Chronic Ethanol Consumption Increases Hepatic Sinusoidal Contractile Response to Endothelin-1 in the Rat

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Recent evidence suggests that hepatic stellate cells function as liver-specific pericytes that are highly contractile in response to endothelin-1 (ET-1). Liver injury has been shown to lead to "activation" of stellate cells producing a phenotypic change to a more myofibroblastic cell type including loss of vitamin A and increased contractility. The present study was undertaken to test the effects of short-term chronic ethanol consumption (36% of total calories for 5 weeks according to the Lieber-DeCarli protocol) on hepatic vitamin A storage, expression of smooth muscle α-actin, and sinusoidal contractility in Sprague-Dawley rats. Using *in vivo* epifluorescence video microscopy, we quantified the number of sites of vitamin A fluorescence (purportedly stellate cells) and assessed sinusoidal microhemodynamics at baseline and during a 20-minute infusion period of ET-1 (1 pmol$*$100 g body weight [bw]$^{-1}*$min^{-1}). Retinol and retinyl palmitate were measured after the experiment by means of high-pressure liquid chromatography (HPLC). A highly significant decrease in liver retinyl palmitate level (control: 622.5 $\pm$ 50.9; ethanol: 273.0 $\pm$ 38.0 μg/g liver; $P < .001$) was found that correlated with a decrease in sites of vitamin A fluorescence (control: 531.4 $\pm$ 76.1; ethanol: 141.1 $\pm$ 30.2$*$mm^{-2}; $r = .82$, $P < .001$). Concomitantly scattered expression of smooth muscle α-actin in sinusoids was observed. Although sinusoidal hemodynamics were not affected at baseline, a significant increase in sinusoidal contractility on endothelin-1 infusion (e.g., sinusoidal resistance [% of baseline value]: control: 10 minutes: 288.7 $\pm$ 71.7, 20 minutes: 200.5 $\pm$ 46.9; ethanol: 10 minutes: 1,916.0 $\pm$ 701.7, 20 minutes: 656.8 $\pm$ 103.3; $P < .05$ and .01, respectively) was observed. These data indicate that chronic ethanol consumption in this moderate model initiates stellate cell activation. Increased sinusoidal responsiveness to the vasoconstrictor ET-1 *in vivo* may contribute to the increased susceptibility of ethanol-fed rats to secondary stresses that increase ET-1 expression, such as endotoxemia. (HEPATOLOGY 1995;22:1565-1576.)

Regulation of total liver blood flow has classically been viewed in the context of sympathetic innervation of hepatic arterioles and portal venules.[1] The significant pressure decrease in hepatic sinusoids[2] as well as the presence of contractile structures in sinusoidal lining cells[3] suggests, however, that regulation of liver blood flow may also occur at the sinusoidal level.

For instance, hepatic stellate cells (HSCs; also termed lipocytes, fat storing-cells, or Ito cells), a cell type that plays an important role in vitamin A storage and extracellular matrix production, also express intermediary filaments such as desmin suggesting that they belong to a muscle cell lineage.[4] These cells are located in the perisinusoidal space of Disse and have long stellate appendages that wrap around the abluminal surface of the sinusoidal endothelium.[5] The characteristic morphology as well as the observation that HSCs in culture are contractile in response to several constrictors, such as thromboxane A_2, prostaglandin $F_{2\alpha}$, or endothelin-1 (ET-1), have led to the proposal that HSCs may act as liver-specific pericytes, modulating blood flow distribution at the sinusoidal level.[6-8] Consistent with this concept, our recent work has shown that ET-1 produces sinusoidal constriction in the isolated perfused rat liver *in situ* that colocalizes with the body of HSC[9,10] and leads to redistribution of sinusoidal blood flow even in normal liver *in vivo*.[11]

Evidence suggests that HSC under pathological conditions, such as associated with alcohol-induced liver disease become more myofibroblastic in nature. This process termed "activation" leads not only to increased formation of extracellular matrix by HSC but also to expression of smooth muscle-α actin.[12] In cultured HSC it has been shown that activation results in change in phenotype with loss of vitamin A[13] and increased contractility.[14] Although *in vitro* assays of contractility allow unambiguous study of a specific cell type, for instance HSC, they are severely hampered by the fact

Abbreviations: HSC, hepatic stellate cell; ET-1, endothelin-1; RBC, red blood cell; FITC, fluorescein isothiocyanate; bw, body weight; VQ, volumetric blood flow; V_{RBC}, red blood cell velocity; R_s, sinusoidal resistance; HPLC, high-pressure liquid chromatography.

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that HSCs lose their three-dimensional arrangement around the sinusoids and are exposed to proteolytic enzymes during the isolation procedure.

In this study, we examined the effects of short-term chronic ethanol consumption on sinusoidal contractility, blood flow distribution, and hepatic vitamin A storage in the intact rat liver *in vivo*. Our results suggest that this model of chronic alcohol consumption does not produce overt microvascular changes as are typically associated with the later development of liver fibrosis or cirrhosis but leads to increased responsiveness to sinusoid constrictor actions of ET-1. This enhanced responsiveness is consistent with an early state of activation of the hepatic stellate cells.

MATERIALS AND METHODS

Animals and Diet. Experiments were performed in male Sprague-Dawley rats (n = 8/group; Charles River Laboratories, Wilmington, MA) that were housed in individual cages on a 12-hour dark-light schedule. Experiments were performed in compliance with the institutional guidelines and the National Research Council's criteria for humane care as outlined in "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health publication no. 86-23, revised 1985).

All rats were fed a complete liquid diet for 5 to 6 weeks according to the Lieber-DeCarli protocol using a commercially available liquid diet (protein: 16.8%; fat: 17.9%; carbohydrate: 53.3%; BioServe, Frenchtown, NJ). Ethanol and control diets are matched except for isocaloric replacement of carbohydrates by ethanol. Ethanol was introduced gradually into the diet and reached the final amount of 36% of total calories by day 8 of the diet. Rats that received the ethanol-containing diet (ethanol group) had free access to their diet, whereas animals in the control group received the control liquid diet in amounts that were limited to the intake of the pair-fed rats from the ethanol group on the preceding day.

In Vivo *Microscopy*

Fluorescent Cell Labeling. Five to eight milliliters of blood of a heparinized donor animal was obtained by aortic puncture under pentobarbital anesthesia. Red blood cells (RBCs) were separated by centrifugation and plasma and buffy coat were discarded. RBCs were labeled according to the technique of Zimmerhackl et al[15] with fluorescein isothiocyanate (FITC) on celite as described in detail elsewhere.[11] Labeled RBCs were resuspended to a hematocrit of approximately 50% and stored after addition of citrate-phosphate-dextrose (1.4:10) and refrigerated for up to 5 days.

Animal Preparation. Eight animals in each group were used for *in vivo* microscopic assessment of sinusoidal microcirculation and vitamin A–associated autofluorescence. In one animal of the ethanol group, cannulation of the portal vein failed and in one animal in the control group the chosen lobule was lost during ET-1 infusion period and microvascular measurements but not the vitamin A autofluorescence data were discarded from evaluation. Experiments in ethanol-fed rats and the pair-fed controls were performed on the same day.

