Pseudocapillarization and Associated Energy Limitation in the Aged Rat Liver

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Age-related impairment of drug metabolism by the liver is consistent with hepatocyte hypoxia, suggestive of the development of a diffusional barrier to oxygen supply. Because the effects of aging on the diffusional pathway (sinusoidal endothelium and space of Disse) have not been described, we performed comparative studies on the livers of Fischer F344 rats aged 4 to 7, 12 to 15, and 24 to 27 months. Light-microscopic examination revealed no evidence of fibrosis, cirrhosis, or other specific pathology. In contrast, scanning and transmission electron-microscopic examination revealed that aging is associated with pseudocapillarization of the sinusoidal endothelium, indicated by defenestration with reduced porosity, thickening of the endothelium, infrequent development of basal lamina, and only minor collagen deposits in the space of Disse. Furthermore, immunohistochemistry studies showed strong expression of collagen IV, moderate expression of factor VIII–related antigen, and weak expression of collagen I along the sinusoids of livers from old rats ($P < .0001$). *In vitro* ^{31}P magnetic resonance spectroscopy analysis showed that aging is associated with changes in high-energy phosphate and other metabolites, consistent with hepatocyte hypoxia. Aging in the liver is associated with changes in the sinusoidal endothelium and space of Disse that may restrict the availability of oxygen and other substrates. (Hepatology 2001;33:537-543.)

The effect of aging in the liver is often considered to be of a lesser degree than in other organs.[1-4] The major recognized changes in the aging liver include reduction in liver mass and hepatic blood flow[1,4,5]; however, it has been concluded that there are few other significant structural or biochemical changes in the liver.[1,2] On the other hand, even subtle age-related changes may have profound consequences for the rest of the body. For example, any age-related impairment in hepatic drug and xenobiotic detoxification could partly explain the susceptibility of elderly persons to adverse drug reactions or illnesses with toxic etiology.[5]

In vivo, the hepatic clearance of many drugs is reduced in elderly persons. Traditional theories have attempted to attribute this to age-related reduction of liver mass and blood flow.[6] However, in a recent review,[5] we noted that there appears to be selective reduction of the clearance of drugs that undergo phase I metabolism, associated with preservation of the clearance of drugs that undergo phase II metabolism. Even more puzzling, the *in vitro* activities of phase I enzymes are maintained into old age.[7] Because these paradoxes cannot be attributed to changes in blood flow and liver mass alone, we suggested an explanation based on oxygen supply, as the activities of phase I enzymes are highly oxygen-dependent because they require oxygen as a substrate.[8] We hypothesized the development of a barrier to oxygen diffusion, leading to functional intracellular hypoxia in the hepatocytes of the aging liver. This provides a plausible mechanism for impairment of *in vivo* phase I metabolism in association with unchanged phase II metabolism (which is less dependent on oxygen delivery) and *in vitro* phase I activity (in which oxygen delivery is not constrained).

The nature of such an oxygen barrier is unknown. In the normal liver, the sinusoidal endothelium and space of Disse do not pose a significant barrier to the transport of solutes such as oxygen.[9-11] In cirrhosis, capillarization of the sinusoidal endothelium has been proposed to provide an anatomic basis for a barrier to oxygen transport, the so-called "oxygen-limitation theory" of cirrhosis.[12] In marked contrast to cirrhosis, the effect of aging on the ultrastructure of the sinusoidal endothelium and space of Disse has not been well characterized. Most structural studies have reported changes of hepatocytes, including increased hepatocyte volume, polyploidy, lysosomes, and lipofuscin,[1] or changes in mitochondria[13] and endoplasmic reticulum[14]; however, all studies have failed to detect any evidence of classical pathologic processes.

In this study, light microscopy, immunohistochemistry, and scanning and transmission electron microscopy were used to investigate the effects of age on the diffusional pathway for hepatic substrates, with particular attention paid to the sinusoidal endothelium and space of Disse. In addition, we looked for evidence of hepatocyte hypoxia using *in vitro* ^{31}P magnetic resonance spectroscopy to examine high-energy phosphate metabolite status.

MATERIALS AND METHODS

Rats. Male Fischer F344 rats were obtained from the National Institute of Aging and were sampled while in quarantine. These rats were specific pathogen–free. They were barrier-maintained with a 12-hour light/dark cycle and fed sterilized NIH 31 rat chow. The rats

Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate.

