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Thromboxane inhibitors attenuate inflammatory and fibrotic changes in rat liver despite continued ethanol administrations

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Abstract

Background—Thromboxane levels are increased in rats fed ethanol whereas thromboxane inhibitors reduce alcoholic liver injury. The aim of this study is to determine whether thromboxane inhibitors could attenuate already established alcoholic liver injury.

Methods—Rats were fed ethanol and liquid diet for six weeks by intragastric infusion to induce liver injury after which ethanol was continued for two more weeks and the rats were treated with either a thromboxane synthase inhibitor (TXSI) or a thromboxane receptor antagonist (TXRA). Liver pathology, lipid peroxidation, nuclear factor-kappa-B (NF- κ B) activity, tumor necrosis factor- α (TNF- α), cyclooxygenase-2 (COX-2), and transforming growth factor-beta1 (TGF- β ₁) were evaluated.

Results—Administration of fish oil and ethanol caused fatty liver, necrosis, inflammation and fibrosis accompanied by increased in lipid peroxidation, NF- κ B activity and expression of TNF- α , COX-2 and TGF- β ₁. Treatment with the thromboxane inhibitors ameliorated a certain level of the pathological and biochemical abnormalities. In particular, TXSI in addition to reducing necrosis, inflammation and fibrosis also decrease the severity of fatty liver.

Conclusion—Thromboxane inhibitors attenuated the alcoholic liver injury, inflammation and fibrotic changes despite continued ethanol administration. Inhibition of the production of thromboxane by thromboxane inhibitor and receptor antagonists may be a useful treatment strategy in clinical alcoholic liver disease.

Keywords

Thromboxane Inhibitor; Alcoholic Liver Disease; Inflammation; Fibrosis; NF- κ B

INTRODUCTION

Alcoholic liver disease is one of the major causes of morbidity and mortality around the world. In 2004, 3.8% of global deaths were related to the consumption of alcohol (Rehm et al., 2009). In terms of liver diseases, long-term consumption of overdose alcohol (20 g/d) may induce steatohepatitis, alcoholic fatty liver, cirrhosis, and in some cases, hepatocellular carcinoma (Mann et al., 2003). After the alcohol intake, two enzyme systems in the liver, cytochrome P450 2E1 (CYP2E1) and alcohol dehydrogenase (ADH) are activated to oxidize alcohol to acetaldehyde (CH₃CHO), and then it is metabolized to CH₃COO[−] in the

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mitochondria (Beier and McClain, 2010). During the metabolizing processes of alcohol in the liver, reactive oxygen species (ROS) are produced from hepatocytes, inflammatory cells, and non-parenchymal cells to favor the oxidative stress in the liver through CYP2E1 and NADPH oxidases (Mandrekar and Szabo, 2009). Increased oxidative stress not only attacks organelles in the cells (e.g. mitochondria) which leads to membrane damage and lipid peroxidation, but also induces the initiation of inflammation and fibrosis (Cunningham and Bailey, 2001). Kupffer cells, the resident macrophages within the liver, are the main source of pro-inflammatory cytokines (e.g. TNF- α , IL-1 β , and IL-6) and cyclooxygenases (e.g. COX-2) during alcoholic fatty liver through the activation of transcription factor NF- κ B. Local inflammation and subsequent recruitment of neutrophils and lymphocytes triggers the activation of cell death cascades and the production of pro-fibrotic factors and ROS, which further exacerbate fatty liver (Mandrekar and Szabo, 2009; McVicker et al., 2007). It has also been shown that, in hepatocytes, chronic consumption of ethanol induces the production of transforming growth factor-alpha and beta1 (TGF- α and TGF- β 1), which regulate the collagen synthesis by hepatic stellate cells (Cao et al., 2002; Kato et al., 2003).

Thromboxane is a vasoconstrictive eicosanoid synthesized from arachidonic acid metabolism by COX or lipoxygenase. It induces hepatic injury through vasoconstriction, platelet aggregation and induction of pro-inflammatory cytokine during alcoholic fatty liver development (Yokoyama et al., 2005). Treatment of thromboxane synthase inhibitor (TXSI) and thromboxane receptor antagonist (TXRA) prior to alcoholic fatty liver induction ameliorated fatty liver injury through reduction of the expression of TNF- α and TGF- β 1 in rat (Nanji et al., 1997a). However, whether those inhibitors are effective in ameliorating alcoholic fatty liver after the alcoholic fatty liver injury development is unknown. In the current study, we reported that TXSI and TXRA can ameliorate a certain level of already established alcoholic liver injury in a rat model.