The liquid diet was withheld overnight but animals were allowed free access to water. After induction of anesthesia (pentobarbital sodium, 50 $mg*kg^{-1}$ body weight [bw], intraperitoneally), animals were placed supine on a heating pad to maintain body temperature. A tracheotomy was performed to maintain a patent airway but animals were allowed to breathe spontaneously. The right jugular vein and the left carotid artery were cannulated with PE 50 tubing for injection of dyes and pressure measurements, respectively. Subsequently, a laparotomy was performed and the liver ligaments were dissected. The intestines were covered with moist gauze and swept to the left to allow cannulation of the splenic vein with a double lumen catheter allowing simultaneous measurement of portal pressure and constant infusion into the portal vein as we have described in detail previously.[11] The common bile duct was cannulated with PE 10 tubing to allow collection of bile in tared microcentrifuge tubes. The intestines were repositioned into the abdominal cavity and covered with saran wrap to prevent evaporative losses during the protocol or re-exteriorization of the bowel. Subsequently, the rat was turned on its left side and the left liver lobe was exteriorized with its lower surface uppermost and mounted on a lucite plate. A mild suction was applied through holes in the plate to minimize respiratory movement. The exteriorized lobe was covered with a piece of saran wrap to prevent drying or mechanical damage. The whole preparation was then moved to the stage of a Zeiss ACM fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). Fluorescent-labeled RBCs ($0.1\ mL*100\ g\ bw^{-1}$) for the assessment of RBC kinetics and fluorescein-sodium to enhance the autofluorescence of the liver ($0.01\ mg*100\ g\ bw^{-1}$) allowing unambiguous measurements of sinusoid diameter were injected intravenously before microscopy as we have described previously.[11]

In Vivo *Observation of Sinusoidal Hemodynamics and Vitamin A–Associated Autofluorescence.* The liver surface was epi-illuminated with a 100-W mercury lamp with 485-nm excitation and 515- to 565-nm emission band pass filters allowing visualization of FITC-labeled RBCs in sinusoids as well as clear assessment of the sinusoidal boundaries. Once an acinus was brought into focus so that the terminal hepatic venule and zone 2 and 3 of draining sinusoids could be observed, epi-illumination was switched to a second filter block with 366-nm excitation and 450-nm emission band pass filters showing multiple punctate, rapidly photobleaching sites of autofluorescence that are associated with vitamin A in HSCs[16]; each site consists usually of a cluster of smaller fluorescent spots. Previous results from our laboratory using high-power epi- and transillumination techniques showed that individual fat droplets in perisinusoidal cells cluster to form autofluorescence that appears as a single site under lower power observation.[9] Therefore, the "sites" of autofluorescence reflect a close estimate of the number of HSCs that contain detectable amounts of vitamin A. Rats fed a vitamin A–free diet for 21 weeks completely lack this type of fluorescence suggesting that it is indeed specific for retinoids (See Results).[16]

For quantitative assessment of sinusoidal microcirculation and the evanescent sites of vitamin A autofluorescence, experiments were videorecorded using a DAGE-MTI SIT 66 camera and an S-VHS recorder (Panasonic AG 7300). Off line assessment of vitamin A–storing cells per unit area and RBC velocity at a specimen monitor ratio of 840× while sinusoid diameters were measured at 1,350× using digitized frame by frame analysis with Bioquant Meg IV image analysis system (R&M Biometrics, Nashville, TN). This procedure allows direct real-time monitoring of sinusoid diameter changes in response to agonists such as ET-1. To avoid spurious results that might occur because of the irregular walls of the sinusoids, sinusoid diameter was measured by tracing the perimeter of a sinusoid segment and then dividing by the length to

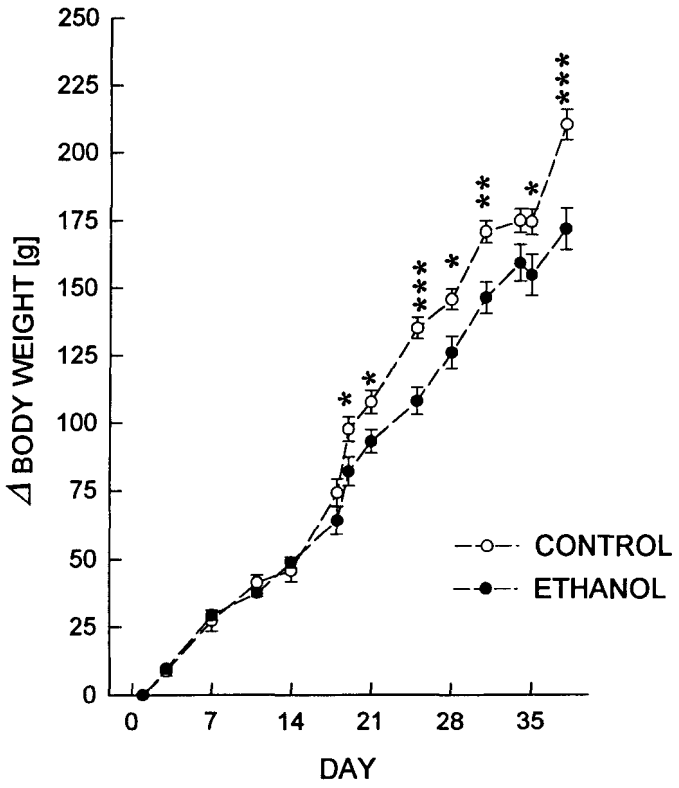

FIG. 1. Changes in bw as compared to bw on day 1 of the study (174.4 ± 2.7 g) in rats fed a liquid diet (control) or a liquid diet in which carbohydrates were partially (36% of total energy) replaced by ethanol (ethanol). Rats fed ethanol had free access to their diet, whereas intake of pair-fed controls was limited to the amount consumed by its paired ethanol-fed rat on the preceding day. Data are mean ±SE for n = 8 animals/group. *denotes $P < .05$, **denotes $P < .01$, ***denotes $P < .001$ between groups for unpaired t-test.

obtain the average width. The average length of sinusoidal segments studied was 54 μm.

Volumetric blood flow (VQ) in sinusoids was estimated from RBC velocity (V_{RBC}) and sinusoidal cross-sectional area ($\pi * r^2$) according to the equation of Gross and Aroesty,[17] i.e.,

$$VQ = V_{RBC} * \pi * r^2$$

Additionally, sinusoidal resistance (R_S) was calculated using the following equation derived from Poiseuille's law:

$$R_S = (8 * \eta * 1)/(\pi * r^4)$$

where η is the blood viscosity and 1 is the length of the sinusoidal segment. Both equations are very simplistic inasmuch as the non-newtonian properties of blood are neglected and a cylindrical shape of the sinusoid and constant viscosity (2.4 cp) is assumed. Therefore, the actual values may not accurately reflect the actual flow and resistance but they allow assessment of relative changes over time and comparison between the experimental groups.[9]

Experimental Protocol. A continuous infusion of 0.9% saline via the double lumen catheter into the portal vein was started in a volume equal to the volume of ET-1 infused subsequently in that experiment by means of an infusion pump (Harvard syringe infusion pump 22, Harvard Instruments, South Natick, MA). The volume infused was always in the range of 25 to 50 $\mu L * min^{-1}$, an amount previously found neither to alter portal pressure nor sinusoidal blood flow.[11] Mean arterial pressure and portal pressure were continuously recorded using a Grass polygraph (Grass Instruments, West Warwick, RI). After an initial stabilization period of 15 minutes the surface microcirculation was recorded for off line analysis of baseline sinusoidal hemodynamics in zone 3. Infusion of ET-1 into the portal vein was started to produce an infusion rate of 1 pmol ET-1 $* 100\ g^{-1} * min^{-1}$. This dose produces a half maximal constriction in the studied zone 3 sinusoids in normal rats on standard pellet diet.[18] The hepatic microvascular measurements were repeated every 5 minutes. Bile was collected, during the whole experiment in 5-minute intervals in pretared microcentrifuge tubes.

At the end of the observation period the left liver lobe was sampled, immediately frozen in liquid nitrogen, and stored at −70°C until assessment of vitamin A and E by means of high-pressure liquid chromatography (HPLC).

HPLC Determination of Vitamin A and E. Retinol, retinyl palmitate, and α-tocopherol in liver were measured using a modification of the HPLC (Shimadzu Co., Kyoto, Japan) method by DeLeenheer et al.[19] This method simultaneously determines retinol, retinyl palmitate, and α-tocopherol levels in tissues. Tissue α-tocopherol was measured to evaluate the effect of ethanol diet on the absorption of another lipid-soluble vitamin. Furthermore, it has been shown that α-tocopherol may have a sparing effect on the expenditure of vitamin A in animals.[20] Liver tissues were homogenized in cold Hank's balanced salt solution and centrifuged at 10,000g for 10 minutes. Methanol was added to aliquots of liver supernatants to precipitate protein. An internal standard (100 $ng * mL^{-1}$ α-tocopherol acetate) was added to all samples to correct for sample recovery. One milliliter of n-hexane was added by interrupted mixing on a vortex mixer for 1 minute. After centrifugation, 500 μL of the organic layer was removed and evaporated in a gentle stream of nitrogen. The residue was dissolved in 100 μL of methanol and the entire volume was loaded onto a reversed-phase (Shim-pack CLC-ODS; 5 μm) HPLC column. Methanol (100%) was used as the mobile phase with a flow rate of 2.5 $mL * min^{-1}$. The column effluent was monitored at 292 nm. Calibration curves for retinol, retinyl palmitate, and α-tocopherol were determined by blotting peak area ratios against known concentration standards. Liver tissue concentrations of retinol, retinyl palmitate, and α-tocopherol were estimated from calibration curves.