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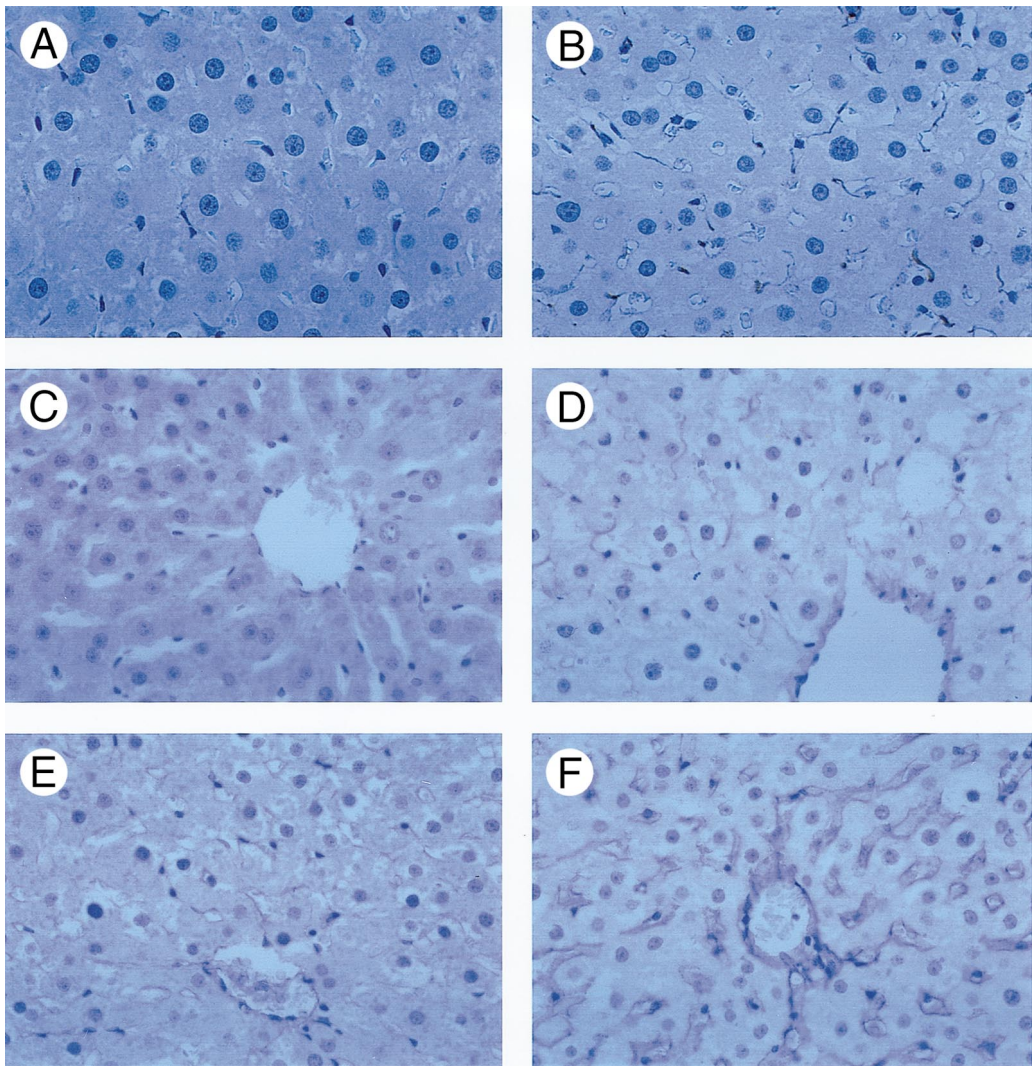

FIG. 1. Immunohistochemistry studies of the liver for factor VIII–related antigen ([A], young rat; [B], old rat), collagen I ([C], young rat; [D], old rat), and collagen IV ([E], young rat; [F], old rat). For each antigen, there is expression along the hepatic sinusoids of the old rat, most apparent for collagen IV.

were selected at random from the National Institute of Aging colony, derived from stock from the National Institutes of Health and Harlan Sprague Dawley Inc. Three age groups were examined: 1) young rats aged 4 to 7 months (n = 18; body weight, 241 ± 25 g); 2) middle-aged rats aged 12 to 15 months (n = 17; 415 ± 19 g); and 3) old rats aged 24 to 27 months (n = 15; 430 ± 27 g). The studies were approved by the Australian National University Animal Experimentation Ethics Committee and the University of Sydney Animal Ethics Committee. Anesthesia was induced in all studies using pentobarbitone sodium (60 mg/kg, intraperitoneally).

Light Microscopy and Immunohistochemistry. Sections of all livers (n = 50) were stained with hematoxylin-eosin for light microscopy. Masson trichrome and reticulin stains were also performed. Immunohistochemistry was used to determine expression of factor VIII–related antigen, collagen I, and collagen IV in 10 young and 8 old livers. Sections were treated with proteinase K after deparaffinization and blocking of endogenous peroxidase with 3% hydrogen peroxide. Incubating sections with 10% normal horse serum or rabbit serum prevented nonspecific binding. For factor VIII–related antigen, sections were sequentially incubated with mouse anti-human factor VIII–related antigen (von Willebrand Factor, Dako, Carpinteria, CA), biotinylated horse antiserum to mouse IgG and streptavidin (Vector Laboratories, Burlingame, CA) labeled with horseradish peroxidase. For collagens I and IV, sections were sequentially incubated with goat anti-human collagen I or collagen IV (Southern Biotechnology Associates, Birmingham, AL), biotinylated rabbit antiserum to goat IgG (Vector Laboratories), and streptavidin labeled with horseradish peroxidase. Peroxidase activity was revealed using 3,3′-diaminobenzidine. After counterstaining with hematoxylin, sections were dehydrated, cleared, and mounted. Only specimens with staining around the portal triad were considered technically suitable for analysis.