MATERIALS AND METHODS

Animal Model and Treatment Groups

Male Wistar rats weighing between 225 and 250 g were fed a liquid diet by continuous infusion through permanently implanted gastric tube as described previously (Tsukamoto et al., 1986). The rats were given their total nutrient intake by intragastric infusion. The percentage of calories derived from fat was 35% of total calories. Vitamins and minerals were given as described previously (Nanji et al., 1997a). The amount of ethanol was modified to maintain high levels of blood ethanol (150 to 300 mg/dL) throughout the day. The amount of ethanol was initially 10 g/kg/d, and was increased up to 16 g/kg/d as tolerance developed. Each ethanol-fed rat had at least two measurements of blood alcohol taken.

Five groups of rats (6 rats per group) were studied to evaluate the effects of thromboxane inhibitors on pathological and biochemical changes. Rats in group 1 were fed a fish oil-ethanol diet for eight weeks before killing. Rats in group 2 were fed the same fish oil-ethanol diet for six weeks. Rats in group 3 and 4, after six weeks on the fish oil-ethanol diet, were administered with TXSI (group 3) or with TXRA (group 4) and continued on the same fish oil-ethanol diet for two more weeks before killing. The vehicle solution for TXSI and TXRA is normal saline (0.9% NaCl). Prior to administration of TXSI or TXRA, a liver biopsy was performed. The rats were anesthetized with 2% isoflurane as previously described (Reinke et al., 2000). In preliminary experiments, we showed that a liver biopsy performed at six weeks had no effect on pathological changes observed after 8 weeks of fish oil-ethanol diet administration (unpublished data). Rats in group 5 received fish oil-dextrose for 8 weeks. In addition, three rats fed fish oil-dextrose for 8 weeks were treated with TXSI and TXRA to determine whether these drugs induced pathological changes in the liver.

Furthermore, to test the effect of the vehicle for TXSI and TXRA drugs on liver pathology, three rats fed fish oil and ethanol for 8 weeks were also administered, via intragastric tube, the vehicle used for the TXSI and TXRA.

The specific TXRA used in our study was ifetroban sodium (Bristol Myers Squibb, Princeton, NJ). Ifetroban sodium is a long-acting orally active TXRA that produces potent and long lasting antagonism of thromboxane-receptor dependent responses (Ogletree et al., 1993). The drug was administered daily as a bolus via intragastric tube at a dose of 3 mg/kg. At the dose administered, intestinal absorption of the drug is over 85% and it retains antagonism of TP-receptor-dependent responses for at least 24 hours after oral administration (Ogletree et al., 1993; Rosenfeld et al., 2001). Ifetroban is devoid of any agonistic activity. The TXSI (CGS 12970; Novartis, Summit, NJ) was administered at a dose of 10 mg/kg/d as a bolus through the intragastric tube. CGS 12970 is a long acting inhibitor of thromboxane production *in vivo* and has no effect on cyclooxygenase or lipooxygenase (Ambler et al., 1985).

All rats were treated according to the guidelines and care on the use of laboratory animals established by the National Institutes of Health.

Histopathological Analysis Including Sirius Red Staining for Collagen

A small sample of liver was obtained by biopsy or at death and fixed in formalin. Hematoxylin-eosin and Sirius Red stain were used for light microscopy. The severity of liver pathology was assessed as follows: steatosis (the percentage of liver cells containing fat), 1+, 25% of cells containing fat; 2+, 26%–50%; 3+, 51%–75%; 4+, 75%. Necrosis was evaluated as the number of necrotic foci per square millimeter; inflammation was scored as the number of inflammatory cells per square millimeter. At least three different sections were examined per sample of liver. The pathologist evaluating these sections was unaware of the treatment that rats had received.

For evaluation of fibrosis around the central veins, sections were stained with Sirius red and analyzed using ImageJ software (NIH, MD). The cross-sectional area of the central vein lumen was measured using the same technique. The area of collagen deposition was divided by the area of the central vein lumen to correct for the size of the lumen and provide a standardized measurement of peri-central vein collagen deposition. The coefficient of variation of parameters measured was determined by assessment of a single central vein on six occasions (<5%). Pericellular fibrosis was estimated as the number of positively staining sites on adjacent hepatocyte surfaces per 100 hepatocytes around the central vein.