Immunohistochemical Staining of Smooth Muscle α-Actin. In additional experiments, expression of smooth muscle α-actin, a marker for HSC transformation, was studied immunohistochemically in two rats fed ethanol or control diet, respectively. After pentobarbital anesthesia and systemic heparinization, livers were flushed via the portal vein with phosphate-buffered saline followed by perfusion with 2% paraformaldehyde in PLP buffer (final concentrations: 0.075 mol/L lysine, 0.0375 mol/L dibasic sodium-phosphate, 10 mmol/L sodium m-periodate; pH: 7.4) at a flow rate of 25 $mL * min^{-1}$ for 5 minutes. Subsequently the livers were cut into blocks of 2 × 5 × 5 mm and stored at 4°C in the same fixative for further immersion fixation until processing. Samples were embedded in paraffin and 15 μm sections were cut. After dewaxing with xylene, slides were incubated with H_2O_2 to block endogenous peroxidase activity. Subsequently, samples were incubated at 4°C overnight with a monoclonal mouse anti–smooth muscle α-actin- antibody (Boehringer Mannheim, Indianapolis, IN; dilution: 1:400). A biotinylated horse anti-mouse antibody (Vector Labs, Burlingame, CA; dilution: 1:1,000) was used as secondary antibody for strep-

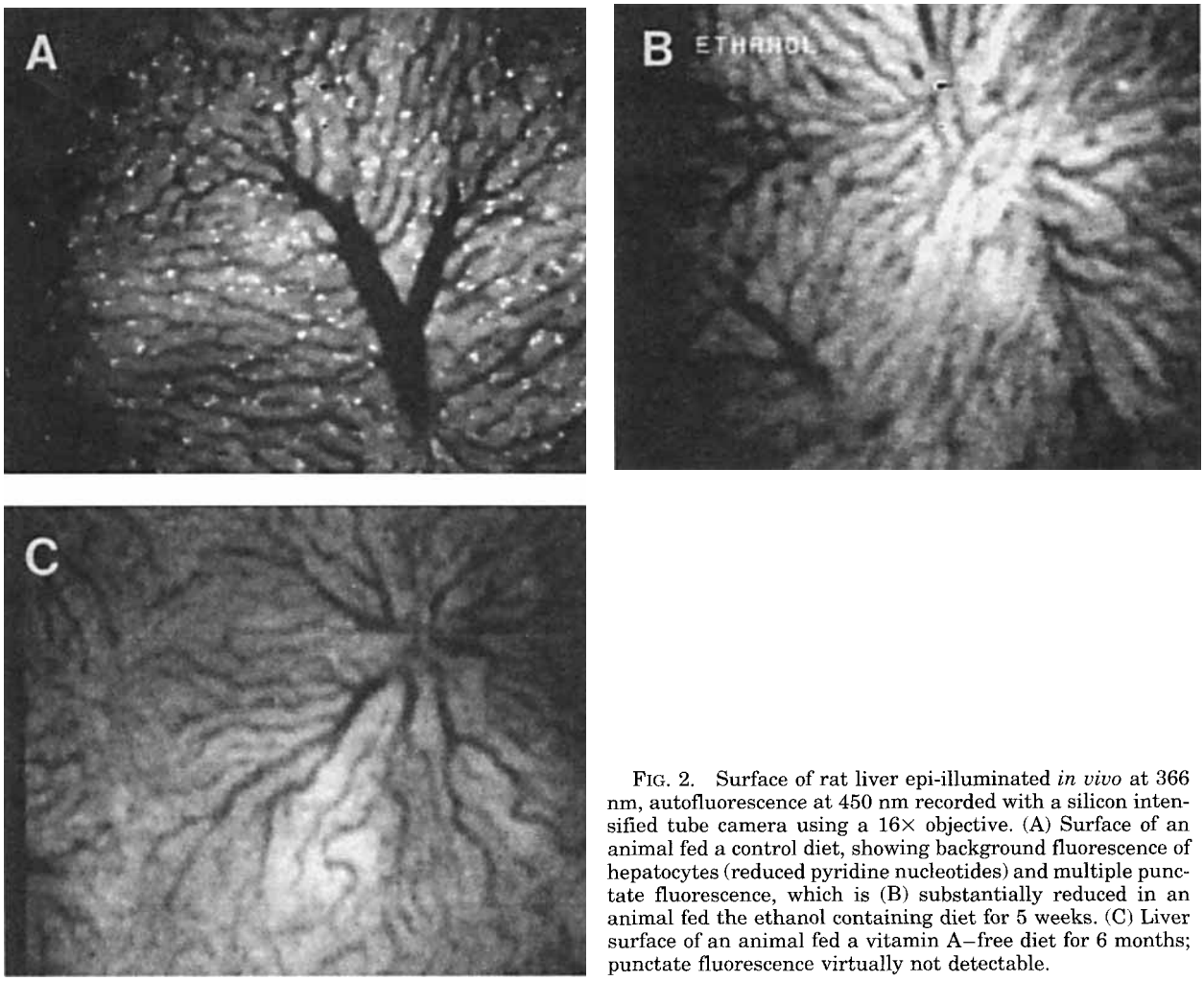

FIG. 2. Surface of rat liver epi-illuminated *in vivo* at 366 nm, autofluorescence at 450 nm recorded with a silicon intensified tube camera using a 16× objective. (A) Surface of an animal fed a control diet, showing background fluorescence of hepatocytes (reduced pyridine nucleotides) and multiple punctate fluorescence, which is (B) substantially reduced in an animal fed the ethanol containing diet for 5 weeks. (C) Liver surface of an animal fed a vitamin A–free diet for 6 months; punctate fluorescence virtually not detectable.

tavidine-biotin-complex peroxidase staining. The peroxidase activity was detected using diaminobenzidine and the sections were counterstained with hematoxylin. All of these procedures were performed in an automated immunostaining apparatus (Techmate 1000, Bio Tek, Santa Barbara, CA).

Statistical Analysis. Data are presented as individual data for correlation analysis, as mean ± SEM, or as a frequency distribution. To account for baseline variability the microvascular data are normalized and expressed as percentage of baseline; raw data at baseline are provided in Table 1. Statistical differences from baseline within each group were determined by repeated measures ANOVA followed by post hoc Student-Newman-Keuls test for all pairwise comparisons. Differences between the two groups were tested using the unpaired *t*-test. For correlation analysis the Pearson product moment correlation was used. When criteria for parametric tests were violated the respective nonparametric test (i.e., Friedman repeated measure ANOVA on ranks and Mann Whitney *U*-test) were used as appropriate. A $P < .05$ was considered significant.

TABLE 1. Effect of Ethanol (36% of total energy for 5 to 6 weeks) on Hepatosecretory Function and Sinusoidal Microhemodynamics at Baseline

	Unit	Control	Ethanol
Bile flow	$mg*100\ g^{-1}*5\ min^{-1}$	25.7 ± 1.6	25.7 ± 4.1
Sinusoid density	$\#perf\ sin*mm^{-1}$	50.7 ± 2.5	50.0 ± 3.6
Sinusoid diameter	μm	9.3 ± 0.4	10.5 ± 0.4
R_s	$10^{16}\ dyn*s*cm^{-5}$	11.8 ± 2.5	7.8 ± 1.3
V_{RBC}	$\mu m*s^{-1}$	374 ± 24	319 ± 24
VQ	$pL*s^{-1}$	28.1 ± 3.9	33.2 ± 4.8

NOTE. The liquid diet was withheld overnight before the experiments. Data are mean ± SE for n = 7 to 8 animals/group.