Electron-Microscopy Studies. Transmission electron-microscopy studies were performed on livers of 8 young, 7 middle-aged, and 7 old rats. Livers were perfused via the portal vein with heparinized saline (5,000 IU in 1 L) to flush blood from the liver, then with fixative containing 3% glutaraldehyde, 2.5% paraformaldehyde, 2 mmol/L calcium chloride, 2% sucrose, and 0.1 mol/L cacodylate buffer at a constant perfusion pressure of 10 cmH_2O for 10 minutes. From each liver, a total of 12 blocks of tissue were taken, with 6 blocks from each of the left and right lobes. Blocks were sampled at random for light-microscopic assessment. Three blocks per liver were finally studied, selected randomly from those satisfying requirements for quality of fixation and tissue integrity. At least 1 block was included from each liver lobe.

Fifty ultrathin (70-90 nm) sections were taken at random from each block for initial scanning (8,000×) using a Philips CM 120 Transmission Microscope. A technically eligible pool of sections resulted from the low-power scanning process, and 10 sections were chosen at random for ultrastructural measurement from each liver. In each of the 10 sections, representative fields were chosen by an operator blinded to tissue category, and 5 electron-micrographic measurements (17,000×) of the space of Disse and sinusoidal endothelial cells were made using Mitutoyo vernier dial calipers. Assess-

