Alterations in Thromboxane Synthase and Thromboxane A₂ Receptors in Experimental Alcoholic Liver Disease¹

AMIN A. NANJI, AMIR RAHEMTULLA, LILI MAIO, SHAMSUDDIN KHWAJA,² SHUPING ZHAO, STEVEN R. TAhan and PETER THOMAS

Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School (A.A.N., A.R., L.M., S.K., S.Z., S.R.T.) and Laboratory of Cancer Biology, Department of Surgery, Beth Israel Deaconess Medical Center and Harvard Medical School (P.T.), Boston, Massachusetts

Accepted for publication March 17, 1997

ABSTRACT

We have previously shown that hepatic thromboxane production is increased in experimental alcoholic liver disease. The present study was designed to investigate the cell type in liver responsible for increased thromboxane synthesis and the role of the thromboxane receptor system in the pathogenesis of liver injury. Male Wistar rats were divided into four groups and fed a liquid diet with dextrose or ethanol for 2, 4 and 8 weeks. Medium chain triglycerides or corn oil provided the dietary fatty acids. Kupffer cells, endothelial cells and hepatocytes were isolated from rats fed the different diets for 4 weeks. Liver histopathology, thromboxane synthase mRNA and protein, thromboxane levels and thromboxane receptor mRNA were evaluated in each group. In rats fed corn oil and ethanol, an increase in thromboxane synthase and liver levels of thromboxane metabolites were significantly higher than in the corn oil-dextrose-fed group and were correlated with the presence of pathological changes in the liver. Kupffer cells showed increased expression of thromboxane synthase. In rats fed medium chain triglycerides and ethanol, the levels of thromboxane synthase mRNA and protein were significantly lower than in the corn oil-ethanol-fed groups (P < .01) and liver injury was absent. However, the levels of thromboxane synthase mRNA, protein and thromboxane were significantly higher in the medium chain triglyceride-ethanol-fed rats than in the respective dextrose-fed controls. Among the different cell types, thromboxane A₂-receptor mRNA levels were highest in the Kupffer cells in corn oil-ethanol-fed rats. The increase in thromboxane synthase in Kupffer cells together with an increase in thromboxane receptor levels suggests than thromboxanes may contribute to liver injury in ethanol-fed rats.

Evidence shows that dietary lipid can modulate the severity of alcohol-induced liver injury. None of the histologic features of alcoholic liver injury develop in rats fed ethanol and saturated lipid, whereas fatty liver, necrosis, inflammation and fibrosis develop in rats fed ethanol and lipids enriched in polyunsaturated fatty acids (Nanji et al., 1989; Nanji and French, 1989). One of the polyunsaturated fatty acids that promotes alcoholic liver injury is linoleic acid (Nanji and French, 1989). To gain a better understanding of the role of linoleic acid in ALD, we investigated the metabolism of linoleic acid to arachidonic acid and its subsequent conversion to eicosanoids in the intragastric feeding rat model for ALD (Nanji, 1993). Our studies showed that increased production of TXB₂ and decreased production of prostaglandin E₂ by liver nonparenchymal cells correlated with the presence of pathologic liver injury in ethanol-fed rats (Nanji et al., 1994b). Additionally, a significant correlation was observed between plasma levels of TXB₂ and severity of liver injury in rats fed corn oil and ethanol (Nanji et al., 1993b). A significant correlation was also seen between pathologic severity and levels of endotoxin in plasma (Nanji et al., 1993b). The Kupffer cell is believed to be the major site of thromboxane production in the liver in response to stimuli such endotoxin (Decker, 1990; Winwood and Arthur, 1993). A role for thromboxanes in promoting necroinflammatory changes in alcoholic liver injury is further supported by recent observations that show that inhibition of thromboxane synthesis or blocking the action of thromboxane at the receptor level leads to a significant decrease in the degree of necrosis and inflammation in corn oil-ethanol-fed rats (Nanji et al., 1997).

In the current study, we extended our observations relating thromboxanes to ALD. First, we investigated the cell type

ABBREVIATIONS: ALD, alcoholic liver disease; CD, corn oil-dextrose; CE, corn oil-ethanol; MCT, medium chain triglycerides; MCTD, medium chain triglycerides-dextrose; MCTE, medium chain triglycerides-ethanol; TX, thromboxane; RT-PCR, reverse-transcription polymerase chain reaction; HEPES, N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid.