RESULTS

Effect of Ethanol on Body Weight, Baseline Sinusoidal Hemodynamics, and Baseline Secretory Function. Changes in body weights of ethanol-fed rats and their pair-fed controls as compared with day 1 of the study are shown in Fig. 1. Although the weight gained by the animals within the first 2 to 3 weeks was similar in both groups, a progressively higher increase in body weight in the late course of the experiment in pair-fed controls was observed.

Assessment of baseline sinusoidal hemodynamics showed no significant differences among the treatment groups (Table 1). The lack of overt microvascular im-

TABLE 2. Levels of Vitamin A and Vitamin E as Assessed Using HPLC and Sites of Vitamin A Fluorescence as Assessed by Epifluorescence Microscopy in Livers of Ethanol-Fed Rats and Pair-Fed Controls

	Unit	Control	Ethanol
Retinol	$\mu g * g^{-1}$ liver	23.0 ± 2.7	17.8 ± 2.1
Retinyl Palmitate	$\mu g * g^{-1}$ liver	622.5 ± 50.9	273.0 ± 38.0*
Vitamin A autofluorescence	$* mm^{-2}$	531.4 ± 76.1	141.1 ± 30.2*
α-tocopherol	$\mu g * g^{-1}$ liver	13.2 ± 2.0	21.3 ± 1.8†

NOTE. Data are mean ± SE for n = 7 to 8 animals/group.
* $P < .001$.
† $P < .05$ as compared with contols.

pairment at baseline thus reflects the mild nature of this model without the characteristic microcirculatory disturbances associated with overt fibrosis. Likewise, hepatocellular secretory function as reflected by the bile flow was not affected at baseline.

Effect of Ethanol on Hepatic Vitamin A and E Content and Vitamin A–Associated Autofluorescence. In rats fed the ethanol-containing diet a profound decrease in liver retinyl palmitate content was observed that correlated with a decrease of vitamin A–associated sites of autofluorescence per unit area ($r = .82$, $P < .001$; Table 2, Fig. 2 and Fig. 3). Although the storage pool of vitamin A, i.e., retinyl palmitate was significantly depleted, no significant decrease in the active form, i.e., retinol was observed. The decrease in vitamin A was paralleled by an increase in vitamin E (Table 2) when comparing the group means, however, no significant correlation of individual vitamin A and E levels was found (retinyl palmitate vs. α tocopherol: $r = -.33$, $P = .23$; retinol vs. α tocopherol: $r = -.31$, $P = .27$). In additional experiments, Sprague-Dawley rats that were matched with respect to age and body weight at the beginning of special diet feeding (n = 6 to 8/group) were fed a vitamin A–free diet (BioServe, Frenchtown, NJ) or a respective control diet for 5 or 21 weeks. Interestingly, rats fed the vitamin A–free diet for 5 weeks were found to have only a moderate decrease (−15%) of retinyl palmitate as compared with their controls, whereas ethanol-fed animals lost 55% of retinyl palmitate levels compared with the respective pair-fed animals (Fig. 4). Vitamin A was not detectable in the diet by HPLC and feeding of the diet for a prolonged period of time (21 weeks) did deplete the vitamin A stores (retinyl palmitate 82% lower than in controls and retinol no longer detectable).

Expression of Smooth Muscle α-Actin. In control animals smooth muscle α-actin was restricted to the wall of portal vessels and terminal hepatic venules; no staining was observed in sinusoidal lining cells. In contrast, in livers from rats fed the ethanol-containing diet scattered expression of smooth muscle α-actin was found in sinusoids in addition to staining of large vessels. At these sites typically clusters of strongly positive staining cells were observed (Fig. 5). However, the appearance of α-actin–positive cells was not nearly as pervasive as the disappearance of sites of vitamin A fluorescence (Fig. 2 and Fig. 5), suggesting some degree of activation without overt phenotypic transformation. The morphology of these smooth muscle α-actin–positive cells is suggestive of HSCs (Fig. 5).

Systemic and Portal Pressure in Response to ET-1. Systemic and portal pressure at baseline and during infusion of ET-1 are summarized in Table 3. Consistent with our previous reports,[11,18] no systemic pressor response accompanied the portal venous infusion of ET-1 at a rate of 1 $pmol * 100\ g^{-1} * min^{-1}$, whereas a moderate portal pressor response reaching statistical significance in the control group was observed. No significant differences in systemic or portal pressures were observed between the treatment groups at any time point.

Bile Flow During Infusion of ET-1. ET-1 had an overall cholestatic effect in both groups that reached significance at 15 and 20 minutes on start of the infusion (Fig. 6). No significant differences were noted between

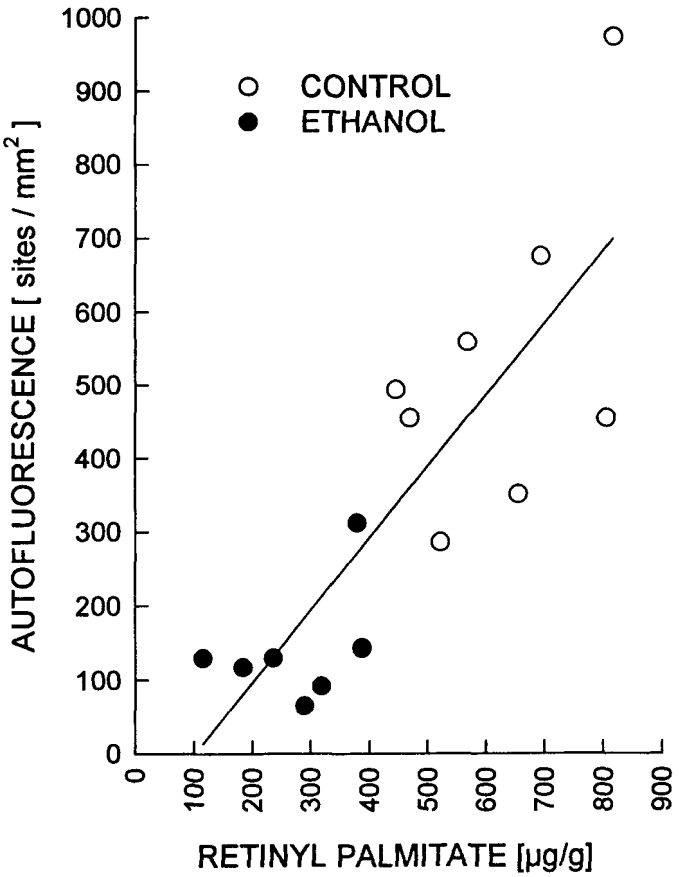

FIG. 3. Relationship between retinyl palmitate in liver homogenate (as determined by HPLC) and sites of vitamin A–associated autofluorescence on liver surface as assessed by *in vivo* epifluorescence microscopy (excitation wavelength 366 nm, emission 450 nm). A significant correlation ($r = .82$; $P < .001$) was found between sites of vitamin A autofluorescence (HSC with a vitamin A content above a threshold concentration that allows discrimination of punctate fluorescence from background) and retinyl palmitate.

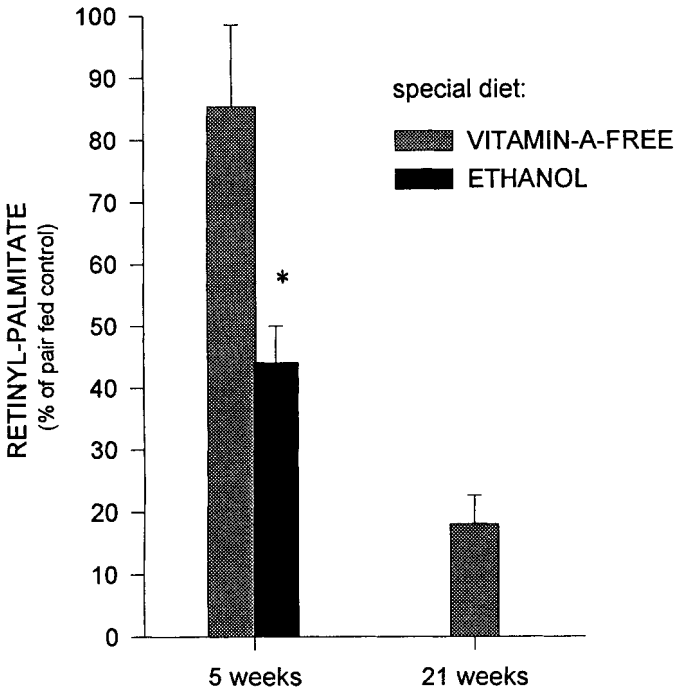

FIG. 4. Retinyl palmitate levels (% of respective pair-fed controls) of animals fed either an ethanol-containing diet (36% of total calories) for 5 weeks or a vitamin A–free diet for 5 or 21 weeks. *$P < .02$ compared with rats fed ethanol for the same period of time.

the two diets. This dose of ET-1 was previously found not to produce a cholestatic effect but rather to have a choleretic effect in animals fed standard pellet food *ad libitum,*[18] suggesting an effect of the specific diet or of starving effects associated with this model on hepatocellular secretory function.