Measurement of Blood Alcohol and Serum Alanine Aminotransferase (ALT)

Rat blood was collected from the tail vein, and ethanol concentration was measured using an alcohol dehydrogenase kit from Sigma-Aldrich (St. Louis, MO). ALT was measured using an automated analyzer (Boehringer Mannheim Hitachi 747, Indianapolis, IN).

Measurements of Conjugated Dienes, Thiobarbituric Acid Reacting Substances (TBARS), 8-Isoprostane and 4-Nitrophenol Hydroxylase

Lipid was extracted according to the method of Bligh and Dyer (Bligh and Dyer, 1959) and conjugated dienes were measured by the method of Recknagel and Glende (Recknagel and Glende, 1984). TBARS and 4-nitrophenol hydroxylase were measured as previously described (Nanji et al., 1997a). 8-isoprostane in plasma was measured using an immunoassay kit (Cayman chemical, Ann Arbor, MI). The blood sample was obtained from the aorta and immediately centrifuged, and the plasma was stored at –70°C until analysis. 8-

isoprostane levels in plasma have been previously shown to correlate well with liver conjugated diene levels in dextrose- and ethanol-fed rats (Nanji et al., 1994).

Measurement of Plasma Endotoxin Levels

Blood samples were collected in endotoxin-free vials (Sigma-Aldrich) and centrifuged at 400 *xg* for 15 min at 4°C. Samples were then diluted 1:10 in pyrogen-free water and heated to 75°C for 30 min to remove inhibitors of endotoxin from plasma. The *Limulus* Amoebocyte Lysate Test (Kinetic-QLC; Whittaker Bioproducts, Walkersville, MD) was used for endotoxin measurements. Samples were incubated at 37°C for 10 min with *Limulus* amoebocyte lysate. The substrate solution was added and incubated for 20 min. The reaction was stopped by adding 25% acetic acid and the samples were read spectrophotometrically at 410 nm.

TGF- β_1 Assay in Plasma

The levels of TGF- β_1 in plasma were measured using a radioimmunoassay kit method (NEN Research Products, Boston, MA). In this assay, the TGF- β_1 in the sample was converted to the activated form using an acidification-neutralizing process. The concentration of TGF- β_1 was determined from a standard curve prepared by using serial dilutions of the standard obtained from the manufacturer.

Determination of NF- κ B and I κ B α

In this assay, the fractionation of the liver tissues and the measurement of NF- κ B activation were done as previously described (Liu et al., 1995).

The specificity of binding was determined by prior addition of 100-fold excess of unlabeled competitor consensus oligonucleotide. Supershift experiments were performed on 5% non-denaturing gels using antiserum directed against the p50 subunit of NF- κ B (Santa Cruz Biotechnology, Santa Cruz, CA). Densitometric analysis of NF- κ B activation was performed using laser scanning densitometry by a Molecular Dynamics Densitometer and Image Quant Software (Molecular Dynamics, Sunnyvale, CA). Western blot analysis for I κ B α was conducted using 50 μ g of cytosolic protein. Samples were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel and incubated with the primary antibody against I κ B α (Santa Cruz Biotechnology) at a dilution of 1:500. Membranes were incubated with a anti-rabbit secondary antibody. Antibody-reactive bands were visualized by the use of an enhanced chemiluminescence assay using reagent from NEN Life Science Products.

RNA Extraction from Liver Tissues and RT-PCR Analysis of COX-1, COX-2, TGF- β_1 , TNF- α , and β -actin

To examine the expression of COX-1, COX-2, TGF- β_1 , and TNF- α in liver tissue, total RNA was isolated by using illustraTM RNeasy spin mini kit (GE healthcare, UK). The integrity of RNA was assessed by agarose gel electrophoresis and ethidium bromide (EB) staining. The sequences of primer pairs for target genes have been previously published (Nanji et al., 1997a). Amplification was performed in an automated thermal cycler at 94°C for 60 sec, 50°C for 90 sec, and 72°C for 120 sec for 35 cycles, followed by an extension at 72°C for 10 min. All amplification reactions were performed in parallel in the same heating block to ensure compatible conditions. To normalize signals from the different RNA samples, we amplified 2 μ l of the same reverse-transcriptase reaction with β -actin-specific primers. PCR products and molecular weight markers were subjected to electrophoresis on 1% agarose gels and visualized by means of EB staining. The bands on gels were analyzed by ImageJ software.

