The 21-Aminosteroid 16-Desmethyl Tirilazad Mesylate Prevents Necroinflammatory Changes in Experimental Alcoholic Liver Disease

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ABSTRACT
We investigated the potential of 16-desmethyl tirilazad mesylate, a member of 21-aminosteroids, to ameliorate alcohol-induced liver injury. Four groups (five rats/group) of male Wistar rats were studied. One group of rats was fed fish oil and ethanol (FE) for 4 weeks, and a second group received isocaloric amounts of dextrose instead of ethanol (FD). The third (FE-LAZ) and fourth (FD-LAZ) groups received the addition of 10 mg/kg/day of 16-desmethyl tirilazad mesylate (U74389) daily via intragastric tube. Liver samples were analyzed for histopathology, nonheme iron, lipid peroxidation and levels of mRNA for tumor necrosis factor-α (TNF-α) and cyclooxygenase-2 (COX-2). Concentrations of endotoxin and 8-isoprostane were measured in plasma. Membrane ATPases were measured in isolated membrane red cells. FE rats developed fatty liver, necrosis and inflammation. Treatment with the 21-aminosteroid resulted in prevention of necroinflammatory changes, but the degree of fatty liver was unchanged. The absence of necroinflammatory changes in the FE-LAZ group was accompanied by a decrease in levels of nonheme iron, lipid peroxidation, TNF-α mRNA and COX-2 mRNA. Ethanol administration decreased membrane Ca^{++}-ATPase and calmodulin-stimulated Ca^{++}-ATPase, and the decrease was reversed by 21-aminosteroid treatment. These data indicate that the improvement in the degree of necrosis and inflammation in the rats treated with the 21-aminosteroid may be explained, at least in part, by reduced levels of proinflammatory stimuli such as lipid peroxidation, TNF-α and COX-2. Membrane stabilization may also, by reducing lipid peroxidation, play an additional role in preventing liver injury.

The mechanism or mechanisms contributing to alcohol-induced liver damage remain uncertain. There is increasing evidence that alcohol toxicity is associated with increased oxidative stress and free radical-associated injury (Cederbaum, 1989; Nanji et al., 1994a; Reinke et al., 1987). Several lines of investigation indicate that the generation of oxygen metabolites such as superoxide (O_2^-), hydrogen peroxide and hydroxyl radicals is believed to be important in the pathogenesis of alcoholic liver injury (for reviews, see Nanji and Zakim, 1996; and Nordmann et al., 1992). The notion that free radical-mediated injury and enhanced lipid peroxidation are common features of many different diseases has stimulated enthusiasm for the potential use of antioxidants as therapeutic agents.

Recently, a new class of antioxidant agents, the 21-aminosteroids (lazaroids, tirilazads), was developed (Hall et al., 1994; Hall and Travis, 1988). A growing body of evidence has confirmed the ability of 21-aminosteroids to limit free radical-mediated organ injury (Bagchi et al., 1995; Liu et al., 1994; Taylor et al., 1996). The exact mechanisms by which 21-aminosteroids protect against organ injury are unknown. One hypothesis is that the beneficial effects of 21-aminosteroids are due to their antioxidant- and free radical-scavenging properties (Braughler et al., 1987; Hall et al., 1994). The other proposed mechanism is stabilization of biological membranes (Wang et al., 1996).

The present study was designed to test the effectiveness of the compound U74389, one of a novel series of 21-aminosteroids, in ameliorating alcoholic liver injury. We used the intragastric feeding rat model for alcoholic liver disease to determine the potential protective effect of U74389 (16-desmethyl tirilazad mesylate). The intragastric feeding model is ideally suited to this kind of study because rats fed ethanol with unsaturated fatty acids develop fatty liver, necrosis and inflammation (Nanji et al., 1994b; Nanji and French, 1989). This model thus allows correlation between improvement in

ABBREVIATIONS: CaM, calmodulin-stimulated Ca^{++} pump ATPase; COX, cyclooxygenase; FD, fish oil and dextrose; FE, fish oil and ethanol; FE-LAZ, fish oil plus ethanol plus the 21-aminosteroid U74389 (lazaroid); FD-LAZ, fish oil plus dextrose plus the 21-aminosteroid U74389 (lazaroid); NF-κB, nuclear factor-κB; TBARS, thiobarbituric acid reactive substances; TNF, tumor necrosis factor; RT, reverse transcription; PCR, polymerase chain reaction.
biochemical markers such as lipid peroxidation and histological evidence of liver injury (Nanji et al., 1995).