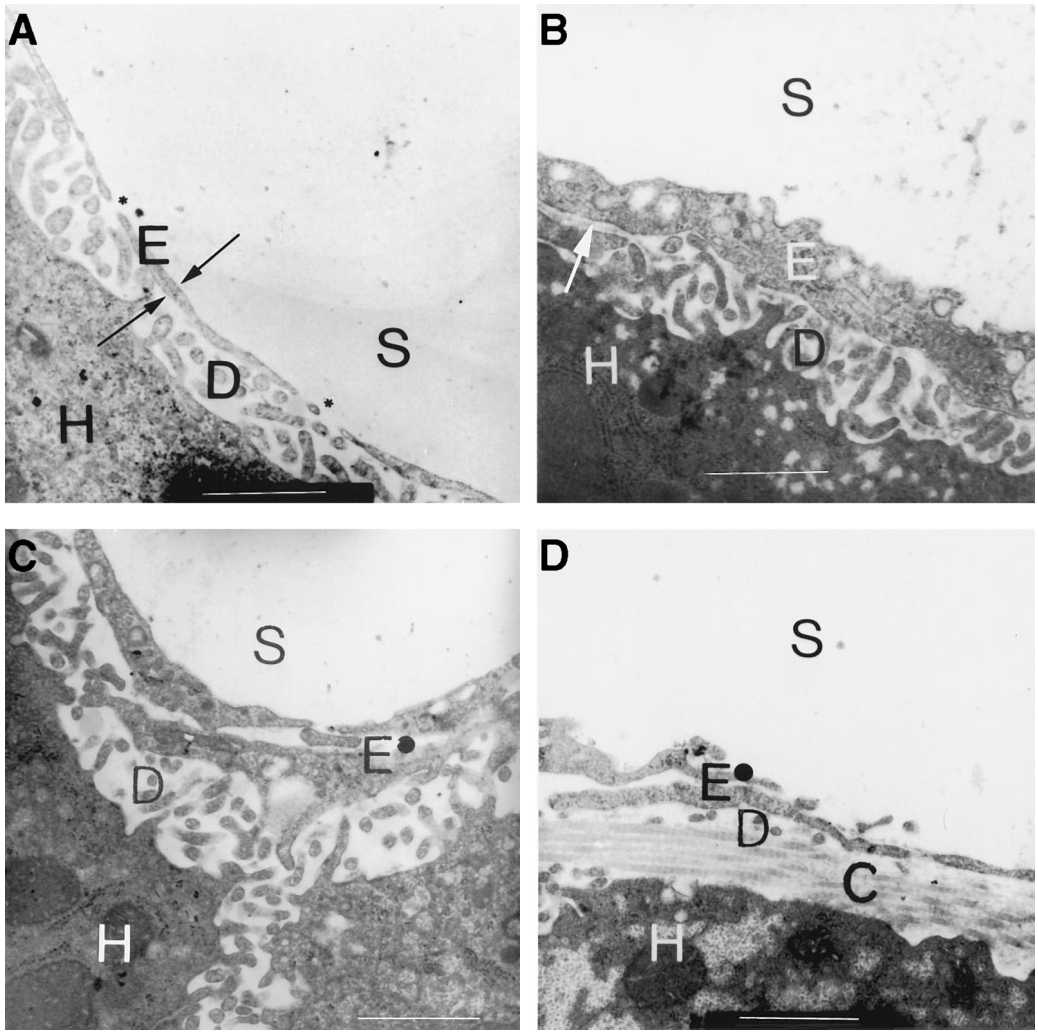

FIG. 2. Transmission-electron micrographs from livers of rats aged 4 to 7 months (A) and 24 to 27 months (B-D). The space of Disse (D) is occupied by villous projections of the hepatocytes (H) and separated from the sinusoid (S) by the endothelial cell (E). The thickness of the endothelial cells ([A], →←) increases with age. The number of fenestrations (*) in the endothelial cells is reduced in number in the older rats. There is evidence of basal lamina deposition ([B], ↑) and collagen deposition ([D], [c]). *Scale bar* = 1μm.

ment and quantitation of endothelial membrane continuity were made using the KS-400 3.00 package. The number of fenestrations was counted manually, and collagen was quantified by grid-point counting.[15] Measurement error was estimated as a 5.9% error of accuracy using 3 blinded, independent observers.

Scanning electron-microscopic studies were performed using the same sampling technique as the transmission electron-microscopic studies, but with sampling confined to 8 young and 7 aged livers. Random specimens of perfused liver tissue were osmicated (1% OsO_4/0.1 mol/L cacodylate buffer [pH 7.3]) for 2 to 3 hours and dehydrated in an ethanol gradient to 100%. Tissue pieces were critical-point–dried using a Baltec CPD 030, and then mounted on aluminum SEM stubs and sputter-coated with gold. The tissue was examined on a Philips XL30 Scanning Microscope (5,000×) to locate and characterize zone 1 and zone 3 sinusoids suitable for further examination. Five tissue blocks were examined per animal, and 10 images were taken from each animal (20,000×). The images were analyzed using the Zeiss KS 4.00 Image Analysis program. The program uses a "region-growing" technique, which separates fenestrae from endothelial cells on the basis of pixel grey levels. Porosity was estimated from some 10,000 fenestrations assessed from a total area of 0.98×10^9 nm^2, with the operator blinded to tissue category during tissue image sampling and analysis.

^{31}P Magnetic-Resonance Spectroscopy. This technique has been described previously.[16,17] Spectroscopy was performed on the livers of 10 young, 10 middle-aged, and 8 old rats. Briefly, at laparotomy under anesthesia, the liver was sampled by freeze-clamping *in situ* using aluminum tongs precooled in liquid nitrogen. Perchloric acid extractions were performed and samples were eluted through a Chelex 100 column. ^{31}P spectra were acquired on a Varian VXR-300 spectrometer at 121.4 MHz at 25°C using a 45° pulse, 6-second relaxation delay, gated broad-band proton decoupling, and approximately 2,000 scans. Assignments were based on literature values and confirmed by the addition of authentic compounds. Absolute metabolite levels were based on 3 integrations of the same spectrum.

Statistics. Results were pooled for each animal, and the data are presented as mean ± SD. The *z* test was used to compare the categorical immunohistochemistry data from young and old rats. The Student *t* test was used to compare the young and old data in the scanning electron-microscopic study. ANOVA with the post-hoc Bonferroni *t* test (SigmaStat version 2, SPSS Inc.) was used to compare the young, middle-aged, and old data from the transmission electron-microscopic and nuclear magnetic resonance studies.

RESULTS

Light Microscopy. Examination by light microscopy (n = 50) showed that there were no age-related differences using Masson trichrome or reticulin stains. Binucleate hepatocytes and vacuolation were noted. There was no evidence of inflammation, neoplasia, fibrosis, cirrhosis, or other pathologic processes (Fig. 1B).

Electron Microscopy. Transmission-electron microscopy showed ultrastructural changes in the sinusoidal endothelium with age. In rats aged 4 to 7 months, the endothelium was thin, had no basal lamina, and contained many open pores

called fenestrations (Fig. 2). However, aging is associated with thickening of the endothelium and reduced numbers of fenestrations (Fig. 2). In addition, we noted the development of a rudimentary basal lamina in 3 of 7 old rats studied and increased deposits of collagen within the space of Disse (Fig. 2).

