PPAR-alpha agonist treatment increases trefoil factor family-3 expression and attenuates apoptosis in the liver tissue of bile duct-ligated rats

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Background/aims: Peroxisome proliferators-activated receptor alpha activation modulates cholesterol metabolism and suppresses bile acid synthesis. The trefoil factor family comprises mucin-associated proteins that increase the viscosity of mucus and help protect epithelial linings from insults. We evaluated the effect of short-term administration of fenofibrate, a peroxisome proliferators-activated receptor alpha agonist, on trefoil factor family-3 expression, degree of apoptosis, generation of free radicals, and levels of proinflammatory cytokines in the liver tissue of bile duct-ligated rats. Materials and Methods: Forty male Wistar rats were randomly divided into four groups: 1 = sham operated, 2 = bile duct ligation, 3 = bile duct-ligated + vehicle (gum Arabic), and 4 = bile duct-ligated + fenofibrate (100 mg/kg/day). All rats were sacrificed on the 7th day after obtaining blood samples and liver tissue. Liver function tests, tumor necrosis factor-alpha and interleukin 1 beta serum levels, degree of apoptosis (TUNEL) and tissue malondialdehyde (malondialdehyde, end-product of lipid peroxidation by reactive oxygen species) in liver tissue were evaluated. Results: Fenofibrate administration significantly reduced serum total bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, and tumor necrosis factor-alpha and interleukin-1p levels. Apoptosis and malondialdehyde were significantly reduced in the fenofibrate group. Trefoil factor family-3 expression increased with fenofibrate treatment in bile duct-ligated rats. Conclusions: The peroxisome proliferators-activated receptor alpha agonist fenofibrate significantly increased trefoil factor family-3 expression and decreased apoptosis and lipid peroxidation in the liver and attenuated serum levels of proinflammatory cytokines in bile duct-ligated rats. Further studies are needed to determine the protective role of fenofibrate in human cholestatic disorders.

Key words: PPAR-alpha, trefoil factor family, bile duct-ligated rats, cholestasis

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Materiell-metod: Dört gruba ayrılan 40 adet Wistar rate, 1. gruba sham (ölçmesiz), 2. gruba safra yolu ligasyonu, 3. gruba safra yolu ligasyonu+ vehicle (gum Arabic), 4. gruba ise safra yolu ligasyonu+ fenofibrat (100 mg/kg/day) uygulandı. Tüm ratlar 7. gününde bukma örnekleri alınarak, safra organlarının işlev fonksiyonunu, serum total bilirubin, aspartat aminotransferaz, alanin aminotransferaz, alkalin fosfataz, gamma-glutamal transferaz ve proinflamatuar sitokin düzeylerini değerlendirilmiştir.

Bulgular: Fenofibrat, serum total bilirubin, aspartat aminotransferaz, alanin aminotransferaz, alkalin fosfataz, gamma-glutamal transferaz, malondialdehid ve proinflamatuar sitokin düzeylerini anlamlı olarak azalttı. Trefoil faktör aileşi-3 ekspresyonu ile apoptosis derecesi de anlamlı oranla azaldı. 

Sonuç: Peroxisom proliferator aktive reseptör-alfa agonisti (fenofibrat) öncelikle trefoil faktör aileşi-3 ekspresyonunu artırır. Ayrancı, fenofibrat, safra yolu ligasyonu uygulamasını ile oluşan proinflamatuar sitokin düzeylerini azaltarak, insandan kolesterolik hastalıklar için yeni bir öneri sunulmuştur.
INTRODUCTION

Trefoil factor family (TFF) domain peptides consist of three members that play a role in mucosal defense and repair (1-3). TFF peptides are synthesized and secreted by mucin-secreting epithelial cells lining the gastrointestinal tract. Three mammalian family members have been identified (1): TFF1 (pS2), TFF2 (spasmolytic polypeptide, SP) and TFF3 (intestinal trefoil factor, ITF). TFF peptides are expressed in several tissues of the human body, especially in the gastrointestinal tract, under normal circumstances. Recent compelling evidence has emerged from experimental and clinical studies indicating a pivotal role of TFFs in the oncogenic transformation, growth, and metastatic extension of common human solid tumors, including gastric cancer (4,5).