Received for publication October 18, 1996.

¹This study was supported in part by grant CA 44583 from the National Institutes of Health.

²A student fellow of the American Liver Foundation at the time the study was conducted.
in the liver responsible for enhanced thromboxane synthesis in ALD. We used immunohistochemical analysis to identify thromboxane synthase in liver tissue, and the RT-PCR to assess the relationship between TX synthase mRNA and liver levels of TXB2 and its metabolites. To further define the cell type and mechanisms involved in enhanced TX synthesis, we isolated the individual cell types from the livers of rats fed ethanol or dextrose with either saturated fat or corn oil. In each group, TX synthase mRNA expression in whole liver and the different cell types was evaluated and related to the immunohistochemical analyses for TX synthase and the presence of pathologic liver injury.

Some of the actions of TXA2 which are likely to be important in the pathogenesis of ALD include a reduction in hepatic blood flow, platelet aggregation and formation of plasma membrane blebs on hepatocytes (Oates et al., 1988; Smith, 1992). There is overwhelming evidence that shows that many of these biological actions of TXA2 are mediated by its specific receptor on the cell surface (Armstrong and Wilson, 1995; Morinelli and Halushka, 1991; Negishi et al., 1993). In view of the potential importance of thromboxanes in liver injury, it is of interest to investigate the role of the TXA2-receptor system in ALD. TXA2 receptors are found in a variety of tissues and cell types in mammals (Negishi et al., 1993). In the liver, recent investigations have identified TXA2-receptors on the hepatic sinusoidal endothelial cells (Ishiguro et al., 1994). Activation of these receptors leads to alterations in the sinusoidal microcirculation which is important in the pathogenesis of ALD (Lieber, 1994; Tsukamoto et al., 1990). To further define the role of TXA2-receptors in ALD, we used RT-PCR to investigate the changes in TXA2-receptor mRNA concentrations in the livers and individual cell types in rats fed different dietary fats with either ethanol or dextrose. Alteration in TXA2-receptor mRNA levels were related to pathologic liver injury.

**Materials and Methods**

**Animal model and treatment groups.** Male Wistar rats weighing between 225 and 250 g were fed a liquid diet via permanently implanted gastric cannulas as described previously (Tsukamoto et al., 1990). The rats were administered their total nutrient intake by intragastric infusion. The rats were fed freshly prepared diets and either MCT or corn oil provided the fatty acids which contributed 35% of total calories. The fatty acid composition of the diet has been described previously (Nanjí et al., 1994a). Vitamins and minerals were given as described previously (French et al., 1993). The liquid diet was infused at a rate of 180 ml/kg b.wt./day to achieve adequate weight gain (1 ml = 1 kcal). Ethanol was infused to maintain blood alcohol levels between 150 and 300 mg/dl (33–66 μmol/l). The amount was initially 10 g/kg/day and was increased up to 16 g/kg/day as tolerance developed. All animals received humane care in compliance with the National Institutes of Health criteria for care of laboratory animals.

In the first experiment, rats fed the different dietary fats (MCT or corn oil) with dextrose or ethanol had evaluation of pathologic changes (fatty liver, necrosis and inflammation), liver TXB2 and TX synthase by immunohistochemical analysis. The animals were divided into four different experimental groups: MCTD (as a source of saturated fatty acids), MCTE, CD (unsaturated fatty acids) and C). Each experimental group was fed the liquid diet containing either dextrose or ethanol for periods for 2, 4 or 8 weeks after which time they were sacrificed.

For the second experiment in which mRNA levels of TX synthase and TX-receptors were evaluated in liver tissue and the various cell types, rats in the four different experimental groups (MCTD, MCTE, CD, CE) were fed the liquid diet containing dextrose or ethanol for 4 weeks after which they were sacrificed. Individual cell types (Kupffer, endothelial and hepatocytes) were isolated as described below.