Quantification showed a reduction in the number of fenestrations in the endothelial membrane from 2.7 ± 1.1 fenestrations per 10 μm of endothelium (n = 8) in young rats, to 1.1 ± 0.7 (n = 7) in middle-aged rats and 0.9 ± 0.8 (n = 7) in old rats (P = .002). The thickness of the endothelium increased from 0.23 ± 0.05 μm (n = 8) in younger rats and 0.25 ± 0.04 μm (n = 7) in middle-aged to 0.32 ± 0.08 μm (n = 7) in old rats (P = .01). Collagen occupied 4.8% of the space of Disse in old rats (n = 7) compared with 1.0% in young rats (n = 8; $P < .01$).

Scanning electron-microscopic examination of the sinusoidal endothelium confirmed patterns of change with aging seen using transmission electron microscopy (Fig. 3). The porosity of the sinusoidal endothelium was significantly reduced from 4.1% ± 2.3% (n = 8) to 2.5% ± 1.2% (n = 7; P = .0001) with congruent results from zone 1 (4.2% ± 2.0 % vs. 2.5% ± 1.3%; P = .003) and zone 3 (4.0% ± 2.7% vs. 2.5% ± 1.1%; P = .02).

Immunohistochemistry. Immunohistochemistry revealed expression of the factor VIII–related antigen in the sinusoidal cells in all old rats (n = 8) and no expression in young rats (n = 10; P = .00005) (Fig. 1A and 1B). Collagen I was weakly expressed along the sinusoids of all old rats (n = 6) and none of the young rats (n = 10; $P < .0001$) (Fig. 1C and 1D). Collagen IV was strongly expressed along the sinusoids of all old rats (n = 7), and was either weakly expressed (n = 6) or not expressed (n = 4) in the young rats (P = .00005) (Fig. 1E and 1F). In the aged rats, there was no lobular gradient in the expression of any antigen.

^{31}P Magnetic-Resonance Spectroscopy. There were marked changes in the spectra at different ages (Fig. 4) as summarized in Table 1. Adenosine triphosphate (ATP), ATP/Pi, ATP/adenosine diphosphate (ADP), and ATP/(ADP.Pi) were significantly lower in old and middle-aged rats compared with young rats. The spectra also allowed determination of other metabolites (Table 1). There was an increase in phosphomonoesters by 24 to 27 months. There were also age-related changes in phospholipid metabolism, with an increase in phosphocholine and phosphoethanolamine and a reduction in phosphodiesters including glycerophosphoethanolamine and glycerophosphocholine.

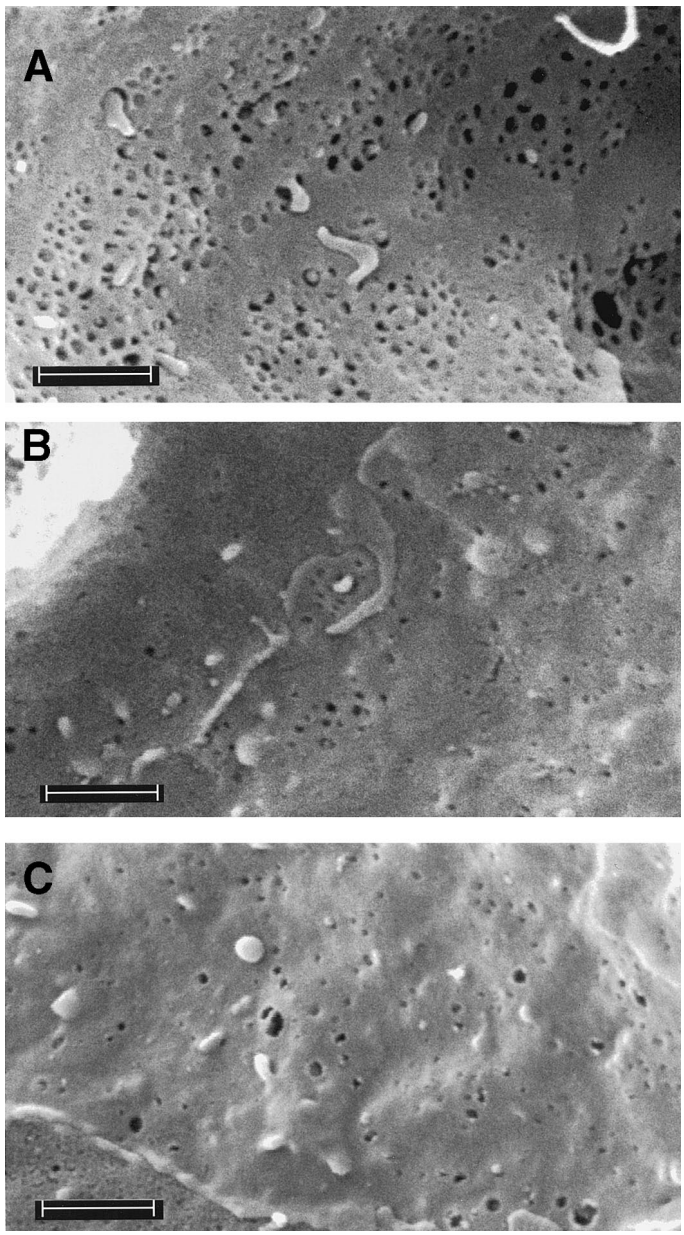

FIG. 3. Scanning-electron micrographs from livers of rats aged 4 to 7 months (A) and 24 to 27 months (B, C). There is defenestration of the aged sinusoidal endothelium.