Effect of Ethanol on Sinusoidal Vasoreactivity. ET-1 is a potent constrictor in the portal circulation causing presinusoidal and sinusoidal constriction. The dose of ET-1 used in this study has been previously shown to produce a half-maximal constriction of zone 3 sinusoids at 10 to 13 minutes into portal venous infusion of the peptide in rats fed standard rat chow *ad libitum.*[18] An example of the peak endothelin response in the sinusoids of a normal rat is shown in Fig. 7. In rats fed the control diet a significant constriction of sinusoids was observed that waned after 15 to 20 minutes in spite of continued infusion of ET-1. In contrast, the sinusoidal constriction in rats fed ethanol was faster developing and more profound and sustained as compared with the control group (Fig. 8). Accordingly, the increase in R_S was pronounced in the ethanol group with an approximately 10-fold increase compared with the respective baseline values (Fig. 9).

RBC Kinetics and Sinusoidal VQ. Analysis of the V_{RBC} as reflected by the velocity of FITC-labeled RBC showed in both groups the characteristic tight normal distribution of V_{RBC} at baseline (Fig. 10). With ET-1 infusion a marked heterogeneity of sinusoidal flow velocity was observed with occurrence of sinusoids exhibiting high-flow velocities, whereas other sinusoids were found to have sluggish or no flow. This heterogeneity is consistent with our previous reports and most likely reflects the combined presinusoidal and sinusoidal sites of action of ET-1 in this model.[11]

Sinusoidal VQ as an index of nutrient delivery to the sinusoidal cells was calculated to be 28.1 ± 3.9 and 33.2 ± 4.8 $pL*s^{-1}$ in controls and ethanol group, respectively, which is in good agreement with our previous report in rats fed standard pellet diet *ad libitum.*[18] Infusion of ET-1 in animals fed the control diet led to a slowly developing overall decrease in mean volumetric flow, whereas animals fed the ethanol diet exhibited a profound and progressive decrease in mean sinusoidal blood flow (Fig. 11). Although mean volumetric flow is a sensitive parameter for changes in microvascular flow inasmuch as it reflects changes in V_{RBC} and sinusoidal width, its use to predict impairment of nutrient delivery to individual sinusoids in increasingly heterogenous systems is limited. As reflected by the frequency distribution for V_{RBC}, infusion of ET-1 induced flow heterogeneity so that, irrespective of the overall volumetric flow, sinusoids with brisk or even increased flow were found in direct juxtaposition to sinusoids that became stagnant of blood flow.

DISCUSSION

In the present study we have investigated the effect of short-term chronic ethanol consumption on sinusoidal contractility, hepatic vitamin A storage, and sinusoidal expression of smooth muscle α-actin. The 5-week period of ethanol feeding in this study obviously did not produce overt microvascular disturbances characteristic of liver fibrosis or cirrhosis. Under the latter conditions the structural changes associated with deposition of extracellular matrix components led to a remarkable heterogeneity of sinusoidal perfusion with occurrence of "slow" and "fast" sinusoids.[21] Therefore, the tight normal distribution of V_{RBC} at baseline in the present study along with the lack of significant differences in baseline microvascular parameters and hepatocellular secretory function emphasizes the mild nature of this model.

In contrast to the lack of overt microvascular disturbances at baseline, a significant increase in sinusoidal contractility in response to ET-1 was observed. Although the potent vasoconstrictive effects of ET-1 in portal circulation are well recognized,[22] direct sinusoidal constriction and the role of HSC as liver-specific pericytes has been a source of recent controversy.[6,7,14] In contrast to earlier reports that showed contractility of cultured HSC in response to various vasoconstrictors and proposed a possible contribution of HSC in regulation of liver blood flow, Rockey et al recently showed that freshly isolated HSC from normal rats fail to contract in an *in vitro* assay. However, HSC isolated from fibrotic livers or those after several days in culture were highly contractile on addition of ET-1.[14] These authors concluded that contractility is confined to "activated" HSC. In contrast to these experiments performed solely

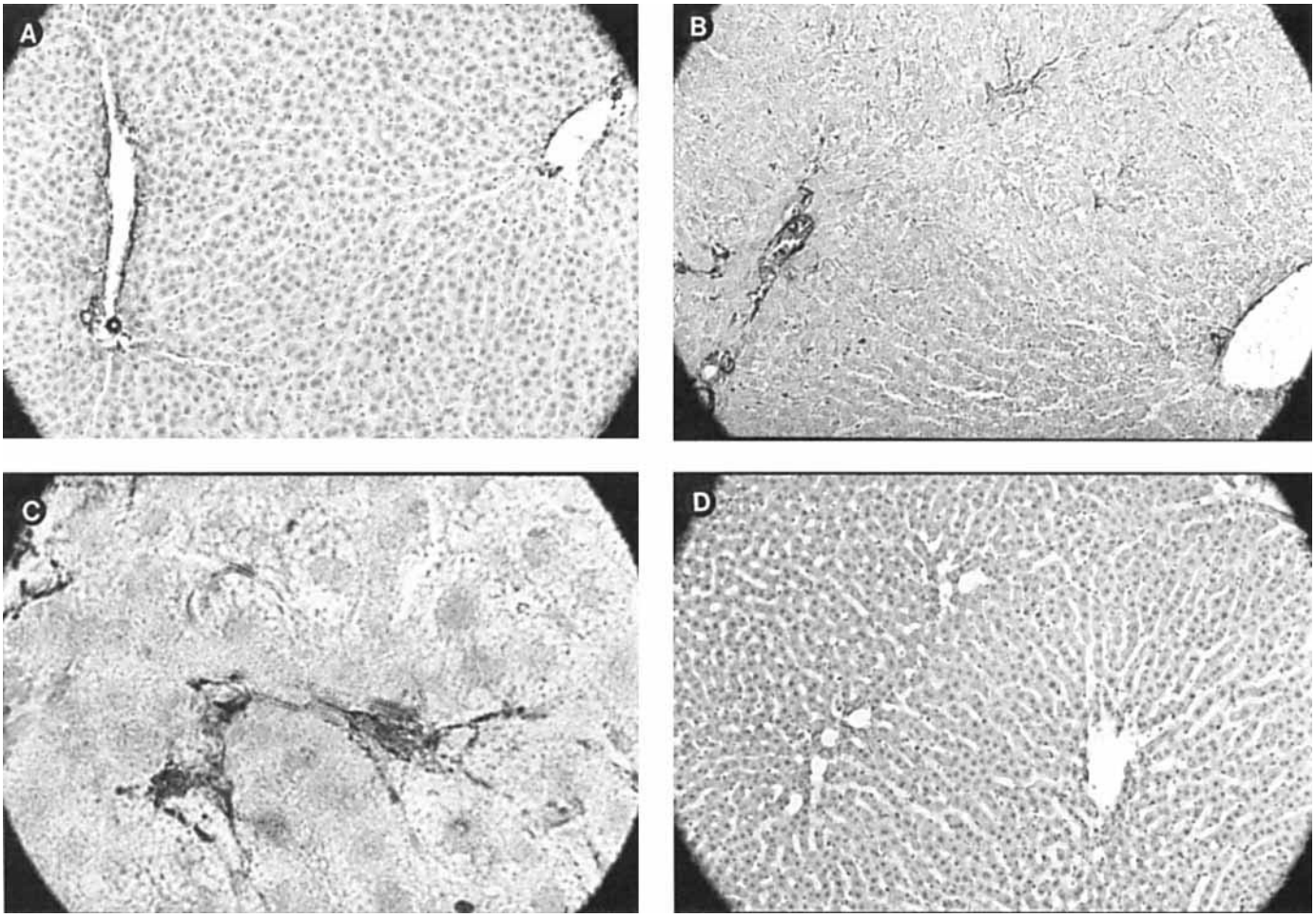

FIG. 5. (A) Immunostaining for smooth muscle specific α-actin in liver of pair-fed control animal. Staining is restricted to the vessel walls of larger vessels particularly in portal tracts, whereas sinusoids are consistently negative (20× objective). (B) Expression of smooth muscle α-actin in vessel walls as well as focal expression in sinusoids after 5 weeks of ethanol feeding (20× objective). (C) High magnification (100× objective) of panel B. (D) Negative control (primary antibody omitted). Slides are counterstained with hematoxylin.