**Isolation of Kupffer cells, endothelial cells and hepatocytes.** Cells were isolated from anesthetized rats by previously described procedures (Petrick et al., 1994; Toth et al., 1985) and isolation buffers described by Seglen (1973). After intravenous administration of sodium heparin (100 U), the livers were exsanguinated in situ by portal vein perfusion with Ca2+-free buffer. Livers were excised, minced and subsequently incubated in 0.05% collagenase in buffer (0.1 M HEPES with 0.39% sodium chloride, 0.05% potassium chloride, 0.05 M calcium chloride, pH 7.6) at 37°C for 45 min. The resulting cell suspension was strained, pelleted and resuspended in fresh collagenase buffer with gentle shaking for 30 min. The cell suspension was centrifuged (×3) at 50 × g for 2 min. The pelleted fraction contained mainly hepatocytes. The cells remaining after centrifugation were washed several times with Hanks’ balanced salt solution and centrifuged in a 17.5% metrizamide gradient in Hanks’ balanced salt solution. This fraction contained approximately 65% Kupffer cells with the balance being liver endothelial and stellate cells. Further purification of Kupffer cells was done by incubation for 2 hr in 48-well tissue culture dishes at 37°C in a humid atmosphere of 5% carbon dioxide for 16 hr. The medium consisted of RPMI-1640, 10% fetal bovine serum with 2 mM l-glutamine and 50 μg/ml penicillin/streptomycin. Adherent cells formed a monolayer on the culture dish and >85% of these cells were macrophages. The different cell types were identified by morphology, and immunohistochemical markers which included peroxidase, acid phosphatase, α1-antichymotrypsin, ED1 and cytokeratin (Alpini et al., 1994). Endothelial cells were the nonadherent cells from the Kupffer cell preparation and were plated onto type I collagen-coated dishes. The cells were stored frozen at −70°C.

**Histopathology.** A small sample of the liver was harvested when the rats were sacrificed and formalin-fixed. Hematoxylin and eosin stain was used for light microscopy. The severity of liver pathology was assessed as follows: steatosis (the percentage of liver cells containing fat) was scored 1+ when <25% of the cells contained fat; 2+, with 26 to 50% fat; 3+, with 51 to 75% fat; and 4+, with >75% fat. Necrosis was scored as the number of necrotic foci per square millimeter, and 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 the sections was unaware of the treatment groups when assessing the histology.

**Blood alcohol levels.** Blood was collected from the tail vein, and ethanol concentration was measured with an alcohol dehydrogenase kit from Sigma Chemical Co. (St. Louis MO).

**TXB2 and 2,3-dinor-TXB2 levels in liver.** Because the measurement of TXB2, the chemically stable hydrolysis product of TXA2, is subject to artifactual increases (Lawson et al., 1988), it has been suggested that measurement of a longer-lived stable metabolite such as 2,3-dinor-TXB2 represents a better measure of thromboxane production (Lawson et al., 1986). Approximately 1 g of liver from the rats in each of the different experimental groups treated for 4 weeks was rapidly homogenized in 10 ml of ice-cold methanol for 30 sec. After centrifugation, the supernatant was dried and resuspended in 0.1 ml of potassium phosphate buffer (pH 7.4) and purified by elutriation through an octadecyl silyl SEP-PAK C18 cartridge (Waters Associates, Milford, MA). The 80% methanol eluent was assayed for TXB2 and 2,3-dinor-TXB2 (Cayman Chemical, Ann Arbor, MI).