It is also becoming increasingly apparent that in addition to promoting direct toxicity, reactive oxygen intermediates initiate and/or amplify inflammation through up-regulation of cytokines and proinflammatory mediators (Suzuki et al., 1997). We have recently shown that up-regulation of TNF-α and COX-2 is associated with increased lipid peroxidation in alcoholic liver injury (Nanji et al., 1997). Thus, it was important to determine the effect of the 21-aminosteroids on these two inflammatory mediators. Finally, because 21-aminosteroids demonstrate membrane-stabilizing effects, we evaluated its effect on membrane ATPases. We have previously shown that ethanol administration causes alterations in membrane ATPases that correlate with the presence of pathological liver injury (Sadrzadeh et al., 1994a).

Materials and Methods

Experimental design. The experimental animals were male Wistar rats weighing between 225 and 250 grams (Harlan-Sprague Dawley, Indianapolis, IN). Four groups of rats (five rats per group) were studied: FE, FD, FE-LAZ and FD-LAZ. The 21-aminosteroid U74389 (generously donated by Upjohn Co., Kalamazoo, MI) was administered orally at a dose of 10 mg/kg/day at the same time each day. The rats were maintained according to the National Institutes of Health guidelines on the care and use of laboratory animals.

Animal model. All animals were fed liquid diets containing ethanol or isocaloric dextrose via continuous infusion through permanently implanted gastric tubes as described previously (French et al., 1986; Tsukamoto et al., 1990). The rats were administered their total nutrient intake via intragastric infusion. The percentage of calories from fat was 35% of total calories. The fatty acid composition of the diets has been described previously (Nanji et al., 1994a). The diets were prepared fresh daily and was supplemented with vitamins and minerals (French et al., 1993). The liquid diet (1 kcal/ml) was infused at a rate of 180 ml/kg/day to achieve adequate weight gain. An appropriate amount of ethanol was infused to maintain blood alcohol levels between 150 and 300 mg/dl; this amount was initially 10 mg/kg/day and was increased to 15 mg/kg/day. All animals were killed 1 month after the initiation of feeding. When the animals were killed, a sample of liver was obtained for histopathological analysis; the remainder of the liver was rapidly excised, washed with ice-cold 1.15% (w/v) KCl and cut into small pieces, which were transferred to plastic vials and placed in liquid nitrogen. The vials were stored at −80°C.

Histological analysis. A small sample of liver was obtained through biopsy or at death and fixed in formalin. 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), 1+, <25% of cells containing fat; 2+, 26% to 50%; 3+, 51% to 75%; and 4+, >75%. Necrosis was evaluated as the number of necrotic foci/mm²; inflammation was scored as the number of inflammatory cells/mm². At least three different sections were examined per sample of liver.

Measurements of blood alcohol. Blood was collected from the tail vein, and ethanol concentration was measured using an alcohol dehydrogenase kit from Sigma Chemical (St. Louis, MO).

Evaluation of lipid peroxidation. Lipid peroxidation was evaluated by measurements of TBARS in liver and 8-isoprostan concentrations in plasma. Levels of TBARS were measured according to the method of Ohkawa et al. (1979). 8-Isoprostanes were measured with an immunosassay 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. We have previously shown that 8-isoprostane levels in plasma correlate extremely well with levels of conjugated dienes in liver (Nanji et al., 1994a).