DISCUSSION

The first major finding in this study was that aging is associated with ultrastructural and immunohistochemical changes in the sinusoidal endothelium and space of Disse. The second major finding was that there is a reduction in the energy status of the aged liver consistent with intracellular hypoxia. Previously, we proposed that there must be a diffusion barrier to hepatic oxygen uptake to explain the impairment of xenobiotic metabolism seen in human and animal aging.[5] If the structural changes that we have described constitute a functional barrier to oxygen diffusion, then a series of therapeutic implications follow from the situation established in cirrhosis.[5,18-21]

Age-related changes in the sinusoidal endothelium and space of Disse have not been reported previously. The rats we examined are a standard model (F344 rats from the NIA, NIH) and age range (4-27 months) for the study of aging,[22] and were free of liver diseases such as fibrosis and cirrhosis. Examination by standard light-microscopic techniques only revealed those few changes that are well recognized in the aged rat liver, such as binucleate hepatocytes and vacuolation.[1,2,23] These results are consistent with past studies, all of which conclude that there are few gross structural changes in the liver with age.[1,2,23]

Previous electron-microscopic studies have not reported specific investigations of sinusoidal structures in the intact aged liver[14,24-27]; however, our assessment by electron microscopy and immunohistochemistry revealed significant age-related changes within the sinusoidal endothelium and space of Disse. Scanning electron microscopy revealed a significant reduction in the porosity of the aged sinusoidal endothelium. Transmission electron microscopy confirmed the reduction of