in isolated cells in an *in vitro* assay, we[9,10,11,18] and others[23] have reported that the sinusoid is clearly contractile in normal livers *in situ* and *in vivo*. It is noteworthy that the use of intravital video microscopy, such as in our studies, allows the direct observation of changes in sinusoid dimensions with a high degree of both spatial and temporal resolution. Moreover, our previous work has shown that ET-1–mediated sinusoidal constriction colocalizes with vitamin A–associated autofluorescence of HSC *in situ* in the isolated liver and is mediated predominately by ET_A receptors.[10] This receptor subtype has been shown to be exclusively present on HSCs among the different liver cell types in rats.[24] Therefore, we conclude that even quiescent

TABLE 3. Systemic and Portal Pressure at Baseline and During the Ensuing 20-Minute Infusion Period of 1 pmol ET-1 * 100 g^{-1} * min^{-1}

	0 Min	5 Min	10 Min	15 Min	20 Min
Mean arterial pressure (mm Hg)					
Control	131 ± 8	130 ± 7	127 ± 8	128 ± 8	132 ± 7
Ethanol	131 ± 3	134 ± 4	131 ± 5	129 ± 7	129 ± 6
Portal Pressure (mm Hg)					
Control	7.1 ± 0.5	8.3 ± 0.4*	8.6 ± 0.5*	8.7 ± 0.5*	8.9 ± 0.4*
Ethanol	7.7 ± 0.4	8.6 ± 0.8	8.3 ± 0.4	8.2 ± 0.5	8.6 ± 0.4

NOTE. Data are mean ± SE for n = 7 to 8 animals/group. No significant difference was noted between the groups at any time point.
* $P < .05$ as compared to respective baseline value for repeated measure ANOVA and *post hoc* Student-Newman-Keuls test.

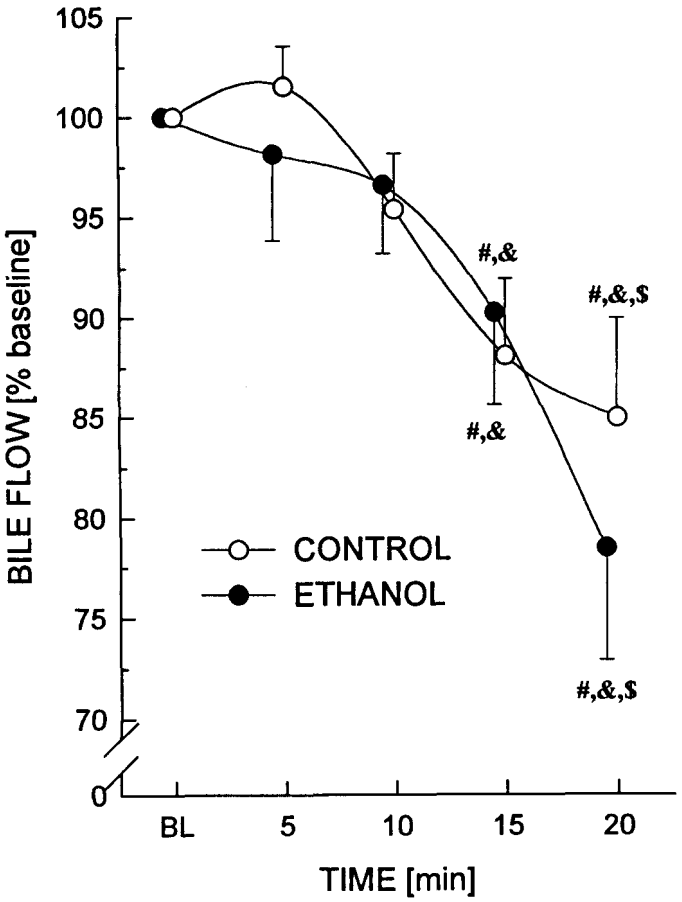

FIG. 6. Time course of bile flow response during portal infusion of 1 pmol ET-1*100 g^{-1}*min^{-1} in % of respective baseline value. Data are mean ± SE for n = 7 to 8 animals/group. #$P < .05$ as compared with baseline, &$P < .05$ as compared with 5 minutes, \$$P < .05$ compared with 10 minutes for repeated measure ANOVA followed by Student-Newman-Keuls test.

HSCs are contractile in response to ET-1 and are likely to act as liver specific pericytes even in the normal liver. However, the aforementioned *in vitro* observations suggest that the HSC becomes significantly more contractile on activation. Two facets of HSC activation, i.e., loss of vitamin A and the occurrence of smooth muscle α-actin, at least in some of the sinusoidal lining cells were observed in the present study. However, ethanol itself does not produce fibrosis in the rat. Our results would suggest that the Lieber-DeCarli protocol, in spite of the lack of overt fibrosis, leads to HSC "activation" in terms of early loss of vitamin A and increased contractility but only moderate transdifferentiation into myofibroblasts as reflected by the scattered strong staining for smooth muscle α-actin. These changes are consistent with the currently proposed cascade model of HSC activation[25] and earlier ultrastructural studies by Mak et al showing that ethanol leads to phenotypic alterations in HSC, such as decrease in lipid droplets and proliferation of the rough endoplasmic reticulum, in baboons fed the Lieber-DeCarli diet suggesting their transformation into transitional cells.[26] Similar changes have been reported in a model of pig serum–induced liver fibrosis in the rat where an early increase in number and desmin staining of HSC preceded overt fibrosis and expression of smooth muscle α-actin by these cells.[27]

The notion that HSCs indeed lose their vitamin A storage capacity when they undergo a phenotypic change toward a myofibroblast is supported by several lines of evidence: It has been shown that HSC in culture become "activated" within days producing collagen and glycosamino-glycans while losing their vitamin A; for instance, stimulation of HSC transdifferentiation in culture with acidified myofibroblast like cell-conditioned medium leads to a significant decrease in retinyl palmitate as early as 6 days after seeding as compared with cells cultured in the absence of the medium.[13] Moreover, recent evidence in a similar *in vitro* system, where transdifferentiation of HSC was stimulated by adding Kupffer cell–conditioned medium, suggests that the underlying mechanism for vitamin A loss during "activation" is the stimulation of intracellular retinyl ester hydrolase activity leading to retinol release into the medium.[28] Therefore, the present results suggest that the profound decrease in retinyl palmitate despite ample dietary supply in ethanol-fed rats, which significantly exceeds the vitamin A depletion achieved by feeding a vitamin A–free diet for the same time, reflects ethanol-induced mobilization of retinoids from HSC. The significant decrease in the number of sites of vitamin A autofluorescence in the ethanol-fed animals compared with the focal expression of α-actin in sinusoids, suggests that loss of vitamin A is an early indicator of activation in this model. Assessment of vitamin A content by means of intravital microscopy has recently been validated by Suematsu et al and when combined with microspectrophotometry allows at least semiquantitative measurements of vitamin A in individual sites of autofluorescence.[16] It is of interest that α-to-