Statistical Analysis

All data are expressed as means $\pm$ SD unless otherwise indicated. Differences between groups were analyzed using analysis of variance (ANOVA) followed by post-hoc multiple comparison according to the Student-Newman-Keuls method. Statistical significance was set at $p < 0.05$.

RESULTS

Effects of Thromboxane Inhibitors on Liver Pathology

There was no significant difference in weight gain before and after switching to diets containing fish oil and ethanol and the thromboxane inhibitors (Table 1). Although the weight gain tended to be lower in the 6 to 8 week period in the ethanol-treated animals when compared to the thromboxane inhibitor-treated groups, the difference was not significant. Also there was no difference in weight gain in the ethanol-treated groups when compared to the fish oil-dextrose treated rats. Furthermore, the weight gain in the 6 week and 6 to 8 week period in the rats fed with thromboxane-inhibitors and fish oil-dextrose was similar to the weight gain in other groups (data not shown). Blood alcohol levels ranged between 150 and 400 mg/dL were similar among the groups (Table 1).

Feeding a fish oil-ethanol diet for 6 (group 2) or 8 weeks (group 1) caused fatty liver, necrosis and inflammation (Table 2). Of note is that the severity of pathological changes in the fish oil-ethanol groups (3 and 4) prior to treatment was not different from the severity of pathological changes in group 1 and 2. Control animals fed with fish oil-dextrose diet (group 5) showed no pathological changes. Significant improvement in liver pathology occurred when the animals were administered with thromboxane inhibitors (group 3 and 4). Necrosis and inflammation was significantly reduced in these two groups. However, fatty liver was decreased only in the TXSI group (group 3; Table 2).

In the histological aspect, chronic administration of ethanol (6 weeks or 8 weeks) induced obvious fatty droplet accumulation and cellular necrosis in the liver. However, following treatment of TXSI or TXRA effectively reduced those hepatic injuries (Fig. 1).

In addition to the above changes, the administration of the thromboxane inhibitors affected the amount of collagen and extent of pericellular fibrosis (Fig. 2). There was about a 40–50% decrease in the amount of central vein collagen and pericellular fibrosis in both thromboxane-inhibitor-treated groups. In the TXSI-treated group, the amount of central vein collagen (% area) decreased from $1.4 \pm 0.2\%$ to $0.7 \pm 0.2\%$ (**: $p < 0.01$). Similarly, in the TXRA-treated group, collagen decreased from $1.3 \pm 0.2\%$ to $0.7 \pm 0.1\%$ (**: $p < 0.01$). Pericellular fibrosis was also significantly reduced in both treated groups. In the TXSI-treated group, pericellular fibrosis decreased from $18 \pm 6\%$ to $8 \pm 2\%$ (*: $p < 0.02$) and in the TXRA-treated group, from $20 \pm 4\%$ to $9 \pm 3\%$ (*: $p < 0.02$). The amount of collagen and pericellular fibrosis was not significantly different in the biopsies obtained after 6 weeks in comparison to the values seen in animals fed with fish oil and ethanol and killed at 6 and 8 weeks (group 1 and 2). The amount of collagen in rats killed at 6 weeks was $1.3 \pm 0.3\%$ and $1.5 \pm 0.3\%$ at 8 weeks. Pericellular fibrosis was $19 \pm 4\%$ at 6 weeks and $22 \pm 5\%$ at 8 weeks. (n = 6 per group).

Rats fed fish oil-dextrose and the TXSI and TXRA for 8 weeks showed no pathological changes. Rats fed with fish oil and ethanol for 6 weeks and then treated with the vehicle and ethanol for two weeks had pathological changes that were similar to rats that were continuously with fed fish oil and ethanol for 8 weeks (group 1) (data not shown).

Modulation of Endotoxemia and Lipid Peroxidation by Thromboxane Inhibitors

The effect of thromboxane inhibitors on the known mediators of alcoholic liver injury e.g. endotoxin and lipid peroxidation was evaluated in the rats at the completion of the administration of experimental diets. Concentrations of endotoxin were not affected by the thromboxane inhibitors. Levels of conjugated dienes, TBARS and 8-isoprostane were decreased in the rats administered with TXSI but not TXRA (Table 3). Part of the decrease in the degree of lipid peroxidation can be explained by the decrease in 4-nitrophenol hydroxylase hydroxylation (which is reflective of CYP2E1 activity) seen in the TXSI group but not in animals receiving TXRA (Table 3).