**RNA extraction from liver tissue and cells and analysis of mRNA for TX synthase, TXA2-receptors and β-actin by RT-PCR.** To examine the expression of TX synthase, TXA2-receptors and β-actin in both liver tissue and cells, total RNA was isolated according to the guanidium isothiocyanate method (Chomczynski...
and Sacchi, 1987). The total RNA concentration of each sample was determined from absorbance at 260 nm, and the quality of each RNA preparation was determined by agarose-formaldehyde gel electrophoresis and ethidium bromide staining. We reverse-transcribed 0.5 to1 µg of total RNA by adding 30 µl of a master mix with reverse transcriptase buffer (0.6 mmol/l MgCl₂, 15 mmol/l KCl, 10 mmol/l Tris HCl [pH 8.3]), 40 pmol of downstream primer, 0.5 mmol/l dNTP mixture, 1 U/µl RNase inhibitor and 13.3 U/µl Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL, Grand Island, NY) (final concentrations indicated). Samples were incubated, first for 60 min at 42°C and then at 75°C for 10 min, then chilled on ice. Then 2 µl of each sample was added to 20 µl of 1.5 mmol/l MgCl₂, 50 mmol/l KCl, 10 mmol/l Tris HCl (pH 8.3), 0.2 mmol/l of each dNTP and 0.01% gelatin, 5 U/100 µl Taq DNA polymerase (Perkin Elmer Cetus) and 50 pmol of sense primer and 10 pmol of antisense primer. The sequences of primer pairs, 5’ and 3’, and predicted sizes of the amplified PCR fragments are shown in table 1. Amplification was performed in an automated thermal cycler at 94°C for 60 sec, 50°C for 90 sec and 72°C for 2 min for 35 cycles, followed by 72°C for 10 min. To measure the efficiency of the extraction of RNA and of reverse transcription, we amplified 2 µl of the same reverse transcriptase reaction with β-actin-specific primers as an internal control. PCR products and molecular weight markers were subjected to electrophoresis on 1% agarose gels and visualized by means of ethidium bromide staining. Location of the predicted PCR products was confirmed by using a 100-base pair ladder (GIBCO-BRL) as a standard size marker. For quantitation, the expression of the products was quantitated using densitometric scan analysis. The index of the various mRNA signals was standardized against that of the β-actin signal from the same RNA.

Varying the number of PCR cycles did not change the relative differences between samples, which indicated that our PCR conditions were not within the plateau phase of amplification. Each experiment included a negative control (sample RNA that had not been subjected to reverse transcription). This sample did not yield a PCR product, confirming the absence of extraneous genomic DNA or PCR product contaminating the samples.

**Thromboxane synthase in liver by immunohistochemistry.** For identification of cells staining for TX synthase, 6-µm-thick sections were prepared from paraffin blocks, after deparaffinization through graded ethanol. The sections were then washed in phosphate-buffered saline. Immunocytochemical staining for TX synthase was performed by an antibody against thromboxane synthase (Cayman Chemical, Ann Arbor, MI) and by the avidin-biotin complex method (Vector Laboratories, Burlingame, CA). The number of positively stained cells were counted and the numbers expressed as cells/mm². The nature of positively stained sinusoidal cell was further defined by characteristic morphology and staining with vimentin which in these animals stained Kupffer cells (Marugg et al., 1990; Nanji et al., 1996). These cells failed to stain positively for desmin.

**Statistical analysis.** Analysis of variance and multiple comparison with the Student-Neuman-Keuls method were used for determination of statistical significance. Pearson’s correlation coefficient (r) was used for evaluation of associations.

<table>
<thead>
<tr>
<th>Oligonucleotide Primers</th>
<th>Size of PCR Product</th>
<th>bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thromboxane A₂ receptor</td>
<td>5’ TGC TGC AGA CGC TAG CTG TC-3’</td>
<td>244</td>
</tr>
<tr>
<td>Thromboxane synthase</td>
<td>5’ GAT TGG CAC CGT CCT TCA GG-3’</td>
<td>450</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5’ GAT GGT GGG TAT GGG TCA GAA GGA-3’</td>
<td>630</td>
</tr>
</tbody>
</table>

**Results**

There was no significant difference in the amount of weight gained in the different experimental groups. There was also no significant difference in blood alcohol levels (mg/dl) (mean ± S.E.) in the ethanol-fed groups at 4 and 8 weeks. At 4 weeks, the blood alcohol levels were: MCTE, 216 ± 39 (47 ± 8.5 µmol/l); CE, 231 ± 41 (50 ± 9 µmol/l); at 8 weeks they were: MCTE, 241 ± 32 (52 ± 7 µmol/l); CE, 251 ± 30 (54 ± 6.5 µmol/l). **Histopathology.** Only rats fed corn oil and ethanol for 4 and 8 weeks developed pathologic liver injury (table 2, fig. 1). None of dextrose-fed rats or rats in the MCTE group developed pathologic changes (fig. 2).