RNA extraction from liver tissue and analysis using RT-PCR. To examine the expression of TNF-α, COX-1 and -2 and β-actin in liver tissue, total RNA was isolated according to the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). RNA (0.5–1 µg) was reverse-transcribed and amplified as described previously. The sequences of primer pairs and predicted sizes of amplified fragments have been shown previously (Nanji et al., 1997). 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 an additional 10-min extension period at 72°C. To account for variations in the amount of reverse-transcribed RNA between samples, all data were normalized to β-actin, which was measured by the same technique. For identification of PCR products, aliquots from each PCR were electrophoresed on 1% agarose gel and visualized by ethidium bromide staining. The gels were analyzed by laser scanning densitometry using a Molecular Dynamics Densitometer and Image Quant Software (Sunnyvale, CA). Each experiment included a negative control (sample RNA that had not been subjected to RT).

Measurement of nonheme iron. Nonheme iron was determined in liver homogenate, with ferene S, as an indicator with the molar absorptivity of 35,500 M⁻¹ cm⁻¹ at 594 nm (Artis et al., 1982). The liver was homogenized in NaCl solution (7 mM NaCl/100 mg of tissue) and centrifuged at 1000 × g for 10 min. The clear supernatant (150 µl) was mixed with deionized H₂O (150 µl) and 150 µl of thiourea/ascorbate solution (4.4% and 2.68% in deionized H₂O). Trichloroacetic acid (150 µl of 40% solution) was added to the mixture vortexed and centrifuged for 30 to 60 sec. The supernatant (500 µl) was then mixed with 125 µl of fresh ferene S solution (35 mg of ferene S in 10 ml of 50% ammonium acetate solution). The mixture was incubated at room temperature for 5 to 10 min, and the absorbance was read at 594 nm. Control experiments were carried out to ensure that the measured nonheme iron was not from nonspecific iron released from ferruginous compounds during the procedures.

Measurement of membrane ATPases. For ATPase determinations, blood was collected from the aorta into heparinized tubes and centrifuged immediately at 4°C to separate the red blood cells. Red blood cell membranes were prepared according to the method of Farrance and Vincenzi (1977), and ATPases were measured using a microtiter plate assay (Sadrzadeh et al., 1993).

Statistical analysis. Results are presented as mean ± S.D. unless otherwise indicated. Analysis of variance and multiple comparisons, with the Student-Newman-Keuls method were used for determination of statistical significance. Pearson’s correlation coefficient (r) was used for evaluation of associations.

Results

In each of the groups studied, the rats increased their weight at a constant rate; there was no difference in weight gain among the groups. There also was no difference in blood alcohol levels (mean ± S.E.M. mg/dl) in the two ethanol-fed groups (FE, 217 ± 36; FE-LAZ, 238 ± 27).

Histopathology. Feeding of the fish oil-ethanol diet for 1 month resulted in fatty liver, necrosis and inflammation (figs. 1 and 2). There was no evidence of pathological changes in the dextrose-fed groups. In the fish oil-ethanol-fed rats who were treated with the U74389 (FE-LAZ), there was complete absence of both necrosis and inflammation (figs. 1 and 3). However, the degree of fatty liver was not affected by drug treatment.

Nonheme iron and lipid peroxidation. As previously reported, nonheme iron levels were significantly increased in ethanol-fed rats compared with control animals (fig. 4) (P < .01). Of note was the increase in nonheme iron levels by
U74389 treatment in the dextrose-fed groups. Treatment with U74389 in the ethanol-fed group led to a significant decrease in nonheme iron levels (P < .01). The level of nonheme iron in the FE-LAZ group decreased to that seen in the FD-LAZ group but not to that seen in the FD group. Our hypothesis that treatment of ethanol-fed rats with U74389 should result in decreased levels of lipid peroxidation is supported by measurements of TBARS and 8-isoprostane. The levels of both TBARS and 8-isoprostane were significant lower in ethanol-fed rats after treatment with U74389 (fig. 4). U74389 treatment had no effect on lipid peroxidation in dextrose-treated rats.