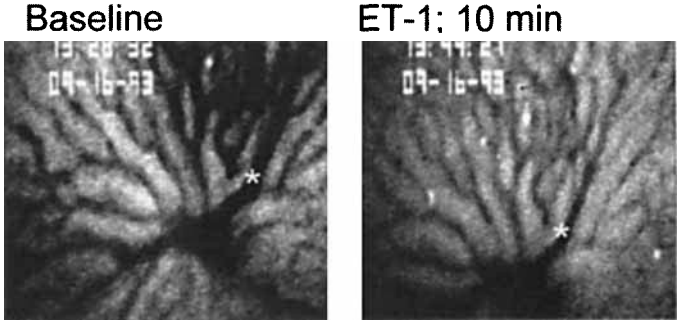

FIG. 7. Video micrographs of the liver microcirculation at baseline and 10 minutes into the infusion of 1 pmol ET-1*100 g^{-1}*min^{-1} into a normal rat *in vivo*. The peak response to endothelin is typically observed at this time (see Fig. 8). The two images are of the same field, which moved slightly between the recording of the two images. For reference, the asterisk marks approximately the same site in 1 sinusoid before and during the ET-1 infusion. Note the substantial narrowing of the sinusoids during the endothelin infusion.

copherol levels were significantly higher, whereas retinyl palmitate levels were decreased in ethanol-fed rats, compared with pair-fed controls. Other studies have observed a similar inverse relationship between tissue vitamin A and E levels. For example, Pelissier et al[29] reported a 69% increase in liver vitamin E content in vitamin A–deficient rats relative to vitamin A–sufficient animals. Likewise, Paquette et al,[30] reported that vitamin A deficiency caused a significant increase in α-tocopherol in lung (23%) and liver (53%) of C3H/HeJ mice.

Although the highly significant decrease in liver vitamin A content as well as the scattered sinusoidal expression of smooth muscle α-actin in ethanol-fed rats in our model points toward the HSC in mediating the increased contractile response, a contribution of other sinusoidal cells, namely Kupffer cells or endothelium, cannot be excluded. This may include expression of contractile structures or altered release of (secondary) mediators by these cells. For instance, Kupffer cells are a potent source of vasoactive mediators, and have been shown to release eicosanoids on endothelin stimulation.[31] The recent report by Adachi et al showing a beneficial effect of gadolinium chloride inactivation of Kupffer cells on hepatocellular injury as early as in the first week of the ethanol feeding in the more severe chronic Tsukamoto-French model would suggest that

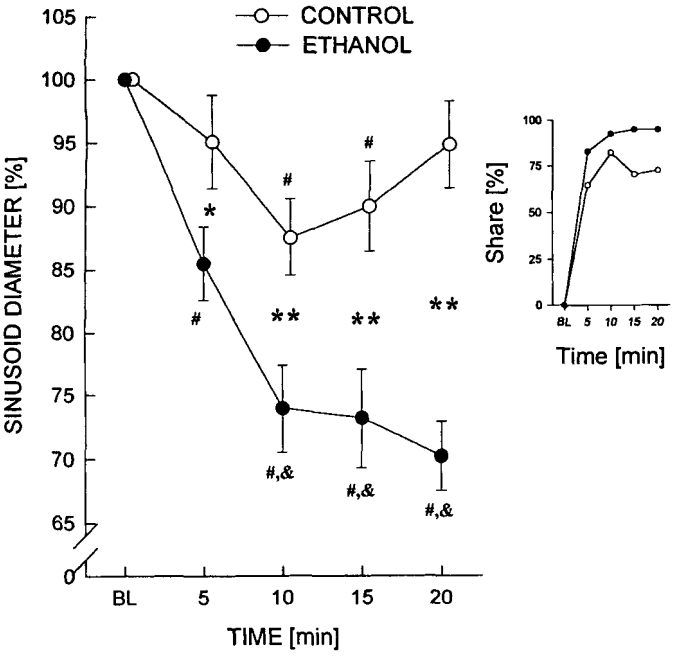

FIG. 8. Time course of sinusoidal diameter during portal infusion of 1 pmol ET-1 * 100 g^{-1} * min^{-1}. Data are mean ± SE for 7 animals/group and are normalized to account for baseline differences (see Table 1). *$P < .05$, **$P < .005$ between groups for unpaired t-test or Mann-Whitney U-test, respectively. #$P < .05$ as compared with baseline, &$P < .05$ as compared with 5 minutes for repeated measure ANOVA and *post hoc* Student-Newman-Keuls test. Inset: Percentage of sinusoids that constrict in response to ET-1 infusion relative to respective baseline value.

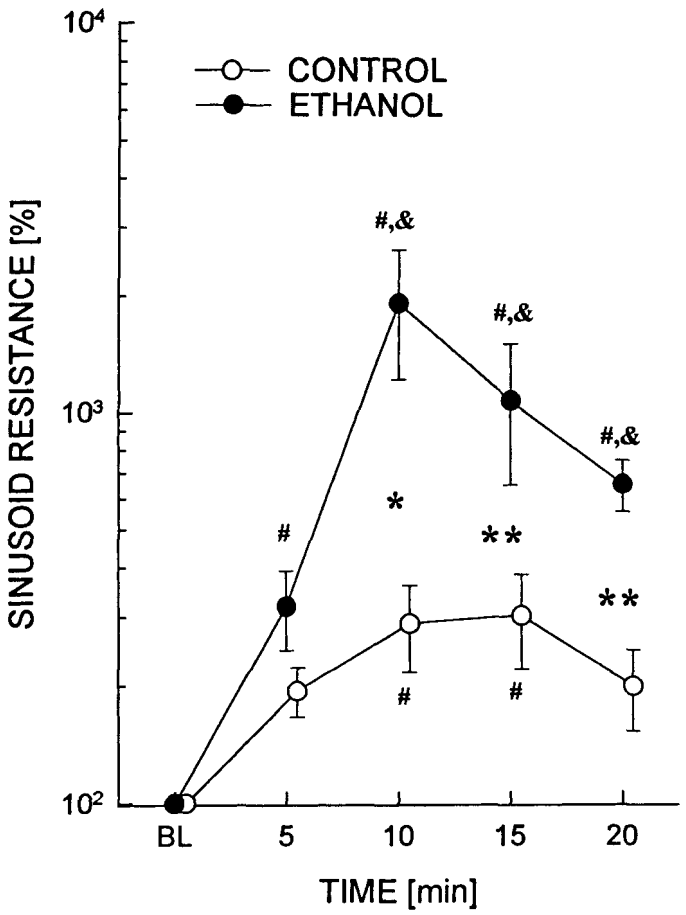

FIG. 9. Changes in sinusoidal resistance during portal infusion of 1 pmol ET-1 * 100 g^{-1} * min^{-1}. For details of calculation see Materials and Methods. Note that resistance is plotted on a log scale. Data are mean ± SE for n = 7 animals/group. *$P < .05$, **$P < .005$ between groups for unpaired t-test or Mann-Whitney U-test, respectively. #$P < .05$ as compared with baseline, &$P < .05$ as compared with 5 minutes for repeated measure ANOVA and *post hoc* Student-Newman-Keuls test.

the Kupffer cell function is altered in very early stages of alcohol-induced liver disease.[32] It seems likely that alterations in Kupffer cell function or responsiveness, such as increased cytokine production on endotoxin stimulation,[33] may contribute to change in phenotype of HSC via release of inflammatory mediators, because Kupffer cell–conditioned medium and proinflammatory cytokines are potent stimuli for HSC activation.[13,28,34]

In contrast to the increased responsiveness of the sinusoid, the presinusoidal sites of ET-1 action were not affected by the ethanol diet. For instance, the portal pressure response tended to be greater in control rats, which is consistent with our previous work in the isolated perfused liver showing that the portal pressure increase largely depends on presinusoidal resistance changes.[10] The increase in presinusoidal resistance leads typically to a decrease in sinusoidal V_{RBC}.[11] Therefore, the high incidence of sinusoids with very