**Liver thromboxane synthase (mRNA and immunohistochemistry) and liver TXB₂ and 2,3-dinor-TXB₂ levels.** The number of sinusoidal lining cells, identified as Kupffer cells based on morphologic characteristics, that stained positive with the antibody against thromboxane synthase, were increased in rats fed corn oil and ethanol (table 3, fig. 3). The number of positively stained cells increased significantly between 2 and 4 weeks in the CE group (P < .01, table 3). An increase in TX synthase positive cells was also seen in the MCTE group compared with the dextrose-fed control group; however, the degree of increase was not as great as was seen in the CE group in the same time periods (fig. 4). Occasional positively staining cells (<0.1/mm²) were seen in rat livers before feeding.

The relationship between the number of TX synthase positive cells, total liver TX synthase mRNA levels and liver concentrations of TXB₂ and 2,3-dinor-TXB₂ in rats fed the different diets for 4 weeks is shown in figure 5. In rats fed MCTD and CD, a faint band was seen for TX synthase mRNA. In CE rats, the levels of TX synthase mRNA (normalized for β-actin) were about six to seven times higher than the levels in the dextrose-fed controls and about two to three times higher than the level in the MCTE group (P < .01) (fig. 5B). Densitometric analysis of β-actin mRNA (internal control) showed similar levels in each cell type in all of the groups studied, which reduced the likelihood that the isolation procedure led to significant degradation of mRNA (data not shown). The significant correlation (r = 0.94, P < .01) (fig. 6) between the levels of TX synthase mRNA and the number of TX synthase-positive cells suggests that the increase in mRNA levels may, in part, account for the increase in TX synthase protein levels. The reason for the increase in TX synthase mRNA (i.e., enhanced transcription or decreased mRNA degradation) cannot be deduced from this study. The increase in TX synthase mRNA and protein probably accounted for the increase in thromboxane levels seen in...
TABLE 2
Pathologic changes in rats fed different dietary fatty acids with dextrose and ethanol

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Duration of Feeding</th>
<th>Fatty Liver (0–4)</th>
<th>Necrosis</th>
<th>Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wk</td>
<td>foci/mm²</td>
<td>cells/mm²</td>
<td></td>
</tr>
<tr>
<td>MCTD</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.02 ± 0.01</td>
<td>0.03 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0</td>
<td>0.03 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>MCTE</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.02 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.02 ± 0.01</td>
<td>0.03 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.03 ± 0.01</td>
<td>0.04 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.03 ± 0.01</td>
<td>0.04 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>CE</td>
<td>2</td>
<td>0.6 ± 0.5</td>
<td>0.04 ± 0.02</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.0 ± 0.6a</td>
<td>0.5 ± 0.07a</td>
<td>2.8 ± 0.3a</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>3.6 ± 0.5a</td>
<td>0.8 ± 0.1a</td>
<td>4.9 ± 1.1a</td>
</tr>
</tbody>
</table>

* P < .05 vs. MCTD, MCTE, CD at all time periods and CE at 2 weeks.
* P < .05 vs. CE at 4 weeks.

Table 2

<table>
<thead>
<tr>
<th>Duration of Feeding</th>
<th>MCTD</th>
<th>MCTE</th>
<th>CD</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.0 ± 0.5</td>
<td>2.1 ± 1.0a</td>
<td>0.3 ± 0.2</td>
<td>2.8 ± 1.9</td>
</tr>
<tr>
<td>8</td>
<td>0.3 ± 0.2</td>
<td>2.0 ± 1.2a</td>
<td>0.5 ± 0.5</td>
<td>12.0 ± 3.6a</td>
</tr>
</tbody>
</table>

* P < .05 vs. dextrose-fed control group.
* P < .01 vs. other groups at same time period.
* P < .02 vs. CE at 2 weeks.

The present study identified the Kupffer cell as the most likely source of the enhanced synthesis of thromboxane in ethanol-fed rats. The stimulus for enhanced synthesis of thromboxane is likely to be linoleic acid (Nanji et al., 1989). In subsequent studies, we determined that conversion of linoleic acid to arachidonic acid led to the enhanced synthesis of thromboxane by nonparenchymal liver cells (Nanji et al., 1993b). Furthermore, plasma levels of thromboxane B₂ correlated with the severity of liver injury. These observations led to the hypothesis that thromboxane(s) are important in the pathogenesis of alcoholic liver injury. In this respect, it was of interest to identify the cell type(s) responsible for increased synthesis of thromboxanes. The present study identified the Kupffer cell as the most likely source of the enhanced synthesis of thromboxane A₃ in ethanol-fed rats. The stimulus for enhanced synthesis of thromboxane A₃ by Kupffer cells is probably endotoxin which originates from the cell wall of gram-negative bacteria and is increased in the plasma of rats fed corn oil and ethanol (Nanji et al., 1989).