The biliary diseases all appear to have an inflammatory component affecting the epithelium, not dissimilar to changes seen in inflammatory bowel disease. The biliary system has many physiological and structural correlates with the luminal gastrointestinal tract, including contact of the mucosal surface with toxic substances, a common embryological ancestor (foregut endoderm), and it tends to be affected by related diseases (e.g. primary sclerosing cholangitis and inflammatory colitis). There is, therefore, a similar need for maintenance of mucosal integrity and cellular repair in the biliary epithelium, as in the luminal gastrointestinal tract (6). There are scarce data regarding the role of TFF in biliary tract normal physiology and pathology. Limited studies have shown that TFF1 is present in inflamed, non-neoplastic gallbladder mucosa and in carcinoma of the gallbladder and extrahepatic biliary tract (7). Another study showed that TFF3 expression is increased in graft-versus-host disease in the biliary epithelium after allogeneic hematopoietic cell transplantation (8).

Inflammation of the biliary epithelium can be controlled by nuclear receptors such as the glucocorticoid receptor (GR), FXR, or the peroxisome proliferator-activated receptors (PPARs) in various epithelial cells (9-11). PPAR-alpha (PPARα) activation inhibits the increase in tumor necrosis factor-alpha (TNF-α) expression triggered by lipopolysaccharide (LPS) (12). Activation of PPARα also results in the expression of the cholesterol extruder, ABCA1, while the accumulation of cholesterol in biliary epithelial cells (BEC) is known to elicit inflammation (13). These observations suggest that PPARα activation protects BEC from excessive inflammation.

In the literature, the effect of PPARα agonists on TFF3 expression has not been studied in the biliary epithelium. Thus, we aimed to study the effects of fenofibrate, a PPARα ligand, on the expression of biliary epithelium in bile duct-ligated (BDL) rats.

MATERIALS AND METHODS

Ethics and Animals

The study was performed on 40 male Wistar Albino rats weighing 230–270 g, and conducted following the experimental protocol approved by the Committee for Research and Animal Ethics of Gazi University. Animals were housed in stainless steel cages under controlled temperature and humidity, and with 12-hour dark/light cycles. All rats were allowed at least one week of adaptation to the laboratory before the experimental procedure began. They were allowed free access to a commercial standard chow and water ad libitum. Rats were randomly assigned to four experimental groups of 10 rats each as follows:

Group 1 (Sham, n=10): underwent a sham operation;
Group 2 (BDL, n=10): underwent common bile duct ligation (BDL);
Group 3 (BDL + vehicle, n=10): underwent BDL and administered gum arabic by gavage;
Group 4 (BDL + fenofibrate, n=10): underwent BDL and given fenofibrate by gavage.

Sham-operated rats served as a control group.

Operative Procedures

Each rat was weighed and anesthetized by intraperitoneal administration of 40 mg/kg ketamine (Ketalar®, Parke Davis, Eczacibasi, Istanbul, Turkey) and 5 mg/kg Xylocaine (Rompum®, Bayer AG, Leverkusen, Germany). The abdomen was shaved and disinfected with 10% povidone iodine. Following a midline incision, the common bile duct was exposed a double-ligature with 5-0 silk was performed, and the bile duct was sectioned between the ligatures. In the sham-operated animals, the common bile duct was freed from surrounding soft tissue without ligation and transaction. Abdominal incision was closed in layers with 4-0 Dexon and 2-0 nylon. Animals received standard rat chow during the experiment.
Preparation and Administration of Fenofibrate

Fenofibrate (Lipofen, Nobel Drug Industry, Istanbul) was dissolved in 3% aqueous sterile solution of gum Arabic. Fenofibrate solution was administered as a single dose of 100 mg/kg via gavage for the first to sixth postoperative days (14). The oral dose and duration of the gum arabic of group 3 was the same used in the treated groups.