Effect of Thromboxane Inhibitors on Activation of NF- κ B

To evaluate the activation of NF- κ B electrophoretic mobility shift assays of nuclear extracts from whole liver were carried out. Nuclear localization of NF- κ B was increased in the fish oil-ethanol fed groups (Fig. 3). Activation of NF- κ B was reduced in both the thromboxane-inhibitor-treated groups when compared to the fish oil-ethanol fed groups (Fig. 3).

To determine whether the activation of NF- κ B might be accompanied by degradation of I κ B α , protein expression of I κ B α was measured by Western blot. A marked decrease or absence of I κ B α protein was observed in the fish oil-ethanol groups (Fig. 3). The levels of I κ B α in the thromboxane inhibitor groups were lower than those in the fish oil-dextrose group ($p < 0.05$) but higher than those in the fish oil-ethanol group, which is consistent with the degree of NF- κ B activation in these groups.

The protein/DNA complex was further characterized by using competition and supershift assay. A 100-fold excess of non-radioactive NF- κ B oligonucleotide completely abrogated the complex formation; addition of STAT-3 oligonucleotide had no effect. Antibody against p50 was used for supershift assays to demonstrate the specificity of NF- κ B complex as previously described (data not shown) (Nanji et al., 1999).

Effect of Thromboxane Inhibitors on COX-2 and TNF- α

As mentioned earlier, we have proposed that lipid peroxidation and endotoxin activate NF- κ B which leads to induction of COX-2 and TNF- α (Nanji et al., 1997a). Thus we also measured the effects of the thromboxane inhibitors on the mRNA expression for COX-1, COX-2, and TNF- α by RT-PCR. Administration of the thromboxane inhibitors led to an approximately 50% decrease in the levels of TNF- α and COX-2 mRNAs (Figs. 4). There was no significant difference of COX-1 mRNA expression among all groups.

Effect of Thromboxane Inhibitors on TGF- β_1 mRNA and TGF- β_1 Levels in Plasma

Fish oil-ethanol treatment significantly increased both mRNA and plasma levels of TGF- β_1 . Treatment of thromboxane inhibitors after the development of liver injury led to a significant decrease in mRNA level and plasma concentration of TGF- β_1 (Fig. 5).

DISCUSSION

A major problem in the treatment of alcoholic liver disease remains the adherence of patients to abstinence from alcohol. Many studies have been carried out to determine the effectiveness of therapy in reducing the progression of liver injury in alcoholics (Beier and McClain, 2010). The experimental model used in the present study has striking similarities to the clinical setting in which alcoholic liver disease occurs. The rats in the current study had alcoholic liver injury before the application of a therapeutic regimen. The data showed that administration of thromboxane inhibitors reduced the indices of necrosis, inflammation and fibrosis even though alcohol administration was continued. Of interest is that the

reduction in the degree of fatty liver occurs only in the TXSI-treated group but not in the TXRA-treated group. The improvement in the severity of fatty liver in the TXSI-treated group may be due, in part, to the inhibition of CYP2E1. The study by Morimoto et al. showed that CYP2E1 is important in the pathogenesis of fatty liver in ethanol-treated rats (Morimoto et al., 1995). In the present study, a decrease in CYP2E1 protein expression was seen in association with a reduction in the degree of fatty liver in the TXSI-treated group. The effect of TXSI on CYP2E1 may be due to the fact that thromboxane synthase, being a member of the cytochrome P450 family, may cause some degrees of inhibition in CYP2E1 activity in addition to thromboxane synthase. This hypothesis clearly needs to be experimentally verified.

Other causes of fatty liver after alcohol administration include increased hepatic synthesis of fatty acids, decreased transport of triglycerides from the liver and disordered mitochondrial beta-oxidation (Lieber, 2004). The effect of thromboxane synthase inhibition on these metabolic processes is not known but may be relevant to the reduction in fatty liver in the TXSI-treated group.