The present study identified the Kupffer cell as the most likely source of the enhanced synthesis of thromboxane in ethanol-fed rats. The stimulus for enhanced synthesis of thromboxane is likely to be linoleic acid (Nanji et al., 1989). In subsequent studies, we determined that conversion of linoleic acid to arachidonic acid led to the enhanced synthesis of thromboxane by nonparenchymal liver cells (Nanji et al., 1993b). Furthermore, plasma levels of thromboxane B₂ correlated with the severity of liver injury. These observations led to the hypothesis that thromboxane(s) are important in the pathogenesis of alcoholic liver injury. In this respect, it was of interest to identify the cell type(s) responsible for increased synthesis of thromboxanes. The present study identified the Kupffer cell as the most likely source of the enhanced synthesis of thromboxane A₃ in ethanol-fed rats. The stimulus for enhanced synthesis of thromboxane A₃ by Kupffer cells is probably endotoxin which originates from the cell wall of gram-negative bacteria and is increased in the plasma of rats fed corn oil and ethanol (Nanji et al., 1989).
1993b; Adachi et al., 1995). Kupffer cells are the primary effector cells in the liver which respond to endotoxin by producing many inflammatory mediators (Decker, 1990). Arachidonic acid metabolites, such as thromboxanes, are prominent mediators produced by macrophages in response to endotoxin (Decker, 1990). The importance of Kupffer cell-derived mediators in the pathogenesis of alcoholic liver injury in the intragastric feeding rat model is further demonstrated by the study of Adachi et al. (1994) who showed that inactivation of Kupffer cells by gadolinium chloride prevented alcoholic liver injury.

The mechanism(s) by which thromboxane contributes to liver injury is unknown. TXA₂ is a potent vasoconstrictor and platelet aggregatory agent (Hamberg et al., 1975; Morinelli and Halushka, 1991). Vasoconstriction of the hepatic sinusoids can aggravate the hypoxia already caused by enhanced oxygen consumption and impaired oxygen utilization seen in ethanol-fed animals (Israel and Orrego, 1987; Lieber et al., 1989; Tsukamoto and Xi, 1989). Platelet aggregation leads to release of secretory products which cause cell injury (Schrö and Braun, 1990). TXB₂ causes bleb formation in isolated hepatocytes (Horton and Wood, 1990); the formation of plasma membrane blebs is a consequence of toxic or ischemic cell injury (Gores et al., 1990). The actions of TXA₂ are generally believed to be mediated by specific TXA₂ receptors (Negishi et al., 1993). The TXA₂-TXA₂ receptor system is one of the factors involved in the regulation of the microcirculation in the hepatic sinusoid (Ishiguro et al., 1994). We found increased levels of TXA₂ receptors in all cell types studied, i.e., Kupffer cells, endothelial cells and hepatocytes, in rats fed corn oil and ethanol. The largest increase was seen in the Kupffer cells, with lesser degrees of increase seen in endothelial cells and hepatocytes. TXA₂ receptors have been de-

Fig. 3. Liver from a rat fed corn oil and ethanol for 8 weeks stained for TX synthase. Note the positive staining in Kupffer cells lining the hepatic sinusoid (arrow).

Fig. 4. Liver stained for TX synthase from a rat fed MCT and ethanol for 8 weeks. There are occasional Kupffer cells that stain positively for TX synthase.