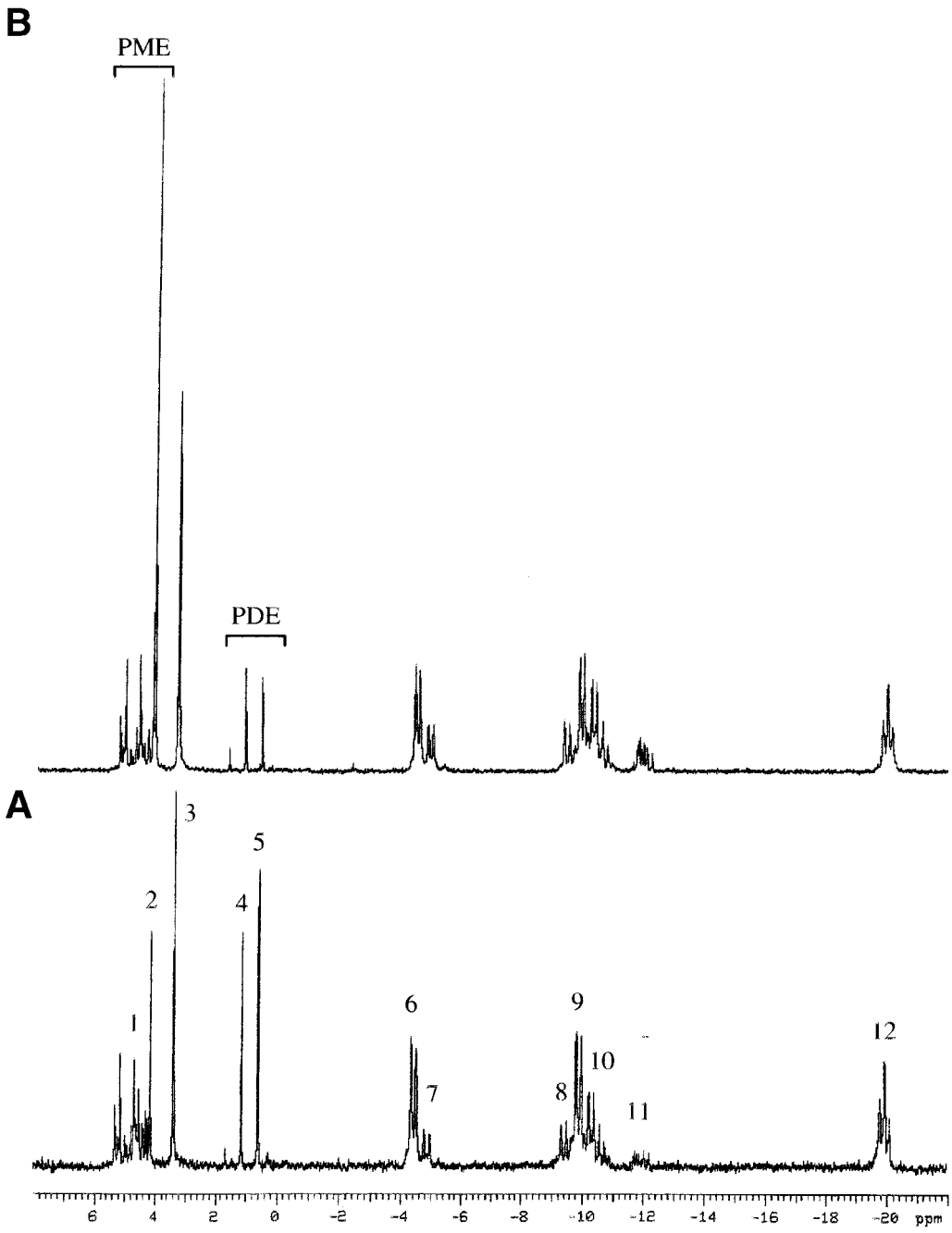

FIG. 4. ^{31}P-Nuclear magnetic-resonance spectra from perchloric acid extracts of liver. There are significant differences in the spectra of rats aged 4 to 7 months (A) and those aged 24 to 27 months (B). The results of the spectral analyses are shown in Table 1. The peaks are: 1, phosphoethanolamine; 2, phosphocholine; 3, inorganic phosphate; 4, glycerophosphoethanolamine; 5, glycerophosphocholine; 6, ATP (γP); 7, ADP (βP); 8, ADP(αP); 9 ATP (αP); 10, diphosphodiesters, mainly NAD(H) and NADP(H); 11, disphosphodiesters, mainly UDP-glucose; 12, ATP (βP); PME, phosphomonoester region 5.5-3.6 ppm; PDE, phosphodiester region 2.0-1.0 ppm.

fenestrations, and also revealed that the endothelium was thickened and associated with occasional development of a basal lamina and increased deposits of collagen in the space of Disse. These changes resemble, in part, those changes termed "capillarization" seen in cirrhosis; however, we suggest that the term "pseudocapillarization" should be used to differentiate these age-related ultrastructural changes from that seen in cirrhosis.[28,29] In contrast to capillarization associated with cirrhosis, the aged liver did not display bridging fibrosis or nodular regeneration, periportal or pericentral fibrosis, nor any loss of hepatocyte microvilli, and only minor deposits of collagen in the space of Disse. Furthermore, basal lamina formation was inconsistent, and neither immunohistochemistry nor scanning electron microscopy revealed any evidence of a zonal gradient in the changes.

Immunohistochemical studies showed that factor VIII–related antigen (von Willebrand Factor), collagen I, and collagen IV were expressed along the sinusoids of the old rats. Endothelial cells in tissue beds outside the liver are associated with expression of the factor VIII–related antigen, a glycoprotein mediating attachment of platelets after endothelial injury. It is not expressed in sinusoidal cells of normal young liver, but is found in cirrhotic liver, where it is the standard immunohistochemical marker of sinusoidal endothelial change in capillarization.[30] In addition, we noted weak collagen I expression, a marker of extracellular matrix deposition,[31] along the sinusoids of the old livers. There was also a dramatic increase in collagen IV expression with aging consistent with the development of a basal lamina.[31] These results provide additional evidence that aging is associated with the develop-

TABLE 1. Effect of Aging on Metabolites Detected by ^{31}P-Nuclear Magnetic Resonance in the Liver

	4-7 mo (n = 10)	12-15 mo (n = 10)	24-27 mo (n = 8)
Bioenergy phosphate metabolites			
ATP (μmol/g)*	2.81 (0.33)‡	2.29 (0.29)	2.25 (0.26)
ATP/Pi*	0.97 (0.22)‡	0.73 (0.10)	0.70 (0.11)
ATP/ADP†	3.30 (0.72)‡	2.56 (0.35)	2.53 (0.81)
ATP/(ADP.Pi)†	1.15 (0.36)‡	0.82 (0.16)	0.78 (0.25)
Other metabolites			
Phosphomonoesters (μmol/g)*	6.61 (0.43)§	6.68 (1.00)	8.18 (0.98)
Phosphodiesters (μmol/g)*	1.56 (0.30)‡	0.88 (0.26)	0.56 (0.11)
Phosphocholine (μmol/g)*	2.51 (0.60)§	2.33 (0.63)	4.18 (0.72)
Glycerophosphocholine (μmol/g)*	0.82 (0.22)‡	0.45 (0.13)	0.33 (0.09)
Phosphoethanolamine (μmol/g)†	0.45 (0.12)§	0.43 (0.13)	0.63 (0.18)
Glycerophosphoethanolamine (μmol/g)*	0.74 (0.10)‡	0.44 (0.13)	0.23 (0.06)

NOTE. Values in parentheses are SD.
* ANOVA, $P \leq .001$.
† ANOVA, $P < .05$.
‡ Significant difference between rats aged 4 to 7 months vs. rats aged 12 to 15 and 24 to 27 months.
§ Significant difference between rats aged 24 to 27 months vs. rats aged 4 to 7 and 12 to 15 months.

ment of a sinusoidal endothelium that has undergone a basic biologic change, with properties typical of that found in capillaries of extrahepatic tissues.