It is well accepted that endotoxin and lipid peroxides are hepatotoxic and levels of both increase in alcohol-induced liver injury (Thurman, 1998). We did not see any effect of the thromboxane inhibitors on levels of endotoxin. Also, levels of lipid peroxides were not affected in the TXRA-treated group but were reduced in the TXSI-treated group. As pointed out previously, this effect could, in part, be ascribed to the inhibition of CYP2E1 activity by the TXSI. One possible explanation for the observed reduction in severity of necrosis, inflammation and fibrosis in the thromboxane inhibitor-treated group is the reduction in the levels or downstream effects of the oxidant molecules, the isoprostanes. Isoprostanes are free radical-catalyzed products of unsaturated fatty acids and their appearance in biological fluids reflects oxidant stress *in vivo* (Meagher et al., 1999). The mechanism(s) of action of isoprostanes and their biological effects have not been completely worked out; however, some isoprostanes act as ligands for prostanoid receptors such as the thromboxane receptors (Morrow, 2006). Part of the effect of activation of thromboxane receptor is the generation of the necro-inflammatory responses by up-regulation of TNF- α production, promoting leukocyte adhesion, and causing hepatocyte injury (Yokoyama et al., 2005). The reduction in the degree of necrosis and inflammation by inhibition of the production of isoprostanes (by TXSI) or the action of isoprostanes at the level of the thromboxane receptor (by TXRA) is consistent with the hypothesis that isoprostanes may mediate the inflammatory response through prostanoid receptors. However, specific drugs targeted to the isoprostane receptor would be required to confirm this hypothesis.

In addition to provoking a necro-inflammatory response, activation of thromboxane receptors may also contribute to the long-term effects of ethanol such as fibrosis. In the kidney, for example, thromboxane promotes extracellular matrix formation at sites of inflammation (Kohan, 1992). The mechanism involved in enhanced matrix synthesis is believed to be due to increased synthesis of TGF- β_1 (Negrete et al., 1995). The observation that the thromboxane inhibitors reduce the hepatic expression of TGF- β_1 and the degree of fibrosis provides a link between activation of thromboxane receptors and fibrosis. Although the effect of thromboxanes has not been studied in relationship to mechanisms of hepatic fibrosis, studies using hepatic stellate cells showed that thromboxane A₂ caused activation and contractility of hepatic stellate cells (Comporti et al., 2009).

The levels of thromboxane A₂ are much lower in fish oil-fed animals when compared to corn oil-fed animals (Nanji et al., 1997a). However, thromboxanes such as thromboxane A₃ generated from n-3 fatty acids could also act at the thromboxane receptor. Furthermore, isoprostanes may also stimulate fibrosis through activation of the thromboxane receptor. It is

important to note that most of the studies with isoprostanes have been carried out with the F2 isoprostanes, 8-epi-PGF_{2α} or 8-iso-PGF_{2α} which are derived from esterified arachidonic acid (Pratico et al., 2001). Since the fatty acids in fish oil, namely eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) easily undergo peroxidation, attempts have been made to determine whether isoprostane-like compounds can be formed from EPA and DHA (Nourooz-Zadeh et al., 1997). F3 and F4 isoprostanes are derived from EPA and DHA, and in the case of DHA, F4 isoprostanes cause alterations in membrane function (Roberts et al., 1998). However, their role in the pathogenesis of cell injury is uncertain.

Based on our previous observation that hepatic fibrosis associated with increased levels of isoprostanes can occur in the absence of necro-inflammatory changes in animals fed fish oil and dextrose (Nanji et al., 1997b), we tested the hypothesis that the thromboxane inhibitors would reduce the degree of fibrosis in this model. The results in the present study indicate that both of the thromboxane inhibitors (TXSI and TXRA) reduced the deposition of centrilobular collagen, supporting the notion that the reduction in fibrosis is related, at least in part, with the inhibition of the thromboxane signaling pathway. As mentioned previously, isoprostanes can mediate their action through the thromboxane receptor.