Fig. 5. Numbers of TX synthase positive cells/mm² (A), TX synthase mRNA (B) and liver levels of TXB₂ and 2,3-dinor-TXB₂ (C) in rats sacrificed at 4 weeks from the different experimental groups. The number of TX synthase positive cells was significantly higher (P < .01) in the corn oil-ethanol group (9.8 ± 5.0/mm²) versus the dextrose-fed control group (CD, 0.3 ± 0.2) and rats fed MCTE (2.1 ± 1.0). The number of cells in MCTE were higher (P < .01) than MCTD (0.2 ± 0.2). TX synthase mRNA was highest in CE (6.0 ± 0.9 arbitrary units) compared with CD (1.0), MCTE (2.8 ± 1.1) and MCTD (0.2 ± 0.05) (fig. 6B). The increase in liver levels of TXB₂ and 2,3-dinor-TXB₂ in the CE group (fig. 6C) reflected the increased expression of TX synthase. The level of TXB₂ (pg/mg protein) (212 ± 36) and 2,3-dinor-TXB₂ (239 ± 44) was higher (P < .01) than in the CD group (64 ± 21 and 64 ± 19), MCTE group (64 ± 21 and 73 ± 26) and MCTD group (17 ± 9 and 16 ± 6). The levels in MCTE were significantly greater than in the MCTD group (P < .01).
identified TXA2 receptor mRNA in hepatocytes, which suggests that TXA2 receptor binding sites are also present on the hepatocyte surface. This observation may account for the ability of TXB2 to cause hepatocyte blebbing (Horton and Wood, 1990).

Correlation between TX synthase mRNA and the number of TX synthase positive cells/mm² ($r = 0.94$, $P < .01$). TX synthase mRNA was measured in whole liver by RT-PCR and assessed densitometrically (see “Materials and Methods”). When the saturated fat (MCT) and corn oil-treated groups were assessed separately, the correlation between the two parameters was significant in each group. $r = 0.90$, $P < .01$ in the MCT group; $r = 0.95$ in the corn oil group. The fold-increase in the different groups was related to the corn oil-ethanol-fed rats, with the highest increase in the CE group (4.6 ± 1.7) than in the MCTD (0.3 ± 0.03), MCTE (0.9 ± 0.2) and CD (1.0) groups. TX2-receptor mRNA levels were the highest in the Kupffer cells in the CE group (13.0 ± 4.0). Ethanol administration in the MCT group also significantly increased TXA2-receptor mRNA levels in Kupffer cells.

Whether up-regulation of receptor synthesis contributes to hepatocyte necrosis, as in corn oil-ethanol-fed rats, remains to be studied.

In summary, our results show that the Kupffer cell is the most probable source of the enhanced production of TXA2 in experimental ALD. Increased levels of thromboxane metabolites have also been seen in the portal vein in human liver disease including ALD (Garcia-Valdecasas et al., 1995). However, other measurements such as endothelin levels were not carried out and the cell type responsible for the production of thromboxane was not identified. Our findings in an experimental model of alcoholic liver injury lend credence to the hypothesis that the combination of enhanced production of TXA2 and enhanced synthesis of TXA2-receptors in the various cell types in the liver may contribute to enhanced cytokine production by Kupffer cells, microcirculatory disturbances and hepatocyte necrosis. This view is further supported by previous studies that show that inhibition of thromboxane synthesis or thromboxane action at the receptor level reduces the expression of TNF-$\alpha$ in the liver and the severity of necroinflammatory changes in experimental ALD.

Acknowledgments

The technical help provided by Dianna Peters is very much appreciated.

References


Fig. 6. Correlation between TX synthase mRNA and the number of TX synthase positive cells/mm² ($r = 0.94$, $P < .01$). TX synthase mRNA was measured in whole liver by RT-PCR and assessed densitometrically (see “Materials and Methods”). When the saturated fat (MCT) and corn oil-treated groups were assessed separately, the correlation between the two parameters was significant in each group. $r = 0.90$, $P < .01$ in the MCT group; $r = 0.95$ in the corn oil group.

Fig. 7. Densitometric analysis of TXA2-receptor mRNA (normalized for $\beta$-actin) in the liver and individual cell types in the different experimental groups. The livers were obtained from rats sacrificed at 4 weeks. The TXA2-receptor mRNA levels are significantly higher ($P < .01$) in the liver and various cell types in the CE-fed rats than in the other treatment groups. The fold-increase in the different groups was related to the corn oil-ethanol group as control. The TX2-receptor mRNA levels in the liver were significantly higher in the CE group (4.6 ± 1.7) than in the MCTD (0.3 ± 0.03), MCTE (0.9 ± 0.2) and CD (1.0) groups. TX2-receptor mRNA levels were the highest in the Kupffer cells in the CE group (13.0 ± 4.0). Ethanol administration in the MCT group also significantly increased TXA2-receptor mRNA levels in Kupffer cells.