hepatic steatosis. This might indicate that the in-vitro results described herein are not relevant in-vitro. Nevertheless, CTGF is regulated by various factors like TNF, IL-6 and glucose which are affected in non-alcoholic fatty liver disease (NAFLD) (Gressner and Gressner, 2008; Gressner et al., 2009) suggesting that liver CTGF levels result from the concerted activity of multiple regulatory pathways. Unfortunately, respective sera of the patients have not been collected, and therefore, it could not be calculated whether systemic adiponectin shows a correlation with hepatic CTGF.

Liver CTGF mRNA is significantly increased in NASH liver. CTGF is well known to be induced in fibrotic liver tissue (Hayashi et al., 2002; Paradis et al., 1999; Paradis et al., 2001). However, histological analysis of liver tissues excluded fibrosis and α-SMA mRNA which is typically induced in fibrotic liver is not upregulated in the NASH livers studied herein. Beside CTGF, TGFβ3 is also higher in NASH livers. These findings suggest that CTGF and TGFβ3 mRNA levels are already increased in the inflamed steatotic liver and may render the liver more vulnerable to fibrosis (Schaffer et al., 2005; Tilg and Moschen, 2010). Whereas enhanced CTGF expression may at least in part be related to low systemic adiponectin, TGFβ3 synthesis is not suppressed by this adipokine in primary human hepatocytes (Wanninger et al., 2011a) and monocytes (own unpublished data). Whether increased TGFβ3 mRNA levels are a secondary result of higher CTGF levels has to be investigated in future studies.

Current data indicate that adiponectin lowers CTGF by a pathway involving AdipoR2 and PPARG. Data on AdipoR2 mRNA expression in steatotic and NASH liver reported so far are inconsistent. AdipoR2 mRNA is found unchanged, reduced or increased in NASH liver whereas its levels are not affected in simple steatosis (Kaser et al.,...
In human NASH liver AdipoR2 protein is found reduced in hepatocytes (Kaser et al., 2010; Nannipieri et al., 2009; Uribe et al., 2008) and AdipoR2 mRNA is significantly lower in the NASH livers analyzed herein. These data suggest that low systemic adiponectin and reduced AdipoR2 may contribute to higher CTGF levels in NASH. PPAR\(\alpha\) mRNA is similarly expressed in the liver samples analyzed in the current study but levels have also been found increased and decreased in NAFLD (Kohjima et al., 2007; Mitsuyoshi et al., 2009). So far it is unclear why such controversy exists regarding PPAR\(\alpha\) and AdipoR2 mRNA levels in NAFLD. It may be related to the grade of liver inflammation and fibrosis, extremely high BMI of some of the cohorts studied or ethnic differences of the cohorts analyzed so far. PPAR\(\alpha\), AdipoR2, TGF\(\beta\), \(\alpha\)-SMA and CTGF mRNA levels do not correlate with each other arguing against common mechanisms regulating the expression of these genes. Further, hepatic mRNA expression is not associated with levels of liver enzymes measured in serum or BMI of the donors. Liver enzyme concentrations are not suitable to diagnose NASH (Gressner et al., 2006; Zhang et al., 2010) and are not consistently increased in the serum of NASH patients.

Serum CTGF in patients with chronic hepatitis C virus infection is a suitable marker for assessing liver fibrosis and may also be increased in the serum of NASH patients (Gressner et al., 2006; Zhang et al., 2010). However, at least hepatic CTGF is already higher in NASH patients without fibrosis and further studies have to show whether measurement of circulating CTGF is a valuable tool to identify liver fibrosis in these patients.

In summary the current study shows that adiponectin interferes with TGF\(\beta\) signaling by 1) reducing phosphorylation of SMAD2 and 2) by downregulating CTGF. So far there are no established therapeutic strategies for NASH and enhancement of adiponectin receptor signaling pathways or treatment with selective PPAR\(\alpha\) agonists may serve as a potential beneficial approach in the treatment of metabolic liver disease.

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**Fig. 3.** Adiponectin inhibits TGF\(\beta\) activity. (A) SMAD2 and P-SMAD2 in PHH preincubated with adiponectin for 1 h and subsequent incubation with TGF\(\beta\) (4 ng/ml) (B) SMAD3 and P-SMAD3 in PHH preincubated with adiponectin for 1 h and subsequent incubation with TGF\(\beta\) (4 ng/ml). (C) Quantification of the data of three independent experiments partly shown in A (arbitrary units, au). (D) Quantification of the data of three independent experiments partly shown in B (arbitrary units, au). (E) SMAD2 and P-SMAD2 in PHH preincubated with WY14643 (150 \(\mu\)M) for 1 h and subsequent incubation with TGF\(\beta\) (4 ng/ml) (F) SMAD3 and P-SMAD3 in PHH preincubated with WY14643 (150 \(\mu\)M) for 1 h and subsequent incubation with TGF\(\beta\) (4 ng/ml).

**Fig. 4.** CTGF mRNA is increased in non-fibrotic NASH. (A) CTGF mRNA in healthy liver (Con), fatty liver (FL) and NASH liver. (B) CTGF in PHH incubated with 0.3 mM palmitic acid (PA) or oleic acid (OA) for 24 h. (C) AdipoR2 mRNA (D) PPAR\(\alpha\) mRNA (E) \(\alpha\)-SMA mRNA and (F) TGF\(\beta\) mRNA in healthy liver (control), fatty liver (FL) and NASH liver.
Conflict of interest statement

The authors declare that there are no conflicts of interest.

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References

Bauer, S., et al., 2010. Low-abundant adiponectin receptors in visceral adipose tissue of humans and rats are further reduced in diabetic animals. Archives of Medical Research 41, 75–82.


Bugianesi, E., et al., 2005. Plasma adiponectin in nonalcoholic fatty liver is related to hepatic insulin resistance and hepatic fat content, not to liver disease severity. The Journal of Clinical Endocrinology and Metabolism 90, 3498–3504.


Wanninger, J., et al., 2011c. MMP-9 activity is increased by adiponectin in primary human hepatocytes but even negatively correlates with serum adiponectin in a rodent model of non-alcoholic steatohepatitis. Exp. Mol. Pathol. 91, 603–607.