NF-κB is a ubiquitous transcription factor that is implicated in the activation of many genes including those involved in alcoholic liver injury (Gobejishvili et al., 2006). Our data confirmed previous observations that activation of NF-κB occurs in the fish oil-ethanol fed rats and is accompanied by IκBα degradation (Nanji et al., 1999). TNF-α and COX-2 are among several genes up-regulated secondarily to the activation of NF-κB (Yokoyama et al., 2005). Thromboxane inhibitors significantly reduced NF-κB activation and levels of COX-2 and TNF-α mRNA in ethanol-fed rats. Although it is difficult, if not impossible, to delineate the effect of thromboxane inhibitors on the individual parameters evaluated in the present study, it is likely that the effect of these inhibitors is due to the inhibition of the downstream effects of isoprostanes and thromboxanes. Oxidative stress, as measured by isoprostane generation, activates NF-κB (Baeuerle, 1998). Thus a reduction in oxidative stress, as seen in the TXSI-treated group, could explain at least part of the decrease in NF-κB activation. A growing body of evidence places the combined role of TNF-α and COX-2 at the center of multiple mechanisms of tissue and liver injury via production vasoactive and pro-inflammatory mediators (Smith and Langenbach, 2001). This is an important idea in the context of alcoholic liver injury because the current experiments showed that alcoholic liver injury activated NF-κB and induced TNF-α and COX-2 and that amelioration of liver injury is accompanied by down-regulation of these induced parameters. Although we did not, in the present study, evaluate the specific cell types expressing NF-κB, COX-2, and cytokines, based on published reports, we expect that the Kupffer cell is the major cell type showing activation of NF-κB and up-regulation of cytokines (Tacke et al., 2009). We cannot, however, exclude the contribution of the endothelial cells, hepatic stellate cells and hepatocytes since these cell types also response to pro-inflammatory stimuli (Tsukamoto, 1999). The contribution of hepatocytes to activation NF-κB is potentially important since they are the major cell type within the liver. NF-κB controls both anti-apoptotic and pro-apoptotic genes, as well as genes involved in liver regeneration (Schmid and Adler, 2000). Thus, the progression of disease in alcohol-fed rats probably represents a balance between genes up-regulated in different cell types secondary to the activation of NF-κB.

In conclusion, our results show that thromboxane antagonists improve the pathological changes such as necrosis, inflammation and fibrosis, despite continued administration of ethanol. The improvement in pathology was accompanied by a decrease in activation of NF-κB and expression of TNF-α, COX-2, and TGF-β₁. The absence of reductions in levels of endotoxin and lipid peroxidation, at least in the TXRA group, shows that it is possible to

attenuate the toxic potential of ethanol by inhibiting events distal to events triggered by endotoxin and lipid peroxidation.

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Figure 1.

Histological changes of rat liver after ethanol administration for 6 or 8 weeks followed by fish oil-ethanol and the thromboxane synthase inhibitor (FE-TXSI) for 2 weeks or fish oil-ethanol and thromboxane receptor antagonist (FE-TXRA) for 2 weeks. Liver sections were stained with H&E staining. Compared to control (FD) group, ethanol caused obvious hepatic fatty infiltration, necrosis and inflammation which were alleviated by treatment of TXSI or TXRA. (Magnification = 200 $\times$).

Figure 2.

Representative images of Sirius Red staining and quantitative data for the amounts of collagen and pericellular fibrosis in the rats liver before or after the administration of thromboxane synthase inhibitor (FE-TXSI) or thromboxane receptor antagonist (FE-TXRA) for 2 weeks. All animals underwent liver biopsy after 6 weeks of fish oil-ethanol treatment. The results of the initial biopsy served as a baseline for comparison with the results after treatment with the thromboxane inhibitors.

Figure 3.

Representative EMSA result for NF-κB activation in nuclear extracts and for IκBα protein in cytosolic extracts from livers in the different experimental groups. Extracts from rats in the fish oil-ethanol (FE) group showed increased DNA binding activity. Low DNA binding activity is seen in the fish oil-dextrose (FD) group. Binding is reduced about 50% in both thromboxane inhibitors-treated groups. Activation of NF-κB is associated with decreased IκBα protein levels. * $p < 0.05$ compared to other groups, # $p < 0.05$ vs. FD. (n = 6 per group).

Figure 4.

(A) Reverse-transcription PCR analysis of TNF- α , COX-2, TGF- β_1 , COX-1 and β -actin in liver samples obtained from the following groups: fish oil-ethanol group (FE), fish oil-ethanol group co-treated with thromboxane synthase inhibitor (FE-TXSI) and thromboxane receptor antagonist (FE-TXRA), and the control fish oil-dextrose (FD) group. β -actin is the internal control. (B) Levels of TNF- α and COX-2 mRNAs in liver samples obtained from the various experimental groups. COX-2 and TNF- α mRNA are significantly reduced ($p < 0.05$) in the thromboxane inhibitor-treated groups. * $p < 0.05$ compared to FE. (n = 6 per group).

Figure 5.

Analysis of TGF- β_1 mRNA in liver (A) and TGF- β_1 in plasma (B) in different groups. No difference in TGF- β_1 mRNA is seen between animals fed fish oil-ethanol for 6 or 8 weeks. TGF- β_1 mRNAs are significantly decreased in the thromboxane inhibitor-treated group. The decrease in the TGF- β_1 mRNA levels parallels the decrease in TGF- β_1 levels in plasma (B). Levels of TGF- β_1 in rats fed FE for 6 weeks are 28 ± 4 ng/L and are not different from levels in rats fed FE for 8 weeks (31 ± 6 ng/L). The levels in the thromboxane inhibitor-treated groups are significantly lower than those in the FE-treated groups. * $p < 0.05$ compared to FE groups. (n = 6 per group).