Harvest of Tissue and Blood Samples

Seven days after the surgical procedures, the animals were anesthetized, and re-laparotomy was performed. After blood samples were drawn, the liver was carefully dissected out from its attachment, and totally excised. All rats were then sacrificed by hemorrhage. The blood samples, which were duplicates, were kept at -80°C for biochemical analyses. Liver tissue was fixed in 10% neutral phosphate-buffered formalin, and then embedded in paraffin wax.

Blood Biochemistry

The serum bilirubin level was determined with Cobas Bio (Hoffman La Roche, Basel, Switzerland) using direct bilirubin test (Hoffman La Roche, Basel, Switzerland). The serum activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT) was measured by using commercially available kits (Boehringer, Mannheim, Germany). Total bile acid (TBA) was determined using an analytical kit from Sigma, USA with a 747 automatic biochemistry analyzer.

Assessment of Apoptosis

Apoptosis in liver tissues was detected by measuring the appearance of apoptotic bodies with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay using the ApopTag peroxidase kit (Invitrogen, Carlsbad, CA, USA), and by pathological quantification of apoptotic foci. Approximately 3000 cells a average were counted per sample. This specific assay uses terminal deoxynucleotidyl transferase to attach biotinylated deoxyuridine triphosphate to free 3'-OH DNA ends. Liver tissue sections (5 μm) were prepared using a microtome and placed on glass slides. The sections were deparaffinized in xylene, dehydrated in ethanol, and then incubated with proteinase K 20 μm/ml in phosphate buffered saline (PBS) for 20 minutes (min) at room temperature. After rinsing the specimen twice with PBS, the sections were processed following the instructions of a commercial kit (Dead End Colorimetric Apoptosis Detection System; Promega, Madison, WI, USA). Sections were stained with streptavidin-horseradish peroxidase conjugate, and then counterstained with hematoxylin. The peroxidase-positive cells were identified morphometrically by brown staining nuclei. The numbers of TUNEL-positive cells were counted in 10 random microscopic fields (400 x).

Histopathology

Liver tissues were fixed in 40 mg/ml formaldehyde and embedded in paraffin. For histopathological evaluation, 4-μm slides were stained with hematoxylin-eosin, Masson’s trichrome, periodic acid-Schiff (PAS), and Hall’s stain for bile. Sections were scored by an independent observer blinded to the experimental protocol. The following lesions were scored according to Modified Histological Activity Index (16,17): portal inflammation, focal necrosis, confluent necrosis, interface hepatitis, and focal inflammation. The numbers of biliary canals in five portal sites for each section were also noted.

RNA Isolation and PCR Detection

Frozen liver specimens were used for real-time quantitative polymerase chain reaction (RT-PCR) analysis of TFF3 gene expression. Total RNA was isolated from approximately 100 mg of frozen liver tissue by using RNeasy Mini Kit (Qiagen) following the homogenization of the tissue. The RNA precipitate was dissolved in 75 L of RNase free water. Total RNA was used for the synthesis of single-strand complementary DNA (cDNA) by
using random hexamers and 100 units avian myeloblastosis virus reverse transcriptase (Q-Biogene, Irvine, CA, USA) for 60 min at 42°C in a final volume of 20 L. This cDNA pool was used as control for qualitative detection by real-time PCR.

**Real-Time PCR**

The quantification of TFF3 mRNA was determined by real-time PCR, using a Lightcycler Instrument (Roche Diagnostics, Mannheim, Germany). A previously described method using a SYBR green I dye was used after some modifications for real-time PCR (18). Briefly, Fast Start DNA Master SYBR Green mix (Roche Diagnostics) containing the hot start Taq DNA polymerase, 5 L cDNA, 0.5 M of each gene specific primer, TFF3-sense (12F) 5=-TGG TCC TGG CCT TGC TGT-3= and TFF3-antisense (79R) 5=-GGC ACA CTG GTT TGC AGA CA-3= and 3 mM magnesium chloride in a final volume of 20 L in glass capillaries were run in duplicate. The reaction mixture was preheated at 95°C for 10 min, followed by 40 cycles at 95°C for 15 seconds (s) and 60°C for 1 min. GAPDH as housekeeping gene was also amplified in each sample by using GAPDH-sense 5-AAG GTC GGA GTC AAC GGA TTT-3 and GAPDH-antisense 5-ACC AGA GTT AAA AGC AGC CCT G-3 primers as previously described to control the cDNA synthesis and PCR amplification (18).