Membrane ATPases. The results of the present study confirm our previous observations (Sadrzadeh et al., 1994b) that ethanol administration leads to a decrease in Ca\(^{++}\) pump ATPase activity (P < .01) (fig. 5). The reversal of changes in the activities of Ca\(^{++}\) pump ATPase and CaM in the FE-LAZ group supports the hypothesis that treatment with U74389 leads to membrane stabilization. In fact, both Ca\(^{++}\)-ATPase and CaM activities were normalized to levels seen in dextrose-fed control animals (fig. 5). A role for lipid peroxidation in alternating Ca\(^{++}\) pump ATPase activity in ethanol-fed rats is suggested by the inverse correlation between 8-isoprostane levels in plasma and Ca\(^{++}\)-ATPase activity (fig. 6, r = −.80, P < .01) and CaM (r = −.80, P < .01).

Effect of tirilazad mesylate treatment on TNF-α and COX-2 mRNA. The observation that TNF-α and COX-2 mRNAs are detectable in rats fed fish oil and ethanol was confirmed in the present study (fig. 7). In support of the hypothesis that decreased lipid peroxidation would lead to down-regulation of TNF-α and COX-2, our results show that COX-2 and TNF-α mRNAs were not detected in the FE+LAZ group. The levels of COX-1 mRNA were similar in all groups.
The objective of this study was to determine the effect of tirilazad mesylate (U74389) on alcohol-induced liver injury. The effect of the drug was tested in the intragastric feeding rat model for alcoholic liver disease. We (Nanji et al., 1994a) and others (Morimoto et al., 1994) have previously shown that feeding ethanol with fish oil results in the development of fatty liver, necrosis and inflammation. The model also makes it possible to make correlations between biochemical changes and the severity of pathological liver injury. The major observation in the present study was that the 21-aminosteroid U74389 was able to completely prevent necrosis and inflammation in rats fed fish oil and ethanol. The absence of necroinflammatory changes was accompanied by down-regulation of the proinflammatory mediators TNF-α and COX-2 and decreased levels of lipid peroxidation. There is a growing body of evidence that implicates TNF-α in alcoholic liver disease and hepatocellular injury (McClain et al., 1993; McClain and Cohen, 1989; Nanji et al., 1994c). It is also recognized that COX-2, via production of vasoactive and proinflammatory compounds, is important in tissue and hepatocellular injury (Dinchuk et al., 1995; Nanji et al., 1997). Of note is that the levels of plasma endotoxin, another stimulus for TNF-α and COX-2 up-regulation (Hempel et al., 1994; Hla et al., 1993), were not altered by U74389 treatment. This observation is important because it suggests that endotoxin...
and lipid peroxidation are two independent mediators of the necroinflammatory process in alcoholic liver injury. In recent years, it has become increasingly apparent that in addition to promoting direct toxicity, reactive oxygen intermediates may initiate and/or amplify inflammation through up-regulation of several different genes involved in the inflammatory response (Chaudri and Clark, 1989; Gossart et al., 1996; Schreck and Baeuerle, 1991). One of the consequences of enhanced lipid peroxidation is the up-regulation of TNF-α and COX-2 (Feng et al., 1995). Our previous studies have shown that TNF-α and COX-2 in Kupffer cells are up-regulated in ethanol-fed rats showing evidence of necroinflammatory changes (Nanji et al., 1997). Activation of certain transcription factors such as NF-κB are important in regulating expression of proinflammatory cytokines (Baeuerle and Henkel, 1994). It has been recently shown that activation of NF-κB and up-regulation of proinflammatory cytokines occur in ethanol-fed rats exhibiting necroinflammatory changes in the liver (Pham et al., 1996). Antioxidants such as 21-aminosteroids can protect not only against direct oxygen radical-mediated toxicity but also through their ability to inhibit the activation of NF-κB and subsequent production of proinflammatory mediators (Suzuki et al., 1997). Such a protective effect is suggested by the present study in the down-regulation of TNF-α and COX-2 in ethanol-fed rats treated with one of the 21-aminosteroids, tirilazad mesylate.