Table 1

Blood alcohol levels and weight gain before and after administration of thromboxane inhibitors

Experimental group	Blood alcohol level (mg/dL)	Weight gain (against the 1 st week, grams)	
		First 6 weeks	Week 6–8
Group 1 (FE-8 wks)	238 ± 58	50 ± 14	16 ± 6
Group 2 (FE-6 wks)	229 ± 62	52 ± 9	--
Group 3 (FE-TXSI)	250 ± 57	46 ± 11	29 ± 9
Group 4 (FE-TXRA)	238 ± 60	53 ± 10	32 ± 11
Group 5 (FD-8 wks)	0	55 ± 10	22 ± 8

Values represent mean ± SD, n = 6

FE: rats fed fish oil and ethanol;

FE-TXSI: rats fed fish oil and ethanol for 6 weeks and then administered the thromboxane synthase inhibitor (TXSI) with the fish oil-ethanol diet for another 2 weeks;

FE-TXRA: rats fed fish oil and ethanol for 6 weeks and then administered the thromboxane receptor antagonist (TXRA) with the fish oil-ethanol diet for another 2 weeks;

FD: fish oil and dextrose.

Table 2

Severity of pathological changes in the treatment groups

Group n = 6	Feeding time (wks)	Steatosis 0–4+	Necrosis (foci/mm ²)	Inflammation (cells/mm ²)
Group 1 (FE-8 wks)				
Fish oil-ethanol	8	4.0 ± 0.0	1.0 ± 0.4	23.2 ± 5.9
Group 2 (FE-6 wks)				
Fish oil-ethanol	6	4.0 ± 0.0	0.9 ± 0.3	22.8 ± 6.2
Group 3 (FE-TXSI)				
Fish oil-ethanol	6	4.0 ± 0.0	0.8 ± 0.3	24.9 ± 6.9
TXSI-fish oil-ethanol	2	2.0 ± 0.5 ^b	0.4 ± 0.1 ^a	11.3 ± 3.2 ^b
Group 4 (FE-TXRA)				
Fish oil-ethanol	6	3.7 ± 0.5	0.9 ± 0.2	24.1 ± 5.8
TXRA-fish oil-ethanol	2	2.6 ± 0.4	0.3 ± 0.1 ^c	10.9 ± 3.0 ^b
Group 5 (FD)				
Fish oil-dextrose	8	0.0 ^d	0.02 ± 0.01 ^d	0.1 ± 0.1 ^d

^a $p < 0.02$ vs. Fish oil-ethanol in the same group^b $p < 0.05$ vs. Fish oil-ethanol in the same group^c $p < 0.01$ vs. Fish oil-ethanol in the same group^d $p < 0.01$ vs. All fish oil-ethanol fed groups

Table 3
Evaluation of endotoxin, lipid peroxidation and 4-nitrophenol hydroxylase activity in the experimental groups

Group n = 6	Endotoxin (pg/ml)	TBARS (nmole/mg protein)	Conjugated dienes, A ₂₃₂	8-isoprostane (pg/ml)	4-nitrophenol hydroxylase (nmole/min/mg)
1) FE (8 wks)	81 ± 18	1.47 ± 0.31	0.49 ± 0.16	467 ± 81	3.16 ± 0.72
2) FE (6 wks)	83 ± 14	1.56 ± 0.37	0.54 ± 0.17	483 ± 97	2.98 ± 0.68
3) FE-TXSI (8 wks)	76 ± 19	0.62 ± 0.19 ^b	0.20 ± 0.09 ^b	184 ± 39 ^b	1.14 ± 0.17 ^b
4) FE-TXRA (8 wks)	78 ± 12	1.36 ± 0.29	0.47 ± 0.15	446 ± 79	3.01 ± 0.87
5) FD (8 wks)	12 ± 5 ^a	0.37 ± 0.12 ^a	0.20 ± 0.08 ^b	149 ± 41 ^b	1.12 ± 0.18 ^b

^a $p < 0.01$ vs. other groups

^b $p < 0.01$ vs. groups 1, 2 and 4