**Statistics**

All results were analyzed and are given as the mean ± standard deviation (SD). Comparisons among multiple groups were performed using Kruskal-Wallis test. If there was a significant difference between groups, further paired comparisons were done using Mann-Whitney U test. Correlations between TFF3 and histopathological findings were calculated using Spearman correlation test. We divided the conventional significant p value (0.05) by the total number of groups (n=4) to find the true p value for this study. For this reason, the significant value of p in this study was accepted as below 0.0125 (0.05 divided by 4 = 0.0125).

**RESULTS**

No deaths were observed during the experiment. All animals with BDL were obviously jaundiced 3 days after the operation. The jaundice was confirmed by measuring the serum total bilirubin concentration on the 7th day after BDL (Table 1). BDL results in severe bile acid-induced liver injury. BDL is associated with intrahepatic bile acid overload and consequent liver injury.

**BDL-Induced Hepatopathology and Cholestasis**

Serum AST, ALT, GGT, ALP, and TBA levels were significantly elevated in all animals in which BDL was performed when compared to sham-operated rats, and the same parameters were considerably lower in the fenofibrate group than the other BDL groups (p<0.01). No significant difference was observed in the biochemical parameters between the control and BDL-vehicle groups. Similarly, although serum direct bilirubin levels were significantly elevated in all BDL animals compared with shams, it was lower after BDL in PPARα-activated rats than the other BDLs (p=0.01).

<table>
<thead>
<tr>
<th>Table 1. Change in concentrations of laboratory data (mean±SD)*</th>
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<tr>
<td><strong>Sham</strong></td>
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<tr>
<td>Bilirubin (mg/dl)</td>
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<td>ALT (IU/L)</td>
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<td>AST (IU/L)</td>
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<td>GGT (IU/L)</td>
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<td>IL-1β (pg/ml)</td>
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<td>Tissue MDA levels (nmol/mg protein)</td>
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<td>TFF-3 mRNA expression (n) %</td>
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</table>

*Comparisons between groups were done using Kruskal-Wallis test. Paired comparisons were done using Mann-Whitney U test.

*p<0.01 versus sham group (Mann-Whitney U after Kruskal-Wallis test)

*p<0.01 as compared to BDL groups (Mann-Whitney U after Kruskal-Wallis test)

*p<0.01 as compared to BDL + vehicle groups (Mann-Whitney U after Kruskal-Wallis test)

Proinflammatory Cytokines
Serum TNF-α and IL-1β levels in animals with BDL were significantly higher than in the sham group (p<0.01, Table 1). There were no significant differences in terms of the cytokines between the BDL and BDL-vehicle groups. Fenofibrate treatment significantly decreased the serum TNF-α and IL-1β levels in rats with BDL (p<0.01, Table 1).

Tissue MDA levels
The levels of MDA, which is the last product of oxidative injury, were significantly increased after BDL when compared with the sham group (p<0.001). Fenofibrate treatment decreased the MDA levels significantly when compared with BDL and BDL-vehicle groups (p=0.01, Table 1).

Histopathologic Examination
Histopathological examination showed that portal inflammation, interface hepatitis, lobar and confluent necrosis, and bile duct number in rats that underwent BDL were significantly higher than in sham-operated rats. Except the bile duct number, all histopathologic parameters in rats administered fenofibrate markedly decreased in comparison to control and BDL-vehicle rats. It was apparent that fenofibrate treatment caused a significant increase in number of bile ducts (p<0.01, Table 2, Figure 1). The reduction in hepatic necrosis and increase in the number of bile ducts suggest that fenofibrate has regenerative effects.

Apoptosis
Hepatocyte apoptosis in the animals with BDL was significantly higher than in the sham group (p<0.01). PPARα activation significantly decreased apoptosis in rats with BDL compared to control and BDL-vehicle groups (p<0.01).