The question arises as to whether these structural changes in the endothelium with aging have any functional significance. The normal hepatic sinusoidal endothelium provides a minimal barrier to the transfer of oxygen.[10,11] However, a significant permeability barrier to the transfer of oxygen is conferred by endothelium in capillaries found in extrahepatic tissues that structurally resemble those we have observed in the aged liver.[32,33] Our ^{31}P-nuclear magnetic-resonance studies on freeze-clamped samples showed significant changes in the bioenergy phosphate metabolite pools (Table 1) consistent with reduced hepatic arterial blood flow, intracellular hypoxia, or impaired mitochondrial function. Other evidence suggestive of intracellular hypoxia includes the age-related reduction in the clearance of drugs that undergo phase I metabolism and reduction in oxygen consumption by the aged liver.[5] However, it has been reported that the ATP/ADP ratio does not change with age in isolated mitochondria of rat hepatocytes,[13] and similar patterns of change have been observed in rat livers rendered hypoxic experimentally[34] and in cirrhotic rat livers.[16] Therefore, our results are consistent with, but do not prove, the presence of intracellular hypoxia in aged livers secondary to impaired diffusion.

On the other hand, it is important to note that we found that the changes in the high-energy phosphate metabolites occurred between the ages of 4 to 7 and 12 to 15 months, preceding most of the endothelial changes that occurred between the ages of 12 to 15 and 24 to 27 months. It is possible that other changes in the diffusional pathway unrelated to endothelial morphology produce intracellular hypoxia that then leads to sinusoidal endothelial changes, potentially via hypoxia-induced cytokines such as vascular endothelial growth factor.[35] In addition, the deposition of collagen within the space of Disse that we noted may provide another stimulus for defenestration.[36]

It will be important to determine whether pseudocapillarization influences the transport of oxygen. The carbon monoxide wash-in technique that we developed[10] will help to resolve this issue both in aging and cirrhosis. Even so, in the heart and hindlimb, it has been recognized that capillaries are a barrier to oxygen transfer[32,33] and the importance of structural and functional limits for oxygen supply to other tissues such as the muscle are established.[37]

In his initial exposition of the free radical theory of aging, Harman[38] postulated that age-related oxidative changes in the cells of the circulatory system might interfere with the flow of oxygen into tissues. It is not known whether oxidants induce pseudocapillarization in the liver; however, agents such as alcohol[39] and endotoxin[40] delivered via the portal vein have been shown to produce capillarization. Accordingly, exposure to such toxins, and possibly oxidants, could contribute to the changes in aging liver that we report here. Strategies aimed at maintenance of hepatic metabolism of xenobiotics or their binding within the gut lumen could offer a basis for modification or prevention of age-related pseudocapillarization.

The beneficial effects of caloric restriction on longevity[41] may reflect net minimization of reduction in hepatic arterial flow, and consequently oxygen delivery, after eating. Because eating is associated with increased oxygen demand to cope with metabolism and detoxification of food and other gut contents, restricting the frequency, amount, and type of food intake might minimize this imbalance in hepatic oxygen supply and demand, and thus optimize hepatic metabolic activity.

It could be speculated that these age-related functional changes in the liver potentially hold pathologic implications for the whole animal. The liver has a pivotal role in detoxification of xenobiotics from bowel microorganisms and the environment in pathogenetic classes such as carcinogens, neurotoxins, and drugs generating adverse reactions. Age-related changes in hepatic detoxification are associated with increased prevalence of adverse drug reactions[5] and may be responsible for those diseases such as Parkinson's disease[42] with toxic pathogenesis. Furthermore, defenestration of the endothelium is thought to contribute to hyperlipidemia and atherosclerosis[43]; therefore, these findings provide an alternate explanation for the association of old age with some aspects of vascular disease.

In cirrhosis, strategies to improve liver oxygenation such as oxygen supplementation[44] and increasing hepatic arterial blood flow[19] lead to improved oxygen utilization and aug-

mentation of phase I drug metabolism. Similar strategies might improve liver function in old age.

In conclusion, we have found that aging is associated with significant changes in the sinusoidal endothelium and space of Disse, which we have termed "pseudocapillarization" to differentiate these age-related changes from those seen in cirrhosis. The structural changes are associated, possibly causally, with changes in high-energy phosphate metabolites consistent with intracellular hypoxia.

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