Evidence suggests that 21-aminosteroids are able to limit free radical-mediated organ injury (Braughler and Pregenzer, 1989). Although the exact mechanism or mechanisms by which these compounds inhibit cell injury have yet to be elucidated, one proposed mechanism is inhibition of lipid peroxidation by scavenging free radicals and thus blocking the lipid chain reaction, a mechanism analogous to that of α-tocopherol (Hall et al., 1994). The inhibition of lipid peroxidation in the present study was reflected in the decrease in levels of TBARS and 8-isoprostane in 16-desmethyl tirilazad mesylate-treated group. The formation of 8-isoprostane occurs in vivo via a non-COX free radical-catalyzed mechanism of unsaturated fatty acids (Morrow et al., 1992). The alterations in lipid peroxide levels in ethanol-fed and drug-treated rats closely mirrored alterations in levels of nonheme iron in the liver. The contribution of nonheme iron to enhanced oxidative stress in ethanol-fed rats has been suggested by several investigators (Bacon and Britton, 1990; Bonkovsky et

**Fig. 6.** Significant inverse correlations between 8-isoprostane levels in plasma and red cell membrane Ca++-ATPase \( (r = -.80, P < .01) \) and CaM \( (r = -.80, P < .01) \).

**Fig. 7.** RT-PCR analysis of mRNAs for TNF-α, COX-2, COX-1 and β-actin in liver samples obtained from the different groups. COX-2 and TNF-α mRNAs were detected only in fish oil-ethanol-fed rats. None of the rats in the other groups had detectable TNF-α or COX-2 mRNA. COX-1 mRNA was present in all groups at the same levels. β-actin was used as a control.
Iron supplementation in a high-fat/ethanol diet caused a marked increase in 4-hydroxyxenonalen and malondialdehyde levels in the liver (Tsukamoto et al., 1995). Furthermore, we have shown that a reduction in nonheme iron levels by the use of oral iron chelators results in decreased lipid peroxidation and improvement in liver pathology (Sadrozadeh et al., 1994). Whether the decrease in nonheme iron levels in ethanol-fed rats treated with U74389 was a direct effect of the drug or a result of inhibition of lipid peroxidation cannot be deduced from the present study. There is evidence that superoxide, generated as a result of ethanol metabolism, can mobilize free iron from ferritin (Shaw et al., 1988; Shaw and Jayatilleke, 1992). Thus, a reduction in free radical levels by free radical scavengers would be anticipated to result in lower nonheme iron levels.

The possible protection against liver injury by U74389 through a membrane-stabilizing effect is supported by measurements of Ca++ pump ATPase and CaM; the normalization of both Ca++ pump ATPase and CaM by U74389 is consistent with a membrane-stabilizing effect. We have previously shown that chronic ethanol administration leads to a decrease in red cell membrane Ca++ pump ATPase activity (Sadrozadeh et al., 1994), and there is evidence that nonheme iron, which promotes lipid peroxidation, can lead to inhibition of Ca++ pump ATPase (Wei and Sadrzadeh, 1994). Although we measured red cell membrane ATPases rather than hepatocyte membrane ATPases, previous studies in ethanol-treated rats have shown similar changes in hepatocytes and red cell membranes in response to ethanol (Israel et al., 1970). Wang et al. (1996) have shown a membrane-stabilizing effect of 21-aminosteroids and protection against liver injury in rats subjected to hepatic ischemia and reperfusion. These investigators also showed a decreased inflammatory response in the livers of rats treated with a 21-aminosteroid. Thus, the decrease in the necroinflammatory reaction in the animals treated with U74389 could in part be due to a membrane-stabilizing effect. In addition, membrane stabilization will affect the propagation phase of lipid peroxidation and therefore indirectly reduce hepatic lipid peroxidation. Reduction in free radical production by inflammatory cells could also result from this membrane-stabilizing effect.

In conclusion, our results show that the improvement in necroinflammatory changes in fish oil-ethanol-fed rats treated with a tirilazad mesylate were accompanied by a reduction in the degree of lipid peroxidation and in expression of TNF-α and COX-2. The reduction in TNF-α and COX-2 expression most likely contributed to the reduction in the severity of pathological change. Although the decrease in lipid peroxidation is believed to be the mechanism for the improvement in liver pathology, a membrane-stabilizing effect of tirilazad mesylate is also likely to be important.

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References


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