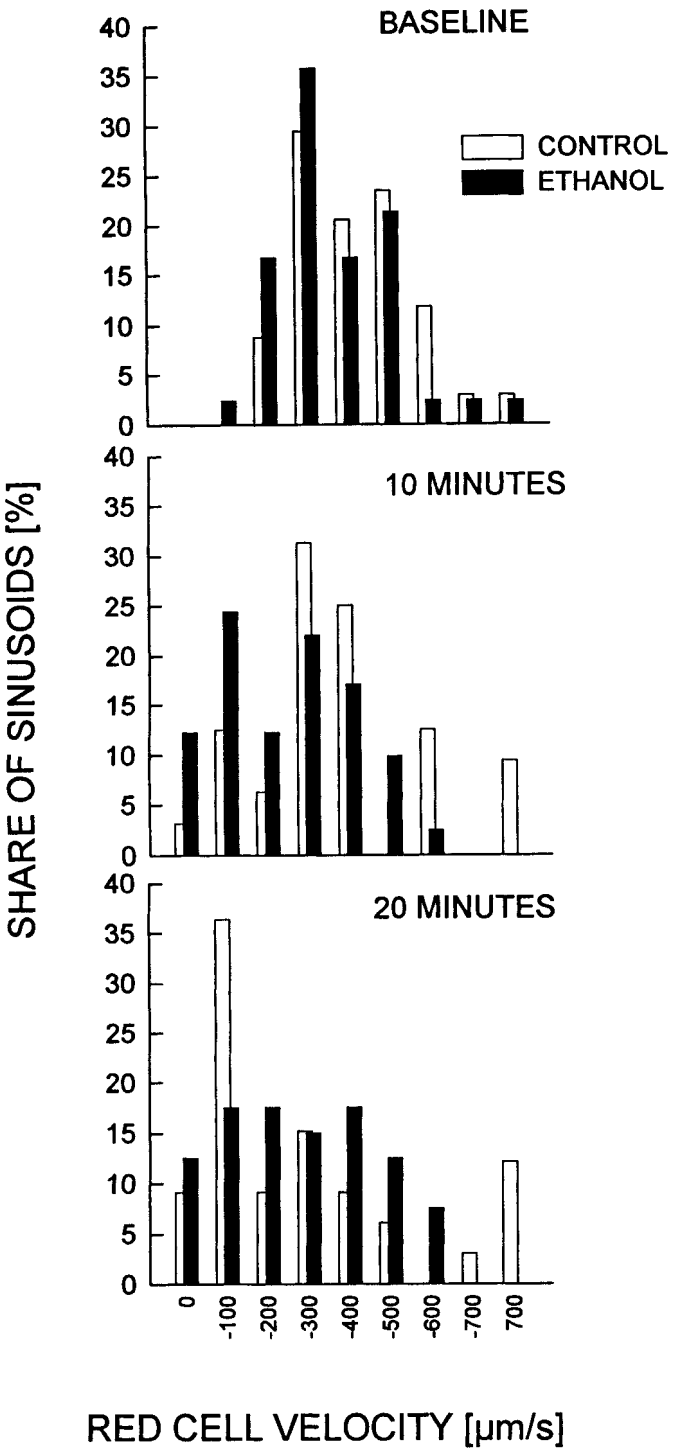

FIG. 10. Frequency distribution for V_{RBC} in zone 3 sinusoids over increments of 100 $\mu m * s^{-1}$ at baseline, and at 10 and 20 minutes into portal infusion of 1 pmol ET-1 * 100 $g^{-1} * min^{-1}$. Fraction of sinusoids represents number of sinusoids as percentage of total zone 3 sinusoids studied exhibiting flow velocities within given range. V_{RBC} in each sinusoid represents average velocity of two individual labeled RBCs.

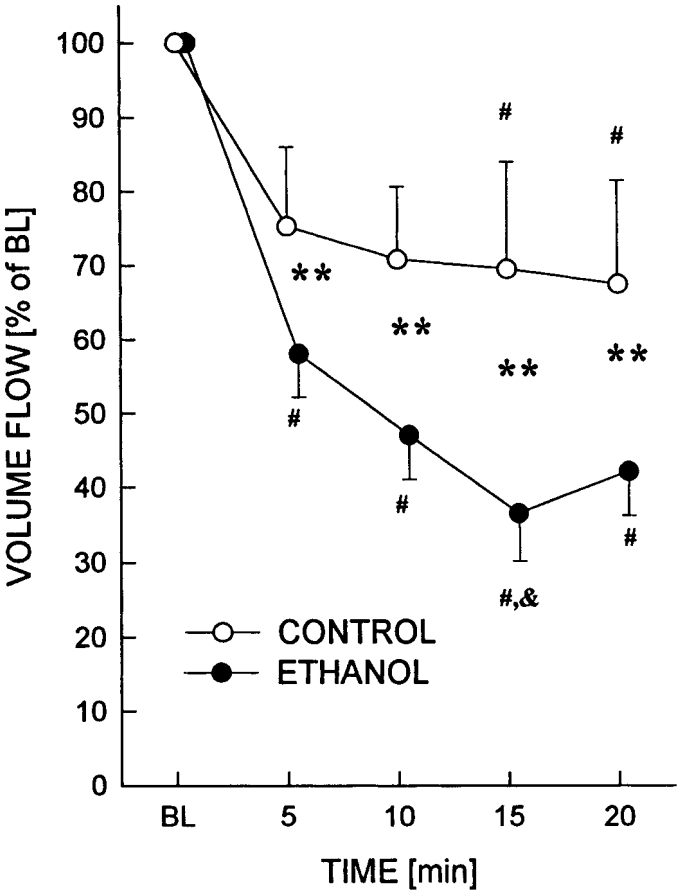

FIG. 11. Time course of volumetric blood flow in hepatic sinusoids as calculated from V_{RBC} and sinusoidal cross-sectional area during infusion of 1 pmol ET-1 * 100 $g^{-1} * min^{-1}$. Data are mean ± SE for n = 7 animals/group. $^{**}P < .005$ between groups for unpaired *t*-test or Mann-Whitney *U*-test, respectively. $\#P < .05$ as compared with baseline, $\&P < .05$ as compared with 5 minutes for repeated measures ANOVA and *post hoc* Student-Newman-Keuls test.

sluggish flow velocities in the control group at 20 minutes into ET-1 infusion when sinusoidal width had recovered again to almost baseline levels would suggest that presinusoidal sites are largely responsible for impaired sinusoidal flow at the late time point in pair-fed controls.

Considering the importance of the microcirculation for oxygen delivery to the parenchymal cells, one would expect that the more profound impairment of microvascular flow in ethanol-fed rats leads to severely depressed secretory function. However, this was not the case. The very complex nature of the response, i.e., involvement of presinusoidal and sinusoidal sites of action, the heterogeneity of the microvascular response, and the concurrent choleretic effect of ET-1, may partially explain the lack of a significant difference in bile flow response between the groups. Furthermore, *in vivo* microscopy using the epi-illumination technique is limited to the surface microcirculation. Because the vessels supplying the peripheral regions of the lobes are of higher order than the ones that supply

the hilus region, the response may be more profound in the surface region. This is supported by previous work from Ji et al who reported that on stimulation of α-sympathetic nerves in the isolated perfused liver shutdown occurs in the surface microcirculation while total blood flow is constant, reflecting increased flow in parts toward the hilus of the lobule.[35] Therefore, it seems likely that during ET-1 infusion the microcirculatory changes are more profound at the surface and large parts of the liver, even in the ethanol-fed rats, may be well perfused explaining the lack of significant differences in bile flow response despite the significant difference in surface microvascular response.

In conclusion, we have shown that in rats fed ethanol for only 5 weeks a significant decrease in vitamin A–positive stellate cells and scattered sinusoidal expression of smooth muscle α-actin are observed coincident with significant increase in sinusoidal contractility. Interestingly, this period of ethanol exposure was not sufficient to produce overt disturbances of liver microcirculation at baseline, but led to an increased responsiveness of the sinusoid to the constrictor actions of ET-1. These findings suggest that early on in the development of alcohol-induced liver disease a "priming" occurs. Because failure of the hepatic microcirculation is a major determinant of liver injury,[36] these findings may partially explain the increased susceptibility of ethanol-fed rats to secondary stresses, such as endotoxin injection,[37] because endotoxin is a stimulator of ET-1 release.[38] The extensive vitamin A depletion that was observed in ethanol-fed rats after only 5 weeks as well as the scattered sinusoidal expression of smooth muscle α-actin reflect some degree of activation of HSCs despite the lack of fibrosis. We propose that activation of the HSCs contributes to the increased responsiveness of hepatic sinusoids to ET-1.

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