Detection of TFF3 mRNA Using Real-Time PCR
Real-time PCR was performed on 40 frozen liver tissues in the four groups. The expression of GAPDH was detectable in all samples indicating that liver tissue samples contained undegraded mRNA.

TFF3 expression was not observed in the sham group. However, we detected TFF3 expression in 3 rats in the BDL group, 4 rats in the BDL+vehicle group and 8 rats in BDL+fenofibrate group (p<0.01, Table 1).

TFF3 expression was correlated with bile duct number/5 portal field (r=0.812, p=0.0014). However, TFF3 expression was not correlated with degree of portal inflammation, interface hepatitis, confluent necrosis, or lobar necrosis.

DISCUSSION
The most important factors for obstructive jaundice are accumulation of bilirubin, bile acids, and cholesterol in the liver (19). Bile acid synthesis has

![Figure 1. TUNEL-positive cells per field. Apoptosis in rats with BDL was significantly higher than in sham-operated rats (p<0.01, Kruskal-Wallis test). BDL: Bile duct ligation. BDL + F: BDL + fenofibrate. BDL + V: BDL + vehicle.](image-url)
been reported to increase after BDL in animals. Weis and Dietschy (20) showed that after acute bile duct obstruction, cholesterol synthesis increased five-fold. The contribution of epithelial cells to wound healing has been extensively studied for barriers such as the skin and colon (21). However, we are unaware of studies in the literature devoted to understanding these processes in the biliary tree, despite the fact that BEC must protect the underlying stroma and other liver cells from the extremely toxic actions of bile salts.

If the molecular mechanisms of BEC repair reactions were better understood, some biliary diseases, such as preservation injury-related strictures, might be avoided altogether if the donor could be pretreated to either prevent the damage and/or promote effective BEC repair (22). TFFs contain one or more trefoil motifs composed of six cysteine residues. These proteins influence the rheological properties of mucus gels and contribute to optimal protection of the intestinal mucosa from injury (23-26). Trefoil peptides also enhance intestinal epithelial restitution primarily by stimulating epithelial cell spread and migration, which accelerates mucosal epithelial recovery after injury (27). Each of the TFF proteins is differentially regulated in different parts of the gastrointestinal tract: TFF1 and TFF2 are expressed primarily in the stomach, and TFF3 predominates in the small and large intestines (28). This and previous studies have shown that TFF1 and TFF3 are constitutively expressed in the large bile ducts of mouse and human livers (29).

Inflammation can be controlled by nuclear receptors such as the GR, FXR or the PPARs in various epithelial cells (30). Recently, a phosphatidylcholine (PC), 16:0-18:1 PC, was identified as the endogenous ligand of PPARα (31). PPARα activation inhibits the increase in TNF-α expression triggered by LPS (12). Activation of PPARα also results in the expression of the cholesterol extruder, ABCA1, while the accumulation of cholesterol in BEC is known to elicit inflammation (13). These observations suggest that PPARα activation protects BEC from excessive inflammation.

In our study, administration of fenofibrate, a PPARα agonist, attenuated biliary damage and increased the expression of TFF3 in the liver tissue of BDL rats. We also noted that pro-inflammatory markers and free radical end products were also decreased in the fenofibrate group. These findings support the previous data regarding the anti-inflammatory effects of PPARα agonists in biliary cells.

Interestingly, the expression of TFF3, which is a marker of biliary repair process, is increased with administration of the PPARα agonist. This is the first time the positive effect of PPARα agonists on the biliary repair process has been shown in the literature.

TFF3 expression was also correlated with bile duct number/5 portal fields in the histological examination. This finding is in accordance with previous data about TFF3, which has a role in the biliary epithelial repair process. However, we did not detect any correlation between TFF3 expression and the other histological findings (e.g. portal inflammation, necrosis...). This might be partly explained by the fact that TFF3 is not involved in inflammation but synthesis of the BEC. These findings should be investigated in further specific trials.

In summary, we found that the PPARα agonist (fenofibrate) attenuated biliary epithelial damage via decreasing inflammation and stimulating TFF3 protein expression and the biliary repair process in BDL rats. These data might be beneficial for further human trials in cholestatic liver diseases.

REFERENCES


